GDAŃSK UNIVERSITY OF TECHNOLOGY | FACULTY OF CHEMISTRY

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Scientific discipline: Chemistry

DOCTORAL DISSERTATION

Title of PhD dissertation: New methodological solutions for the determination of health-promoting compounds and assessment of the possibility of using Kaffir lime (*Citrus Hystrix*) fruit waste as raw materials for industrial purposes

Title of PhD dissertation (in Polish): Nowe rozwiązania metodyczne do oznaczania związków prozdrowotnych oraz ocena możliwości wykorzystania odpadów z owoców limonki Kaffir (*Citrus Hystrix*) jako surowców do celów przemysłowych

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DESCRIPTION OF DOCTORAL DISSERTATION

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Keywords of doctoral dissertation in English: Kaffir lime; citrus wastes; citrus by-products; valorization; bioactive properties; prohealth properties; antioxidant assays; gas chromatography; instrumental techniques

Summary of doctoral dissertation in Polish: Rozprawa doktorska dotyczy opracowywania i wykorzystania nowoczesnych technik analitycznych do oznaczenia wybranych związków chemicznych w próbkach soku i limonki Kaffir oraz produktów ubocznych. Szczególny nacisk położony został na oznaczenie związków z grupy terpenów, będących główną frakcją chemiczną owoców cytrusowych. Ponadto, skupiono się także na oznaczeniu związków, które charakteryzują się licznymi właściwościami prozdrowotnymi i bioaktywnymi. Były to przede wszystkim: polifenole, antocyjany, flawonoidy, flawanole, taniny, karotenoidy i ksantofile, chlorofil A i B, witamina C, mikro- i makroelementy. Do oceny właściwości przeciwutleniających wykorzystano testy: CUPRAC, DPPH, ABTS i FRAP. W pracy badano także produkty uboczne, które można otrzymać z owoców cytrusowych, takie jak pektyny, czy olejki eteryczne. Otrzymane wyniki porównano z wynikami otrzymanymi dla innych owoców, by zweryfikować założenia, iż limonka Kaffir jest dobrym źródłem związków o dobrych właściwościach prozdrowotnych i funkcjonalnych. Ostatnim celem była ocena możliwości zastosowania odpadów limonki Kaffir jako surowców w różnych gałęziach przemysłu. Rozprawa doktorska oparta jest na dziesięciu artykułach opublikowanych w recenzowanych czasopismach naukowych oraz jednym rozdziale książkowym, których doktorantka jest autorem.

Summary of doctoral dissertation in English: The doctoral dissertation concerns the development and use of modern analytical techniques to determine selected chemical compounds in samples of Kaffir lime juice, pulp and by-products. Particular emphasis was placed on the determination of compounds from the terpene group, which are the main chemical fraction of citrus fruits. In addition, there was also a focus on determining compounds that have numerous health-promoting and bioactive properties. These were primarily: polyphenols, anthocyanins, flavonoids, flavanols, tannins, carotenoids and xanthophylls, chlorophyll A and B, vitamin C, and micro- and macroelements. The following tests were used to assess the antioxidant properties: CUPRAC, DPPH, ABTS and FRAP. The work also examined by-products that can be obtained from citrus fruits, such as pectins and essential oils. The obtained results were compared with those obtained for other fruits to verify the assumptions that Kaffir lime is a good source of compounds with good health-promoting and functional properties. The last goal was to assess the possibility of using Kaffir lime waste as raw materials in various industries. The doctoral dissertation is based on ten of the author's own scientific articles published in peer-reviewed scientific journals and one book chapter.

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I am deeply grateful to Professor Żaneta Polkowska, who took me under her wing after the passing of my supervisor. She provided me with truly maternal care, fought for me like a lioness, and pushed me forward, ensuring that I never gave up. In difficult moments, she always took matters into her own hands and led me to the successful completion of my doctorate. I am especially grateful for her patience, encouragement, and unwavering belief in my abilities. Her mentorship has not only shaped my academic growth but has also been a testament to the true dedication of a scholar and educator.

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Abbreviations and acronyms

2-ABT	2-aminobenzothiazole
3D-FL	three-dimensional fluorescence
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
Asc	ascorbic acid
CE	catechin equivalent
CGE	cyaniding-3-glucoside equivalent
CUPRAC	cupric ion reducing antioxidant capacity
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	differential scanning calorimetry
DW	dry weight
FI	fluorescence intensity
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalent
GAL	gallic acid
GC	gas chromatography
GC×GC	two-dimensional gas chromatography
HM	high-methoxyl
has	human serum albumin
LM	low-methoxyl
LOD	limit of detection
LOQ	limit of quantification
MP	microwave plasma
MS	mass spectrometry
OES	optic emission spectrometry
RI	retention index
SPME	solid phase microextraction
TE	Trolox equivalent
TPTZ	tripyridyltriazine
UV	ultraviolet

Contents

1 Introduction

Citrus fruits are the most popular family of fruits available in almost all countries. They are commonly imported into many countries where their cultivation is made impossible by climatic conditions. The citrus genus, which belongs to the *Rutaceae* family according to the Tanaka classification from 1961, has 156 species. In turn, the Swingle classification from 1943 includes 16 species. However, only four of them (pomelo, citron, mandarin and papeda) are counted as primary fruits. All other citrus fruits are secondary fruits, being successive hybrids of primary fruits. Due to its ability to reproduce apomictically, its numerous species mutations, and natural and artificial hybridization processes, it is still problematic to create an accurate classification of the genus ‘citrus’.

The impact of consuming citrus fruits on human health is well known. Due to the large amount of vitamins – especially vitamin C, antioxidants, polyphenols, carotenoids and flavonoids – drinking citrus juices has a positive effect on the human body. Citrus juices such as orange and grapefruit juice are rich sources of vitamin C, a powerful antioxidant that can boost the immune system and protect against oxidative stress [1]. The flavonoids found in citrus fruits have been linked to reducing the risk of cardiovascular diseases by improving blood vessel function and reducing inflammation [2]. Citrus juice consumption has been associated with a lowered risk of stroke due to its potential to improve blood pressure and reduce the formation of blood clots [3]. Regular consumption of citrus juices, particularly orange juice, has been linked to a reduced risk of developing kidney stones, possibly due to their citrate content [4]. The phytochemicals in citrus fruits, such as hesperidin, have anti-inflammatory properties that may help alleviate symptoms in individuals with inflammatory conditions [5]. Citrus fruits and their juices have a low glycaemic index and can help regulate blood sugar levels, making them beneficial for individuals with or at risk of type-2 diabetes [6].

As the most popular family of fruits and having been known about and enthusiastically consumed for many years, citrus has found several applications. Citrus fruits are widely utilized in a variety of ways due to their refreshing flavour, versatility, and nutritional value. Citrus fruits are used especially for food purposes, and in recent years they have also been increasingly used in various industries, primarily pharmaceuticals and cosmetics. Citrus fruits are a primary source of citrus juices such as orange juice, grapefruit juice, and lemon juice. These juices are consumed as beverages or used as ingredients in various recipes. The zest and peel of citrus fruits are used to add flavour to dishes. Lemon zest, for example, can enhance the taste of desserts and savoury dishes. Citrus peel is also used to make candied peel and marmalades. Citrus fruits, particularly oranges, are used to make preserves and marmalades, which can be spread on bread or used as a topping. Citrus flavours are used in various candies, gummies, and sweets. Essential oils extracted from citrus fruits are used in

aromatherapy, perfumes, and flavourings. They can be used as a natural insect repellent. They contain compounds that deter pests. Citrus extracts are used in some cleaning products due to their natural degreasing and fresh scent properties.

Juice is the main product obtained from citruses. Orange juice is the most consumed type of juice in the world. Citrus juice yields fruits that account for half of the fruit weight, and hence a large amount of citrus waste is produced worldwide [7]. In the case of some citrus fruits, the problem is not only the waste from commercial juice production but also the fruit not being distributed and instead being discarded due to regulations restricting production [8]. There are also fruits whose pulp and juice are not consumed because of the unpleasant taste. In the case of these fruits, waste accounts for up to 90%. An example of such a fruit is the Kaffir lime.

The still-growing consumption of juices and the amount of post-production waste generated have been the subject of many considerations. New solutions for their use are being sought that are financially profitable but also environmentally friendly. For a given solution to be economically advantageous, the yield of the products must be relatively high and the process costs low. Therefore, it is important to properly select the waste disposal method for the type of raw material. Production waste can be used in many ways. It is well known that citrus fruits and the waste produced from them are a source of many substances with proven health-promoting properties that can be reused after their isolation from citrus wastes. Citrus fruits are a rich source of essential oils, pectins, minerals, vitamins, and antioxidants such as polyphenols or flavonoids.

2 Theoretical part

2.1 Citrus family

2.1.1 Characteristics of the genus

Citrus fruits are the most popular fruits and are available in almost all countries. The genus Citrus belongs to the *Rutaceae* family according to Tanaka's 1961 classification and has 156 species. In turn, the Swingle classification from 1943 includes 16 species. However, only four of them (pomelo, citron, tangerine and papeda) are considered primary fruits [9]. All other citrus fruits are secondary fruits, which are successive hybrids of the primary fruits. Due to the ability of apomictic reproduction, the numerous mutations of the species, and natural and artificial hybridization processes, creating an accurate classification of the genus Citrus is still a problematic issue.

2.1.2 Production and cultivation of citrus fruits

The genus of citrus is native to South Asia, East Asia, Southeast Asia, Melanesia and Australia. Despite this, China and Brazil are currently the leading producers of citrus. Spain is

dominant in Europe. According to Agrodigital, world citrus production in the 2018/19 season was 101.5 million tonnes. They are widely imported to many countries where their cultivation is impossible due to climatic conditions. Citruses are considered subtropical plants. Citrus trees can grow at between 13 °C and 38 °C. Ideal growing temperatures and cold tolerance vary by species and variety. For example, the ideal temperature for oranges to grow is between 21 °C and 32 °C. Some citrus species are grown in specific places, e.g. the main crops of bergamot oranges are in Italy, in the town of Bergamo. Hence the common name of the fruit.

2.1.3 Application of citrus fruits

As the most popular family of fruits, known and eagerly consumed for many years, citrus fruits have found many applications. Citrus fruits are used especially for food purposes, and in recent years they have also been increasingly used in various industries – primarily in the pharmaceutical and cosmetics industries.

a) Culinary

Most citrus fruits are eaten fresh. Oranges, tangerines and grapefruits are among the most popular citrus fruits consumed directly. The main product obtained from citrus fruits is juice. Orange juice is the most consumed type of juice in the world. Due to their high contents of vitamins, especially vitamin C, antioxidants, polyphenols, carotenoids and flavonoids, drinking citrus juices has a positive effect on the human body. Citrus juice is used directly as a drink or as an ingredient in drinks (lemonades) and as an addition to dishes (sauces or dressings) or cocktails (drinks).

Citrus is also used to produce many types of preserves, such as marmalades and jams. Due to the high content of pectins, fruit peels are also added to preserves, which facilitates the gelling process. Citrus-based alcoholic beverages are also very popular. Lemon tinctures, citrus wines, or Cointreau liqueurs, which are made with bitter oranges, are widely available. For culinary purposes, products isolated from citrus fruits are also commonly used, such as citric acid as an acidity regulator, pectins as gelling agents, or essential oils as flavourings for dishes.

b) Medicinal plants

Citrus fruits are a source of many compounds with a health-promoting effect. For centuries, their properties have been used in folk medicine, especially in Asian countries [10]. Citruses are primarily one of the best natural sources of vitamin C. Vitamin C, or left-handed ascorbic acid, is an essential nutrient for the human body. Vitamin C is needed for many metabolic functions and its deficiency causes scurvy. Vitamin C is considered the main antioxidant

compound in citrus fruits [11]. However, numerous studies report that the share of ascorbic acid in the antioxidant activity is less than 10% [12]. In addition to vitamin C, fruits also contain many other compounds with bioactive properties, such as polyphenols, flavonoids, carotenoids, and terpenes [13]. There are many scientific reports on the bioactive properties of these chemical compounds, which are associated with the following effects: antioxidant, antiviral, and anticancer [14,15]. Studies show that eating citrus fruits may help to prevent many diseases [16]. For this reason, extracts from citrus plants are commonly added to medicines and dietary supplements [17].

c) Cosmetics and pharmaceutical industry

Due to the ever-increasing interest in cosmetics of natural origin, the use of citrus fruits in the cosmetics industry is constantly growing. Citrus essential oils are commonly used, which, thanks to their bioactive properties and pleasant fragrances, are a popular ingredient in creams, lotions, and shampoos and, above all, the key component of perfumes. Essential oils owe their aromatic and bioactive properties to monoterpenes, which are often isolated from citrus fruits. Limonene, linalool or y-terpinene are added to a wide range of cosmetics.

In addition, the white part of the peel – the albedo – is a very good source of pectin. Pectins are added to cosmetics as stabilizing and gelling agents. However, they also have a health-promoting effect – they help regulate cholesterol and carbohydrate metabolism, which is why their use in cosmetics is not limited to their stabilizing function. In addition, the demonstrated antioxidant effect means they are often added to anti-ageing cosmetics [18]. In addition, citric acid, which is commonly isolated from citrus fruits, is used in cosmetics as an acidity regulator.

d) Ornamental plants

Due to their appearance, citrus trees are popular ornamental plants. Citrus can be grown both in pots and in the ground. They do not require special growing conditions. However, the fruits of ornamental trees are often characterized by poor flavour. Many varieties of trees are available in garden stores, the most popular being lemon, orange lime, and tangerine trees.

2.2 Valuable citrus by-products

2.2.1 Terpenes

Terpenes are secondary metabolites that many plants produce to fulfil specific biological and ecochemical functions such as hormone biosynthesis or protection against UV radiation and photo-oxidative stress, but also to act as pest and toxin repellents, growth regulators, pollinator attractors, photosynthetic pigments or electron acceptors. Less volatile, bitter or toxic secondary compounds are produced by plants as protection against microbes and insects [19]. Some terpenes are produced by plants to attract pollinating insects. Citrus fruits are

popular industrially for their production of terpenes. Simpler terpenes (mono- and sesquiterpenes) and terpenoids are the main components of essential oils and are widely used in the food industry. They can be additives to food, cosmetics, hygiene products, and household items, acting not only as flavourings but also as antibacterial and antioxidant agents. Terpenes are used primarily as fragrances in new perfumes and as additives to creams, lotions, or shampoos. In addition, some functionalized terpenes also show bioactivity against some types of cancer and bacterial and viral cells, so interest in such compounds is constantly growing. The terpene profile of essential oils depends not only on the citrus species; under the influence of external factors such as the presence of light or microorganisms, terpenes undergo biotransformation into other chemical compounds. For example, α-terpineol and terpinen4-ol are formed from linalool and limonene during citrus ripening, so are often considered indicators of quality and maturity [20]. It is also worth knowing the degree of fruit ripeness and the terpene metabolic pathway in citrus when determining terpene content [21].

The main terpene compound present in citrus, both in the peel and in the juice, is limonene. Citruses accumulate the limonene at high levels in the oil glands of their peels. Approximately 36 million kg of limonene annually is recovered as a by-product of the citrus industry [22]. It is responsible for the pleasant aroma of citrus. Limonene is often used as a fragrance in cosmetics and cleaning products. It is also added as a flavouring to food. It can be found in cakes, juices, and ice creams. Due to its health-promoting properties, limonene is often used in medicine. It has been used clinically to dissolve cholesterol-containing gallstones. The terpene has also been used to relieve heartburn and gastroesophageal reflux. Clinical trials have also shown an effect in the treatment of breast tumours. Limonene is present in citrus peel and juice alike, with the concentration in the peel being much higher. The high content of limonene was noted in the peel of tangerine at 67,384.04 μg/g [23].

2.2.2 Flavonoids

Flavonoids have been distinguished as a class of polyphenols. They are characterized by a diverse chemical structure resulting from the attachment of substituents and different degrees of oxidation [24]. The attached substituents interfere with the structure of the compound, changing its properties; hence the different roles that flavonoids play in the course of plant life functions. Flavonoids are used as antibiotics and as anti-ulcer and anti-inflammatory agents. They are also used in the treatment of diseases such as hypertension and allergies [25]. They also play an important role in reducing the risk of obesity and diabetes [26].

Mandarins contain the highest levels of the carotenoid β-cryptoxanthin, which is responsible for the orange-yellow colour of the segments in ripe mandarins [27] and has been suggested as an efficient provitamin A source in the *Citrus* genus.

2.2.3 Carotenoids

Carotenoids are a class of natural pigments responsible for the vibrant colours found in various fruits and vegetables. Citrus fruits such as oranges, lemons, and grapefruits are known for their high content of carotenoids, which contribute to their appealing colours and nutritional value. These compounds possess antioxidant properties and play a crucial role in human health by protecting against oxidative stress and reducing the risk of chronic diseases. Citrus is a great source of carotenoids, containing the largest number of carotenoids found in any fruit [28], particularly β-carotene, lutein, and zeaxanthin. β-carotene, a precursor of vitamin A, contributes to healthy vision, immune system support, and overall skin health. A comprehensive review by Rodrigo et al. (2013) provides detailed information on the carotenoid profiles of different citrus species and varieties [29]. The study highlights the presence of various carotenoids, including β-carotene, lutein, zeaxanthin, β-cryptoxanthin, and others. The content and composition of carotenoids can vary depending on factors such as fruit ripeness, growing conditions, and genetic factors. Research by Johnson et al. (2013) showed that diets high in carotenoids are associated with a reduced risk of cardiovascular diseases. The anti-inflammatory properties of carotenoids help lower blood pressure and cholesterol levels, thereby promoting heart health. Regular consumption of citrus fruits has been linked to a decreased risk of coronary heart disease and stroke [30]. Among citrus fruits, the one that typically contains the most carotenoids is the orange. Other citrus fruits such as tangerines, mandarins, and grapefruits also contain carotenoids, but oranges are particularly rich in these beneficial compounds. The specific carotenoid content may vary depending on the variety and ripeness of the fruit. Including a variety of citrus fruits in one’s diet can provide a range of health benefits due to their different nutrient profiles.

2.2.4 Polyphenols

Polyphenols are one of the most numerous groups of active biological ingredients commonly found in plants and the pulp of fruits and vegetables. They are products of plant metabolism, in which they play a protective role against fungal and bacterial infections. Polyphenols owe their unique antioxidant properties to a large number of hydroxyl groups. They are an essential component of the diets of humans and animals alike. Taking into account their positive impact on human health, they have become the subject of numerous studies [25].

There are four classes of polyphenols, and the division criteria are the number of aromatic rings connected and the method of their connection. There are phenolic acids, flavonoids, stilbenes, and lignans. The simplest polyphenols are simple compounds, including phenolic acids such as gallic acid. The more complex structures include lignans. Polyphenolic compounds are commonly used as a food additive for their natural antioxidant action. They

can also be used as stabilizers to strengthen and fix the colour of preparations containing anthocyanins. Moreover, they can act as preservatives, inhibiting the growth of microorganisms [25,31,32].

2.2.5 Anthoyanes

Anthocyanins are the pigments that play the most important role among the flavonoids. They are some of the most common colouring substances in nature [33]. The basic structural unit of anthocyanins is the flavylic cation. Anthocyanins are relatively unstable compounds because they are easily subject to changes that change their colour. They show particular sensitivity to elevated temperature [33]. Consuming anthocyanins may improve eyesight in people who suffer from myopia. They also contribute to improving the cognitive and protective functions of the brain, as they participate in the processes reducing the damage caused by cerebral ischemia. Anthocyanins are also responsible for the regulation of blood sugar levels, hence their positive importance in the prevention of diabetes. Additionally, they reduce the level of cholesterol [34].

2.2.6 Vitamin C

Vitamin C is a water-soluble antioxidant that supports various bodily functions. One of its primary roles is in boosting the immune system by promoting the production of white blood cells, which help the body defend against infections and illnesses. Additionally, vitamin C aids in collagen synthesis, contributing to the health of skin, tendons, ligaments, and blood vessels.

The rich vitamin C content in citrus fruits offers numerous health benefits. Regular consumption has been associated with reduced risk factors for chronic diseases such as cardiovascular disease, hypertension, and certain cancers. Furthermore, the antioxidant properties of vitamin C combat oxidative stress, helping to mitigate cellular damage caused by free radicals.

The vitamin C content in citrus fruits varies slightly between types and cultivars. Oranges, for instance, are well-known for being an excellent source of this essential nutrient.

2.2.7 Pectins

Pectins are a group of complex carbohydrates known as polysaccharides and are found in the cell walls of plants. They play a crucial role in providing structural support to plant tissues and have various applications in the food industry due to their unique properties. One of the most significant properties of pectins is their gelling ability. When combined with sugar and acid, pectins form a gel, which is essential in the production of jams, jellies, and fruit preserves. The

ability to form gels is influenced by the degree of methylation of pectins, which affects their molecular structure and gel strength. Pectins are classified into two main types: high-methoxyl (HM) and low-methoxyl (LM) pectins. HM pectins require higher sugar content and lower pH levels to gel, whereas LM pectins gel in the presence of calcium ions and lower sugar concentrations. This differentiation allows food manufacturers to select the type of pectin that best suits their desired product texture.

Aside from their gelling properties, pectins also act as natural thickeners and stabilizers, contributing to the texture and mouthfeel of various food products. Additionally, pectins are water-soluble, making them suitable for a wide range of applications in the food and beverage industry.

Pectins also have health benefits, as they are dietary fibres that can help with digestion and promote a feeling of satiety. They may also have a positive impact on cholesterol levels due to their ability to bind to bile acids.

2.2.8 Essential oils

Essential oils are volatile fragrances found in the excretory tissues of many plants. They are commonly isolated from plants for their numerous uses. Due to their fragrance properties, they are the main fragrance components of perfumes. They are also used in aromatherapy, where their bioactive properties are used. In the case of citrus, the essential oils are mainly found in the peel. In the aroma profile of citrus oils, limonene is the predominant chemical compound. The presence of other chemical compounds makes the fragrance range of citrus oils very diverse. The most popular methods of obtaining essential oils from citrus peel are hydrodistillation and steam distillation. Processes such as extrusion, extraction with volatile solvents, extraction with non-volatile solvents (i.e. maceration), and cold absorption in fats are also used. Essential oils are commonly used in the cosmetics industry. Thanks to their proven bioactive properties, they are also used in medicine. They have been used as a natural antioxidant, antifungal, and antibacterial food additive, including for food preservation.

2.3 Kaffir lime

A specific representative of the citrus genus is Kaffir lime, popularly known as papeda. It is one of the few citrus fruits in which the main part intended for culinary purposes is not the juice, which is very astringent. The consumed elements of the fruit are mainly the leaves and the outer part of the peel, which are integral to Asian cuisine.

2.3.1 Characteristics

Kaffir lime (*Citrus hysteria, Citrus hystrix*) belongs to plants from the *Rutaceae* family (*Rutaceae*). The small evergreen Kaffir lime shrub or tree grows 3 to 12 metres tall and has distinctive leaves that have a petiole almost as large and wide as the leaf blade. The plant comes from Southeast Asia [35]. It is most common in Indonesia, Malaysia, the Philippines, Laos, Thailand, and Vietnam [36]. In Poland, it is known under many names, including papeda, Kaffir lime, Thai lime, Angel wings (due to the shape of the leaves), and Makrut [37]. Kaffir lime fruit reach up to 4 cm long. They are covered with a thick and wrinkled green skin. The plant is grown in gardens, occasionally as a minor crop in mixed orchards [38].
The scientific classification of Kaffir lime is presented below:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Sapindales

Family: *Rutaceae*

Genus: *Citrus*

Species: *C. hysteria*

Binomial name: Citrus hystrix DC.

Common names: Kaffir lime, makrut lime, Thai lime, caffre lime, kieffer lime, kuffre lime, makrut, magrood, lima, leech-lime.

2.3.2 Applications

The main elements of the fruit used in cooking are the leaves and the outer part of the peel, which are spices added to many Asian dishes. The leaves – fresh, frozen, and dried – are used primarily in the cuisine of Laos, Cambodia, Indonesia, and Vietnam. The outer part of the rind is the main ingredient of Thai curry paste. Candied fruit is also eaten in some countries.

The juice is not consumed directly due to its overly tart taste; therefore it is not commercially produced. In Thailand, it is sometimes used as a cleaner for clothes and hair [39]. In Cambodia, Kaffir lime pieces are added to holy water during religious ceremonies. However, these are occasional applications. Kaffir lime juice is also used in folk medicine in some Asian countries. According to folk beliefs, it has the ability to kill lice.

2.4 The problem of citrus waste management

An important problem in the production of fruit products, such as juices or jams, is the problem of post-production residues. Global citrus production (orange, lemon, lime, sweet orange, etc.) is more than 121×10^6 t per year, while the industrial juice sector generates around 25×10^6 t of citrus waste [40]. In the case of citrus whose peel is not directly consumed, the waste can be about 80% of the weight of the whole fruit. The Food and Agriculture Organization of the United Nations estimated that, in 2013, the amount of industrial waste from orange juice production was 14.3 million tonne

s [41]. Therefore, solutions are sought to make it possible to use citrus post-production waste. It is estimated that currently around 80–90% of citrus waste is processed into useful products such as dried pulp, molasses, pectins, essential oils, citric acid, limonene, animal feed, and biologically active materials. In the selection of an appropriate citrus waste processing process, it is important to assess the possibility of obtaining satisfactory yields and quality of the products obtained. Therefore, an important element is to determine the content of individual groups of compounds in waste before the process of its management [42].

2.4.1 Effects of citrus waste generation

The problem of citrus production residues is unfavourable for many reasons. The economic aspects related to waste management, storage, and disposal are extremely important. Food processing plants face the problem of proper disposal of post-production waste. This is an economically unfavourable phenomenon. It is also connected with the need to have appropriate infrastructure for the storage of waste generated in the production process. The utilization of post-production waste therefore reduces the costs of its disposal or storage.

Due to the highly organic nature of this waste, improper disposal practices are considered harmful to the environment, aquatic life, and human health. Organic waste deposited in landfills may, due to biochemical and biological processes such as fermentation, cause the release of many chemical compounds into the atmosphere that may cause an odour nuisance. The storage of citrus waste entails several unfavourable phenomena, such as the emission of landfill gases or leachates, the formation of which is subject to strict regulations and generates many costs. The most popular methods of citrus waste management are composting, anaerobic digestion, incineration, thermolysis, and gasification. However, various types of waste are generated during these processes, including solid waste, liquid waste, and distillation residues.

In addition, citrus waste is sometimes stored and discharged not only to the designated places; for example, sewage is directly discharged into lakes and ponds or discharged into the municipal sewage system. The discharge of post-production wastewater into lakes can cause

serious changes in the aquatic ecosystem, especially when lake resources are insufficient to adequately dilute the wastewater. Waste discharged to municipal sewers can contaminate underground water sources and cause damage to pumps and pipelines, clogging of sand beds and foaming in primary settling tanks. Sewage is also discharged into forests, which defoliates trees, possibly due to loss of oxygen in the soil around tree roots [7].

2.4.2 Possibilities of citrus waste management

a) Production of animal feed

One popular use of citrus waste is as animal feed. This is very economical, as it reduces not only the disposal costs of citrus waste but also the purchase costs of conventional animal feed. The main waste from citrus processing is fresh juice extraction residues, representing between 492 and 692 g/kg of: fresh fruit (of which the individual parts of the fruit yield are: 600–650 g/kg of peel, 300–350 g/kg of pulp, and 0–100 g/kg of seeds [43]); dried citrus pulp (which is made by collecting, liming, pressing, and drying the peel, pulp, and seed residues); and citrus meal, which is separated after the drying process. Citrus waste can be used as a high-energy feed in ruminant feeds to support growth and lactation, with less negative impact on rumen fermentation than starch-rich feeds [44]. In addition, they are suggested to inhibit the growth of *Escherichia coli* and *Salmonella* in feed [45]. In the case of using waste from plant materials, the key property is their nutritional and energy value. However, the use of citrus waste has an additional advantage, namely the content of vitamins and micro- and macroelements, the presence of which in animal feed is an additional value.

b) Production of biogas and bioethanol

Along with the development of biofuel production processes, new solutions are sought that will allow biofuels to be obtained more efficiently and cheaply. In most cases, starchy crops such as potatoes, cassava maize, or cereals are used as a raw material. This approach, however, raises a lot of controversies, because raw materials that could be consumed by humans are used. It also increases the cost of biofuel production. One of the economically advantageous raw materials for the production of biofuels is citrus waste. This approach also has several disadvantages: pre-treatment is necessary, which requires energy input; the low pH of citrus waste inhibits hydrogenase uptake activity; and the presence of terpene compounds with bactericidal activity inhibits the fermentation process. However, efforts are still being made to improve methods of producing biofuels from citrus waste [46]. One solution is to dispose of essential oils that can be reused.

c) Essential oils production

Production of essential oils from citrus wastes is a promising and environmentally friendly approach to utilize by-products generated by the citrus industry. Citrus fruits such as oranges, lemons, and grapefruits are widely consumed worldwide, and their peels, seeds, and other discarded parts can be rich sources of valuable essential oils.

The process of extracting essential oils from citrus wastes involves several steps. Initially, the collected peels and other discarded parts undergo washing to remove any dirt or contaminants. Once cleaned, the waste material is then dried to reduce its moisture content, enhancing the efficiency of the extraction process.

The next stage involves steam distillation, which is the most common method used to extract essential oils from citrus wastes. In this process, the dried citrus waste is subjected to steam, which helps release the essential oil from the plant material. The steam and essential oil mixture is then condensed and separated. Essential oils, being less dense than water, float to the top and are collected, while the remaining water (now infused with the essence of the citrus) is called “hydrosol” and can also be utilized for various purposes.

Citrus essential oils obtained from waste have numerous applications across various industries. They are extensively used in the fragrance and cosmetics industry for their pleasant aromas and potential skincare benefits. Additionally, these oils are used as flavourings in the food and beverage industry, imparting a natural citrus flavour to products.

Moreover, essential oils from citrus wastes exhibit antimicrobial properties, making them valuable in pharmaceuticals and cleaning products. They also possess antioxidant and anti-inflammatory attributes, which have led to their incorporation into health and wellness products.

The production of essential oils from citrus wastes brings multiple benefits. It reduces environmental pollution by diverting organic waste from landfills and converting it into value-added products. This sustainable approach can lead to a potential economic advantage for citrus-producing regions, as it creates additional revenue streams and reduces waste management costs.

d) Pectin production

The inner part of the citrus peel called the “albedo” is a rich source of pectin. Pectins are polysaccharides commonly found in the cell walls and intercellular spaces of plants. Currently, the main source of pectins obtained on an industrial scale are citrus fruits containing about 30% of pectins on a dry-matter basis. There are many methods for extracting pectin from

citrus, including extraction with water, ultrasound, buffers, enzymes, and microorganisms. Acid extraction is the most common method of extracting pectins, being the fastest, most efficient method [47]. The annual pectin consumption is estimated at 45,000 tonnes per year [48]. Citrus wastes such as peels, pulp, and seeds are abundant by-products of the citrus processing industry and offer a valuable source for pectin production. Pectins extracted from citrus wastes find numerous applications in the food industry, functioning as gelling agents, stabilizers, and thickening agents in products like jams, jellies, confectionery, and dairy items. Moreover, pectin's natural and biodegradable properties make it an eco-friendly alternative to synthetic thickeners and stabilizers. In the pharmaceutical industry, they are used to treat diseases such as hypoglycaemia and gastrointestinal disorders.

The production of pectins from citrus wastes not only adds value to the by-products of the citrus industry but also contributes to sustainability and waste reduction efforts, making it a beneficial and environmentally conscious process.

e) Production of citric acid

Citric acid is the most popular acidity regulator used in the food industry. Global production exceeded 2,000,000 tonnes in 2018 [49]. Citric acid concentrations in citrus fruits range from 0.005 mol/L in oranges and grapefruit to 0.30 mol/L in lemons and limes; these values vary within species, depending on the variety and the conditions in which the fruit was grown. Citric acid is a valuable organic acid widely used in the food, beverage, pharmaceutical, and cosmetics industries. Traditionally, it has been produced through microbial fermentation or chemical synthesis. However, there is growing interest in utilizing citrus wastes such as peels, pulp, and seeds as a sustainable and cost-effective source for citric acid production.

The process involves several steps: first, the citrus waste is collected and treated to remove impurities. Next, it undergoes pretreatment to release the sugars and other organic compounds. Then, the waste is subjected to microbial fermentation using citric-acid-producing microorganisms like *Aspergillus niger*. During fermentation, citric acid is produced and accumulated. After the desired citric acid concentration is reached, the fermentation broth is processed to separate and purify citric acid crystals.

Utilizing citrus wastes for citric acid production not only reduces environmental pollution but also adds value to the by-products of the citrus industry, promoting a circular economy approach. This sustainable strategy contributes to the conservation of resources while meeting the increasing demand for citric acid in various applications.

2.4.3 The problem of Kaffir lime waste management

In the case of some citrus, the problem is not only waste from commercial juice production but also fruit that is not subject to distribution and is thrown away due to regulations limiting production [8]. There are also fruits whose pulp and juice are not eaten because of the unpleasant taste. In the case of these fruits, waste is up to 90%. An example of such a fruit is the Kaffir lime. The leaves are the most commonly used part of the plant in a culinary context. The outer part of the rind is used to prepare the curry paste. In contrast, the juice in traditional medicine in some Asian countries is often used in shampoo or as a clothing cleaner. There is still little research on the health-promoting properties of this fruit, which is the reason for its use in folk medicine. Due to the pleasant sensory properties and the large amount of fruit left after collecting the leaves, Kaffir lime fruits can often be found in toilets, where they are used as air fresheners. However, there is no industrial production of juices, and the described uses are occasional. Thus, Kaffir lime fruit, which is cultivated mainly for its leaves, may be a good source of valuable chemicals with health-promoting and sensory properties. The use of Kaffir lime fruit as a raw material for obtaining chemical compounds for industrial purposes could be an element of the management of generated waste.

3 Research goals

Citrus waste management is an important economic and environmental issue. This aspect is more complicated in the case of Kaffir lime fruit, the main part of which is consumed is its aromatic leaves, whereas the fruit is largely wasted. The main research hypothesis is that Kaffir lime is a good source of raw materials for different branches of industry. The first objective was to assess the possibility of using Kaffir lime fruit waste (*Citrus Hystrix*) as raw material for industrial purposes, especially for the food, cosmetics, agricultural, and pharmaceutical industries. Emphasis was placed on a holistic approach to the study of the fruit. Both Kaffir lime juice and peel were tested directly, but so too were by-products such as pectins and essential oils.

Reports on the use of Kaffir lime fruit in folk medicine allowed a thesis about its therapeutic effect to be formulated. In the case of citrus, essential oils extracted from the peel are an extremely important product, which is why it was decided to study the terpene profile of the oil so that knowledge of the content of individual terpenes might explain the bioactive effect of the fruit. Due to the thickness of the peel, it was decided to analyse the pectins isolated from the skin of the fruit and to determine their characteristics. In addition, it was decided to determine the content of selected groups of bioactive compounds, such as polyphenols, flavonoids, anthocyanins, chlorophylls, and vitamin C. To confirm the therapeutic effect of

individual parts of Kaffir lime fruit, total antioxidant activity and binding to human serum albumin (HSA) were assessed.

In addition, the work aims to develop analytical methodologies for the determination and characterization of selected chemical compounds present in samples of Kaffir lime juice, pulp, and peel as potential raw materials for use in various industries. The subject of research is mainly bioactive chemical compounds, such as terpenes, flavanols, flavonoids, tannins, vitamin C, anthocyanins, chlorophyll A and B, carotenoids, pectins, and micro- and macroelements. During the research, efforts were made to use solvent-free analytical techniques as well as solvent-free extraction techniques, which are in line with the principles of green analytical chemistry.

Great emphasis was placed on the determination of compounds from the terpene group. According to current knowledge, these compounds are the main group of bioactive compounds derived from citrus. The tests were carried out in peel, essential oils, juice, and pulp of Kaffir lime. In the case of oils, the main focus was on the terpene profile, because this feature is the most important for the potential use of oils for industrial purposes. However, the main goal was to develop and validate a dedicated analytical methodology for the determination of terpenes in Kaffir lime samples. In the first step, the terpene content in the peel and pulp was compared. And then the focus was on validating the methodology for determining terpenes in Kaffir lime juice samples. It was decided to conduct a detailed examination of the juice because this product is the main post-production waste from Kaffir lime and is used less than the peel or leaves. It was known to be used in folk medicine, so it was decided to evaluate other potential uses resulting from its properties.

In the last step, the content of selected terpenes was compared with representatives of other species and genera. In the case of terpenes, certain selection criteria were applied to the selection of comparison fruits. The selection criteria for one comparison fruit were that it be of the same species as Kaffir lime but a different variety: accordingly, we selected *Citrus aurantifolia*, the variety of lime commonly available in Poland. The other three comparison citrus fruits: pomelo, grapefruit, and key lime, were selected on the basis of their capacity to fill a gap in the research literature: there were few available studies on the determination of terpenes for these three genera at the time. Then, selected bioactive and antioxidant properties were compared with a representative of the same species but a different variety (*Citrus aurantifolia*) and representatives of different species: *Momordica charantia* (criteria for qualifying for research: similar appearance to Kaffir lime and same geographical origin), *Actinida deliciosa*, *Actinidia erinanatha*, *Actinidia arguta* (criterion for qualifying for research: fruits classified as "superfruits", known for their large amount of bioactive compounds and

good antioxidant properties), *Durio zibethinus L.* (criterion for qualifying for research: same geographical origin as Kaffir lime). The schematic research plan with reference publications (numbers) is presented in Figure 1.

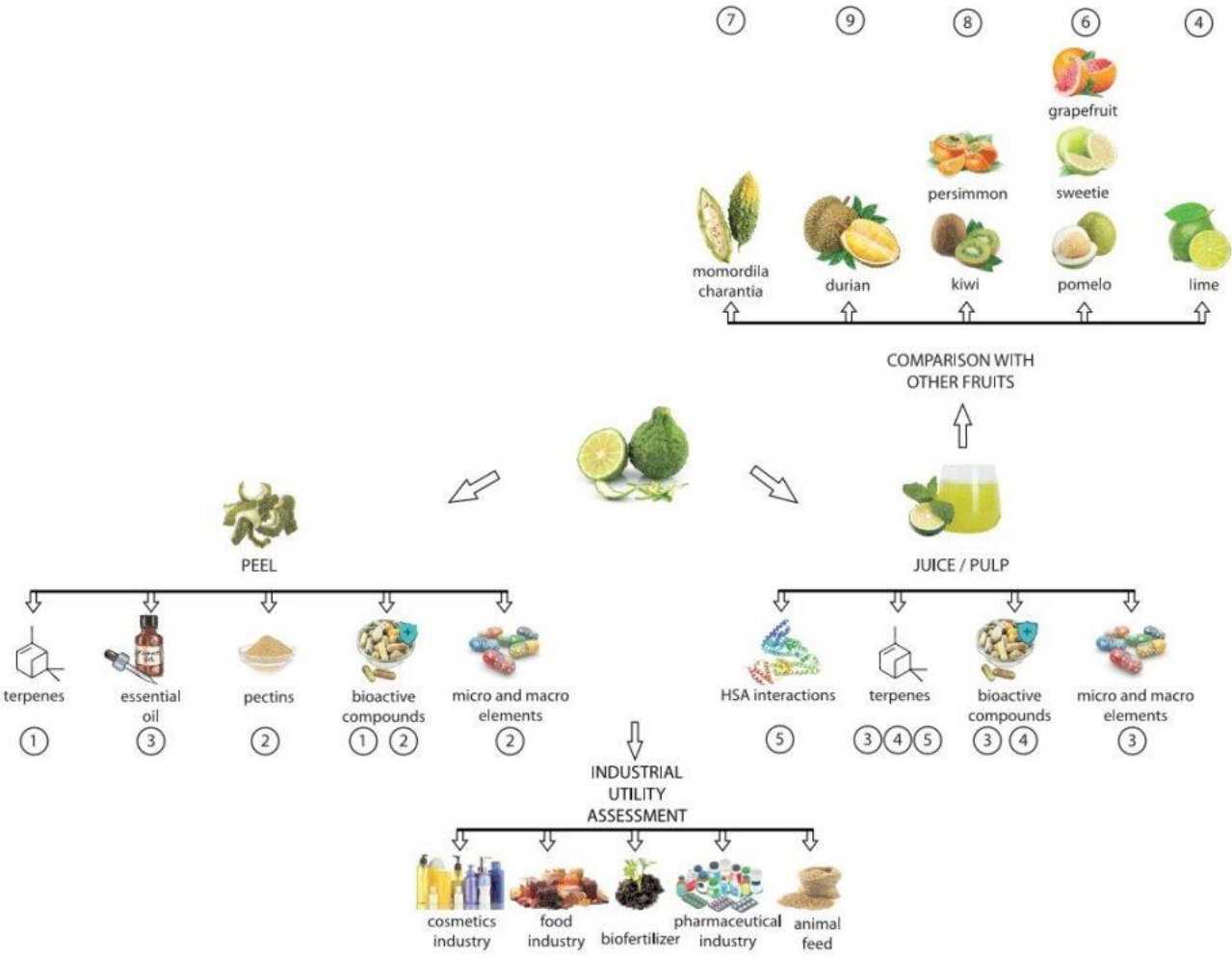


Figure 1. Schematic plan of the research with corresponding articles (numbers of articles in the circle)

To verify the research hypothesis, it was decided to pursue the following research tasks:

1. **Chemical, physicochemical and biochemical characterization of Kaffir lime peel and by-products**

a) Development of the method of extraction and characterization of pectins obtained from Kaffir lime
b) Extraction and determination of the aroma profile of Kaffir Lime essential oils
c) Determination of the content of selected groups of bioactive compounds
d) Determination of total antioxidant capacity
e) Determination of the content of micro- and macroelements

2. **Chemical, physicochemical and biochemical characterization of Kaffir lime juice**

a) Determination of selected terpenes
b) Determination of the content of selected groups of bioactive compounds
c) Determination of the content of micro- and macroelements
d) Study of the binding effect of human serum albumin by bioactive compounds present in Kaffir lime juice

3. **Development of analytical methodology for the determination of selected terpenes in Kaffir lime samples**

a) Literature review comprising the application of gas chromatography techniques in food analysis
b) Determination of the profile of volatile compounds present in Kaffir lime samples
c) Development and validation of analytical methodology to determine the profile of Kaffir lime samples using gas chromatography

4. **Comparison with other fruits**

a) Comparison of the content of selected terpenes in samples of Kaffir lime with samples of a selected representative of the citrus genus
b) Comparison of the content of selected groups of bioactive compounds in Kaffir lime samples with samples of a selected representative of another type
c) Comparison of the content of selected bioactive compounds in Kaffir lime samples with samples of a selected representative of other species

4 Research description

4.1 Chemical, physicochemical and biochemical characterization of Kaffir lime peel and by-products

Hypothesis: Kaffir lime peel as citrus waste is a good source of many compounds with health-promoting or functional properties that can be used in various industries.

4.1.1 Development of the method of extraction and characterization of pectins obtained from Kaffir lime

Detailed results regarding the extraction and characteristics of pectins from Kaffir lime have been described in detail in the second article comprising this thesis, *Molecules*, 2023.

4.1.1.1 Optimization of the extraction method

There are many methods of extracting pectin from citrus fruits, including extraction with water, using ultrasound, buffers, enzymes, or microorganisms. Acid extraction is the most popular method of pectin extraction, being the fastest, most efficient method [47]. The extraction process is most often carried out using citric, sulphuric, hydrochloric or nitric acid at a pH ranging from 1 to 3 and at an elevated temperature from 70 ºC to 100 ºC. However, research shows that, in some cases, pectins may degrade at temperatures above 90 ºC [50]. Pectins are extracted using alcohol, with ethanol, methanol and isopropanol being the most common. Research shows that extraction efficiency often depends on the type of raw material and the solvent used [51]. For dry citrus peels, methanol is the most effective precipitating solvent [52]. The research aimed to find the optimal conditions for the extraction of Kaffir lime pectin.

Based on the research carried out, it can be concluded that the selection of citric acid to obtain a given pH value during pectin extraction seems to be a good solution, as it allows a very good product efficiency of 22% to be attained. When nitric acid was used, an extraction efficiency of less than 8% was attained. These results are consistent with those obtained by Shah, where the best extraction efficiency of pectin from microwave-dried Kaffir lime was obtained for citric acid [53]. In the case of temperature, with its increase, an increase in pectin extraction efficiency was noted. However, it was not decided to increase the temperature above 90 °C due to the possible degradation of the obtained pectins [50]. However, in the case of pH, as it increased, a decrease in the yield of extracted pectins was observed. The

obtained results show that Kaffir lime peel is a rich source of pectin, so it can be considered for use in commercial pectin production alongside other citrus fruits.

4.1.1.2 Physicochemical characteristics of the obtained pectins

It has been proven that the physicochemical properties of pectins can vary significantly depending on the extraction methods [54]. Temperature is an important parameter during the extraction process. The research aimed to determine the effect of extraction conditions on the physicochemical properties of Kaffir lime pectin, namely galacturonic acid content, degree of methylation, antioxidant activity, and thermodynamical properties.

The degree of pectin methylation is an important factor in controlling the binding time of pectins, their ability to combine with metal ions, and their ability to form gels. High-methyl pectins gel in an acidic environment with a high sugar content, while low-methyl pectins require divalent ions to form gels but can be used over a wider pH range (3–6) and with a lower sugar content (30–40%). This is an important issue from the perspective of the use of pectins in the food industry. The conducted research shows that the extraction conditions did not have a significant effect on the degree of esterification of the obtained pectins, which was 3–4% in all cases. This means that the pectins extracted from Kaffir lime belong to the group of low methoxylpectins and would be a good option for the food industry, especially for low-calorie jams and as a stabilizer in dairy products.

Galacturonic acid content is a key determinant that affects the properties of pectins. The content of galacturonic acid may affect the chemical and sensorial properties of the matrix such as pH, total acidity, microbial stability, sweetness, and global acceptability [55]. Also, the quality of extracted pectins may be assessed based on galacturonic acid content. Commercially available pectins have a galacturonic acid content greater than 65% [56]. The minimum content of uronic acid in the food industry is also 60% [57]. The content of Galacturonic acid was from 312.8±2.2 to 650±75 mg GAL/g and depended on the extraction conditions used. Only the use of acid extraction at 60 °C allowed the requirements for the quality of pectins for use in the food industry to be met. However, under these conditions, the yield of the obtained pectins was the lowest. Therefore, considering both parameters – galacturonic acid content and extraction efficiency, obtaining pectin from Kaffir lime for the food industry can be assumed to be only a moderately attractive solution. However, it can still be considered for use in the cosmetics industry, where low-methoxyl pectin is used as a structure provider in pastes, ointments, oils, and creams, and the restrictions are not so demanding.

Thermal analysis of polysaccharides is an important test in assessing their stability and shelf life for further use in the food industry. The influence of extraction temperature and raw material on the thermodynamic properties of the obtained pectins was examined using differential scanning calorimetry (DSC). Based on the results, increasing the temperature of

extraction and the addition of ultrasounds influence methoxyl groups, which in turn translates into the functional properties of the obtained pectins, as described above.

Recent scientific reports indicate that polysaccharides from plants, including pectins, have certain antioxidant activity on free radicals and thus could be considered novel potential antioxidants.

4.1.2 Extraction and determination of the aroma profile of essential oils from Kaffir lime

Detailed results regarding the extraction and determination of the aroma profile of essential oils from Kaffir lime have been described in detail in the second article comprising this thesis, *Molecules*, 2023.

a) Essential oil extraction

The hydrodistillation method, where raw materials are directly immersed in boiling water, were chosen to extract oils from Kaffir lime peel. The boiling water acts as a protective barrier and prevents the extracted essential oil from overheating. Thanks to the use of an eco-friendly solvent (i.e., water), an efficiency of 1.78% was achieved, which is relatively high compared to the literature data [58].

b) Determination of terpene profile of Kaffir lime essential oils

Due to their pleasant sensory properties and the health-promoting properties resulting from the presence of bioactive compounds, essential oils are widely used in the cosmetics and pharmaceutical industries. Essential oils consist mainly of terpenes, which have different bioactive properties; therefore, determining the content of individual terpenes in an essential oil makes it possible to determine the health-promoting properties of a given oil. A terpene profile chart is a tool for understanding the effects, flavours, aromas, and/or important information about common terpenes present in a sample. It presents the main terpenes that affect the properties of the raw material. The terpene profile of the obtained oil was determined using the GC-MS technique. The terpene profile is shown in Figure 2. The conducted research shows that the main terpenes that may influence the health-promoting and aromatic properties of Kaffir lime oil are Sabinene, β-pinene, and Limonene, which have numerous bioactive properties that are presented in the figure. For this reason, Kaffir lime oil can be used in the production of sprays, mouthwashes, and acne-control cosmetics, which is confirmed by research on the therapeutic effects of this oil [59]. The main terpenes contained in Kaffir lime oil create a tart citrus aroma. These properties also find an application in aromatherapy.

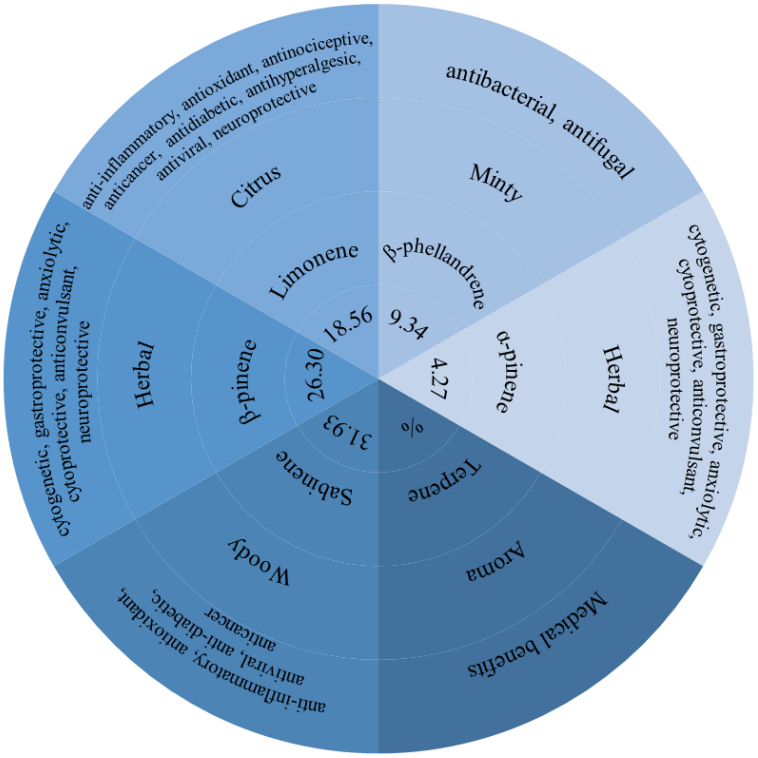


Figure 2. Terpene profile chart of Kaffir lime essential oil

4.1.3 Determination of the content of selected groups of bioactive compounds in Kaffir lime peel

Detailed results on the determination of the content of selected groups of bioactive compounds of Kaffir lime have been described in detail in the second and fourth articles comprising this thesis, *Molecules*, 2023. The research was conducted in cooperation with prof. Shela Gorinstein from the Hebrew University of Jerusalem.

As part of the research, the content of bioactive compounds was determined: polyphenols, flavonoids, vitamin C, anthocyanins, chlorophyll a and b, and carotenoids. These are chemical compounds with proven health-promoting effects, including antioxidant, antiviral, anticancer and anti-inflammatory properties [60]. Table 1 presents the results of the determinations of bioactive compounds in the water extract of Kaffir lime peel. The obtained results were compared with literature data on other fruits, which showed that Kaffir lime peel is a relatively good source of polyphenols, flavonoids, and carotenoids, an average source of anthocyanins and a very good source of vitamin C compared to the peels of other citrus fruits. These properties mean that Kaffir lime peel can be used as functional animal feed. Moreover, Kaffir lime peel extract can be successfully used in the cosmetics and pharmaceutical industries as a source of natural vitamin C.

Table 1. Bioactive compounds expressed as dry weight in Kaffir lime peel samples

Parameter	Value
Polyphenols [mg GAE/g DW]	39.9±3.1
Flavonoids [mg CE/g DW]	2.72±0.25
Vitamin C [mg Asc]	2.43±0.19
Anthocyanins [mg CGE/kg DW]	24.8±1.8
Chlorophyll a [µg/g DW]	185.5±8.1
Chlorophyll b [µg/g DW]	60.4±3.2
Xanthoproteins + Carotenes [µg]	12.02±.102

DW – *dry weight*, CE – *catechin equivalent*, GAE – *gallic acid equivalent*, Asc – *ascorbic acid*, CGE – *cyaniding-3-glucoside equivalent*

4.1.4 Determination of total antioxidant content of the water extracts of Kaffir lime peel

Detailed results on the determination of total antioxidant content in the first and second articles comprising this thesis, *Industrial Crops and Products*, 2018; *Molecules*, 2023. The research was conducted in cooperation with prof. Shela Gorinstein from the Hebrew University of Jerusalem.

a) Methods based on the reduction of metal ions, e.g. iron (FRAP) or copper (CUPRAC)

The methods used were based on the reduction of metal ions, e.g. iron (ferric reducing antioxidant power [FRAP] assay) or copper (cupric ion reducing antioxidant capacity [CUPRAC]). The method for determining the ability to reduce iron (III) ions (FRAP) and the method for determining the ability to reduce copper (II) ions (CUPRAC) are based on reaction mechanisms. The CUPRAC test evaluates the ability of antioxidants in a sample to reduce Cu^{2+} to Cu^{+} in the presence of a chelating agent. The FRAP test is based on the principle of reduction of the iron-tripiridyltriazine (TPTZ) complex (Fe^{3+}-TPTZ) to ferrous tripiridyltriazine (Fe^{2+}-TPTZ) by antioxidants contained in the sample at low pH. The results of the determination of the total antioxidant power of Kaffir lime peel samples using the FRAP and CUPRAC methods are presented in Table 2. The results of the antioxidant activity were compared with the results obtained for the peel of other citrus fruits, showing that the Kaffir lime's peel antioxidant capacity is slightly higher than that of other citruses, based on FRAP results (e.g., 29.34 for *Citrus paradisi* [60]). Also, in the case of the CUPRAC test, the results for Kaffir lime peel were higher than those for the citrus fruits compared in the article.

Table 2. Total antioxidant capacity of Kaffir lime samples using FRAP and CUPRAC methods

Matrix	FRAP, mmole TE/g DW	CUPRAC mmole TE/g DW
Kaffir lime peel	48.7±4.6	76.98±8.1

DW – *dry weight*, TE – *Trolox equivalent*

b) Method based on synthetic radical scavenging capacity (DPPH)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is used to predict the activity of antioxidants through the mechanism by which they inhibit lipid oxidation, i.e., by scavenging DPPH radicals. The methods are based on the ability to capture a synthetic radical. The results of determining the total antioxidant power of Kaffir lime samples using DPPH methods are presented in Table 3. The results of the antioxidant activity were compared with the results obtained for the peel of other citrus fruits and showed that the Kaffir lime peel antioxidant capacity is slightly lower than that of the other citruses, based on DDPH results.

Table 3. Total antioxidant capacity of Kaffir lime samples using the DPPH method

Sample	DPPH mMTE/g DW
Kaffir lime peel	12.02±1.02

DW – *dry weight*, TE – *Trolox equivalent*

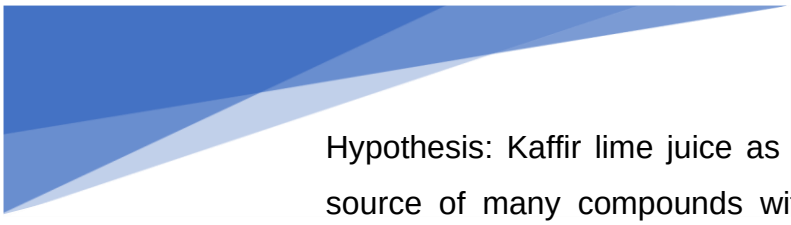
Many studies show that citrus peels are a good source of antioxidants and can be a functional food. Moreover, among all parts of citrus fruits, the peels contain the highest amounts of antioxidants [61]. Taking into account the results obtained during the conducted research, Kaffir lime peel is a very good source of antioxidants, and its use as a source of compounds with strong antioxidant activity is a good way to extract value from citrus wastes.

4.1.5 Determination of the content of micro- and macroelements in Kaffir lime peel samples

Detailed results on the determination of the content of micro- and macroelements in Kaffir lime peel samples have been described in detail in the second article comprising this thesis, *Molecules*, 2023.

Another important group of compounds present in citrus waste is that of micro- and macroelements. Although there are no processes aimed at obtaining them from citrus waste, knowledge of their content is an important element in the context of using citrus waste as an ingredient in the agricultural or pharmaceutical industry. They are necessary for the proper development of organisms and take part in many metabolic processes. Therefore, the determination of microelements (chromium, zinc, fluorine, iodine, manganese, vanadium, iron) and macroelements (chlorine, phosphorus, magnesium, potassium, sulphur, sodium, calcium)

in Kaffir lime peel samples was performed using the MP-OES (formerly called MP-AES) technique. Nine microelements (Fe, Zn, Cu, Mn, Co, Ni, Cr, Mo, and V), four macroelements (Mg, Ca, K, and Na), and seven ballast substances (Cd, Hg, Pb, Al, V, Sr, and Pt) were determined. Among microelements, iron 32.72±0.39 mg/kg DW (dry weight) had the highest concentration. The calcium content was 9416±34 mg/kg DW, the highest in the case of macroelements. The research showed that its contents of individual elements make Kaffir lime peel a good potential raw material for use in the food, agriculture, pharmaceutical, and cosmetics industries. It can be used to produce biofertilizers, dietary supplements, or animal feed.



Hypothesis: Kaffir lime juice as citrus waste is a good source of many compounds with health-promoting or functional properties that can be used in various industries.

4.2 Chemical, physicochemical, and biochemical characteristics of Kaffir lime juice

4.2.1 Determination of the content of selected groups of bioactive compounds in Kaffir lime juice

The results of the study of the effect of binding human serum albumin by bioactive compounds present in Kaffir lime juice have been described in detail in the third and fifth articles comprising this thesis, *International Journal of Molecular Science*, 2023; *Journal of Luminescence*, 2018. The research was conducted in cooperation with prof. Shela Gorinstein from the Hebrew University of Jerusalem.

The results of the determination of selected bioactive compounds Kaffir lime juice samples are presented in Table 4.

Table 4. Bioactive compounds expressed as dry weight in Kaffir lime juice samples

Parameter	Value
Polyphenols [mg GAE/g DW]	23.16±2.18
Flavonoids [mg CE/g DW]	0.62±0.05
Flavanols [µg CE/g DW]	91.48±8.54
Vitamin C [mg Asc]	1.74±0.17
Tannins [mg CE/g DW]	1.92±0.19
Anthocyanins [mg CGE/kg DW]	63.45±5.15

Chlorophyll a [µg/g DW]	260.08±7.65
Chlorophyll b [µg/g DW]	729.00±16.54
Xanthoproteins + Carotenes [µg]	217.11±5.32

DW – *dry weight*, CE – *catechin equivalent*, GAE – *gallic acid equivalent*, Asc – *ascorbic acid*, CGE – *cyaniding-3-glucoside equivalent*

Comparing literature data, Kaffir lime juice has a similar polyphenol content to *Citrus reticulata* (24.98 mg GAE/g dw) [62] at 23.16±2.18 mg GAE/g dw, which is one of the highest among citrus juices. Compared to other lime studies, for almost all other health-promoting compounds, the content in Kaffir lime was higher. The only exception was vitamin C. Given its high content of bioactive compounds, Kaffir lime juice can be considered a good source of these compounds and can be explored for its health-promoting values in food products, diet supplements or raw materials for the pharmaceutical industry.

In the same research, the antioxidant properties of Kaffir lime juice were also assessed using four tests: ABTS, CUPRAC, DPPH, and FRAP. Comparing antioxidant capacity with literature data for other citrus fruits [63,64], the results obtained for the ABTS and CUPRAC tests in the conducted research for Kaffir lime were higher than for the rest of the fruits (*C. sinensis* (L.) Osbeck, *C. reticulata, C. paradisi, C. .limon*), which proves the very good antioxidant properties of Kaffir lime juice. Similarly to other citrus fruits, the high antioxidant capacity is the result of the high content of polyphenols and vitamin C.

4.2.2 Determination of the content of micro- and macroelements in Kaffir lime juice samples

The results of the determination of the content of micro- and macroelements in Kaffir lime juice samples have been described in detail in the third article comprising this thesis, *International Journal of Molecular Science*, 2023.

The aim of the study was to determine the main mineral compounds in Kaffir lime juice samples. As already mentioned in the case of the determination of mineral compounds in the peel, although there are no processes of extraction of micro- and macro-elements from citrus fruits, knowledge of their content is advisable when using them as raw materials in various industries.

To carry out the determination, the methodology used employed the MP-OES technique (formerly MP-AES), which had previously been subjected to a validation process. Iron, with a concentration of 16.578±0.029 mg/kg, was the most abundant microelement. Similar values were obtained for Argentinian lemon [65]. In the case of macroelements, the highest concentration was magnesium – 1034.8±4.8 mg/kg. Both of these compounds have a positive effect on the human body – iron is responsible for transporting oxygen to the tissues in the

body, while magnesium is a catalyst for protein, fat, and carbohydrate metabolism, affecting nerve conduction, the cardiovascular system, and muscle contractility. The relatively high mineral content is an additional advantage when considering the use of Kaffir lime juice in various industries, especially for the production of cosmetics or dietary supplements.

4.2.3 Study of the effect of binding human serum albumin by bioactive compounds present in Kaffir lime juice

The results of the study of the effect of binding human serum albumin by bioactive compounds present in Kaffir lime juice have been described in detail in the third and fifth articles comprising this thesis, *International Journal of Molecular Science*, 2023; *Journal of Luminescence*, 2018. The research was conducted in cooperation with prof. Shela Gorinstein from the Hebrew University of Jerusalem.

In order to determine the therapeutic effect of bioactive compounds contained in Kaffir lime juice, tests were carried out on their impact on human serum albumin (). HSA is the most common protein in human plasma. The most outstanding property of IHSA is its ability to reversibly bind multiple endogenous and exogenous ligands. The interaction of HSA with small molecules such as drugs, medicines, or bioactive compounds can significantly influence their absorption, distribution, metabolism, and toxicity and increase their bioavailability. At the same time, the functions and structure of the protein may also change. Therefore, the binding to HSA allows us to control the concentration of active compounds and understand the processes underlying their distribution and elimination [66].

The fluorescent properties of Kaffir lime juice aqueous solution and human serum albumin are shown in Figure 3. According to literature data, the binding properties of the new antiplatelet drug ticagrelor are approximately 28.8%.[67]. However, the addition of Kaffir lime juice to HSA showed a 37.7% increase in binding (sum of peaks a and b) (Table 5). The strong antioxidant affinity for HSA and the synergism of bioactivity are the main indicators for the use of Kaffir limes for health purposes. These study results may be useful in determining their pharmacokinetic profiles in the future development of healthy foods.

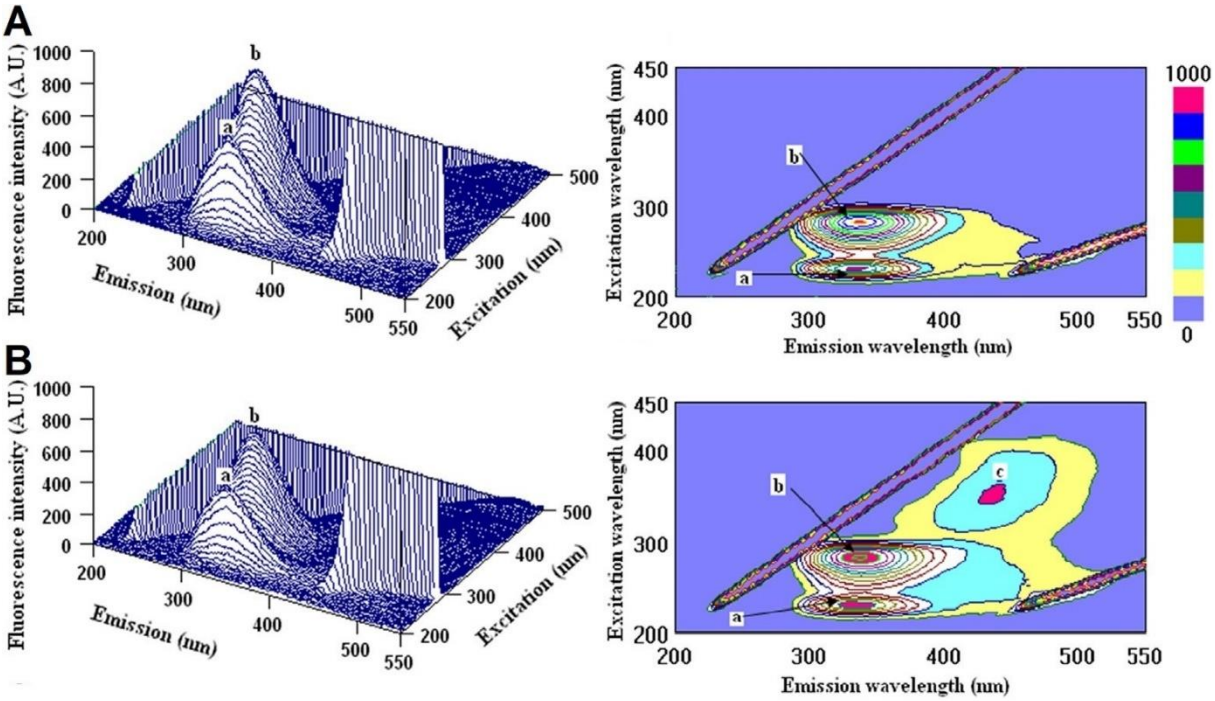


Figure 3. Fluorometric measurements in three-dimensional fluorescence analysis (3D–FL) of: (A) HSA; (B) Kaffir lime; and their corresponding surface spectra. The values of peaks a, b and c are given in the table below.

Table 5. Fluorescence and binding properties of Kaffir lime juice

Parameter	Kaffir lime juice	HSA
FI (peak a). AU	123.45±10	570.21±9.2
FI (peak b), AU	709±12	852±11
FI (peak c), AU	168.32±6.3	-
Binding to HSA, peak a %	20.96±1.5	-
Binding to HSA, peak b %	16.74±1.2	-

FI – *fluorescence intensity* in arbitral units (AU)

Then, the research was extended to the determination of β-pinene, the main terpene that is a component of Kaffir lime juice (Table 6). In this case, even higher values of parameters regarding the binding of bioactive compounds of Kaffir lime juice with human serum albumin were obtained, which confirms the therapeutic effect of this juice. It was also shown that the results are similar to those between paracetamol and HSA under physiological conditions [68]. Additional results were also compared with those obtained for the interaction of HSA with one of the important thiazole derivatives, 2-aminobenzothiazole (2-ABT) which was widely used as a structural unit in the synthesis of antioxidants, anti-inflammatories, herbicides, or antibiotics [69]. Figure 4 shows the fluorimetric measurements of Kaffir lime juice and HSA.

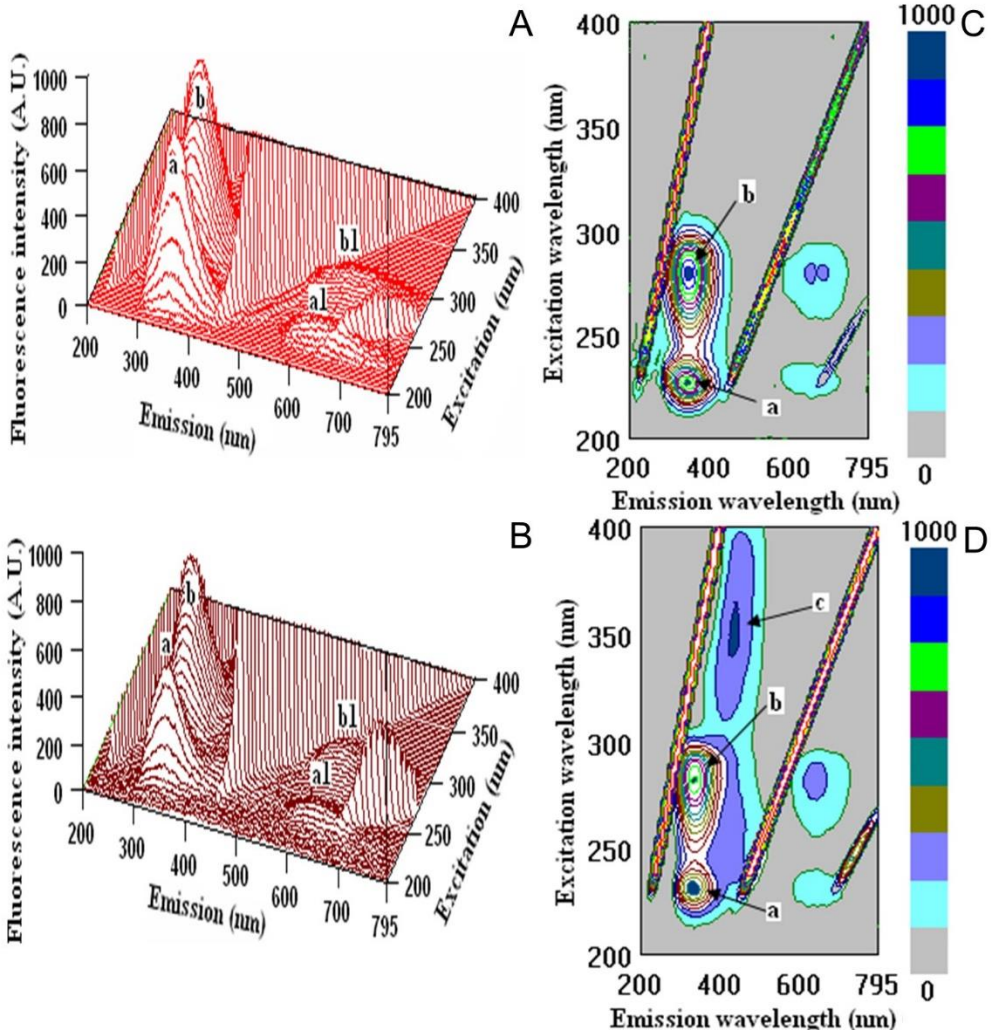


Figure 4. Fluorometric measurements in the 3D fluorescence analysis (3D–FL) of: (A) has, and (B) HSA + Kaffir lime juice after interaction and cross images of: (C) HSA; (D) HSA+ Kaffir lime juice. The values of peaks are given in the table below.

Table 6. Fluorescence and binding properties of Kaffir lime juice and β-pinene

Peaks	Indices	HSA	Kaffir lime juice	Binding to HSA [%]	β-Pinene	Binding to HSA [%]
a	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	227/349	231/334	-	228/349	-
	Int F_0	765.90±58.14	481.24±42.11	37.2±3.31	497.18±45.71	35.09±2.52
a1	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	-	233/637	-	-	-
	Int F_0	-	95.40±8.13	-	-	-
b	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	279/353	282/339	-	280/354	-
	Int F_0	875.01±79.11	723.63±7.63	17.3±1.50	760.21±68.24	13.12±1.21
b1	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	-	283/644	-	-	-
	Int F_0	-	129.78±11.2	-	-	-
c	$\lambda_{ex}/\lambda_{em}$ (nm/–m)	-	347/436	-	-	-
	Int F_0	-	169.44±13.14	-	-	-

Fo – *fluorescence intensity* in arbitral units (AU)

Based on the high value of B-pinene binding to HSA, it was found that the binding affinities of Kaffir lime juice are related to the bioactive properties of terpenes. The results showed that, thanks to high binding to HSA values, Kaffir lime juice is well transported in the circulatory system after consumption. Taking into account this fact and the many bioactive properties of terpenes, Kaffir lime juice is a good candidate as a raw material in the pharmaceutical industry.

4.2.4 Development of an analytical methodology for the determination of selected terpenes in Kaffir lime samples

4.2.4.1 Determination of the profile of volatile compounds present in Kaffir lime samples

The first stage of the research was to determine the profile of volatile compounds present in Kaffir lime juice samples. To select the best techniques for extraction and analysis, a literature review was conducted on the applications of gas chromatography techniques in food. The details have been described in the chapter being a part of the thesis (6.1.1), *Comprehensive Foodomics*, Elsevier, 2020. Based on previous literature reports, it was found that, as in all citrus fruits, their matrix is a mixture of many chemical compounds, often with similar properties. These are primarily terpene compounds. Many terpenes have a similar structural structure, which translates into similar physicochemical properties. This causes these chemicals to co-elute during chromatographic analysis. To eliminate this problem, it was decided to use the two-dimensional gas chromatography technique GC×GC. Solid phase microextraction (SPME) was chosen as the method for isolating and enriching analyses. Using this type of isolation and enrichment of analytes eliminates the need to use solvents, which is one of the key aspects of green analytical chemistry. The conducted research confirmed the thesis that the volatile profile of Kaffir lime samples consists mainly of chemical compounds from the terpene group. The high content of terpenes in the juice may prove useful if the juice were to be used to produce vinegar, which is a solution that has been considered in recent years [70]. Detailed results on the determination of the profile of volatile compounds present in Kaffir lime samples have been described in detail in the fourth article comprising this thesis *Food Control*, 2018. As shown in Figure 5, terpenes represent nearly 88% of all volatile substances present in the Kaffir lime samples. For this reason, special emphasis was placed on developing an analytical methodology for the determination of chemical compounds from this chemical group.

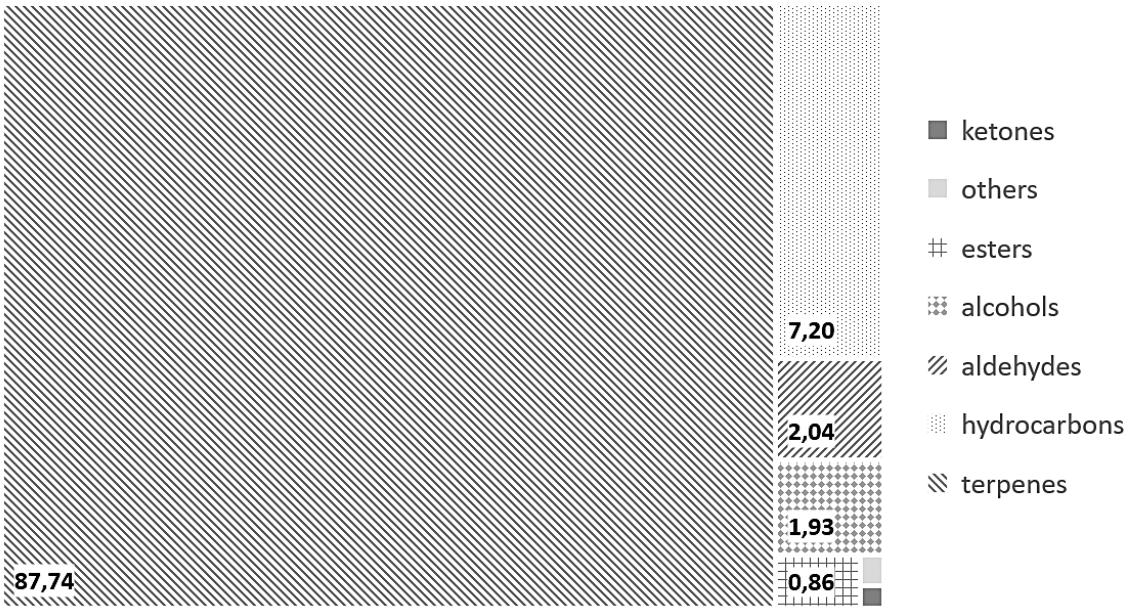


Figure 5. The volatile profile of Kaffir lime juice samples

4.2.4.2 Selection of the main compounds from the terpene group in Kaffir lime samples

The next stage, presented in the fourth and fifth articles comprising this thesis *Food Control*, 2018; *Journal of Luminescence*, 2018, was the selection of the main chemical compounds from the terpene group as preparation for the next stage, which is the optimization and validation of the targeted analytical methodology. Research conducted using the SPME-GC×GC-TOFMS technique shows that the main terpenes in Kaffir lime juice samples were a-Pinene, Limonene, Camphene, Linalool, Terpinen-4-ol, Myrcene, g-Terpinene, and a-Terpineol. It should be borne in mind that, for such a complex matrix as food, especially fruit, the results obtained are only indicative. In the case of fruit, the test result may be influenced by many parameters such as growing conditions, storage or transport conditions, geographical origin, and, above all, degree of ripeness. When determining terpenes, the degree of ripeness has an extremely important impact because these compounds undergo numerous transformations under the influence of storage conditions (temperature, time, presence of enzymes or microorganisms), creating various metabolic pathways. In addition, the metabolism of particular groups of chemical compounds depends on the species and conditions (i.e., during a pathogen attack). Nevertheless, thanks to the obtained results, it was possible to select the potentially main compounds of the volatile fraction of Kaffir lime, constituting the basis for designing the further part of the study. In the same studies, terpinen-4-ol was designated as a potential marker of the aroma of Kaffir lime juice.

4.2.4.3 Development and validation of analytical methodology for determining the main terpenes of Kaffir lime samples using gas chromatography

Based on chromatographic analyses, it was found that, in the case of selected compounds from the terpene group, which are the main compounds in the volatile fraction of Kaffir lime *Food Control*, 2018, it is possible to achieve satisfactory chromatographic separation using one-dimensional gas chromatography. Therefore, the next step was to optimize the analytical methodology for the quantification of the main terpenes in Kaffir lime samples. Detailed results on the development and validation of analytical methodology for determining the profile of Kaffir lime samples using gas chromatography have been described in the third article comprising this thesis, *International Journal of Molecular Science*, 2023.

Table 7. Selected validation parameters obtained based on the analysis of reference substances corresponding to the main terpenes identified in the volatile fraction of Kaffir lime

Terpene	RI	a	b	R^2	n	LOQ [µg/g]	LOD [µg/g]	Range		CV	Rec. [%]
								Min.	Max.		
Camphene	957.91	0.0322	-0.1579	0.9956	7	25	8.3	25	252.5	3.07	125.88
Limonene	1031.96	0.0283	-0.3372	0.9954	7	19	6.8	19	424.8	2.85	108.92
ß-Pinene	981.04	0.0335	0.0125	0.9949	7	39	13	39	1101	2.88	108.99
α-Phellandrene	1008.20	0.0324	-0.1394	0.9995	7	17	5.6	17	213.6	6.12	46.10
α-Pinene	942.93	0.0284	-0.1267	0.9954	7	26	8.5	26	433.3	0.22	94.11
α-Terpinene	1021.58	0.0542	-0.5572	0.9935	7	31	10	31	211.9	9.17	93.98
α-Terpineol	1183.68	0.0242	-0.116	0.9913	7	25	8.2	25	235.9	3.33	127.02
γ-Terpinene	1058.39	0.0542	-0.5572	0.9915	7	23	7.8	23	215.4	2.50	51.78
Terpinen-4-ol	1174.64	0.02	-0.2083	0.9944	7	28	9.2	28	471.21	1.97	105.45
Terpinolene	1085.55	0.0264	0.0175	0.9966	7	38	12.8	38	172.2	3.82	38.33

RI_{sample} – *Retention Index obtained during the analysis*, R^2 – *Coefficient of determination*, LOQ – *limit of quantification*, LOD – *limit of detection*, CV – *coefficient of variation*, Rec. – *recovery*

In the first stage, a temperature programme was selected. The best separation was obtained for the following parameters of the temperature programme: initial temperature 60 °C, increased at a rate of 7.5 °C/min to 150 °C, then 15 °C/min to 250 °C and maintained for 2 min. The total analysis time was 18 minutes. The effects of four independent factors on the extraction efficiency of major terpenes from Kaffir lime juice were evaluated using FFD (Fractional Factorial Design). The factors that were subject to optimization were: extraction time, extraction temperature, sample weight and the weight of salt added to the sample. The optimal results were obtained for the following values: 30 min, 45 °C, 3 g, and 0.5 g, respectively. For validation purposes, a model liquid was prepared to correspond to the juice samples in terms of sugar content (the representative sugar was sucrose), citric acid and

vitamin C (which is the main vitamin found in citrus juice). The values of citric acid, vitamin C, °Brix and pH were 74.74±0.50 g/kg, 22.31±0.53 mg/100 ml, 10.35±0.70, and 2.406±0.086, respectively. The obtained values of validation parameters are presented in Table 7.

4.2.4.4 Determination of selected terpenes in real samples of Kaffir lime juice with the use of optimized and validated analytical method using the HS-SPME-GC-TOFMS technique

Using the determined optimal extraction parameters and a previously selected temperature programme, chromatographic analyses were performed, on the basis of which calibration curves were prepared enabling the quantitative determination of selected terpenes in Kaffir lime samples. The internal standard method using borneol was chosen as the calibration method.

4.3 Comparison with other fruits

Hypothesis: Kaffir lime juice is a better source of many prohealth compounds as compared with many popular fruit juices/pulps.

4.3.1 Comparison of the content of selected terpenes in Kaffir lime samples with samples of a selected representative of the citrus genus

Detailed results regarding the determination of main terpenes in Kaffir lime and selected citruses have been described in detail in the fourth, fifth, and sixth articles comprising this thesis, *Food Control*, 2018; *J. Lumin.*, 2018; *Molecules*, 2020.

In order to estimate the terpene content, it was decided to analyse another fruit from the *Rutaceae* family, a citrus species. The key lime (*Citrus aurantifolia*) fruit available in Poland was selected. Sweetie (*Citrus grandis* × *Citrus paradisi)*, pomelo (*Citrus maxima)* and white grapefruit (*Citrus paradisi)* were also selected for comparison. The concentrations of selected terpenes in analysed citruses are presented in the table below.

Table 8. Concentrations of selected terpenes in the samples of different citrus fruits

No.	Chemical compound	Concentration±SD [mg/kg]				
		Citrus hysteria	*Citrus aurantifolia*	*Citrus grandis* × *Citrus paradisi*	*Citrus maxima*	*Citrus paradisi*
1	α-Pinene	3.07±0.03	1.04±0.01	0.8241±0.0096	<LOQ	2.851±0.015
2	Limonene	10.78±0.17	50.5±2.1	5.298±0.058	2.75±0.54	15.79±0.30

3	Citronellal	<LOQ	0.55±0.01	<LOD	<LOD	<LOD
4	Aromadendrene	1.00±0.07	<LOQ	<LOD	<LOD	<LOD
5	Camphene	4.86±0.67	3.38±0.34	<LOD	<LOD	<LOD
6	Linalool	20.13±0.71	3.45±0.09	<LOD	<LOD	<LOD
7	Nerol	<LOQ	2.77±0.10	<LOD	<LOD	<LOD
8	*trans*-Geraniol	<LOQ	1.86±0.10	<LOD	<LOD	<LOD
9	β-Pinene	<LOQ	2.10±0.11	<LOD	<LOD	<LOD
10	Terpinen-4-ol	44.79±1.09	1.96±0.06	<LOD	<LOD	<LOD
11	Myrcene	22.36±0.95	24.89±0.96	4.1±0.14	<LOQ	3.22±0.029
12	γ-Terpinene	25.01±0.28	19.41±0.69	7.27±0.34	<LOQ	2.566±0.026
13	α-Terpineol	1.50±0.06[a]	0.68±0.02[a]	20.96±0.70	<LOQ	87.9±2.0
14	Citral	<LOQ	20.91±0.60	<LOD	<LOD	<LOD
15	Ocimene	<LOD	<LOD	1.600±0.097	<LOQ	2.057±0.078

In research regarding *Citrus grandis* × *Citrus paradisi*, *Citrus maxima*, *and Citrus paradisi*, it was shown that among these three fruits, terpenes constitute the largest percentage in the volatile fraction of grapefruit, at almost 70%. In the case of Kaffir lime it was below 90%, and Key lime more than 50%. Moreover, in the first article of the thesis *Industrial Crops and Products*, 2018, a detailed literature comparison of the content and bioactive properties of selected terpenes contained in citrus fruit samples is presented.

Based on the results of further quantitative determinations, it can be concluded that *Citrus hysteria* and *Citrus aurantifolia* contain the highest concentration of terpenes in the juice. The bioactive properties of Kaffir lime caused by the content of individual terpenes and the resulting health-promoting effects have been described in the first article of the thesis *Industrial Crops and Products*, 2018. Despite the limited direct consumption of Kaffir lime juice compared to other citrus fruits, it is worth considering it as an addition to dishes.

4.3.2 Comparison of the content of selected bioactive compounds in Kaffir lime samples with samples of a selected representative of the citrus genus

Detailed results regarding the comparison of the content of selected bioactive compounds in Kaffir lime samples with samples of a selected representative of the citrus genus have been described in detail in the fourth and fifth articles comprising this thesis *Food Control*, 2018; *J. Lumin.*, 2018.

Table 9 presents the results of the determination of selected bioactive compounds and antioxidant activity of Key and Kaffir lime juice samples based on *Food Control*, 2018. Very similar results were obtained during research for *J. Lumin.*, 2018. Citrus fruits are one of the best sources of antioxidants among fruits [71]. This is primarily due to the high content of

vitamin C. Based on the results, it can be briefly stated that all parameters other than vitamin C are higher in the case of Kaffir lime. An almost twofold difference was noted in the case of polyphenols, flavonoids and tannins. Differences in chlorophyll content were slight. Significant differences were noted when determining the total antioxidant capacity of both fruits. Based on FRAP, CUPRAC, ABTS and DPPH analyses, improved antioxidant properties of the aqueous Kaffir lime extract were demonstrated, which suggests the potential use of fruit extracts in the design of dietary supplements. Among the citrus fruits tested by Guimarães (limes, oranges, lemons, grapefruits), *Citrus aurantifolia* juice had the highest DDPH values [72]. In the same study, key lime also had the highest flavonoid content. Taking into account the above facts, it can be concluded that Kaffir lime juice is one of the best sources of antioxidants among citrus fruits.

Table 9. Comparison of the content of selected bioactive compounds in aqueous extracts of juices of two lime varieties

Parameter	***Citrus Hystrix***	***Citrus aurantifolia***
polyphenols, mgGAE/g DW	23.2±2.2	11.9±7.7
flavanols, µgCE	91.5±8.5	64.4±5.4
flavonoids, mgCE	0.620±0.050	0.360±0.050
tannins, mgCE	1.92±0.19	0.780±0.070
vitamin C, mgAsc	1.74±0.17	2.44±0.11
anthocyanins, mgCGE/kg	63.5±5.2	47.3±4.1
chlorophyll a, mg	468.5±12.1	432±11
chlorophyll b, mg	260.1±7.7	245.8±5.3
xanthophylls + carotenes, mg	217.1±5.3	195.4±4.4
FRAP, mMTE/g DW	28.5±2.4	14.68±1.3
CUPRAC mMTE/g DW	124.3±5.4	28.0±2.7
ABTS mMTE/g DW	161.6±8.5	57.5±5.6
DPPH mMTE/g DW	33.3±3.2	12.0±1.2

DW – *dry weight*, TE – *Trolox equivalent*, GAE – *gallic acid equivalent*, CA – *catechin equivalent*, CGE – *cyaniding-3-glucoside equivalent*

4.3.3 Comparison of the content of selected bioactive compounds and selected antioxidant activity assays in Kaffir lime samples with samples of a selected representative of other species

Detailed results regarding the determination of the content of selected bioactive compounds and selected antioxidant activity assays in the selected representative of other species have

been described in detail in the seventh, eighth, ninth and tenth articles comprising this thesis, *Food Control*, 2019; *Molecules*, 2021; *Applied Sciences*, 2021; *Microchemical Journal*, 2021.

Literature reports show that geographical origin influences the content of health-promoting compounds in fruits and vegetables. This is because similar growing conditions (sunshine, temperature, etc.) influence the formation of specific groups of chemical compounds [73]. In some cases, this also translates into a similar colour of fruits and vegetables from the same growing areas, which is influenced by the content of chemical pigments such as carotenoids and chlorophylls. First, Kaffir lime was compared with the fruits of small and large varieties of bitter melon, due to them having the same geographical origin and similar appearance (green fruits, with strongly wrinkled skin). A comparison of the content of bioactive substances and antioxidant activity of Kaffir lime juice and *Momordica charantia*'s water extracts is presented in Table 10. The greatest differences were noted in the content of polyphenols, flavanols, and anthocyanins. Moreover, Kaffir lime juice turned out to be more similar in terms of the content of bioactive compounds to the larger variety of *Momordica charantia* fruit. When tested for antioxidant activity, in each case, Kaffir lime showed the highest values (the highest of all tests in the case of ABTS and CUPRAC assays).

Table 10. Comparison of the content of bioactive substances and antioxidant activity of Kaffir lime juice and *Momordica charantia*'s water extracts

Parameter	***Citrus Hystrix***	***Momordica charantia small***	***Momordica charantia large***
polyphenols, mgGAE/g DW	23.2±2.2	11.58±1.11	12.76±1.21
flavanols, µgCE/g DW	91.5±8.5	0.093±0.01	49.90±4.19
tannins, mgCE/g DW	1.92±0.19	2.96±0.29	6.80±0.65
vitamin C, mgAsc/ g DW	1.74±0.17	1.15±0.11	1.81±0.18
anthocyanins, mgCGE/kg	63.5±5.2	170.33±14.18	577.78±41.3
chlorophyll a, mg/g DW	468.5±12.1	624.00±43.07	350.00±31.05
chlorophyll b, mg/g DW	260.1±7.7	519.50±42.12	229.00±21.12
xanthophylls + carotenes, mg	217.1±5.3	229.50±22.37	206.50±21.54
FRAP, mMTE/g DW	28.5±2.4	25.90±2.34	23.48±2.56
CUPRAC mMTE/g DW	124.3±5.4	52.36±5.32	47.67±4.23
ABTS mMTE/g DW	161.6±8.5	39.60±3.76	35.54±3.12
DPPH mMTE/g DW	33.3±3.2	26.04±2.43	23.35±2.2

DW – *dry weight* TE – *Trolox equivalent*, GAE – *gallic acid equivalent*, CA – *catechin equivalent*, CGE – *cyaniding-3-glucoside equivalent*

Another fruit from the same geographical region is the durian, a fruit often called the “king of fruits”, not only because of its specific smell but also because of its several health-promoting properties. In almost every case, Kaffir lime juice turned out to contain more bioactive compounds and better antioxidant activities than all Durian varieties. The exception was flavonoids, the content of which was 2–3 times lower than in *Durio zibethinus* extracts.

It was similar in the case of another fruit grown in Southeast Asia – the dragon fruit, classified as a superfruit due to its numerous health-promoting properties, including the high content of polyphenols. Only the *amarilla* variety had a comparable polyphenol content. The remaining dragon fruit varieties showed lower polyphenol content and lower antioxidant activity than Kaffir lime juice.

Kiwi fruits are valued for their strong antioxidant and anti-inflammatory properties [74]. Vitamin C (ascorbic acid) is the most important and most publicized nutrient in kiwi fruit [75]. When comparing three varieties of this fruit with Kaffir lime juice, it turns out that only the A. eriantha variety shows better antioxidant properties based on the DPPH and FRAP tests. This is due to the higher vitamin C content than in other varieties – 41.20±4.34 mg Asc/g DW (the correlation between the vitamin C content and DPPH and FRAP was 0.9474 and 0.9342, respectively) [76].

Another fruit known for its high content of biologically active components that exhibit many health benefits such as antioxidant behaviour, radical scavenging activity, and antihypertensive and antiatherosclerosis activities is the persimmon fruit. In the case of comparable values, only the content of flavonols was twice as high in the persimmon fruit samples. The values of all other parameters were lower than in the case of Kaffir lime extract. The results of determination of antioxidants and antioxidant activity of selected fruits are presented in Table 11.

Table 11. Comparison of the content of bioactive substances and antioxidant activity of Kaffir lime juice and *Actinicida deliciosa*, *Actinidia eriantha*, *Actinidia arguta*, *Diospyros kaki*, three varieties of *Durio zibethinus*, and three varieties of pitaya

Parameter	polyphenols, mgGAE/g DW	flavonoids, mgCE	FRAP, mMTE/g DW	CUPRAC mMTE/g DW	ABTS mMTE/g DW	DPPH mMTE/g DW
Citrus Hystrix	23.2±2.2	0.620±0.050	28.5±2.4	124.3±5.4	161.6±8.5	33.3±3.2
A. deliciosa	6.89±0.48	2.08±0.19	11.33±0.92	28.58±2.18	22.46±1.24	13.71±1.21
A. eriantha	35.61±2.15	2.44±0.15	47.27±2.65	116.63±7.21	96.48±6.14	57.87±3.12
A. arguta	8.63±1.05	4.91±0.28	26.91±1.83	71.71±5.02	54.36±3.18	33.09±2.02
Diospyros kaki Thunb.	4.74±0.18	1.21±0.14	9.86±0.61	20.72±1.23	17.961.02	10.45±0.35
D. zibethinus Monthong	4.99±0.43	2.1±0.14	11.7±0.31	56.1±5.8	15.88±0.36	nd
D. zibethinus Chanee	3.31±0.24	1.39±0.07	6.82±0.29	41.01±6.4	11.8±0.16	nd

D. zibethinus Puangmanee	3.17±0.2	1.33±0.07	7.41±0.11	39.7±7.2	113.70±0.15	nd
Pitaya roja	12.42±1.11	nd	nd	39.47±3.39	nd	23.46±1.87
Pitaya amarilla	23.46±1.87	nd	nd	14.48±1.12	nd	8.10±0.67
Pitaya blanca	9.16±0.87	nd	nd	26.55±2.43	nd	14.62±1.21

In summary, in almost all cases, the aqueous Kaffir lime juice extract contained more polyphenols than compared to the juice extract of the other fruits. However, the content of flavonoids was lower than in the other fruits. Antioxidant activity was also higher in almost every case in the Kaffir lime extract samples. This is probably due to the high content of vitamin C. The only result was the fruit of *Actinidia eriantha*, in which the values of individual parameters were higher than or similar to those of *Citrus Hystrix* juice. Comparison with other fruits, including those considered superfruits due to their health-promoting properties, shows that Kaffir lime is a very good source of bioactive compounds and should be included in the diet. However, if not in direct form, the solution may be extracts added to dietary supplements or food products.

5 Conclusion

As a result of the research included in this doctoral dissertation, the characteristics of individual components of Kaffir lime juice, peel and pulp were presented in the context of their use as raw materials in various industries. This ensures a holistic approach to the studied matrix, taking into account all its parts. A set of analytical tools was proposed to assess the content of ingredients such as polyphenols, anthocyanins, flavonoids, flavanols, tannins, carotenoids and xanthophylls, chlorophyll a and b, vitamin C, and micro- and macroelements. Particular emphasis was placed on the development, optimization and validation of the methodology for determining terpenes in Kaffir lime samples, due to the fact that these are the predominant group of compounds in the above-surface fraction of Kaffir lime. The research was supplemented with an assessment of the health-promoting and antioxidant properties of individual parts of the fruit or individual by-products.

The main conclusions and accomplishments from the conducted research are the following:

- Thanks to the use of modern analytical techniques, it was possible to determine many chemical compounds in samples of Kaffir lime juice and pulp and by-products, and to determine health-promoting and antioxidant properties, providing a new insight for food science and technology, as well as many industries.
- Kaffir lime peel is a good source of many valuable chemical compounds such as pectins, essential oils, and bioactive compounds, which can be used in the cosmetics, food and agricultural industries; this will contribute to the valorization of citrus fruit wastes.
- Despite the lack of direct consumption of Kaffir lime juice, it is a valuable source of health-promoting compounds and has strong bioactive properties and a good affinity for human albumin serum, so it can be used in various industries.
- Compared to other fruits, Kaffir lime is a rich source of both antioxidant compounds and micro- and terpene compounds and has good antioxidant properties.

The results obtained during the research for the doctoral thesis not only complement the current state of knowledge about Kaffir lime, but above all provide information for various industries, which constitutes the background for its potential application. The use and valorization of Kaffir lime waste reduces problems with its storage, contributing to cost reduction and eliminating the negative impact on the environment resulting from its processing or storage. Learning about the characteristics of Kaffir lime, especially the health-promoting aspects, may contribute to the popularization of the use of this fruit not only in Asian countries. The use of Kaffir lime as a raw material in the cosmetics or pharmaceutical industry brings a

number of benefits. Not without significance is the natural origin of the raw material, as well as the fact that it meets the requirements of vegan, kosher or Hallal functional food.

6 Publications and chapters used for the doctoral dissertation

6.1 A novel analytical approach in the assessment of unprocessed Kaffir lime peel and pulp as potential raw materials for cosmetic applications

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A novel analytical approach in the assessment of unprocessed Kaffir lime peel and pulp as potential raw materials for cosmetic applications

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ABSTRACT

Volatile fraction of fruits is a rich source of bioactive and aroma compounds, which can be used in the cosmetics industry after meeting relevant criteria. This is particularly evident in citrus fruits, especially in *Citrus hystrix*, in which the headspace consists mainly of terpenes. Due to the insufficient sensitivity of analytical methods, essential oils are used in investigations in contrast to fresh fruits. Therefore, a novel approach in the assessment of unprocessed *Citrus hystrix* was proposed for the first time. It was proven that the application of two-dimensional gas chromatography coupled with time-of-flight mass spectrometry combined with solid phase microextraction gives reliable results in this context. Quantitation of key aroma compounds (α-pinene, limonene, citronellal, linalool, terpinen-4-ol, myrcene, α-terpineol, and citral), in the peel and pulp of fruit after prior assessment of bioactive properties measured as total phenolic content, ferric-reducing/antioxidant power and binding to human serum albumin, gives opportunity to use *Citrus hystrix* as a raw material in the cosmetic industry. Terpinen-4-ol and citronellal appeared to be the most important constituents of *Citrus hystrix* with the highest concentrations in the peel (34.58 ± 0.75 μg/g) and pulp (66.02 ± 0.85 μg/g), respectively. Polyphenols and antioxidant activities and binding properties revealed approximately twice higher bioactivity of Kaffir lime peel than pulp. Fluorescence studies of interaction of polyphenol extracts and some volatile standards with human serum albumin (HSA) showed relatively high binding properties and the correlation between biological activity and the volatile composition. Terpenes are primarily used as components of the fragrances of new perfumes and also as additives to creams, lotions or shampoos. The natural origin of terpenes is recommended in cosmetics industry.

1. Introduction

Kaffir lime (*Citrus hysteria, Citrus hystrix*) belongs to the plants of Ruta family (*Rutaceae*). The fruit is also known as Kaffir lime, Thai lime, Makrut or Angel Wings, due to the shape of the leaves (Wongpornchai, 2012). Most often it occurs in Indonesia, Malaysia, the Philippines, Laos, Thailand and Vietnam (Tunjung et al., 2015). In this region of the world Kaffir lime leaves are, besides ginger and lemon grass, the most important spice added to almost every dish. Kaffir lime juice and pulp is not directly consumed because of their pungent taste. However, it is an excellent source of antimicrobial, antiviral, and antioxidant substances that can be used in the cosmetic industries (Fortin et al., 2002; Rafiq et al., 2016; Tunjung et al., 2015). Kaffir lime peel except for small culinary applications is also industrial waste (Shaha et al., 2013).

Citrus fruits are one of the more readily consumed fruits in the world, not only due to their flavorful, but also pro-health properties. The main part of citrus fruit, which is used on an industrial scale, is the pulp from which juices are squeezed. It is estimated that more than half of the fruit after the production process is an industrial waste. This problem is greater in the case of Kaffir lime, which juice is not consumed directly, so a significant part of the fruit is a post-production waste. For this reason, new methods of waste management are sought. However, still much of the waste remains unused. Other way to utilize fruit peel is the production of bioethanol and biogas (Negro et al., 2016; Taghizadeh-Alisaraei et al., 2017). However, this process can be disturbed by the presence of terpenes, especially limonene, in the fruit peel (Ruiz and Flotats, 2014). There are some reports on the disposal of terpenes, including mainly limonene, from citrus waste. Pourbafrani et al. (2010) proposed a method of removing limonene during the production of biogas from citrus waste. It is important to know exact

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concentrations of terpenes in the sample in order to receive fragrances, essential oils and bioactive ingredients from citrus peel or pulp. It will allow to designate which part of the fruit is more aromatic and its differences. There are no reports on the methodology of fresh Kaffir lime analysis. In general, the essential oils are analyzed. The composition of Kaffir lime peel essential oil (EO) was assessed using gas chromatography and mass spectrometry (GC–MS) (Haiyee and Winitkitcharoen, 2012; Kasuan et al., 2013). The reason of use EOs for the analysis could be too low sensitivity of the analytical devices. Analysis of fresh citrus could prevent the formation of artifacts and loss of analytes, which takes place in the extraction and distillation processes carried out to obtain EOs (Ziino and Romeo, 2004). The solution that enables the analysis of fresh fruit is the application of novel analytical techniques which are characterized by high resolution, as two-dimensional gas chromatography (GC × GC). According to the best of our knowledge, there is a lack of reports about the analysis of volatile fraction peel and pulp of C. *hysteria* using two-dimensional gas chromatography. Lubinska-Szczygieł et al. (2018) analyzed the volatile fraction of lime juice. This technique, in combination with solid phase microextraction and time of flight mass spectrometer, has been used repeatedly for fruit analysis (Dymerski et al., 2015).

The aim of the research was to develop an analytical methodology to identify, determine and compare the contents of volatile compounds present in the samples of Kaffir lime pulp and peel using the two-dimensional gas chromatography technique coupled with time-of-flight mass spectrometry (GC × GC-TOFMS). It will explain the potential causes of different taste of peel and pulp, restrictions on use of this fruit in gastronomy and provide an information, which could be a basis for further elaboration of isolation method for cosmetology applications. For this reason, two-dimensional gas chromatography couples with time-of-flight mass spectrometry was selected as a proper tool for the analysis of complex matrix of Kaffir lime fruit (Dymerski et al., 2013). In order to find correlation between the biological activity and volatile composition total polyphenols, antioxidant activities and binding properties of main volatiles to human serum albumin were carried out. Fluorescence measurements and antioxidant assays were performed.

2. Materials and methods

2.1. Samples and standards solutions

The subject matter was the peel and pulp of Kaffir lime (*Citrus hysteria, Citrus hystrix*). Kaffir lime fruits were bought in Bangkok (Thailand). Fruit samples were imported to Poland in sealed plastic bags in portable fridge maintained at between 10 and 15 °C. The procedure of sample preparation is shown in Fig. 1A. Standard solutions of terpenes: α-pinene, limonene, citronellal, linalool, terpinen-4-ol, myrcene, α-terpineol, and citral (Sigma-Aldrich, St. Louis, MO, USA) were prepared using methanol (Avantor Performance Materials Poland S.A.) as a solvent. Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid); Folin–Ciocalteu reagent (FCR); Tris, tris (hydroxymethy1)aminomethane; $FeCl_3 \times 6H_2O$; were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2, 4, 6-tripyridyl-s-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionised and distilled water were used throughout.

2.2. Apparatus

Agilent 7980A (Agilent Technologies, Palo Alto, CA, USA) two-dimensional gas chromatograph equipped with a liquid nitrogen cooled two-stage cryogenic modulator (Zoex Co., Houston, USA) and MPS (Gerstel Co., Mülheim, Germany) configured as headspace autosampler was used. Liquid nitrogen was used as the cooling medium. The column set consisted of a 30 m × 0.25 mm i.d. × 0.25 µm – Equality 1 (Supelco Bellefonte, PA, USA) primary column and 2 m 0.1 mm i.d. × 0.1 µm – SGWAX (SGE Analytical Science Austin, TX, USA) secondary column. Applied temperature program is shown in Fig. 1B. Modulation period was 4 s (hot pulse of 0.80 s). Helium N6.0 was used as carrier gas and its volumetric flow rate was set up to 1 mL/min. The injector worked in splitless mode at a temperature of 250 °C. A Pegasus® IV time-of-flight mass spectrometer (LECO Corp.) was used as detector. The transfer line and the ion source were maintained at 250. The detector voltage was set to – 1600 V and the MS was operated in electron impact ionization mode (70 eV). Cool time between stages was 1.20 s. In the step of isolation and enrichment of analytes a solid phase microextraction (SPME) was used. SPME 50/30 µm of thickness and 2 cm of length Carobxen/Polydimethylsiloxane/Divinylbenzene (CAR/PDMS/DVB) fiber was used (Sigma-Aldrich, St. Louis, MO, USA). The temperature of extraction was set up to 35 °C, and the time of a single extraction was 35 min. Before the extraction the samples were kept at 40 °C for 2 min and agitated with a magnetic stirrer (700 rpm). Thermal desorption was set up to 250 °C for 5 min to release the analytes from the surface of fiber. Between each analysis, the fiber was cleaned for 2 min at 250 °C.

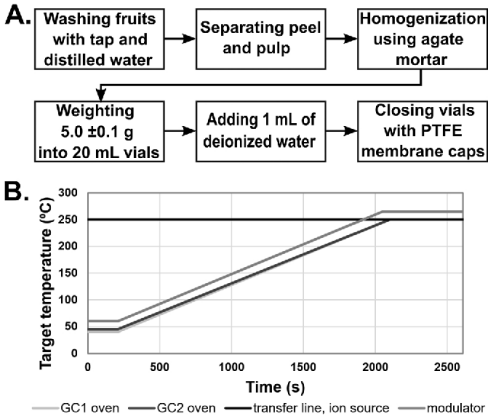

Fig. 1. A Scheme of sample preparation; B. Chromatographic temperature program applied in HS-SPME/GC × GC-TOFMS analytical procedure.

2.3. Data acquisition and qualitative analysis

ChromaTOF (LECO Corp., version 4.44.0.0) software was used to collect data. Tentative identification was done by correlation of retention times of analytes with retention times of authentic standards. The time of flight mass spectrometer (Pegasus 4D), which identified the chemicals time of flight, was produced by LECO Corp. (St. Joseph, MI, USA). Automated peak find and spectral deconvolution have been employed during data treatment. The mass range (*m/z*) from 40 u up to 500 u and the acquisition rate of 125 spectra/s were used to collect data. Major chemicals were identified by comparison of mass spectra with data included in NIST 11 and Wiley library.

2.4. Quantitative analysis

Quantitative analysis was performed using analytical procedure described above. Chemical compounds with the highest peak area in both samples were selected for quantitative determination. The standard addition method was used as a method of quantitative determination. Using this method, it was possible to compensate effects of matrix. Sample was spiked with the terpenes standards mixture to generate nine amendment levels of 0.1, 0.2, 0.5, 1.0, 5.0, 10.0, 20.0, 50.0, 100.0 µL/L (v/v). The analysis was performed in three replicates

for each one of the sample and standard solutions. The concentration of tentatively identified volatile compounds was calculated using calibration curve. For each standard a calibration curve equation and determination coefficient (r^2) was calculated. The analytical parameters: limits of detection (LOD) and quantification (LOQ) were also determined. Values of LOD and LOQ were based on the standard deviation of the intersection of analytical curve (SD) and on the slope of the curve (a). The LOD as and LOQ were estimated as a 3.3·SD/a and 10·SD/a, respectively.

2.5. Determination of bioactive compounds, antioxidant and binding properties

Polyphenols were extracted from lyophilized samples of peel and pulp with water (concentration 20 mg/mL) during 1 h in a cooled ultrasonic bath and were determined by Folin-Ciocalteu method with measurement at 750 nm with spectrophotometer (Hewlett-Packard, model 8452A, Rockvile, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g DW (Singleton et al., 1999).

The total antioxidant capacity (TAC) was determined by Ferric-reducing/antioxidant power (FRAP): FRAP reagent (2.5 mL of a 10 mmol ferric-tripiridyltriazine solution in 40 mmol HCl plus 2.5 mL of 20 mmol $FeCl_3xH_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6) of 900 µL was mixed with 90 µL of distilled water and 30 µL of extract samples as the appropriate reagent blank and absorbance was measured at 595 nm (Benzie and Strain, 1996).

Fluorometric measurements were used for the evaluation of binding properties of citrus extracts and some standards to human serum albumin (HSA). Three dimensional (3D-FL) fluorescence measurements were recorded on a model FP-6500, Jasco spectrofluorometer, serial N261332, Japan. The concentrations of citrus extracts were ranged from 0 to 1.5 mg/mL. The emission wavelength was recorded between 200 and 500 nm for three-dimensional fluorescence spectra. All solutions for protein interaction were prepared in 0.05 mol/L Tris-HCl buffer (pH 7.4), containing 0.1 mol/L NaCl (Dymerski et al., 2015).

2.6. Odor activity value (OAV) and statistical analysis

The odor activity values (OAVs) were calculated by dividing the concentrations of aroma compounds with their sensory thresholds taken from the literature. OAV data were expressed as the mean ± standard deviation of triplicate tests. Two-way analysis of variance (ANOVA) was used to compare the significant differences among the means using STATISTICA 12 under the Fisher test at a p-level of 0.05 (StatSoft, Inc., Tulsa, Oklahoma, USA).

3. Results and discussion

3.1. Comparison of the content of selected classes of chemicals in samples of peel and pulp of Kaffir lime

Aroma of food products is a complicated mixture, sometimes consisting of several compounds (Wardencki et al., 2009). By performing chromatographic analysis, it was possible to detect about 500 chemical compounds in samples of the peel and pulp of Kaffir limes. Twenty two substances were selected as the main chemical compounds. Selected substances created a characteristic aroma profile of the tested samples, namely "fingerprint" (Fig. 2A). Based on the contour plots, it can be observed that both Kaffir lime peel and pulp was characterized by a different composition of the volatile fraction. In addition, for peel samples, selected chromatographic peaks were more intense than for pulp. On this basis, it can be deduced that Kaffir lime peel contained more terpenes. The content of these analytes was more than 70% of all chemical compounds present in the volatile fraction of sample. The heat map (Fig. 2B) shows the averaged results of the analysis of three samples of the fruit. Compound with the largest area of chromatographic peak was citronellal. Beside the citronellal, other terpenes such as terpinene, thujene and limonene in large amounts in the samples headspace were present. The major chemical compounds identified in the sample of Kaffir lime are listed in Table 1. Many studies have been conducted on the content of terpenes in Kaffir lime peel (Jantan et al., 1996; Kasuan et al., 2013; Muhammad et al., 2013; Nor, 1999; Waikedre et al., 2010). Each time the test object was not fresh peel, as in the present research, but essential oil, obtained by the use of different distillation methods. Chanthaphon et al. (2008) conducted the research of volatile fraction of ethyl acetate extract of hydrodistilled essential oil, in comparison with fresh peel used in the present experiment. The received results differ from those obtained in the reviewed study. The percentage of citronellal in the volatile fraction of Kaffir lime peel was about 17% and in case of ethyl acetate extract of hydrodistilled essential oil, 25.96% and 15.67%, respectively. The amount is therefore similar to the content in the oil. The biggest difference occurs in case of β-pinene, whose percentage in the research is about 2% and in the cited paper is about 30%. The conditions for sample preparation and analysis were influenced by the results, as well as ripeness of fruits, vegetative stage of plant, and storage conditions.

Based on the chromatographic peak area of selected compounds, the radar charts presenting the content of selected chemical classes in volatile fraction of Kaffir lime peel and pulp were prepared (Fig. 3A). In all cases the composition of volatile fraction of Kaffir lime peel is characterized by a higher content of individual compounds, than the composition of the volatile fraction of Kaffir lime pulp. The following data apply to all compounds detected in both samples.

Carboxylic acids are the smallest group of detected compounds in Kaffir lime fruits, which are present in amounts less than 0.01% of all compounds. However, when comparing the two samples it can be noticed that the fruit peel contains more carboxylic acids than the pulp. This is the reason of stronger peel smell than of pulp. In many cases, the carboxylic acids are suitable characteristics of fruit sour taste. Carboxylic acids also affect the flavor, giving the acid-fruity aroma. For Kaffir lime, because of the low content, carboxylic acids did not affect its smell. Detected carboxylic acids included caprylic, pelargonic, malic, acetic and citronellic acids.

A bigger amount of aldehydes is contained in the peel of Kaffir lime. The content of all detected compounds is 78%, while in the pulp only 22% of all aldehydes is detected. The compounds are responsilbe for fruity and fresh aroma of the fruits. The major aldehydes detected in Kaffir lime samples were dodecanal, hexanal, 2-hexenal and heptanal. Most detected aldehydes were included in both peel and pulp of the fruit, but with different concentration levels.

In case of ketones, distribution of these compounds is similar to that as in alcohols. In the volatile fraction of Kaffir lime peel 74% of the ketones was detected, while in the pulp only 26%. Acetophenone, fenchon, and 2, 6-dimethyl-3-heptanone is the most common ketones identified in samples. Most detected ketones have long carbon chains, what is associated with a pleasant fruity odor of food products.

Alcohol contents in both samples were different. The peel headspaces contained 69% of all detected compounds belonging to the alcohol class while the rest was present in the volatile fraction of fruit pulp. The most common alcohols found in the studied samples were: 1-hexanol, 2-hexen-1-ol and nonanol.

Esters were the only chemical class which in the peel and pulp occurred in approximate amounts. Among all identified esters of 54% was detected in the peel, while 46% was found in the pulp. The presence of esters in the food makes their aroma fruity. Among the esters the most popular were hexyl acetate and ethyl benzoate.

Terpenes were present in each of the research objects in a very large amount, but when comparing both fractions, it can be observed that peel of the lime fruit had higher amount of these compounds (66%) than the pulp (34%). This can be the justification for the fact that the peel has a much stronger aroma than fruit pulp.

Comparing the content of the chemical classes, it can be observed

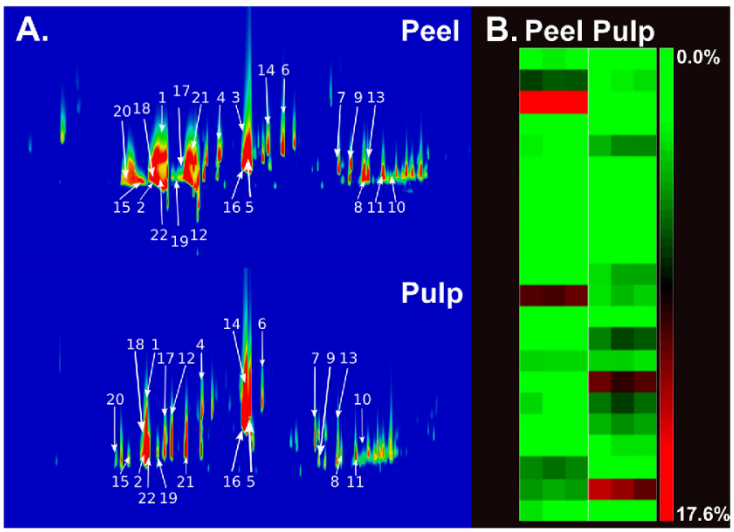

Fig. 2. A The comparison of terpene profiles by the use of GC×GC-TOFMS system (extracted ion contour plots obtained in TIC mode); B. Heat map describing a percentage distribution of selected terpenes in the Kaffir lime pulp and peel (calculated on the chromatographic peak areas); 1. β-pinene, 2. sabinene, 3. citronellal, 4. linalool, 5. α-terpineol, 6. β-citronellol, 7. citronellyl acetate, 8. α-copaene, 9. geranyl acetate, 10. α-cubebene, 11. β-caryophyllene, 12. limonene, 13. germacrene D, 14. α-pinene, 15. camphene, 16. terpinen-4-ol, 17. α-terpinene, 18. myrcene, 19. α-phellandrene, 20. α-thujene, 21. γ-terpinene, 22. β-phellandrene.

Table 1
The major compounds identified in the volatile fraction of Kaffir lime by GC×GC-TOF-MS.

No.	Chemical compound	RT1[a] [s]	Average RT2[b] [s]	Similarity	Unique mass	Aroma descriptors
1	β-Pinene	1186	1.184	854	93	green, musty, pine, resisous, sweet, turpentine, woody
2	Sabinene	1050	2.097	939	93	herbal
3	Citronellal	1310	2.204	886	69	citrus, fatty, floral, lemon, rose
4	Linalool	1332	2.208	745	71	citrus, orange, lemon, floral, waxy, aldehydic, woody
5	α-Terpineol	1402	2.408	790	59	earthy, floral, musky, spicy, woody
6	β-Citronellol	1438	2.289	928	69	citrus, floral, rose
7	Citronellyl acetate	1606	2.132	916	69	citrus, berry, floral, rose
8	α-Copaene	1682	2.070	937	161	woody
9	Geranyl acetate	1642	2.136	916	69	floral, rosy, waxy, herbal and green with a slight cooling nuance
10	α-Cubebene	1766	2.038	870	161	citrus, fruity, radish
11	β-Caryophyllene	1738	2.074	917	93	woody, spicy
12	Limonene	1170	2.026	936	93	citrus, mint, orange, terpenic, xyloid
13	Germacrene D	1694	2.012	907	161	woody, spicy
14	α-Pinene	998	2.034	861	93	pine, terpenic
15	Capmhen	1056	1.984	951	91	arborescent
16	Terpinen-4-ol	1382	2.160	864	71	woody, fruity, herbal, licorice, moldy, musty
17	α-Terpinene	1134	2.044	939	93	woody, oil, fruit, gasoline, lemon,
18	Myrcene	1050	2.188	808	93	terpenic, herbaceous, woody with a rosy celery and carrot nuance
19	α-Phellandrene	1164	2.040	915	93	citrus herbal terpene green woody peppery
20	α-Thujene	962	2.128	899	93	arborescent
21	γ-Terpinene	1272	2.156	914	93	woody, fruity, gasoline, herbal, sweet, terpenic
22	β −Phellandrene	1058	2.200	836	93	minty, terpenic

[a] RT1–first dimension retention time.
[b] RT2–second dimension retention time.

that the chemical compounds of each of these classes in higher amounts occurred in the Kaffir lime peel. The peel is therefore more aromatic part of this fruit. Moreover, peel is a natural barrier to protect the fruit against external factors and against the loss of many chemical compounds.

3.2. Comparison of the amount of key aroma compounds present in Kaffir lime peel and pulp determining the character of overall odor sensation

It is commonly known that terpenes are aroma active chemical compounds. It was shown that terpenes determinate the aroma of citrus fruits (Md Othman et al., 2016). For selected terpenes identified in samples of lime fruit corresponding aroma descriptions (Table 1) were assigned according to the AroChemBase V4 library. It can be observed that the aroma of compounds from the group of terpinene is a woody, earthy, while other chemical compounds are characterized by a citrus or fruity aroma.

The results of quantitative analysis are shown in Fig. 3B. Considering the content of individual terpenes, it can be noticed that the chemical compounds with citrus odor dominated in peel samples. While the greater the content of compounds of the Terpinene group was recorded in the fruit pulp. This explains the pungent and bitter smell of the fruit pulp and a pleasant citrus aroma of peel.

The biggest differences between the contents in the peel and pulp can be observed in the case of citronellal, which was the main chemical compound of the peel. The content was nearly one fifth of the volatile

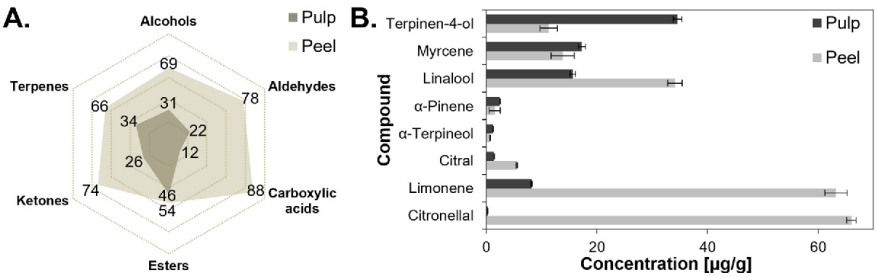

Fig. 3. A Relative amount (in percentage) of class of chemical compounds; B. Concentration of selected terpenes in Kaffir lime.

fraction of lime peel, while in the pulp it occurs in trace quantities, and not possible to be quantitative. Citronellal may be a potential marker of Kaffir lime peel aroma, especially that in other fruits of the Rutacea family (*Citrus limon*) it was at low concentrations (Nhu et al., 2006). Literature reports show that content of citronellal is also high in case of fresh Kaffir lime leaves. Citronellal, the main compounds in Kaffir lime peel, exhibits antimicrobial properties, which may hinder the production of biomass from citrus residues by destroying fermenting bacteria, therefore it has to be removed during the process. The recovered citronellal, due to its antioxidant, anti-inflammatory and redox-protective properties, can be used in the cosmetic and medicinal manufacturing. A pleasant citrus aroma makes citronellal a popular perfume ingredient and food additive. Its high content in Kaffir lime peel (66.02 ± 0.85 μg/g) shows a valuable source of this terpene. The main ingredient of the Kaffir lime pulp is terpinen-4-ol. This compound of woody aroma is used as a flavor and fragrance agent.

To investigate the cause of overall odor sensation character the concentration of aromatic compounds present in Kaffir lime peel and pulp was determined. Calculated concentrations were the basis for determining of the OVAs, which were the odor intensity indicators of these substances. The OAV can be calculated using the following equation:

$$OAV = \frac{C}{C_{OT}} \quad (1)$$

where:

c – concentration of substance [μg/g],

c_{OT} – odor threshold concentration of substance [μg/g].

There are some reports presenting the value of odour threshold (Van Gemert, 2011; Averbeck and Schieberle, 2009; Pino and Mesa, 2006), which were used for OAV evaluation.

Based on Eq. (1) it can be stated that the sensory properties of studied samples were determined by two factors, namely the concentration of chemical compounds in the sample, which characterize the volatile profile of the sample, and the odor thresholds. These features are largely influenced by the structure of the chemical compounds and their physicochemical properties.

The odor activity value (OAV) in the fruit samples can be calculated as ratio of the concentration of the volatile compound in sample compound to its odor threshold in water. By calculating OAVs, it can be determined which compounds present in the fruit have the greatest effect on their aroma. Jirapakkul et al. (2013) found that citronellal, linalool and limonene were the most important contributors to Kaffir lime leaves flavor. The OAVs for the 8 selected terpenes identified in the pulp and peel of Kaffir lime samples. As it can be seen in the pulp samples, it was not possible to calculate citronellal OAV because its concentration was too low. The concentrations of the remaining selected terpenes were significantly higher than their odor threshold. This means that these compounds can be detected, and also recognized by the use of the human nose. Statistical calculations have shown that only OAV for α-terpineol in peel and pulp of Kaffir lime samples did not differ statistically.

α-Pinene has the smallest odor threshold value of 6 μg/L (m/v). Therefore, the small concentration of this substance is perceptible by the human, because OAV is inversely proportional to the odor threshold. The odor thresholds of other terpenes are similar and varies from 25 to 46 mg/L (m/v). This is caused by the fact that these substances are from the same chemical clas of terpenes, and have very similar physicochemical properties. Comparing the OAVs for peel and pulp samples, it can be concluded, that α-pinene, myrcene and α-terpineol, similarly effect on the aroma of Kaffir lime peel and pulp, giving the samples a wood aroma. More differences between OAVs were observed for terpinen-4-ol, linalool and citral. The concentration of terpinen-4-ol in the pulp was 3-fold higher than the concentration of this compound in the peel. Due to the fact that OAV is proportional to the concentration, the same dependence on terpinen-4-ol can be observed in Table 2. Threefold higher value of OAV for this compound caused that Kaffir lime pulp was characterized by intense woody aroma. In the case of linalool and citral, higher concentrations as well as higher OAVs may be observed for lime peel samples. It should be emphasized that higher value of OAV was the reason of more intense aroma. In turn, linalool and citral gave the peel samples a delicate citrus aroma, because the OAVs were not high.

The major differences in OAVs can be observed for citronellal and limonene. It can be explained by more intense and citrus aroma of Kaffir lime peel. Therefore, citronellal and limonene can be used as distinguishing parameters of Kaffir lime peel and pulp in sensory studies. Limonene, citronellal and linalool had the biggest effect on the aroma of Kaffir lime peel giving a citrus aroma, while wood aroma of the pulp was caused by the presence of terpinen-4-ol, myrcene and α-pinene. Furthermore, on the basis of Jirapakkul et al. (2013) studies, it can be concluded that Kaffir lime peel has a similar aroma to the leaves of this fruit.

3.3. Kaffir lime health benefits

Terpenes are the main chemical class of Kaffir lime samples. All of them have complex bioactive properties such as antioxidant, antimicrobial or antiulcer effects. The main health benefits of compounds detected in samples of Kaffir lime are shown in Table 3.

3.3.1. Antioxidant and binding properties of volatiles and bioactive compounds of Kaffir lime

Antioxidant activity is related to food preservation by inhibiting oxidation processes. Consuming food rich in antioxidants is essential for proper functioning of the vascular system, prevent from atherosclerosis, cancer, ischemic and heart disease. The antioxidant activity of Kaffir lime is a result of presence such chemical compounds as γ-terpinene, terpinen-4-ol or camphen. The first two of them were the main components of volatile fraction of *Citrus hystrix* peel, so that peel is a rich

Table 2
Comparison of odor intensity originating from pulp and peel of Kaffir lime.

No.	Chemical compound	r^2	LOQ[b] [μg/g]	LOD[c] [μg/g]	Odor threshold [μg/L]	OAV ± SD[d] [mg/L]	
						Pulp	Peel
1	α-pinene	0.995	1.93	0.64	6[e]	370.8 ± 3.2	256.2 ± 12.9
2	limonene	0.994	2.19	0.72	30[f]	241.4 ± 3.8	2108.0 ± 83.8
3	citronellal	0.992	2.66	0.88	25[e]	< LOQ	2640.6 ± 50.8
4	linalool	0.990	3.02	1.00	40[g]	349.8 ± 12.2	852.4 ± 42.2
5	terpinen-4-ol	0.997	1.53	0.51	41[e]	753.7 ± 18.2	274.6 ± 16.9
6	myrcene	0.994	2.22	0.73	29[g]	531.4 ± 22.4	475.5 ± 36.7
7	α-terpineol	0.996	1.75	0.58	42[e]	38.7 ± 0.2[a]	15.9 ± 0.7[a]
8	Citral	0.990	2.94	0.97	46[e]	39.6 ± 0.3	119.7 ± 0.8

[a] Averages in rows marked with the same letters not differ significantly ($P < 0.05$).
[b] LOQ– limit of quantitation.
[c] LOD–limit od detection.
[d] SD–standard deviation, Mean ± SD of 3 measurements.
[e] Van Gemert (2011).
[f] Averbeck and Schieberle (2009).
[g] Pino and Mesa (2006), Pino and Mesa (2006).

source of antioxidants and may be an important ingredient of diet. The antioxidant properties are also exhibited by linalool, whose high content was determined both in the peel and pulp of Kaffir lime (15.62 ± 0.49 μg/g and 34.10 ± 1.3 μg/g, respectively). The polyphenols and corresponding antioxidant capacities in peel were 1.80 and 1.78 times higher than in pulp (Table 4). This is in line with recent report, where similar results were shown (Park et al., 2014). Terpinen-4-ol and citronellal were similar in their bioactivity with slight higher level in citronellal. The binding properties determined by the decrease of fluorescence intensity after interaction with HSA were in direct relation with the bioactive substance (Table 4, Fig. 4). Comparison of the fluorescence intensity of HSA (Fig. 4A, peaks a, b) with the results of Fig. 4B and C showed that the binding of peel was 1.72 times higher in pulp. The binding properties of citronellal and terpinen-4-ol (Fig. 4D, E) were lower than in the citrus fruit and supported previous results (Shafreen et al., 2017).

3.3.2. Antimicrobial activity

Almost all of the volatiles of Kaffir lime are the agents that fight against microorganisms or stop their growth. Citronellal, the main chemical compound of the pulp, displays the inhibitory and bactericidal activity against *E. coli* and *S. aureus Propionibacterium acnes* (Lee et al., 2013). Kaffir lime is a component of cosmetics and medicines, especially that in recent years the interest in antimicrobial natural drugs has grown.

3.3.3. Anti-inflammatory activity

Anti-inflammatory activity of Kaffir lime is caused by the presence of volatile organic compounds such as α-pinene, β-caryophyllene, sabinene and limonene. The high content makes the consumption of Kaffir lime helps to mitigate the symptoms of such diseases as rheumatism, arthritis, edema, and gout.

3.3.4. Comparison of the composition of bioactive compounds with other citrus fruits

The comparison of content of selected chemical compounds with bioactive activity contained in the samples of Kaffir lime peel and pulp with other fruits was presented in Table 3.

For comparison, eight citrus juices were taken (Lubinska-Szczygieł et al., 2018; Moufida and Marzouk, 2003; Pérez-López and Carbonell-Barrachina, 2006) and six citurs fruit peels (Gancel et al., 2002; Vekiari et al., 2002; Asikin et al., 2012; Fan et al., 2009). All of the terpenes in the table exhibit antioxidant properties (Lu et al., 2014; Melo et al., 2011; Aggarwal et al., 2002; Rajeshwari and Andallu, 2011; Duarte et al., 2016; Dudai et al., 2005; Baik et al., 2008; El-Nekeety et al., 2011). In addition, in the case of citronellal, linalool,

In addition, in the case of citronellal, linalool, citral, camphene and terpinen-4-ol, anti-inflammatory, antimicrobial, and anticancer activities have been also found. Each of these chemical compounds was found in Kaffir lime. Kaffir lime is similar to Mexican lime and Star Ruby grapefruit, in which citronellal, is the main chemical compound of the peel and occurs in the amount of 66.02 ± 0.85 μg/g. In previous reports, the presence of this compound was not found in fruits juices and pulps, except Kaffir lime pulp and Key lime juice. Kaffir lime and bergamot are fruits with the highest content of linalool in juice and pulp comparing to the rest of citrus fruits. Terpinen-4-ol with a wide bioactive activity, in the largest amount was also noted in the samples of Kaffir lime juice and pulp. This compound is characteristic for lime peel, as well as camphene. Despite limes, only lemon and orange contain this chemical compounds, but in smaller amount that in limes. Camphene does not occur in the juice of other citrus fruits than limes. The other compound with the most extensive bioactive activity is citral. Its presence was pointed in the peels of most citrus fruits, and the highest amount was in Mexican lime. There is less of citral in the Kaffir lime peel. In the case of juice, citral was determined only in the Key lime pulp. By comparing the contents of chemical compounds with bioactive activity in the Kaffir lime peel and pulp with other fruits, it can be concluded that the activity is higher in Kaffir lime. The presence of characteristic chemical compounds as camphene, citral, linalool, terpinen-4-ol and citronellal denotes a high antioxidant effect. This is especially due to the presence of last two chemical compounds that are the main in the Kaffir lime peel and pulp and what is confirmed by determination of antioxidant and binding properties of limes (Table 4). This is consistent with the relationship between the content of terpene chemical class and the bioactive properties of Kaffir lime.

4. Conclusions

In case of Kaffir lime, despite their culinary use, the peel and pulp of this fruit is often an industrial waste. Peel and pulp contain important substances due to their potential utilization in cosmetic products. This is connected with bioactive and aroma properties of chemical compounds present in this fruit. It was proven that constituents of *Citrus hystrix* have high biological activity confirmed by indices assessment of total phenolic content, ferric-reducing/antioxidant power and binding affinities to human serum albumin. Therefore, a new analytical method was elaborated to quantify the substances, key in this context, successfully. For the first time it was possible to measure terpene content without solvent extraction or hydrodistillation step before chromatographic analysis with accordance to the roles of green analytical

Table 3
Comparison of the content and bioactive properties of selected terpenes contained in citrus fruits samples.

Health benefits	Chemical compounds										
	citronellal	limonene	linalool	camphene	citral (neral + geranial)	α-terpineol	α-pinene	myrcene	γ-terpinene	terpinen-4-ol
antioxidant	+	+	+	+	+	+	+	+	+	+
anti-inflammatory	+	+	+	+	+		+		+	+
antimicrobial	+	+	+	+	+	+		+		+
anticancer	+		+	+	+		+			+
Ref.	Lu et al. (2014); Melo et al. (2011)	Aggarwal et al. (2002); Rajeshwari and Andallu (2011)	Duarte et al. (2016)	Rajeshwari and Andallu (2011)	Dudai et al. (2005)	Rajeshwari and Andallu (2011)	Rajeshwari and Andallu (2011)	Baik et al., 2008; El-Nekeety et al. (2011)	Rajeshwari and Andallu (2011); Ramalho et al. (2015)	Rajeshwari and Andallu (2011)
Concentration [μg/g or μg/mL]										
Citrus pulps and juices										
Kaffir lime pulp	0.21	8.18	15.62	3.82	1.39	1.23	2.45	17.25	19.24	34.58
Kaffir lime juice (Lubinska-Szczygeł et al., 2018)	nd.	10.78	20.13	4.86	nd.	1.50	3.07	22.36	25.01	44.79
Blood orange juice (Moufida and Marzouk, 2003)	nd.	99.30	0.25	nd.	nd.	7.47	4.42	nd.	nd.	1.68
Sweet orange juice (Moufida and Marzouk, 2003)	nd.	301.88	0.06	nd.	nd.	2.45	22.81	nd.	nd.	nd.
Lemon juice (Moufida and Marzouk, 2003)	nd.	111.07	0.02	nd.	nd.	1.80	0.39	nd.	nd.	nd.
Bergamot juice (Moufida and Marzouk, 2003)	nd.	243.93	34.43	nd.	nd.	–	4.75	nd.	nd.	nd.
Bitter orange juice (Moufida and Marzouk, 2003)	nd.	89.41	1.39	nd.	nd.	0.58	2.65	nd.	nd.	0.16
Key lime juice (Lubinska-Szczygeł et al., 2018)	0.55	50.50	3.45	3.38	20.91	0.68	1.04	24.89	19.41	1.96
Mandarin juice (Pérez-López and Carbonell-Barrachina, 2006)	nd.	59.20	0.17	nd.	nd.	nd.	0.21	1.03	0.05	0.03
Citrus peels										
Kaffir lime	66.02	63.24	34.10	18.39	5.51	0.67	1.54	13.79	13.36	11.26
Mexican lime (Gancel et al., 2002)	10.00	8778.00	62.00	17.00	700.00	95.00	486.00	39.00	nd.	42.00
Star Ruby Grapefruit (Gancel et al., 2002)	7.00	14880.00	22.00	tr.	18.00	12.00	84.00	60.00	1.00	nd.
Mexican lime and Star Ruby Grapefruit hybrid (Gancel et al., 2002)	69.00	7934.00	56.00	nd.	204.00	15.00	61.00	88.00	1.00	1.00
Lemon (Vekiari et al., 2002)	0.98	270.90	4.60	0.44	81.90	7.36	14.90	18.90	78.30	1.04
Shikuwasa (Asikin et al., 2012)	nd.	488.20	4.40	nd.	nd.	0.70	88.00	12.20	153.70	0.50
Orange (Fan et al., 2009)	0.04	5.92	nd.	0.39	0.11	0.05	0.12	0.44	0.02	nd.

Table 4
Antioxidant and binding properties of limes and monoterpenes in water extract.

Indices	Kaffir lime pulp	Kaffir lime peel	Terpinen-4-ol	Citronellal
Polyphenols, mgGAE/g DW	22.14 ± 1.5[b]	39.85 ± 3.1[a]	5.75 ± 0.8 [d]	5.93 ± 0.7[d]
FRAP, mMTE/g DW	27.40 ± 3.6[b]	48.65 ± 4.6[a]	7.11 ± 0.8[d]	7.32 ± 0.6 [d]
Binding to HSA, %	32.4 ± 1.2[b]	55.75 ± 5.5[a]	16.1 ± 1.7 [b]	16.64 ± 1.3 [d]

Values are means ± SD of 5 measurements; Means within a raw with the different superscripts or without superscripts are statistically different ($p < 0.05$; Student's *t*-test). Abbreviations: GAE, gallic acid equivalent; FRAP, Ferric-reducing/antioxidant power; TE, trolox equivalent; HSA, human serum albumin.

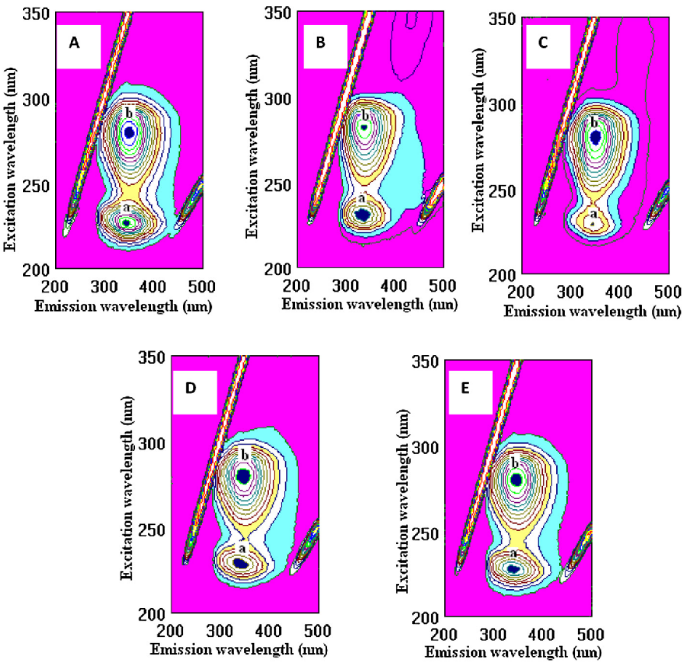

Fig. 4. Corresponding cross spectral images of water extracts of polyphenols and volatile standards in interaction with HSA, A–E, HSA, Kaffir lime peel, Kaffir lime pulp; HSA, Kaffir lime, Key lime. The values of peaks a, b were the measurement s of decreasing of fluorescence intensity during interaction. HSA, human serum albumin (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

chemistry. It was possible to assure relatively higher sensitivity of the proposed method, based on the use of HS-SPME/GC × GC-TOFMS, comparing to application of other common analytical techniques. Quantitation was also the basis for calculation of OAV, which is one of the main criteria during fragrance designing of new cosmetics. In the same time, provided investigation was useful to prove the pro-health properties of quantified substances, which is also significant in the aspect of cosmetic manufacturing. Determination of terpene concentration in *Citrus hystrix* fruit, odor activity value and bioactive activity allows for proper designing of the technological process for new cosmetics, such as creams, perfumes or lotions, which integrants origin from natural source.

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6.2 Determination of the Major By-Products of Citrus hystrix Peel and Their Characteristics in the Context of Utilization in the Industry

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Determination of the Major By-Products of *Citrus hystrix* Peel and Their Characteristics in the Context of Utilization in the Industry

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Abstract: Kaffir lime (*Citrus hystrix*) is a popular citrus in Southeast Asia. Despite the growing interest in the peel of the fruit, the leaves are the most frequently used part of the fruit. The aim of the study was to determine the main by-products of the peel, such as pectins, minerals, essential oil, and bioactive compounds, and to evaluate the possibility of using them in various branches of industry. In the study of the essential oil obtained by hydrodistillation performed using the TGA chromatography technique (GC-MS), sabinene (31.93%), β-pinene (26%), and limonene (19%) were selected as the most abundant volatile compounds. Nine microelements (Fe, Zn, Cu, Mn, Co, Ni, Cr, Mo, and V), four macroelements (Mg, Ca, K, and Na), and seven ballast substances (Cd, Hg, Pb, Al, V, Sr, and Pt) were also determined using the microwave plasma-atomic emission spectrometry technique (MP-AES). In the case of microelements, iron 32.72 ± 0.39 mg/kg DW (dry weight) had the highest concentration. In the case of macroelements, the calcium content was 9416 ± 34 mg/kg DW. Optimization of the pectin extraction was also performed by selecting citric acid and obtaining a yield of 7.6–17.6% for acid extraction and 9.9–28.2% for ultrasound-assisted extraction (UAE), depending on the temperature used. The obtained pectins were characterized by the degree of methylation, galacturonic acid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, and DSC (differential scanning calorimetry) analysis. Among bioactive compounds, the contents of polyphenols (22.63 ± 2.12 mg GAE/g DW), flavonoids (2.72 ± 0.25 mg CE/g DW, vitamin C (2.43 ± 0.19 mg Asc), xantoproteins + carotenes (53.8 ± 4.24 ug), anthocyanins (24.8 ± 1.8 mg CGE/kg DW), and chlorophylls A and B (188.5 ± 8.1, 60.4 ± 3.23 µg/g DW) were evaluated. Antioxidant capacity using (cupric ion-reducing antioxidant capacity) CUPRAC and DPPH assays was also provided with the results of 76.98 ± 8.1, and 12.01 ± 1.02 µmol TE/g DW, respectively.

Keywords: kaffir lime; by-products; essential oil; pectins; bioactive compounds; antioxidant activity; thermodynamic properties; minerals

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1. Introduction

Citrus fruits belong to the group of the largest crops in the world, with an annual production of more than 146 million tons [1]. The largest world citrus producers are China, Brazil, the USA, India, and Mexico [2]. Almost 33% of crops, especially oranges, are industrially processed for juice production. However, more than half of the processed citruses, including peel, are citrus waste, a possible source of environmental pollution [3]. The problem is not only post-production waste, but also fruits discarded for commercial reasons and fruit discarded because of regulations that limit production [4]. The main

ways to use citrus waste are the production of biofuels, in particular, ethanol and biogas; in essential oils; or in the production of cattle feed [5,6]. Valorization of citrus peels for the recovery of by-products seems to be economically and environmentally reasonable. Despite that there are some limitations of citrus peels in industrial applications, such as instability, poor water solubility, and low bioavailability, the future trends seem to be promising [7].

There are also fruits in which pulp and juice are not directly consumed because their taste is too pungent. An example of such a fruit is kaffir lime (*Citrus hystrix*), popular in Asian countries. The leaves are the most frequently used part of the plant in the context of culinary purposes [8]. The outer peel is used to make curry paste. Thanks to the pleasant sensory properties and a large amount of fruit remaining after collecting leaves, kaffir lime fruits can often be found in toilets, where they are used as air fresheners. The candied kaffir lime peel can be found in Cambodia, but it is not very popular. In addition to its pleasant sensory properties, dried kaffir lime peel is an excellent additive to cakes or desserts. Kaffir lime has been used in traditional medicine for treating various illnesses, for example, cold pain [9]. This is confirmed in the literature, where the potential therapeutic effects of *Citrus hystrix* DC on metabolic disorders have been demonstrated [10]. Kaffir lime essential oils are utilized in the production of cosmetics [11]. According to recent literature reports, kaffir lime essential oil peel is safe for the skin in an in vitro model [12]. There are also many articles about the characteristics and use of the leaves of this fruit [13]. However, the research area on kaffir lime peel still needs to be completed.

The inner part of the citrus peel is also a rich source of pectins, which constitute about 30% of its content. Although pectins were discovered more than 200 years ago, their physicochemical and structural properties are still under investigation owing to the great diversity of this family of polymers and the close relationship between structure and function. More and more research is being carried out because of the new sources and conditions of pectin extraction. Plant raw materials are increasingly used for pectin extraction. The pectin content and their characteristics depend on the plant species from which the pectin is isolated and the plants' age or degree of maturation. Lemon peel is particularly rich in pectin, from which 36.71% was extracted [14]. Owing to the thick albedo, good pectin yields have also been obtained from pomelos [15]. Thick, wrinkled kaffir lime's peel appears to be a very promising source of pectin. The composition of pectins is strongly related to their source, extraction, and purification method [14]. Pectins for specific applications are selected depending on their composition and structure (percentage of galacturonides and neutral sugars, degree of esterification, and so on). According to reports, polysaccharides from plants have been considered as a novel potential antioxidant source thanks to their low toxicity and high level of antioxidant capacity, such as their radical scavenging abilities [16]. DPPH radical scavenging assay has been widely used for the determination of the antioxidant activity of pectins [17]. An extremely important aspect of determining pectin properties is also their thermodynamic characteristics, which affect their quality in the final application. Considering the importance of the structural and physical properties in the functionality of pectins, it is important to provide their characterization.

Citrus peels are also good sources of bioactive compounds, such as polyphenols, flavonoids, anthocyanins, or vitamins, showing many properties [18]. Earlier literature reports showed that kaffir lime peel contains a similar polyphenol content to the most commonly consumed orange peel [8,19]. These attributes are commonly used while producing new dietary supplements, cosmetics, or medicines containing plant ingredients. Another important group of compounds present in citrus waste are micro- and macroelements. Although there are no processes aimed at obtaining minerals from citrus waste, the knowledge of their content is important in the context of using citrus waste as animal feed. All these compounds are necessary for the proper development of organisms and are involved in many metabolic processes. According to the best of our knowledge, there is a lack of scientific reports about the content of micro- and macroelements of kaffir lime peel and the state of knowledge of bioactive compounds of kaffir lime has not been exhausted yet.

In this study, major functional components from the by-products of kaffir lime peels, such as essential oils, pectins, micro- and macroelements, and bioactive compounds (polyphenols, flavonoids, vitamin C, anthocyanins, chlorophyll A and B, carotenoids (xanthophylls + carotenes)), were evaluated and characterized. Determination of antioxidant activity FRAP and CUPRAC was carried out. The results provide a full characteristic of the peel of kaffir lime. It is the background for potential industrial applications. Getting to know the health benefits of consuming kaffir lime peel products could contribute to the spread of this fruit, not only in Asian countries. The insight provided could be useful in food science and technology.

2. Results and Discussion

2.1. Essential Oil

2.1.1. Essential Oil Extraction Efficiency

Hydrodistillation is one of the most simple and still the most common methods for the extraction of essential oil (EO) from plant materials. It allows obtaining a relatively high yield of obtained oil with the use of an eco-friendly solvent. Though raw materials are directly immersed in boiling water, the water acts as a protective barrier and prevents the extracted essential oil from overheating to a certain extent [11]. In earlier studies that used the hydrodistillation technique to obtain essential oils from fresh kaffir lime peel, the yield was 0.16–2.10% [20,21]. On the other hand, in our research, 1.78% of the oil was obtained, which is a very good result compared with previous studies. Hongratanaworakit et al. obtained 1.5% of the essential oil by distilling it for 2 h [22]. However, it should be remembered that the efficiency of obtaining essential oils depends not only on the conditions of the extraction process, but also on the harvesting regime, drying mode, and storage period [23]. Among the Citrus species, *C. sinensis* exhibited the maximum oil yield (0.24–1.07%) followed by *C. reticulata* (0.30–0.50%) and *C. paradisi* (0.20–0.40%). So, the kaffir lime is a promising source of essential oil production in terms of the amounts of oil extracted.

2.1.2. Essential Oil Composition

The second important feature when selecting raw materials for obtaining EOs is their composition. Citrus fruits' EOs consist mostly of terpenes, which have several bioactive properties, as well as pleasant aromas, thanks to which the oils are widely used in the pharmaceutical and cosmetic industries [24]. Depending on the terpene profile, essential oils have specific health-promoting and aromatic properties, which is a key element in the stage of designing new drugs and cosmetics. The use of kaffir lime oil in the production of cosmetics has become more and more popular in recent years [11].

Some research on kaffir lime oil composition has been provided so far [25]. In most cases, β-pinene, limonene, and sabinene were major chemical compounds [26,27]. It should be remembered that the content of volatile substances in essential oils depends on the geographical origin of the fruit [25]. Warsito et al. determined citronellal as the major component of *Citrus hystrix* essential oils, with small amounts of terpenes described above [28].

The results of the study of the terpene kaffir lime peel oil profile are shown in Table 1. The most abundant terpene was sabinene, with a percentage of 31.9%. This chemical compound, with a woody aroma, has anti-inflammatory, antioxidant, antiviral, anti-diabetic, and anticancer properties [29], which is an important element for the use of oil for industrial purposes. The contents of β-pinene with a herbal aroma (26.3%) and limonene with a citrus aroma (18.6%) are also very high. These three chemicals play a key role in creating the aroma of kaffir lime oil, which is described in the cosmetic industry as fresh and sharp.

Table 1. Chemical composition of kaffir lime peel essential oil.

No.	Chemical Compound *	Composition (%)
1	α-pinene	4.27
2	Camphene	0.17
3	Sabinene	31.9
4	β-pinene	26.3
5	Myrcene	0.81
6	p-cymene	0.79
7	β-phellandrene	9.34
8	Limonene	18.6
9	β-ocimene	0.27
10	Linalool	0.24
11	Citronellal	0.24
12	Terpinen-4-ol	0.23
13	α-Terpineol	0.33
14	Carveol	0.16
15	Citronellol	0.17

* Owing to the applied standard solution, particular terpene isomers were not distinguished.

Other important compounds making up the composition of kaffir lime essential oil are β-phellandrene, α-pinene, myrcene, and p-cymene, belonging to the group of monoterpene hydrocarbons. The results are consistent with the results obtained by Baccati et al. [30], although with a slight predominance of sabinene over β-pinene in our case. As already mentioned, the difference may be due to the geographical origin of the fruit, the degree of ripeness, or the method of extracting essential oils. The content of many terpenes with several bioactive properties makes kaffir lime oil a mixture with many health benefits (Figure 1).

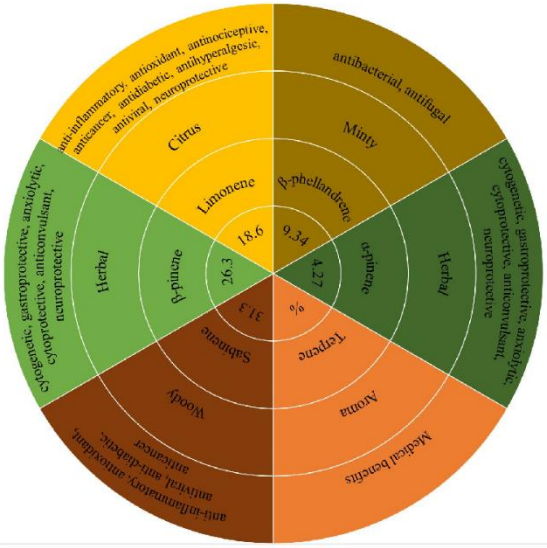

Figure 1. Terpene profile chart of kaffir lime essential oil.

Proven antibacterial and antifungal properties lead to the use of kaffir lime oils in the production of oral sprays, mouthwashes, and acne-control cosmetics [11]. Their use in aromatherapy is also common [11]. Our research is consistent with previous literature reports.

2.2. *Pectins*

2.2.1. Optimization of Pectin's Extraction Conditions

Selection of Temperature and Type of Acid

The figure shows the efficiency of pectin extraction from kaffir lime depending on the extraction conditions and the type of acid used (Figure 2). Based on the conducted research, it can be concluded that the selection of citric acid to obtain the set pH value during pectin extraction seems to be a good solution, as it allows for a very good product yield—22%. When nitric acid was used, an extraction yield of less than 8% was obtained. The results are consistent with those obtained by Shaha et al., where the best efficiency of pectin extraction from microwave-dried kaffir lime was obtained for citric acid [31]. In the case of temperature, along with its increase, the efficiency of pectin extraction increased. A temperature of 90 °C was used as a limit variable in the design of the experiment. In the further part of the experiment, it was not decided to increase the temperature to 90 °C because of the possible degradation of the obtained pectins [32]. Instead, pectins extracted at 60 °C were analyzed.

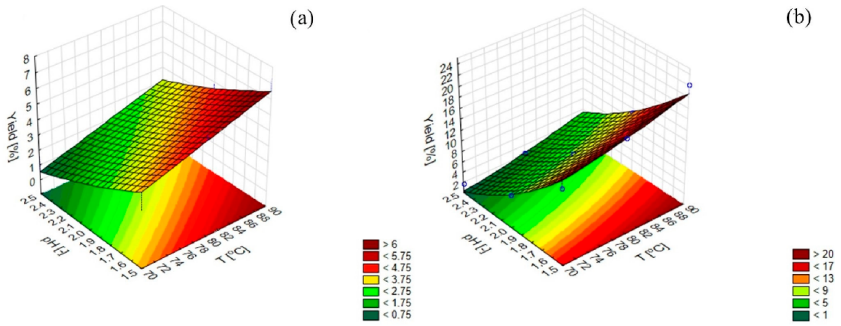

Figure 2. The efficiency of pectin extraction from kaffir lime depending on the extraction conditions for (**a**) nitric acid and (**b**) citric acid.

Determination of the Influence of Ultrasound on the Extraction Efficiency

As mentioned above, in further studies, the extraction temperature was lowered in order not to degrade pectins. Based on the above results, citric acid was selected for extraction.

Pectin yield is one of the most important parameters taken into account while choosing the extraction conditions. The yields of pectins obtained using different extraction conditions are presented in Table 2. Based on the literature research and experiments conducted, citric acid was chosen as an extracting agent, being a better option than nitric acid [31]. In addition, by raising the extraction temperature to 80 °C, a yield of about 18% was obtained. The use of ultrasound enables extractions at lower temperatures, ensuring a higher yield and better quality, but may cause a difference in their chemical structures [33]. In the case of UAE when heated to 60 °C, a yield of 10% was obtained and raising the temperature to 80% allowed to increase the efficiency of this method more than three times. Rodsamran et al. proved a better pectin yield of lime for the conventional method of pectin heating for a higher peel-to-extractant ratio (1:40) for citric acid. The value was 19.63%. Therefore, it seems reasonable to use a high ratio of dried peel to extrahent like in our case (1:50) for citric acid. Sayah et al. studied the yield of pectin from grapefruits and oranges, obtaining results at the level of 22.69–33.69% [34]. In turn, the pectin content of *Citrus reticulata* using subcritical water was determined in a quantity of 19.21–21.95% [35]. Therefore, comparing the pectin content of kaffir lime peel to other citruses, this fruit seems to be a very good source of these chemical compounds.

Table 2. Yields of pectins obtained using different extraction conditions.

Extraction Conditions	Pectin Yield (%)
Acid extraction, 60 °C (60A)	7.6
Acid extraction, 70 °C (70A)	14.1
Acid extraction, 80 °C (80A)	17.6
Ultrasound acid extraction 60 °C (60U)	9.9
Ultrasound acid extraction, 70 °C (70U)	16.0
Ultrasound acid extraction, 80 °C (80U)	28.2

2.2.2. Degree of Methylation

The functionality of pectins in food products largely depends on their degree of methylation and polymerization. High methoxylpectins form a gel at acidic pH (approximately 3) in the presence of a high sugar concentration (approximately 65%), while low methoxylpectins require divalent ions to form gels, but can be used over a wider pH range (3–6) and with a lower sugar content (30–40%). Therefore, owing to dietary restrictions, low methyl pectins are more commonly used in the food industry [36].

The degree of pectin methylation determined by the titration method is presented in the table (Table 3). Based on the results of the analysis, it can be stated that the extraction conditions did not have a significant effect on the degree of esterification of the obtained pectins. In all cases, the degree of esterification was 3–4%. This means that the pectins extracted from kaffir lime belong to the group of low methoxylpectins and would be a good option for the food industry.

Table 3. The degree of esterification of kaffir lime pectins determined by the titration method.

Extraction Conditions	Degree of Methylation (%)
Acid extraction, 60 °C (60A)	3.45 ± 0.51
Acid extraction, 70 °C (70A)	2.81 ± 0.45
Acid extraction, 80 °C (80A)	4.00 ± 0.84
Ultrasound acid extraction 60 °C (60U)	3.81 ± 0.89
Ultrasound acid extraction, 70 °C (70U)	3.87 ± 0.03
Ultrasound acid extraction, 80 °C (80U)	2.91 ± 0.64

2.2.3. Galacturonic Acid Content

Table 4 shows the content of galacturonic acid in the samples of kaffir lime depending on the extraction conditions used. The highest content was recorded in the case of using acid extraction and a temperature equal to 60 °C. As the temperature increased, the content of galacturonic acid in the samples of kaffir lime decreased. Moreover, it was noticed that supporting the extraction with ultrasound also decreased the content of the determined chemical compound. According to the regulations, pectins used in the food industry should contain a minimum of 65% of uronic acid [37], so only the use of a temperature of 60 °C made it possible to meet this criterion. For comparison, orange peel pectins contained 422 mg/g and 1099 mg/g of uronic acid, depending on the extraction method used [37].

Table 4. The content of galacturonic acid in the samples of kaffir lime pectins depending on the extraction conditions used in terms of dry matter.

Extraction Conditions	Galacturonic Acid Content (mg GAL/g)
Acid extraction, 60 °C (60A)	650 ± 75
Acid extraction, 70 °C (70A)	503.2 ± 8.7
Acid extraction, 80 °C (80A)	439.4 ± 6.5
Ultrasound acid extraction, 60 °C (60U)	312.8 ± 2.2
Ultrasound acid extraction, 70 °C (70U)	339.5 ± 1.7
Ultrasound acid extraction, 80 °C (80U)	367 ± 10

2.2.4. DPPH Free Radical Scavenging

DPPH free radical scavenging is a mechanism commonly used for screening the antioxidant activity of plant extracts [38]. The results of DPPH free radical scavenging of pectins extracted from kaffir lime are presented in Figure 3. The antioxidant activities for the extracted pectins with two different methods were increased in a concentration-dependent manner from 1 to 12 mg/mL. It is noticed that there are no several differences between the impact of the temperature of pectin extraction by the conventional method for the antioxidant potential of pectins obtained. However, there was an increase in DPPH• scavenging activity for pectins extracted using ultrasounds at the same temperatures as the conventional method. This is in agreement with previous literature reports that using ultrasound-assisted methods of extraction of pectins affects larger values of antioxidant potential of pectins (higher DPPH• scavenging activity) [39]. Compared with data provided by Gharibzahedi et al., the results for vitamin C at a concentration of 12 mg/mL were much higher (about 85%). On the other hand, in the reported research [40] for solutions of pectin samples from figs with a concentration of 12 mg/mL, a DPPH• scavenging rate of about 60% was obtained. Compared with our results, kaffir lime peel pectins show lower values of these parameters: 33.25 ± 0.17% (80A), 48.13 ± 0.15% (70U), which proves relatively low antioxidant activity. It is thus not suitable for use in biomedical applications. Low antioxidant activity may also indicate the high viscosity of extracted pectins [40]. In turn, that proves the good gelling properties and the possibility of use in the food industry.

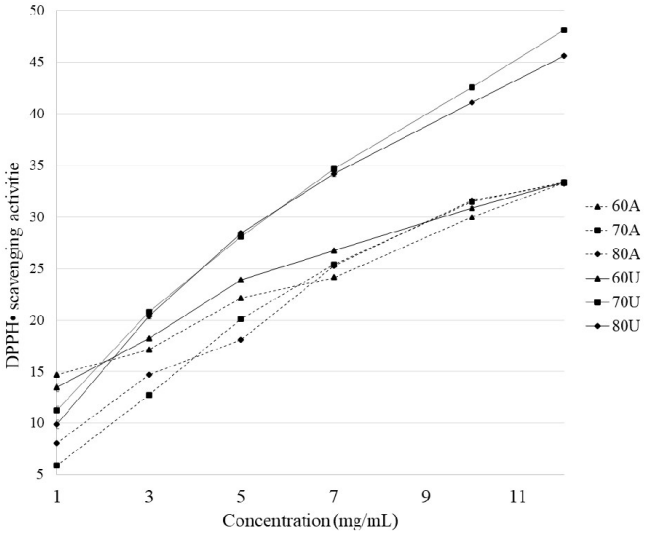

Figure 3. DPPH free radical scavenging of pectins extracted from kaffir lime.

2.2.5. DSC Analysis

In drug development, preformulation plays a key role and provides much information about the product. DSC thermal study is one of the preferred techniques because it provides general information about the thermal transition as well as chemical stability and properties.

The effects of extraction temperature and the addition of ultrasounds in the extraction process on the thermodynamic properties of pectin were examined by DSC between 30 °C and 300 °C. As shown in Figure 2, an endothermic peak and an exothermic peak were observed in the DSC thermograms of all pectin samples. The parameters of the two peaks are listed in Table 5, such as the maximum temperature of the main endothermic peak

(T_{endo}), enthalpy of the main endothermic peak (ΔH_{endo}), degradation temperature (T_{exo}), and degradation enthalpy (ΔH_{exo}).

Table 5. Thermal properties of pectins determined by DSC.

	T_{endo} [°C]	ΔH_{endo} [J/g]	T_{exo} [°C]	ΔH_{exo} [J/g]
Acid extraction, 60 °C (60A)	203.40	334.6	256.21	54.91
Acid extraction, 60 °C (60U)	204.77	191.1	255.87	86.97
Acid extraction, 70 °C (70A)	204.15	320.5	253.49	70.49
Acid extraction, 70 °C (70U)	200.49	276.5	254.42	67.33
Acid extraction, 80 °C (80A)	204.26	151.5	256.82	65.52
Acid extraction, 80 °C (80U)	201.23	262.4	256.84	77.73

T_{endo}, temperature of main endothermic peak (°C); ΔH_{endo}, enthalpy of main endothermic peak (J/g); T_{exo}, temperature of degradation (°C); ΔH_{exo}, degradation enthalpy (J/g).

Because the endothermic phenomenon is assigned to water evaporation [41], a higher melting temperature and melting enthalpy meant more energy was needed to absolutely remove water. There is no clearly visible trend associated with the enthalpy value. The endothermic peak starts above 90 °C, which is connected to water evaporation. In samples extracted at lower temperatures without the addition of ultrasounds, one lower peak (at 130 °C for 60A and 140 °C for 70A—Figure 4) can be seen. For these two samples (60A and 70A), enthalpy is similar and above 300 J/g. Samples after using ultrasound for the extraction have no additional peak and enthalpy is lower between 191.1 J/g and 276.5 J/g for 60U, 80U, and 70U, in order.

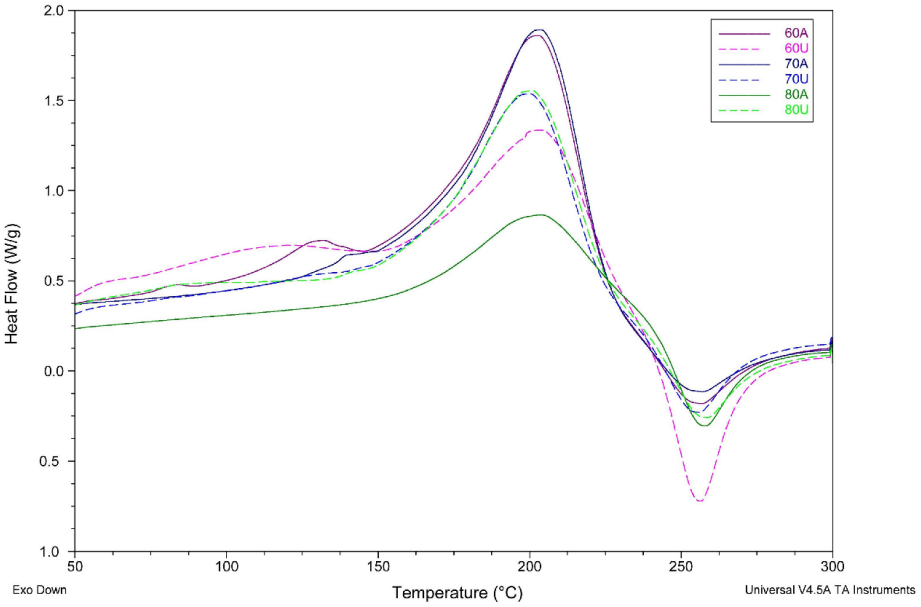

Figure 4. DSC thermograms in the full temperature range of citrus pectins extracted at 60 °C, 70 °C, and 80 °C by (A) citric acid in pH 1.5 and (U) citric acid in pH 1.5 with the addition of ultrasounds, precipitated using methanol.

T_{endo} values ranged from 200.26 to 204.77 °C. The lowest temperature was observed for the sample extracted at 70 °C in citric acid with the addition of ultrasound (70U). The highest temperatures were obtained for the samples without additional ultrasound

treatment (70A and 80A),with the exception of samples extracted at 60 °C (60A and 60U). For these samples, the temperature difference is smaller and the highest temperature of all samples was observed for the sample extracted with the addition of ultrasounds at 60 °C (60U).

The second peak was caused by the degradation of pectin in heat processing [42]. As shown in Table 5, samples showed little differences for T_{exo} from 253.49 °C to 256.84 °C. The temperature T_{exo} range is similar to temperatures reported for exothermic peak for amidated pectins in the literature [41]. Amidated pectins have a lower amount of methoxyl groups, which is consistent with similarities between the literature results of DSC measurements for low-methoxylated pectins [43] and the results for pectin samples extracted for this publication (Figure 5).

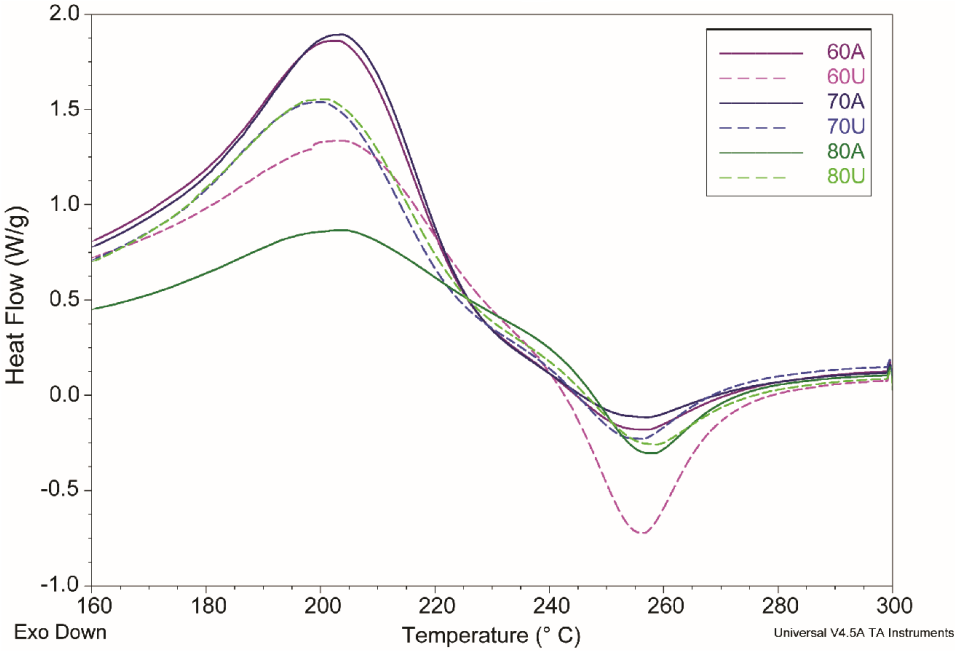

Figure 5. Fragments of DSC thermograms of citrus pectins extracted at 60 °C, 70 °C, and 80 °C by (A) citric acid in pH 1.5 and (U) citric acid in pH 1.5 with the addition of ultrasounds, precipitated using methanol, in the 160 °C–300 °C temperature range.

In Figure 5, it can be noticed that peaks obtained for the pectins described in this paper were more separated than peaks for the low-methoxylated pectins prepared by acidic treatment (LMP-A) described in the literature by Einhorn-Stoll et al. in 2020 [43]. This means lower melting temperatures and a higher amount of water in extracted pectins.

A shift of the DSC curves to a lower temperature (earlier pyrolysis) is characteristic of pectin demethoxylation [41]; this shift was less prominent for samples extracted at higher temperatures or with the addition of ultrasounds (Figure 5). The most prominent shift was observed for the sample 60U, which was extracted at 60 °C with the addition of ultrasounds.

2.3. Bioactive Compounds' Determination and Antioxidant Assays

Citrus fruits are a good source of phytochemical substances. The extraction of these bioactive compounds from citrus wastes can be performed using conventional extraction

techniques (hydrodistillation, maceration, and solvent extraction) and via green extraction approaches (ultrasound-assisted extraction, enzyme-assisted extraction pulse electric field extraction, and microwave-assisted extraction) [44]. The presence of bioactive compounds in citrus waste makes them attractive owing to antimicrobial, anticancer, antidiabetic, antiplatelet aggregation, and anti-inflammatory activities, which is not without significance in the context of use as animal feed. The results of bioactive compound determination and antioxidant potential of kaffir lime peel are presented in Figure 6.

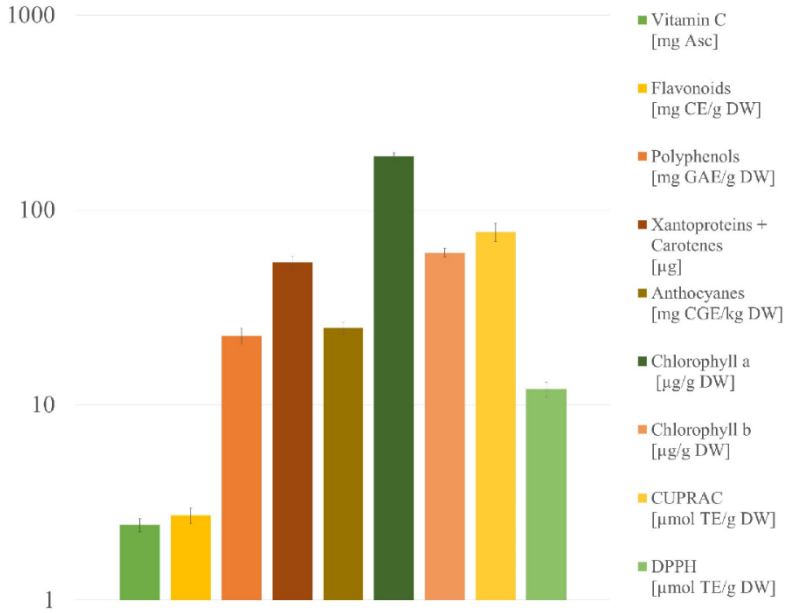

Figure 6. Bioactive compounds and antioxidant potential of kaffir lime peel.

2.3.1. Polyphenols

Polyphenols, as the products of plant metabolism, play a protective role against fungal and bacterial infections. They are an important component of the diet of both humans and animals, so their determination is crucial in the context of using peel wastes as animal feed or natural ingredients for cosmetics. The total polyphenols content (TPC) in kaffir lime was 22.63 ± 2.12 mg GAE/g DW. In our previous reports, the amount of total polyphenols extracted with methanol from kaffir lime juice was 15.79 ± 1.34 mg GAE/g DW [8]. The content of polyphenols in pomelo (*C. grandis*), citron (*C. medica*), mandarin (*C. reticulata*), and grapefruit (*C. Sinensis*) was 14.93 ± 0.21, 8.88 ± 0.34, 23.46 ± 1.19, and 12.68 ± 0.39 mg GAE/g DW, respectively [45]. Kaffir lime peel is thus a better source of polyphenols than juice. Moreover, compared with the peel of other citrus fruits, the amount of total polyphenols in kaffir lime is relatively high.

2.3.2. Chlorophylls

Another important group of bioactive compounds in kaffir lime is chlorophylls. They belong to the group of green natural pigments, commonly used in the medicine and food industries. Thanks to their antioxidant properties, they are used to prepare fortified and functional products. Commercially, chlorophylls are obtained from plants with green leaves, like spinach [46]. Such an approach requires special cultivation of plants for obtaining colorants, designed specifically for this purpose. Recent literature reports mention the use of citrus waste to obtain natural dyes that can be used in industry. The problem with

obtaining pigments from natural raw materials is their high cost. The approach using citrus waste, therefore, seems to be economically and environmentally viable, especially while production would be performed concomitantly with the extraction of pectins or essential oils [47]. Among other citrus fruits, a high total chlorophyll (a + b) content was determined for different lime varieties (*Citrus aurantifolia* Swingle cv. 'Paan')—392.5 µg/g DW [48]. Kaffir lime may be a good source of chlorophylls of all citruses, especially owing to the high content of pectins that can be extracted concomitantly. Kaffir lime waste may be considered as functional animal feed because of its high chlorophyll content, which exerts prominent health benefits when consumed; that is, antioxidant antimutagenic, and antigenotoxic [49].

2.3.3. Flavonoids

Flavonoids, as part of polyphenols, are able to interfere with the structure of the other compound and change its properties, from which their bioactive properties result. For example, they are anti-inflammatory agents because of the diminished formation of proinflammatory mediators (leukotrienes, prostaglandins, and reactive oxygen species) [50]. Citrus fruits, especially their peels, are rich dietary sources of flavonoids. Chatha et al. provided a determination of total polyphenols in citrus fruit peels [51]. Mussambi fruit peel extract contained the highest total flavonoid content (2.98 g CE/100 g DW) among all citruses tested. The results for other citruses were similar and ranged between 2.20 and 2.98 g CE/100 g DW. In our research, kaffir lime peel extract contained 2.72 mg CE/g DW. Thus, compared with other citruses, kaffir lime peel is an average source of flavonoids. However, when the peel is used as a waste animal feed, any content of these compounds adds to its value. It is worth mentioning that apple pomace, commonly used as animal feed, contained 1.19 ± 0.058 mg RE/g DW [52]. In this context, kaffir lime waste is a promising source of flavonoids.

2.3.4. Anthocyanins

Anthocyanins, as part of flavonoids, show many bioactive functions. Research works showed that anthocyanin-rich dried fruits have positive effects on health-promoting markers in humans and other animals, and that is why they may find application in the poultry feed industry [53] Although citrus fruits are not the best sources of anthocyanins, while they are natural purple pigments, much research on this area is provided. Gorinstein et al. determined 4.5 ± 0.3 and 17.5 ± 1.5 mg CGE/kg DW in grapefruit juice and peel, respectively [54]. In our previous research, kaffir lime juice was analyzed and contained 63.45 ± 5.15 mg CGE/kg DW [8]. In turn, in this research, the anthocyanin content in kaffir lime peel content was determined to be 24.8 ± 1.8 mg CGE/kg DW..

2.3.5. Carotenoids (Xanthophylls and Carotenes)

Carotenoids are the pigments responsible for the color of many citrus fruits. Citrus is a complex source of these compounds, with the biggest number of carotenoids found in any fruit [55]. Their content in flavedo depends on the maturity and thus the color of the fruit. Among the Citrus family, mandarin's (*Citrus reticulata*) peel shows a high content of total carotenoids, that is, 2143 ± 25.24 µg/g DW [56]. Moreover, sweet orange peel is a good source of carotenoids, 31.57 ± 0.06 µg/g DW. [57]. *C. limon* (L.) Bur, commonly added to dishes, contains 110.0 ± 1.0 24 µg/g DW. Based on our research, it was stated that kaffir lime peel contains 53.86 ± 4.24 µg/g DW, which is a relatively high value among citrus [3].

2.3.6. Vitamin C

Vitamin C, with a high antioxidant potential, is a common ingredient in cosmetic products. It is also necessary for the proper functioning of the body, so its content in animal feed as well as in diet supplements is highly recommended. Thanks to its antioxidant potential, it can be used topically in dermatology to treat and prevent changes associated with photoaging [58]. Citrus fruit juice is one of the best sources of vitamin C. Among all citruses, key lime peel is found to be a rich source of vitamin C (1.779 ± 0.78 mg Asc/g) [57].

Based on our research, kaffir lime peel showed a higher content of vitamin C, that is, 2.43 ± 0.19 mg Asc/g. Kaffir lime peel extract can be successfully used in cosmetics and drugs as a source of natural vitamin C.

2.3.7. DPPH Free Radical Scavenging and CUPRAC Cupric Reducing Antioxidant Capacity

The antioxidant capacity of fruits is an important indicator of their in vitro potential as health promoters. The CUPRAC test assesses the ability of antioxidants in a sample to reduce Cu^{2+} to Cu^{+} in the presence of a chelating agent. The DPPH test is used to predict the activity of antioxidants through the mechanism by which they inhibit lipid oxidation, i.e., by scavenging DPPH radicals. The DPPH value for kaffir lime peels was 12.02 ± 1.02 µmol TE/g DW. CUPRAS assay results were 76.98 ± 8.1 µmol TE/g DW. *Citrus sinensis* (L.) Osbeck and *C. limon* DPPH values were 18.20 ± 1.62 and 23.07 µmol TE/g DW, respectively [19,59], so the kaffir peel lime antioxidant capacity is slightly lower than other citruses' peels. The situation is slightly different in the case of the CUPRAC test, where the values for the kaffir lime peel were higher than for other citrus fruits' peels (54.8 ± 2.34 µmol TE/g DW for *Citrus reticulata* and 52.31 µmol TE/g DW for *C. paradisi* [60,61]). These results provide an alternative way to make good use of kaffir lime peel to utilize it as a natural antioxidant source.

2.4. Micro and Macroelements

Chemical elements found in living organisms can be divided into microelements, macroelements, and ballast elements. Although the isolation of micro- and macroelements from citrus fruits has not been carried out so far, the current trends do not exclude this possibility, which is a promising prospect. According to Barros et al., citrus fruits are promising sources of mineral elements [62]. The presence of minerals in citrus waste makes it a good substrate for the production of bio-fertilizers. The results of the determination of selected elements in kaffir lime peel are presented in Table 6.

In the case of microelements, their importance lies in the regulation of the activity of enzymes, hormones, vitamins, and other factors determining the course of metabolic processes in organisms. In the samples of kaffir lime peel, it was possible to quantitatively determine five trace elements, i.e., iron, zinc, copper, manganese, and molybdenum. The remaining compounds in this group were below the limit of detection or quantification. Iron had the highest content, i.e., 32.72 ± 0.39 mg/kg DW. To compare, *Citrus maxima* peel extract contains 9.06 ± 0.79 mg/100 g [63]. Iron plays an important role in the supply of oxygen to the organs and muscles of living organisms. Although citrus is not the main dietary source of iron, its presence in fruit intended for animal feed is an added benefit. Second in terms of the content of kaffir lime in the peel, zinc is a recommended micronutrient in fertilizers for the production of corn [64]. The content of zinc was 16.09 ± 0.14 mg/kg DW. The high content of microelements is also an important aspect of the production of dietary supplements using plant materials. Therefore, kaffir lime peel is a good raw material for use in the food, agriculture, pharmaceutical, and cosmetic industries.

Macroelements are building materials for proteins, lipids, sugars, nucleotides, the skeletal system, and the external skeleton of animals. They have been widely used in the prevention and treatment of many diseases [65]. Among the macroelements, the most abundant elements in the peel extract were potassium and calcium, with a concentration of 10,820 ± 130 and 9416 ± 34 mg/kg DW, respectively. This is consistent with other studies. Dibanda Romelle et al. showed that orange and pomegranate peels had calcium as the most abundant mineral analyzed [66]. The high content was also determined in the case of sodium and magnesium, i.e., 1500 ± 28 and 1050.3 ± 7.3 mg/kg, respectively. To compare, *Citrus maxima* peel extract contains 46.12 ± 1.46 and 1.88 ± 0.05 mg/100 g, respectively [63]. The high content of these elements in kaffir lime citrus waste makes them good food for cattle. In turn, the high content of potassium is important in the context of bio-fertilizers, as potassium is one of the basic plant nutrients.

Table 6. Results of the determination of the content of selected elements in kaffir lime peel ($x_m \pm U$, ($k = 2$)).

Microelements Concentration ± U [mg/kg]	
Fe	32.72 ± 0.39
Zn	16.09 ± 0.14
Cu	4.518 ± 0.039
Mn	7.008 ± 0.029
Co	<LOQ
Ni	<LOD
Cr	<LOQ
Mo	0.295 ± 0.017
V	<LOD
Macroelements Concentration ± U [mg/kg]	
Mg	1050.3 ± 7.3
Ca	9416 ± 34
K	10820 ± 130
Na	1500 ± 28
Ballast Element Concentration ± U [mg/kg]	
Cd	<LOD
Hg	0.0145 ± 0.0011
Pb	<LOD
Al.	77.46 ± 0.33
Ba	16.52 ± 0.29
Sr	16.16 ± 0.22
Pt	<LOD

Ballast elements are chemical elements without a known function in metabolism [67] often harmful to humans, and often toxic at higher concentrations. Heavy metals are also among them. The elements are derived directly or indirectly from the polluted environment. In the kaffir lime peel, all of the ballast elements were found in trace amounts without particular importance for the use of citrus waste in the industry. In the case of aluminum and strontium, the concentrations were 77.46 ± 0.33 and 16.16 ± 0.22 mg/kg DW, respectively. Their oral exposure is usually not harmful, so their presence is not a limit while producing animal food from kaffir lime wastes. The presence of mercury and barium is undesirable, but the amounts indicated are relatively small.

3. Materials and Methods

3.1. Materials and Reagents

Kaffir lime fruits were bought in Thailand by the local distribution point in the Pomeranian Voivodship in December 2019 and transported to the laboratory in refrigeration conditions. Fruits for the analysis were taken from four batches, each composed of 70–80 pieces (about 3 kg). Fruits for analysis were washed with tap and distilled water and peeled manually. Fresh peel has undergone further preparation processes, such as drying, freeze-drying, mineralization, extraction, or hydrodistillation. D-(+)-galacturonic acid monohydrate, sulphamic acid, 3-phenylphenol, terpenes mix in methanol, Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid), Folin–Ciocalteu reagent (FCR), gallic acid, 20-azobis-2-methyl-propanimidamide, $FeCl_3 \cdot 6H_2O$, Folin Ciocalteu reagent (FCR), lanthanum (III) chloride heptahydrate, $CuCl_2 \cdot 2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine), 1,1-diphenyl-2- picrylhydrazyl (DPPH), potassium persulfate, and terpenes standards were purchased from Sigma-Aldrich (St. Luis, MO, USA). Methanol, citric acid, sodium hydroxide, and nitric acid were purchased from Avantor Performance Materials Poland S.A. K, Ba, Ca, Cd, Co, Cu, Zn, Ni, Pb, Pt, V, and Mo standards at a concentration of 1000 ± 2 mg/L; Mg standard solution at a concentration 1006 ± 4 mg/L; Fe standard at a concentration 1001 ± 2 mg/L; and Al standard at a concentration 998 ± 5 mg/L were obtained from

Sigma-Aldrich (Darmstadt, Germany). Na standard at a concentration of 10,000 mg/L and Sr standard at a concentration of 1005 ± 5 mg/L in 4% HNO_3 were purchased from MS Spectrum (Poland). Cr standard at a concentration of 1003 ± 3 µg/mL and Mn standard at a concentration of 1000 ± 6 µg/mL were purchased from CPI INTERNATIONAL (Santa Rosa, CA, USA). Nitric acid (65–70% purity) was obtained from Alfa Aestar (Regensburg, Germany). Mercury standard-MSHG at a concentration of 100.10 ± 0.43 µg mL^{-1} in 10% HCl was purchased from Inorganic Ventures, INC (Christiansburg, VA, USA). N-acetyl-L-cysteine was obtained from Sigma Aldrich (Germany). All other chemicals used in the study were of analytical grade.

3.2. Essential Oil Compositions

3.2.1. Hydrodistillation

The essential oil was extracted from fresh kaffir lime peel by the hydrodistillation process. Here, 500 g of kaffir lime peel was crushed and extracted in glass distillation apparatus for 3 h and then separated in a laboratory glass separator. The extraction process was performed three times and the obtained products were combined. To enhance the yield of the extraction, a salt effect using NaCl treatment was performed. The essential oil was dried under anhydrous sodium. The yield of the oil obtained was calculated as a percentage. The essential oil was stored at 4 °C until further use.

3.2.2. GS–MS Analysis

The GC–MS system (Shimadzu, Kyoto, Japan) consisted of a GCMS-QP2010Plus gas chromatograph mass spectrometer. Separation of the oil was performed using DB WAX 52 CB (Agilent Technologies, Santa Clara, CA, USA) chromatographic column (30 m × 0.25 mm id, film thickness 0.25 m). Hydrogen was used as the carrier gas at a constant flow rate of 24.1 mL/min. Injector and MS transfer line temperatures were set at 175 and 220 °C, respectively. The ion source temperature was 220 °C. The initial temperature was kept at 40 °C for 2 min and then gradually increased to 90 °C at a rate of 5 °C/min, then to 220 °C at a rate of 30 °C/min, and was held for 7 min. The total time of analysis was 23.23 min. The samples were injected into the GC–MS system in the split mode (split ratio of 15). The identification of the components was performed based on comparing retention times to the retention times of standards. The relative peak areas of each component in essential oil were calculated by normalization of the peak areas as the percentages of the total essential oil component. The results were all expressed as mean ± SD.

3.3. Pectins' Characteristics

3.3.1. Extraction

Acid extraction (AE) was used for the isolation of pectins from plant material. Fruits for pectin esterification were washed with tap and distilled water. Peel was manually separated for the pulp, cut into small pieces, and dried at 40 °C for 48 h. The dried peel was ground to a powder. For the extraction, 3 g of grounded power was weighed and 150 mL of distilled water was added (solid/liquid ratio 1:50). In order to select the best extraction conditions, an experiment determining the influence of two independent parameters, such as temperature (70–90 °C) and pH for nitric and citric acid on the extraction yield of kaffir lime pectin, was performed.

The pH of the solutions was adjusted to the desired values using citric and nitric acid. Samples were heated under cover at 70, 80, and 90 °C for 1 h. After heating, an equivalent amount of cooled methanol was added to precipitate the pectins and left for 24 h. The coagulated pectins were centrifuged at 10,000× *g* for 30 min and washed with methanol until the pH was neutral. Pectins were dried at 40 °C until a constant mass. For further use, pectins were powdered. In the case of ultrasound extraction (UAE), the same sample preparation and conditions as in acid extraction were applied with the addition of

ultrasound treatment. Pectin yield was calculated as the ratio of the mass of pectin and the sample taken for extraction.

3.3.2. Degree of Methylation

The degree of methylation (DM) of pectin was determined using the titrimetric method of Rodsamran [68]. Here, 0.1 g of dried pectin sample was moistened with 2 mL ethanol and dissolved in 20 mL water at 40 °C. After the pectin solution was completely dissolved, the sample was titrated with 0.1 M NaOH against phenolphthalein at the end-point (V_1). Afterward, 10 mL of 0.5 M NaOH was added, the mixture was shaken vigorously, and then left for 20 min. After that, 10 mL of 0.5 M HCl was added and titrated with 0.1 M NaOH to a faint pink color that persisted after vigorous shaking (V_2). The DM was calculated using the following equation:

$$DM\% = \frac{V_1}{V_1 + V_2} \cdot 100\% \quad (1)$$

3.3.3. Galacturonic Acid Content

Galacturonic acid content was determined based on the Melton method [69]. Here, 5 mg of dried pectin samples were hydrolyzed with 1 mL of concentrated sulfuric acid for 5 min, under constant stirring, and cooled in an ice bath, and the procedure was duplicated. Then, 0.5 mL of distilled water was added, the mixture was stirred for 5 min, then the next portion of water was added and it was mixed again for 5 min. The mixture was diluted to 10 mL with distilled water in volumetric flasks. The samples were separated in a centrifuge model MPW-352 (MPW MED. INSTRUMENTS, Warsaw, Poland) by centrifuging for 10 min at 2000× *g* at room temperature. The supernatant was used for the colorimetric assay as follows. To 400 µL of supernatant or standard (galacturonic acid in water), 40 µL of 4 M sulfamic acid/potassium sulfamate solution (pH = 1.6) was added and vortexed. Then, 2.4 mL of 75 mM sodium tetraborate/sulfuric acid solution was added. The mixture was heated (100 °C) for 20 min and then cooled in ice. Then, 80 µL m-hydroxydiphenyl solution was added to the sample and reagent control tubes. To the sample control, 80 µL 0.5% NaOH was added to determine the sugar coloring. Samples were then vortexed and the absorbance was measured after 10 min at 525 nm in a DR3900 Benchtop VIS Spectrophotometer with RFID Technology (Hach Company, Loveland, CO, USA) against the reagent control (a mixture of reagents from the above procedure without samples of pectins). The determination was performed in three repetitions.

3.3.4. DPPH Free Radical Scavenging

The antioxidant potential of pectins was determined by the DPPH assay using the method previously reported in the literature [39]. In brief, 1 mL of pectin's solutions of concentrations of 1, 3, 5, 7, 10, and 12 mg/mL were mixed with 0.2 mM DPPH methanol solution and incubated for 30 min at 24 °C in the dark. The absorbance was read at 517 nm using a Hach DR3900 spectrophotometer (Hach Company, Loveland, CO, USA) against methanol. The control was prepared by replacing the DPPH solution with anhydrous methanol.

3.3.5. DSC Analysis

Differential scanning calorimetry (DSC Q20, TA Instruments-Waters LLC, New Castle, DE, USA) was used to investigate the thermal properties of the pectins according to the method previously described [35]. Then, 5 mg of dried pectin sample was added into a standard aluminum crucible and sealed by a non-hermetic seal. The crucible was heated from 40 °C to 300 °C at a heating rate of 10 °C/min in a dynamic inert nitrogen atmosphere (75 mL/min). Simultaneously, an empty standard aluminum crucible was used as a reference. Extrapolated peak temperature and enthalpy were calculated with the TA Universal Analysis software, as shown in Figure 7.

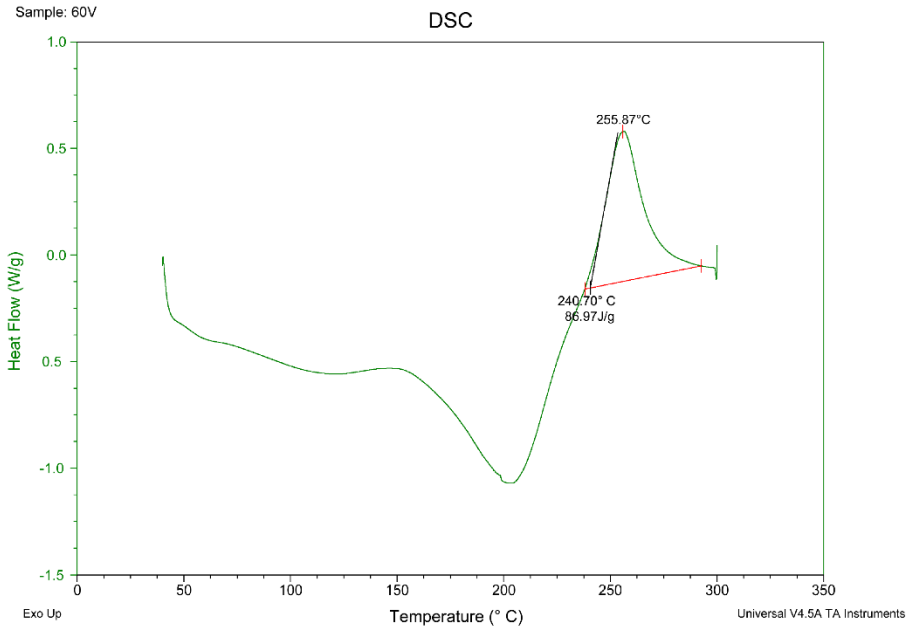

Figure 7. Calculation of the degradation temperature and enthalpy in DSC curves, based on 60U sample.

3.4. Bioactive Compounds' Determination and Antioxidant Assays

Polyphenols were determined using the method described in our previous research with slight modifications [18]. The freeze-dried powders of investigated samples were immersed in methanol (1/10 *w/v*). The filtrate was collected three times with constant stirring of the mixture at every 24 h interval of a 72 h total collection period at room temperature. The extract was then concentrated under reduced pressure at 45 °C using a vacuum rotary evaporator. Then, lyophilized peel samples were determined by the Folin–Ciocalteu method. The absorbance was measured at 750 nm. The results were expressed as mg of gallic acid equivalents (GAEs) per g of dry weight (DW).

Flavonoids were determined by the method described by Papotuis [70]. Samples were extracted using methanol in ultrasounds for 20 min (vortexed every 5 min for 10 s) and centrifuged. Appropriate amounts of H_2O, 5% $NaNO_2$, 10% ACl_3, and 4% NaOH were added and the absorbance was measured at 510 nm using the Hach DR3900 spectrophotometer (Hach Company, Loveland, Colorado, United States). The results were expressed as mg of catechin equivalents (CE) per g of the dry weight of the sample.

For anthocyanin determination, triple extraction with 15 mL of 80% methanol (pH = 2) for 10 min was performed. After each extraction, samples were centrifuged for 5 min and supernatants were collected. Then, 1 mL of combined extracts was filtered through a syringe filter. Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5 and calculated using the following formula: $A = (A_{520nm} - A_{700nm})_{pH1{\cdot}0} - (A_{520nm} - A_{700nm})_{pH4{\cdot}5}$ with a molar extinction coefficient of cyanidin-3-glucoside of 29,600 and a molecular weight of 449.2 g/mol. The results were expressed as milligrams of cyanidin-3-glucoside equivalent (CGE) per g of DW.

Total carotenoids (xanthophyll + carotenes) were extracted with 100% acetone and determined spectrophotometrically at 454 nm, while chlorophylls were extracted with 85%

acetone and determined spectrophotometrically at 660 and 642.5 nm [71]. The results were expressed in µg.

For the CUPRAC assay, 1 mL of copper (II)-neocuproine and NH4Ac buffer solution, extract of sample (or standard) solution, and H_2O was added to a final volume of 4.1 mL. The absorbance at 450 nm was read against a reagent blank. The results were expressed as µmole Trolox/g DW.

Scavenging free radical potentials were analyzed in 3.9 mL of methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) with the samples of lime peel extracts in methanol (0.1 mL). The results were expressed as µmole Trolox/g DW [72].

Total ascorbic acid was determined by CUPRAC assay in water extract (20 mg/mL). The absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm [73].

3.5. Micro- and Macroelements' Content

In order to determine the content of selected elements in the samples, they were subjected to microwave-assisted mineralization. For this purpose, about 1 g of the sample was weighed into a reaction vessel. Then, 8 mL of HNO_3 was added to each reaction vessel. Mineralization proceeded for the first 20 min at 100 °C and the next 20 min at 180 °C. Then, the mineralized samples were placed in 25 mL flasks and supplemented with deionized water to the dash. After mixing, each solution was poured into stoppered plastic tubes. The samples prepared in this way were analyzed using atomic emission spectrometry. The 4210 MP—AES supplied by Agilent was used. Millipore—Milli-Q Water Purification System (USA) and Anton Paar Multiwave Go microwave mineralizer were used. Mercury/MA-3000 supplied by Nippon Instruments Corporation (NIC, Osaka, Japan) was used to analyze mercury using the cold vapor technique and purified dry air was used as the carrier gas.

Determinations were made at several wavelengths for each element. The final choice of the wavelength at which the determination was made was determined by the value of the coefficient R^2 for the calibration curve. The results were expressed as mg/kg DW. Validation parameters are presented in Table 7.

Table 7. Validation parameters of the procedure for the determination of the selected elements in lime samples.

Analyte	WAVELENGTH [nm]	LOD [mg/kg]	LOQ [mg/kg]	Linearity					
				Calibration Range [mg/kg]					
				Min.	Max.	Points	Rep.	Calibration Curve	R^2
Microelements									
Fe	371.993	0.33	1.0	1.0	100	8	4	y = 5510x − 1049	0.9997
Zn	213.857	0.19	0.58	0.58	10	9	4	y = 12014x + 96	0.9995
Cu	327.395	0.026	0.077	0.30	20	6	4	y = 44555x − 1626	0.9999
Mn	403.076	0.0064	0.019	0.019	1.0	5	4	y = 28990x + 44	0.9999
Co	345.351	0.012	0.035	0.050	1.0	5	4	y = 13331x − 2.4	0.9999
Ni	361.939	0.0070	0.021	0.10	20	7	4	y = 5637x − 338	0.9999
Cr	425.433	0.0027	0.0082	0.01	10	8	4	y = 29402x + 29	0.9999
Mo	386.410	0.0060	0.018	0.018	20	9	4	y = 15860x + 29	0.9995
V	437.923	0.0057	0.017	0.017	20	9	4	y = 7795x + 42	0.9997
Macroelements									
Mg	279.553	0.40	1.2	1.2	40	6	4	y = 152325x + 37340	0.9996
Ca	430.253	2.0	6.0	10	250	6	4	y = 898.5x + 1376	0.9995
K	766.491	0.16	0.48	2.5	20	4	4	y = 48347x − 16941	0.9997
Na	568.263	1.1	3.3	10	200	5	4	y = 34.5x − 34.1	0.9998
Ballast substances									
Cd	228.802	0.022	0.066	0.066	20	8	4	y = 23459x − 525	0.9998
Hg	253.700	0.00096	0.0029	0.0029	0.10	10	3	y = 1.001x − 0.15	0.9999
Pb	405.781	0.012	0.035	0.050	5.0	6	4	y = 2776x − 77	0.9999
Al	396.152	0.088	0.26	1.0	100	8	4	y = 20008x + 632	0.9998
Ba	493.408	0.21	0.63	0.63	3.0	4	4	y = 20318x + 5708	0.9962
Sr	421.552	0.0045	0.013	0.013	40	6	4	y = 29277x + 58	1.0000
Pt	265.945	0.075	0.23	0.40	4.0	4	4	y = 3628x + 499	0.9994

3.6. Data Analysis

Microsoft® Excel® spreadsheet was used for data entry and calculations. The data were presented as a mean or mean ± SD (standard deviation) of at least three independent measurements. Where it was appropriate, one-way analysis of variance (ANOVA) was used to compare the mean values. A probability of 5% or less was accepted as statistically significant. Pearson's correlation coefficient was used to determine correlations. The design of the experiment of pectins' extractions was performed using STATISTICA 12 (StatSoft, Inc., Tulsa, OK, USA).

4. Conclusions

Kaffir lime peel, as a citrus waste, is a promising raw material in many branches of the industry thanks to its high content of valuable by-products, whose presence increases its dietary and therapeutic value. The pectin extraction experiment showed that citric acid is a good option for pectin extraction and that the yield of pectins increased with the temperature and lowered with an increase in pH. Kaffir lime peel is a good source of low methyl pectins that could be used in the food industry. According to the DSC results, increasing the temperature of extraction and the addition of ultrasounds have an influence on the structure of the obtained pectins. The main difference may be connected to the different amounts of methoxyl groups. Kaffir lime essential oil contains several terpenes with many bioactive properties and has a pleasant smell and is a good choice for aromatherapy and cosmetology. Because of the high content of bioactive compounds and minerals, kaffir lime peel can be used as animal feed or in the production of bio-fertilizers. In addition, thanks to its pro-health properties and pleasant smell, powdered kaffir lime peel can be an important component of dietary supplements in the form of capsules, as in the case of commercially available orange peel. The high levels of health-promoting compounds, compared with other citrus fruits, make the peel of kaffir lime worthy of consideration as a candidate for widespread use in dried or freeze-dried form. *Citrus hystrix* seems to have great value for utilization in many forms in the food, medical, cosmetic, or agriculture industries. The natural origin of this ingredient makes it even more attractive for these applications.

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6.3 Chemical, Aroma and Pro-health Characteristics of Kaffir Lime Juice—The Approach Using Optimized HS-SPME-GC-TOFMS, MP-OES, 3D-FL and Physiochemical Analysis

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Chemical, Aroma and Pro-Health Characteristics of Kaffir Lime Juice—The Approach Using Optimized HS-SPME-GC-TOFMS, MP-OES, 3D-FL and Physiochemical Analysis

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Abstract: The study aimed to provide the chemical, aroma and prohealth characteristics of the kaffir lime juice. A procedure using solid-phase microextraction with gas chromatography (SPME-GC-TOFMS) was optimized and validated for the determination of terpenes of kaffir lime. Main physicochemical parameters: pH, vitamin C, citric acid and °Brix were evaluated. Micro- and macro elements were determined using microwave plasma optic emission spectrometry (MP-OES). The binding of kaffir lime terpenes to human serum albumin (HSA) was investigated by fluorescence spectroscopy (3D-FL). β-Pinene and Limonene were selected as the most abundant terpenes with the concentration of 1225 ± 35 and 545 ± 16 µg/g, respectively. The values of citric acid, vitamin C, °Brix and pH were 74.74 ± 0.50 g/kg, 22.31 ± 0.53 mg/100 mL, 10.35 ± 0.70 and 2.406 ± 0.086 for, respectively. Iron, with a concentration of 16.578 ± 0.029 mg/kg, was the most abundant microelement. Among the macroelements, potassium (8121 ± 52 mg/kg) was dominant. Kaffir lime binding to HSA was higher than β-Pinene, which may indicate the therapeutic effect of the juice. Kaffir lime juice is a source of terpenes with good aromatic and bioactive properties. Fluorescence measurements confirmed its therapeutic effect. Kaffir lime juice is also a good source of citric acid with potential industrial application. The high content of minerals compared to other citruses increases its prohealth value.

Keywords: kaffir lime; gas chromatography; plasma-optic emission spectrometry; 3D fluorometry; optimization; validation; aroma properties; health benefits

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1. Introduction

Kaffir lime is one of the most popular fruits in Southeast Asia. Unlike many citrus fruits, the main part of the fruit consumed is not the juice but the aromatic leaves. The juice is not consumed directly because of the extremely tart and often bitter taste [1,2]. In traditional medicine in some Asian countries, juice is often used in shampoo because of its antidandruff properties or as a cleanser for clothing [3]. Detailed chemical characteristics of the fruit make it possible to detect specific compounds responsible for the health-promoting effect of the fruit. In the case of citrus, their bioactive effect is due, among others, to the presence of terpenes [4]. Terpenes are secondary metabolites of many plants, produced to fulfil specific biological and ecochemical functions, such as hormone biosynthesis, but also protection against UV radiation and photo-oxidative stress, but also as pest and toxin repellants, growth regulators, pollinator attractors, photosynthetic pigments and electron acceptors [5]. Less volatile, bitter or toxic terpenes are produced by plants as protection against microbes and insects [6]. Terpenes are used primarily as fragrances in new perfumes and as additives to creams, lotions, or shampoos. In addition, some functionalized terpenes also show bioactivity against certain types of cancerous, bacterial, or viral cells.

Therefore the interest in such compounds is constantly growing [7]. It is recommended that the terpenes used in the food, cosmetic and pharmaceutical industries should be of natural origin.

Determination of chemical, aroma and prohealth characteristics of fruits is extremely important from the point of view of their use in various industries. Since terpenes are the main group of chemicals in citrus, much attention is paid to them. Considering the variable factors that may affect the result of the determination of terpenes in fruit juice samples, such as growing conditions, degree of maturity or storage conditions, an optimized and validated methodology for the determination of these compounds should be developed, which will ensure repeatability and reliability of results regardless of external factors. This is especially important for kaffir lime juice, which is not directly consumed. It is known, however, that it is used in folk medicine or as a home cosmetic, but its detailed health-promoting properties have not been fully investigated. Kaffir lime is an underrated fruit and a good source of many chemical compounds, especially since the juice is not consumed due to its tart taste. To use a given raw material in the industry, methodologies for its analysis must be developed. In previous literature reports, terpenes turned out to be the main group of chemical compounds in kaffir lime. It was decided to focus on this group and optimize and validate the analytical methodology for it.

The study of the volatile fraction of citrus fruit is a popular element of food research [8]. However, many of the performed approaches concern the qualitative or semiquantitative analysis of volatile terpenes under non-optimized conditions. For quantitative analyzes, a common approach is to run an untargeted analysis to determine the overall fragrance profile. In the case of kaffir lime, previous untargeted studies have shown that the aroma profile of the juice is primarily terpenes such as γ-Terpinene and Terpinen-4-ol [9]. However, the research was conducted under non-optimized conditions. In line with recent trends, solvent-free extraction techniques such as solid phase microextraction (SPME) should be used in volatile fraction studies. The efficiency of HS-SPME of monoterpenes can be affected by several factors, including the mass of the sample and the addition of salt, which increases the ionic strength, extraction time and temperature. Henceforth, an optimized analytical method for targeted terpenes determination is crucial to facilitate the characterization.

The first objective of the study was the development of an analytical procedure based on the HS-SPME in combination with gas chromatography with time-of-flight mass spectrometry (GC-TOFMS) for targeted analysis of terpenes in kaffir lime juice sample. Optimization of major HS-SPME conditions using fractional factorial design (FFD) was performed. The second objective was the validation of the elaborated procedure and application to real samples. Moreover, a characteristic of kaffir lime, including sugar, citric acid and micro and macroelements, was performed. Bioactive compounds' interaction with albumins significantly affects their transport and biological metabolism [10]. To better understand terpenes' prohealth activity, their binding properties to human serum albumin were measured using three-dimensional fluorescence analysis (3D-FL). The results provide a background for using kaffir lime juice as a functional food or additive cosmetic and pharmaceutical industry. The conducted research complements the previous research on the antioxidant properties of kaffir lime juice and its therapeutic effect on the human body [9,11]. The last goal was to provide the fruit characteristics of major by-products, which can be obtained from kaffir lime or used in citrus waste management processes. For this purpose, a novel analytical method using microwave plasma optic emission spectrometry (MP-OES) was provided. To our knowledge, lime juice research has not been exhausted yet. The paper provides new information about the different branches of science and the different kinds of industry.

2. Results and Discussion

2.1. Terpenes' Content

2.1.1. SPME Optimization

Fractional factorial design is a screening method that assesses the effect of certain factors in minimum runs. It is relatively advantageous compared to the full factorial design as it requires many experiments [12]. The design matrix and the corresponding response of fractional factorial design (FFD) experiments were used to determine the influence of the four independent variables, including the mass of sample (X_1), mass of added salt (X_2), extraction time (X_3), extraction temperature (X_4) for the extraction and enrichment of main terpenes of kaffir lime juice. Based on the experimental design generated by FFD, 11 extraction processes were performed. The response for each run in the experimental Fractional Factorial Design was expressed as the value relative to the maximum yield obtained (%) for each level of main terpenes in kaffir lime juice (Table 1).

Table 1. The Fractional Factorial Design for four factors with their observed responses.

DOE	Extraction Variables				Extraction Yield (Relative % to Maximum Yield)									
	X_1	X_2	X_3	X_4	ß-Pin	Lim	γ-Ter	α-Pin	α-Terpl	Camp	α-Phell	α-Terpin	Terpin	Sum
1	0	0	0	0	11.32	94.77	95.39	89.84	27.10	73.68	3.85	100.00	62.99	97.33
2	0	0	0	0	12.51	100.00	100.00	91.41	19.48	78.41	10.09	98.58	66.52	97.76
3	0	0	0	0	22.86	95.73	96.37	85.63	19.37	74.27	9.68	84.00	64.80	100.00
4	−1	−1	1	1	54.05	44.56	65.40	27.69	24.36	36.77	7.93	27.64	54.63	67.88
5	1	1	−1	−1	18.30	49.33	60.59	79.08	5.99	62.09	100.00	49.61	30.03	74.78
6	1	−1	1	−1	1.18	55.06	92.12	100.00	5.55	88.64	9.62	70.23	48.23	73.98
7	−1	−1	−1	−1	7.49	73.26	72.62	57.35	1.72	50.50	5.95	28.08	29.95	65.44
8	−1	1	−1	1	76.39	30.37	28.10	12.88	35.95	16.46	3.94	15.24	23.17	56.01
9	1	−1	−1	1	12.51	36.94	85.47	72.78	22.79	28.56	9.63	37.30	54.14	63.76
10	1	1	1	1	5.46	72.09	73.04	60.37	100.00	100.00	14.49	59.98	100.00	87.07
11	−1	1	1	−1	100.00	66.73	46.91	26.80	18.09	26.92	4.98	26.80	28.73	84.90

ß-Pin—ß-Pinene, Lim—Limonene, γ-Ter—γ-Terpinene, α-Pin—α-Pinene, α-Terpl—α-Terpineol, Camp—Camphene, α-Phell—α-Phellandrene, α-Terpin—α-Terpinene, Terpin—Terpinolene.

The model's efficiency was evaluated using analysis of variance (ANOVA). Table 2 presents the results of the regression model for independent variables and their interactions. Based on the results, it can be concluded that all of the variables (X_1, X_2, X_3, X_4) are statistically significant ($p < 0.05$) in relation to their influence on the isolation and enrichment of main terpenes of kaffir lime juice. Moreover, the 2-way interaction between variables X_1 (mass of the sample) and X_4 (extraction time) is also significant. High and very close values of regression coefficients (R^2 = 99.82%, adjusted R^2 = 99.12%) evidenced a good correlation between measured and predicted data.

Table 2. Regression model for independent variables and their interactions.

Sum of Peak Areas of Main Terpenes in Kaffir Lime Juice			
	Mean Square	F-Value	*p*-Value
Model	2.92519×10^{15}	141.91	0.007 *
Linear	1.60964×10^{15}	78.09	0.013 *
X_1	8.06023×10^{14}	39.10	0.025 *
X_2	1.25972×10^{15}	61.11	0.016 *
X_3	3.62883×10^{15}	176.04	0.006 *
X_4	7.44009×10^{14}	36.09	0.027 *
2-way interactions	4.85738×10^{14}	23.56	0.041 *

Table 2. *Cont.*

Sum of Peak Areas of Main Terpenes in Kaffir Lime Juice			
	Mean Square	**F-Value**	***p*-Value**
X_1X_2	3.42030×10^{14}	16.59	0.055
X_1X_3	9.73402×10^{13}	4.72	0.162
X_1X_4	1.01785×10^{15}	49.38	0.020 *
R^2	99.82%		
R^2 (adjusted)	99.12%		

* Significant at $p < 0.05$.

To check the adequacy of the experiment of each of the main terpenes in kaffir lime juice, a model for each compound was constructed, and the coefficient of determination R^2 was considered (Table 3). The model's coefficients were confirmed to provide a high correlation between the experimental and predicted values for each terpene.

Table 3. Coefficients of determination of a model of each chemical compound.

Chemical Compound	R^2
ß-Pinene	98.13%
Limonene	99.74%
γ-Terpinene	99.77%
α-Pinene	99.80%
α-Terpineol	99.45%
Camphene	99.83%
α-Phellandrene	99.69%
α-Terpinene	98.30%
Terpinolene	99.88%

To select the optimal conditions of isolation and enrichment of the main terpenes of kaffir lime juice, Multiresponse Prediction (MRP) was performed (Figure 1). MRP is a method for identifying the combination of input variable settings that jointly optimize a set of responses. *X*-axis shows the optimum values of the variables for a desired response. Y-axis expresses various responses and targets achieved by performing experiments with composite desirability (D) and individual desirability (d) values. The vertical red lines on the graph represent the current settings. The horizontal blue lines represent the current response values.

The proposed ordinates and optimal conditions for HS–SPME by MRP are shown in Table 4.

Table 4. The parameters of extraction of terpenes from kaffir lime juice selected during MRP.

Factors	Value	Unit
X_1 sample mass	3.0	g
X_2 mass of salt added	0.5	g
X_3 extraction time	30	min
X_4 extraction temperature	45	°C

2.1.2. Method Validation of HS–SPME GC–TOFMS

The validation parameters are presented in Table 5. The determination coefficient (R^2) ranged from 0.9915–0.9995. The method's precision was evaluated by assessing repeatability (intra-day) and intermediate precision (extra-day). A measure of repeatability, intermediate precision, and reproducibility were the standard deviation and relative standard deviation values. The precision was calculated as the coefficient of variation for all the results obtained in all the analyzed samples using the developed method (expressed

as % CV, $n = 7$). CV ranged from 0.22% (α-Pinene) to 9.17% (α-Terpinene). The coefficient of variation of main components does not exceed 3%, and for all the rest determined compounds, CV is lower than 10%, confirming good repeatability and precision of the method. It is assumed that the developed method meets the requirements when the value of the coefficient of variation does not exceed 15% [13].

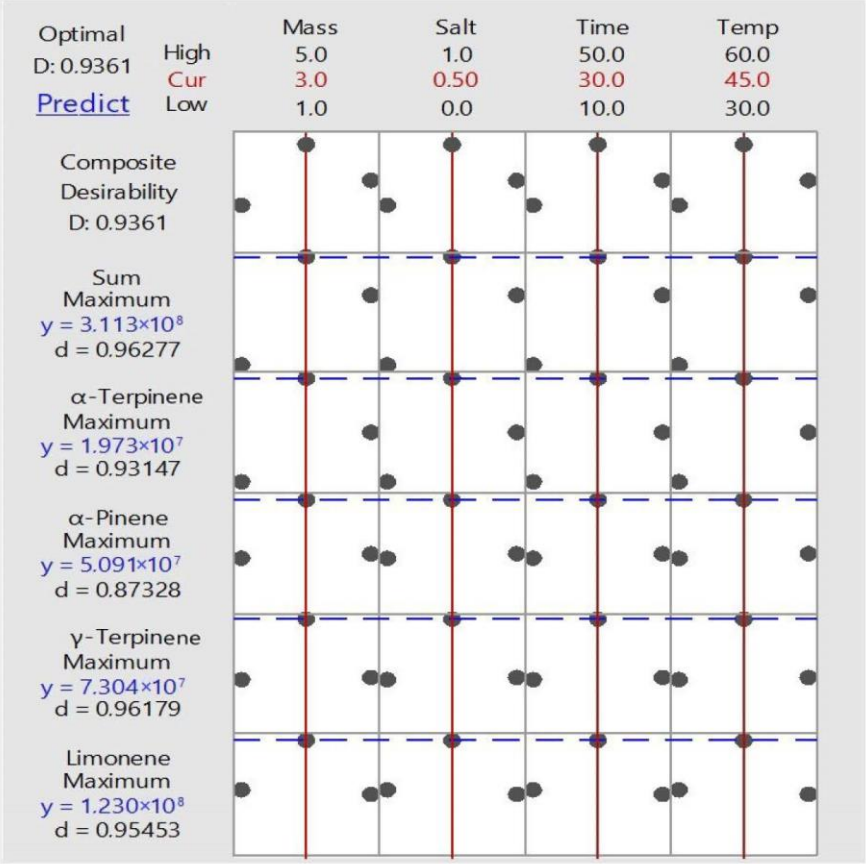

Figure 1. Results of Multiresponse Prediction of the selecting of optimal parameters of extraction of terpenes from kaffir lime juice.

Certified reference material was not available. Consequently, definitive statements cannot be made concerning accuracy. However, the recovery was calculated according to the results of the analysis of model liquids prepared based on the physicochemical analysis of kaffir lime juice—a model liquid was made corresponding to the juice samples in terms of sugar content (sucrose was the representative sugar), citric acid and vitamin C (which is the main vitamin in citrus fruit juice). The recoveries ranged from 38.33% (Terpinolene) to 127.02% (α-Terpineol). These results show that the developed extraction method applies to

assessing studied terpenes. The chromatograms of analysis of the sample and the mixture of terpenes using the optimized method are presented in Figure 2.

Table 5. Selected validation parameters obtained based on the analysis of reference substances corresponding to the main monoterpenes identified in the volatile fraction of kaffir lime.

Terpene	RI_{sample}	$RI_{lit.}$	Ref.	Cond.	a	b	R^2	*n*	LOQ [µg/g]	LOD [µg/g]	Range		CV	Rec. [%]
											Min.	Max.		
Camphene	957.91	960.64	[14]	ZB1, 110 °C	0.0322	−0.1579	0.9956	7	25	8.3	25	252.5	3.07	125.88
Limonene	1031.96	1030.3	[14]	ZB1, 110 °C	0.0283	−0.3372	0.9954	7	19	6.8	19	424.8	2.85	108.92
ß-Pinene	981.04	981.73	[14]	ZB1, 110 °C	0.0335	0.0125	0.9949	7	39	13	39	1101	2.88	108.99
α-Phellandrene	1008.20	1006.7	[14]	ZB1, 110 °C	0.0324	−0.1394	0.9995	7	17	5.6	17	213.6	6.12	46.10
α-Pinene	942.93	941.85	[14]	ZB1, 110 °C	0.0284	−0.1267	0.9954	7	26	8.5	26	433.3	0.22	94.11
α-Terpinene	1021.58	1020	[15]	HP-101	0.0542	−0.5572	0.9935	7	31	10	31	211.9	9.17	93.98
α-Terpineol	1183.68	1179.4	[16]	DB1, 120 °C	0.0242	−0.116	0.9913	7	25	8.2	25	235.9	3.33	127.02
γ-Terpinene	1058.39	1055.8	[14]	ZB1, 110 °C	0.0542	−0.5572	0.9915	7	23	7.8	23	215.4	2.50	51.78
Terpinen-4-ol	1174.64	1170.8	[14]	ZB1, 120 °C	0.02	−0.2083	0.9944	7	28	9.2	28	471.21	1.97	105.45
Terpinolene	1085.55	1079.3	[14]	ZB1, 120 °C	0.0264	0.0175	0.9966	7	38	12.8	38	172.2	3.82	38.33

RI_{sample}—Retention Index obtained during the analysis, $RI_{lit.}$—Retention Index taken from literature, Ref.—literature reference for $RI_{lit.}$, Cond.—conditions of analysis performed during measurements of Retention Indexes taken from literature, R^2—Coefficient of determination, LOQ—limit of quantification, LOD—limit of detection, CV—coefficient of variation, Rec.—recovery.

2.1.3. Real Samples' Determination

Using the determined optimal extraction parameters and an appropriately selected temperature program, chromatographic analyzes were performed, based on which calibration curves were prepared that enabled the quantitative determination of selected terpenes in the samples of kaffir lime. The results of the quantitative determination of the main terpenes in kaffir lime juices are presented in Figure 3.

As previously reported, terpenes are the main chemical class of compounds in kaffir lime juice [9]. However, due to factors such as degree of maturity, geographic origin, growing conditions, and harvest time, the terpene profile of the fruit can vary significantly. In pressed juice, time and storage conditions have an additional influence. In the case of terpenes, which are highly reactive and unstable compounds, the influence of temperature, light, enzymes, and the action of microorganisms is not without significance. Terpenes undergo oxidation processes, creating their metabolic pathway and converting to other terpenes, hydroperoxides, monoepoxides, diepoxides and aldehydes [17]. For example, the precursor to α-Terpineol is Limonene and Linalool [18]. According to literature reports, the biotransformation of β-Pinene leads to the formation of Terpinolene [19], from which Terpinen-4-ol is then formed [20]. Terpinen-4-ol can also be synthesized from α-Terpinene [20]. The main terpenes in kaffir lime juice are β-Pinene and Limonene, which are precursors to the synthesis of other terpenes, with concentrations of 1223 ± 35 µg/g and 545 ± 16 µg/g, respectively. In previous reports, Terpinen-4-ol and γ-Terpinene were the most abundant compounds [9]. This indicates greater oxidation of the samples used for previous tests.

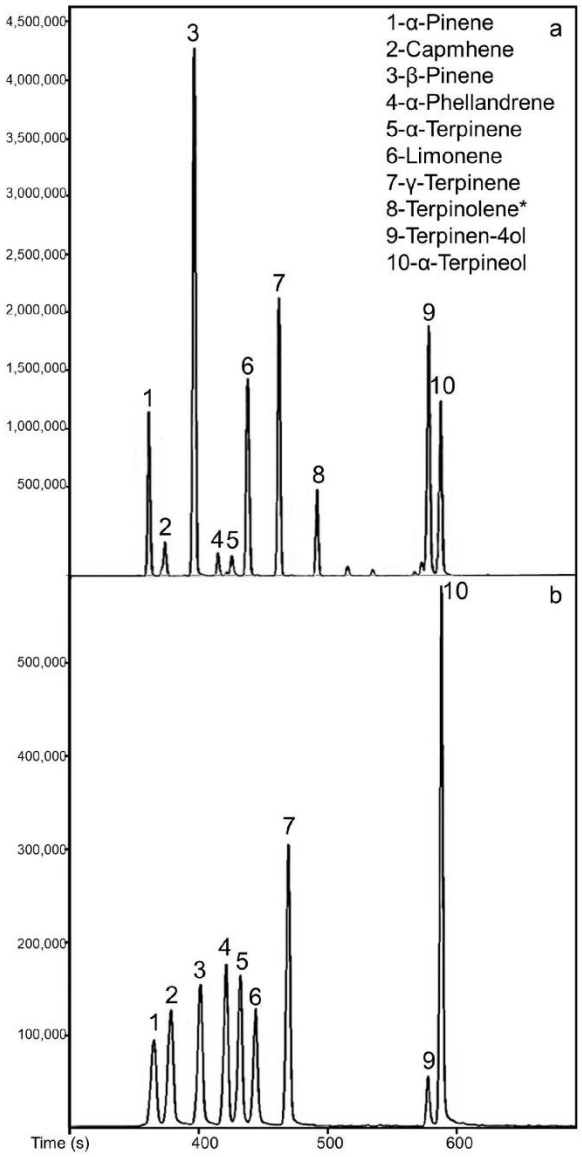

Figure 2. Chromatograms of GC analysis (for the mass 93) of the sample of (**a**) kaffir lime juice, (**b**) terpenes' standard mixture, performed with the use of optimized extraction conditions, * The compound was investigated in a separate analysis. Therefore it is not shown in the chromatogram.

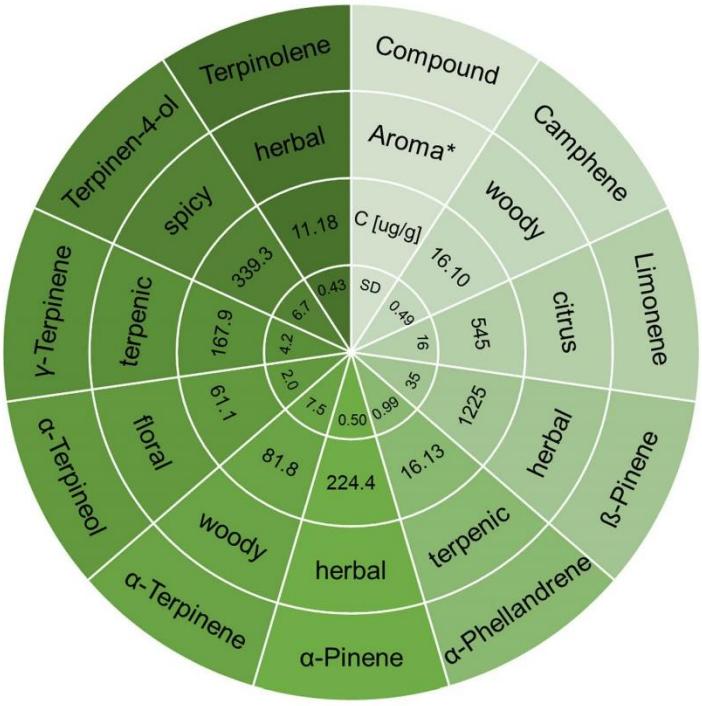

Figure 3. Concentration and aroma properties of main terpenes determined in kaffir lime juice with the use of optimized HS-SPME-GC-TOMFS method, * www.thegoodscentscompany.com (accessed on 14 April 2023).

Different terpene profiles affect the aromas of the samples but also their bioactive properties. While in previous research, the high content of Terpinen-4-ol could suggest possible anti-inflammatory, antioxidant, antimicrobial and anticancer properties of the juice [21], in the case of high ß-Pinene, it has different prohealth activity. Research provided by Salehi et al. also showed its anticoagulant, antitumor, antimalarial, antioxidant, anti-*Leishmania*, and analgesic effects. The authors summarized its cytogenetic, neuro-, cyto- and gastroprotective, anxiolytic, and anticonvulsant activity [22].

To indicate the intensity of the contribution in creating the aroma of the juice, the odour activity values (OAVs) were calculated by dividing the concentrations of selected terpenes and their odour thresholds (OT) taken from the literature. The OAVs, which is a measure of the importance of a compound to the odour of a sample, were the highest in the case of Limonene and ß-Pinene (Table 6). Comparing the differences in aroma properties with our previous results, the high content of these two terpenes makes the smell of kaffir lime juice more citrus, with herbal accents. Terpinen-4-ol and Terpinolene also contributed to the creation of the aroma with relatively high OAVs. With the comparison with previous research concerning Terpinen-4-ol and γ-Terpinene content, it can be concluded that a greater degree of oxidation, caused by temperature, degree of maturity, or the presence of enzymes and bacteria, may cause changes in the aroma of kaffir lime juice, resulting from the greater presence of Terpinen-4-ol with a woody, and γ-Terpinene with terpenic odour description.

Due to the high content of terpenes, which show numerous bioactive activities [9], kaffir lime juice is a potential candidate for use in the cosmetics industry. It can be used as an additive to hair care products, shampoos, soaps, skin creams, gels, and lotions. This is consistent with literature reports that show that the kaffir lime juice is a natural acid, good for protecting the skin of the head as well as cleaning the residue of soap and shampoo [3]. Due to its pleasant sensory properties will work well as an addition to foot creams, ensuring their pleasant smell. Due to the high content of Limonene, which is known for its ability to fight insects [23], kaffir lime juice will also work as an addition to insect repellents, providing not only effective insect removal but also a pleasant smell. The advantage of this solution will also be the natural origin and non-toxicity of the active substance—terpenes from Kaffir lime juice.

Table 6. Aroma intensity properties of kaffir lime juice.

Compound	OT µg/g	OAV	Ref.
Camphene	186	0.086545	[24]
Limonene	0.01	54,488.61	[24]
ß-Pinene	1.5	816.8033	[24]
α-Phellandrene	0.5	27.15541	[25]
α-Pinene	26	8.632425	[26]
α-Terpinene	2.4	34.06655	[27]
α-Terpineol	0.33	185.0893	[28]
γ-Terpinene	1	167.8755	[24]
Terpinen-4-ol	0.34	998.0478	[24]
Terpinolene	0.041	272.705	[24]

2.2. *Physicochemical Characteristics of Kaffir Lime Juice*

The physicochemical characteristics of kaffir lime juice are presented in Table 7. In some countries, citric acid is still produced from citruses as it is economically advantageous [29]. Citric acid is the major organic acid that contributes approximately 90% of the citrus fruit acidity [30]. Among citrus fruits, citric acid is most concentrated in lemons and limes [31]. Previous studies have shown that lemons contain 48.0 g/L of citric acid [31]. The determined kaffir lime's citric acid content is much higher. Literature researches suggest that kaffir lime can be used as an organic demulsifier formulation and to decrease water hardness due to the high content of citric acid [32,33]. Citric acid extracted from citruses is used as an acidity regulator in beverages, detergents, and for applications other than food, such as cosmetics, pharmaceuticals, and the chemical industry. Using directly consumed lemon juice seems less cost-effective than using Kaffir lime juice, which is food waste [9]. Due to the high content of citric acid, which fights scale deposits in bathrooms, but also due to the presence of terpenes with antibacterial properties and pleasant sensory properties, kaffir lime juice seems to be a very good ingredient in preparations for cleaning bathrooms or toilets.

Table 7. Physicochemical properties of kaffir lime juice.

Parameter	Value
Total acidity	7.474 ± 0.050
$C_{citric\ acid}$	74.74 ± 0.50 g/kg
Brix	10.35 ± 0.70
pH	2.406 ± 0.086
°Brix/acidity ratio	1.385 ± 0.050
Vitamin C	22.31 ± 0.53 mg/100 mL

Brix/acid ratio is considered the best objective measurement that reflects the consumer acceptability of juices [34]. Consumer research proves that the higher the °Brix/acid ratio, the more acceptable the juice is by consumers, which may explain the lack of kaffir lime

consumption. To compare, the literature shows that the °Brix/acid ratio of orange juice samples ranges from 15–18 [35].

In the case of the most abundant vitamin in citrus juice—the concentration in kaffir lime juice was determined to be 22.31 ± 0.53. Very similar results were obtained by Najwa et al.—21.58 ± 0.51 mg/100 g [36]. In the same research, orange represented the highest vitamin C content—43.61 ± 1.72 mg/100 g among all citrus fruits. One of the best sources of ascorbic acid, acerola, contains 1500–4500 mg/100 g, which is around 50–100 times more than orange or lemon [37]. Considering the above, Kaffir lime is an average source of vitamin C. However, considering that the juice is waste that can be used to produce dietary supplements or cosmetics, the presence of vitamin C in a relatively large amount is an additional advantage. Vitamin C in cosmetics protects the skin against oxidative stress and fights free radicals.

2.3. *Micro- and Macroelements Content in Kaffir Lime Juice*

Over the past few decades, several studies on nutritional elements have been conducted to determine their role in the human diet. Heavy metals belong to a group of xenobiotics which are the most commonly controlled harmful components of food or other products due to their ability to accumulate along the food chain. Accordingly, their maximum levels have become global quality standards. Right next to elements considered toxic (e.g., Cd, Pb, Hg), some elements are essential and indispensable in the human diet (e.g., Co, Cr, Fe, Mn, Mo, Ni, Sn, Zn, Ca, Mg and K). However, elevated levels of both essential and non-essential elements can also cause health anomalies. This, therefore, leads to the conclusion that it is so important to determine the level of trace elements in kaffir lime juice [38]. The results of the determination of selected elements in kaffir lime juice are presented in Figure 4.

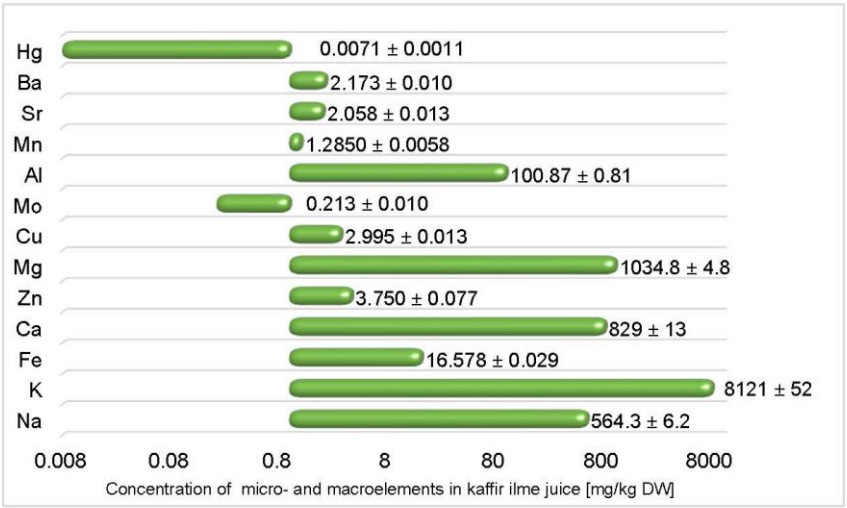

Figure 4. The concentration of micro- and macroelements in kaffir lime juice [mg/kg dry weight (DW)] was determined using the MP-OES technique.

Macroelements such as magnesium, calcium and potassium in food contribute to the normal functioning of the cardiovascular system, the conduction of nerve impulses and the support of the immune system [39]. As part of the study, of all elements determined, the highest concentrations were found for Mg, Ca, and K. These are 1034.8 ± 4.8; 829 ± 13

and 8121 ± 52 mg/kg DW, respectively. To compare, orange and lemon juice contains 100 ± 12.2 and 69.2 ± 4.02; 90 ± 7.65 and 63.5 ± 1.32; 1620 ± 156 and 1257 ± 73.1 mg/L respectively [40]. The last element with the highest content was sodium (564.3 ± 6.2 mg/kg DW). Sodium is responsible for maintaining the acid-base balance, the disruption of which can result in dangerous acidosis, which can cause osteoporosis, among other diseases. As an electrolyte, it is responsible for maintaining the internal water balance, as are potassium, magnesium, and calcium.

Micronutrients are trace elements that every living organism needs to function properly. For humans, the micronutrient requirement is less than 100 mg per day. Micronutrients can have different effects on the body. Iron, a component of hemoglobin responsible for transporting oxygen to the tissues in the body [41] was determined, in this study, in amounts of 16.578 ± 0.029 mg/kg DW. This is the comparable value as determined in the lemon juice samples from Argentina (15.6 ± 1.18 mg/kg). Similar results were also obtained to determine molybdenum—at 0.20 mg/kg [42]. In turn, zinc, which is involved in DNA and RNA synthesis and protein and insulin synthesis, was determined at 3.750 ± 0.077 mg/kg DW in the present study. Several essential micronutrients, including V, Ni, Cr, and Co, were below the defined LOD limits. The high content of microelements is an essential aspect of producing dietary supplements, drugs, animal food or cosmetics from plant raw materials. Therefore, kaffir lime juice may be applied in the food, agriculture, pharmaceutical, and cosmetic industries.

Most of the undesirable elements (Cd, Pb, Pt) in the diet were not determined (<LOD) or were determined at very low levels (Sr, Ba). An example of elements being determined is mercury (2.995 ± 0.013 mg/kg DW). Interestingly, the fourth highest elemental concentration determined was Al (100.87 ± 0.81 mg/kg DW). Aluminum does not show exaggerated toxic effects on the human body. Nevertheless, its excess is not welcome and can cause some damage to health. However, considering that the given values refer to the juice's dry weight (moisture of citrus juices is almost 90% [43]) and the provisional tolerable weekly intake value is 1 mg·kg^{-1} body weight/week [44], the aluminium contained in the samples does not pose a health risk human.

Although micro- and macronutrients are not isolated from citrus fruits, according to Barros et al., citrus fruits are promising sources of mineral elements [45]. Citrus fruits are good sources of minerals, including potassium, calcium and magnesium, compared to other fruits [46]. These compounds are responsible for the body's water and electrolyte balance. Therefore, the addition of kaffir lime juice may have potential use as an addition to rehydration drinks. Compared to other fruits, cherries, considered to be a good source of potassium, contain 2900 ± 50 mg/kg DW. The content of micro- and macroelements is also very important in the potential use of Kaffir lime juice as an additive to cosmetics. In cosmetic preparations, iron supports skin regeneration. In turn, zinc is a strong antioxidant, significantly delaying aging. It positively affects collagen metabolism and is responsible for the growth of hair, nails, and tissue regeneration.

2.4. Fluorescence Properties of Kaffir Lime Juice

Terpenes are nearly present in all-natural products. Human Serum Albumin (HSA) forms stable complexes with several substances. HSA binding can influence the bioactivity of terpenes, and albumin can also be considered and applied as a relatively cheap affinity protein. Therefore, we examined the potential interactions of the main terpene with HSA employing fluorescence spectroscopy. The flexibility of albumin's structure is due to its organization into three domains, I, II and III. Each is subdivided into two subdomains, A and B. Intramolecular disulfide bonds ensure rigidity within each protein subdomain, but allow significant modifications in the shape and size of albumin in response to pH changes or other biophysical influences [47]. Some recent reports showed the interaction of terpenes with HSA, forming complexes, as with many compounds and drugs [48]. The binding of drugs to HSA determines their distribution through systemic circulation and its pharmacological effects on the organism [49], while terpenes are considered drugs and

pharmaceutical agents [50]. Determination of terpenes' binding to the main protein in blood human serum (HSA) is essential for human metabolism. The binding of terpenes to HSA under physiological conditions results from forming a complex. The fluorescence properties of lime extracts are presented in Figure 5.

The measurements were performed at the initial albumin (Alb) with λex/λem (nm/nm) = 227/349 and 279/353 with fluorescence intensity (FI, arbitral units) = 765.90 and 875.01 for peaks a and b, respectively. After interaction of HSA with kaffir lime juice, changes in λex/λem (nm/nm) = 231/334 with FI = 481.24 for peak a and λex/λem (nm/nm) = 282/339 with FI = 723.63 for peak b were detected. According to the decrease in the fluorescence intensities of peaks a and b, the binding properties were calculated (Table 8). The addition of kaffir lime to HSA showed increased binding of 54.5%. The relatively high percentage of binding properties of the bioactive compounds found in kaffir lime juice can be compared with the binding of HSA with different drugs. The interaction between paracetamol and HSA under physiological conditions has been investigated by fluorescence and showed similar results. Moreover, the protein-ligand docking study indicated that paracetamols (two paracetamols bind to HSA) bind to residues located in the subdomain IIIA [51].

The obtained results of kaffir lime juice can be as well compared with the interaction of HSA with one of the essential thiazole derivatives, 2-amino benzothiazole (2–ABT), which is widely used as a structural unit in the synthesis of antioxidants, anti-inflammatories, herbicides, antibiotics, and thermoplastic polymers. The interaction of 2–ABT with HSA under simulated physiological conditions by three-dimensional (3D) fluorescence showed high binding and fluorescence quenching spectra properties [52].

Recent reports showed wide use of the interaction of HSA with several mycotoxins. They proved that HSA binding could be applied as a relatively cheap affinity protein and used in vitro studies, as in our case with kaffir lime juice bioactive compounds [53].

As previously shown, HSA is widely used as an affinity protein. It is essential to show that not only polyphenols of natural products such as plants and fruits having high antioxidant activities form strong polyphenol-protein complexes in vitro and as well in vivo, based on the measurements of the binding properties of the substances under normal physiological conditions, as in the present study, but also terpenes. The levels of glycated proteins in the blood of diabetics are higher than that of non-diabetic subjects. The glycation of proteins is linked to the occurrence of diabetic complications and similar diseases, as it was shown in the reports that the glycation of HSA is believed to reduce the binding affinities for acidic drugs such as polyphenols and phenolic acids [54].

Table 8. Fluorescence and binding properties of kaffir lime juice.

Peaks	Indices	HSA	KL	Binding to HSA [%]	β-Pinene	Binding to HSA [%]
a	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	227/349	231/334	-	228/349	-
	Int F_0	765.90 ± 58.14	481.24 ± 42.11	37.2 ± 3.31	497.18 ± 45.71	35.09 ± 2.52
a1	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	-	233/637	-	-	-
	Int F_0	-	95.40 ± 8.13	-	-	-
b	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	279/353	282/339	-	280/354	-
	Int F_0	875.01 ± 79.11	723.63 ± 7.63	17.3 ± 1.50	760.21 ± 68.24	13.12 ± 1.21
b1	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	-	283/644	-	-	-
	Int F_0	-	129.78 ± 11.2	-	-	-
c	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	-	347/436	-	-	-
	Int F_0	-	169.44 ± 13.14	-	-	-

Abbreviations: F_0, fluorescence intensity in arbitral units (A. U.).

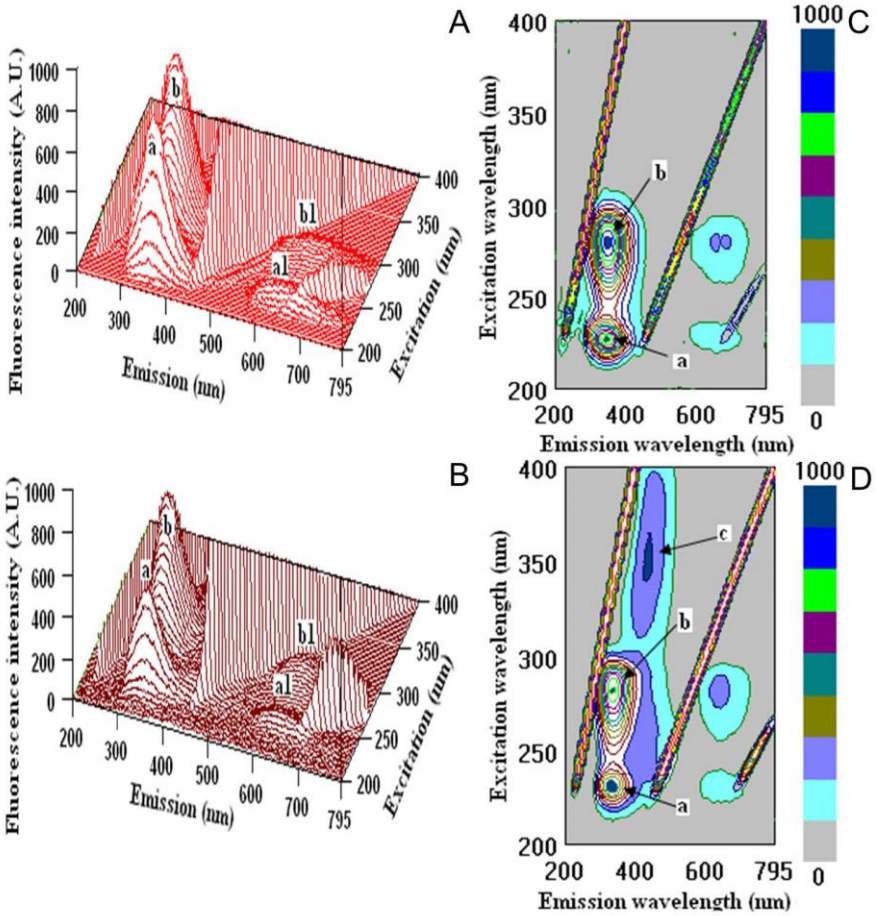

Figure 5. Fluorometric measurements in three-dimensional fluorescence analysis (3D–FL) of: (**A**)—HSA and kaffir lime juice after interaction (**B**) and cross images of: (**C**)—HSA (**D**)—HSA+ kaffir lime juice. The locations of peaks a, b, c, a1 and b1 are shown in the figure and in Table 8 (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The binding affinities of kaffir lime juice are related to the bioactive properties of terpenes. The provided results suggest that terpenes in kaffir lime juice can be effectively transported and distributed in the blood after consumption. This creates new opportunities for the potential use of kaffir lime juice extracts in the pharmaceutical industry.

3. Materials and Methods

3.1. Samples

Citrus Hystrix fruits for analysis were transported from Thailand by the local distribution point in the Pomeranian Voivodship in December 2019. Fruits were provided to the laboratory in refrigeration conditions. From the information provided by the supplier,

it appeared that the fruit was harvested in a similar degree of maturity and that the time since harvest was the same. Fruits were harvested manually. Samples were prepared and analyzed immediately after purchasing. Before the analysis, fruits were cleaned with the tap, rinsed with distilled water, dried using lint free paper towel and peeled manually. The juice was squeezed manually using a plastic juice squeezer to avoid oxidation processes and poured into a glass bottle. Immediately after squeezing, the juice was weighed into 20 mL SPME vials using plastic pipette tips. The vials were closed with caps with silicone Teflon membrane. Limes for the analysis were taken from 4 batches, each composed of 3 kg of fruits (70–80 pieces).

3.2. Reagents

Reagents for micro- and macroelements analysis were purchased from Merck (Darmstadt, Germany). Deionized water (Millipore—Milli-Q Water Purification System (Bedford, MA, USA)). was used throughout the study, and spectroscopically grade nitric acid (65%), supplied by Merck (Darmstadt, Germany), was used. Methanol, terpenes' standards (ß-Pinene, Limonene, γ-Terpinene, α-Pinene, α-Terpineol, Camphene, α-Phellandrene, α-Terpinene, Terpinen-4-ol) were obtained from Sigma Aldrich (Schnelldorf, Germany). All chemicals, standards and reagents were of analytical grade.

3.3. Terpenes' Analysis

3.3.1. Optimization of Headspace Solid-Phase Microextraction (HS–SPME)

Carboxen/Polydimethylsiloxane/Divinylbenzene (CAR/PDMS/DVB) fibre with a thickness of 50/30 mm and a length of 2 cm (Sigma-Aldrich, St. Louis, MO, USA) was used for SPME. This fibre is recommended for flavour compounds by the manufacturer. It shows a strong extraction capacity for terpenic hydrocarbons, aldehydes, ketones and acids [55]. Before the extraction, the samples were kept at 40 °C for 5 min and agitated with a magnetic stirrer (750 rpm). Thermal desorption was set up to 250 °C for 5 min. Between each analysis, the fibre was cleaned at 250 °C for 2 min.

The influence of four independent factors on the yield of extraction of main terpenes from kaffir lime juice was evaluated using Fractional Factorial Design 2^{4-1}_{IV} using Minitab v17.1 Statistics Software (Minitab Inc. State College, PA, USA). A sum of peak areas of main terpenes (namely β-Pinene, Limonene, γ-Terpinene, α-Pinene, α-Terpineol, α-Phellandrene, camphene, α-Terpinene and Terpinen-4-ol) was considered as the response variable in the optimization of solid-phase microextraction—extraction yield. The type of fibre, extraction temperature, pH, sample volume, stirring speed, extraction time and ionic strength are related to SPME. Among these factors, the type of fibre, extraction temperature and extraction time are essential for volatile compound analysis [56]. The salt effect is also important, as it reduces the solubility of hydrophobic compounds and retains ionic strength [57]. According to the literature, terpenes have a high distribution coefficient between the coating and the sample; in this case, the amount of sample is a prominent factor [58]. As mentioned above, the fibre type was selected based on the manufacturer's suggestions. For these reasons, the temperature and the time of extraction, the mass of salt added, and the mass of the sample were subjected to an optimization process (Table 9) and were selected for the FFD. The factors' levels were selected based on previous research (to avoid overloading the detector at a given equipment sensitivity) and based on literature data [9]. Preliminary studies were performed to determine the required range of mass of the sample (X_1), the mass of added salt (X_2), extraction time (X_3), and extraction temperature (X_4). The whole experiment consisted of 11 runs (three replicates at the centres of the design). The experiments were performed in randomized order. The factors were denoted as −1 (low), 0 (central point) and +1 (high) to normalize the variables because of their different ranges and units (Table 9). Analysis of variance (ANOVA) was used to determine the adequacy of the factorial model. To select the optimal extraction conditions, Multi Response Prediction (MRP) was provided.

Table 9. Selected factors and their level of the Fractional Factor Design experiment.

Factors	−1	0	1	Unit
X_1 mass of the sample	1	3	5	g
X_2 mass of salt added	0	0.5	1	g
X_3 extraction time	10	30	50	min
X_4 extraction temperature	30	45	60	°C

3.3.2. Gas Chromatography

Gas chromatograph Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) equipped with a split/splitless injector and Pegasus 4D TOFMS (LECO Corp., St. Joseph, MI, USA) was used for analysis. The extraction step was made using an MPS autosampler (Gerstel Co., Mülheim, Germany). The nonpolar Equity-1 (Supelco, Bellefonte, PA, USA) column 30 m × 0.25 mm i.d. × 0.25 µm film thickness was utilized. The front inlet temperature was 200 °C, and the transfer line and ion source temperatures were set at 250 °C. The injector worked in split mode (ratio 1:100). The separation was achieved using the following temperature program for the oven: initial temperature 60 °C, ramped at 7.5 °C/min to 150 °C, then 15 °C/min to 250 °C and held for 2 min. The total time of analysis was 18 min. Helium (N6.0 class) was used as a carrier gas at a 1.0 mL/min flow rate. The detector voltage was 1716 V Mass spectra were collected from m/z 35–500 at ten spectra per second. The acquisition delay was 300 s. The internal standard method using borneol was chosen as the calibration method. The preliminary identification of the analytes was made by comparing the experimental spectra with those contained in the NIST 11 and Wiley libraries and by comparing the calculated retention indices. (RI) with literature values. RI values were calculated based on the C8–C20 n-alkanes analysis results. For each standard solution, the equation of the calibration curve and the coefficient of determination (R^2) were determined. Validation parameters were also defined: limits of detection (LOD) and quantification (LOQ). The LOD and LOQ values were calculated based on the value of the standard deviation of the signal set (Sa) and the slope angle of the calibration curve (a).

3.4. Physicochemical Characteristics

Physicochemical parameters of the analysed juice included pH, titratable acidity, total soluble solids (°Brix), Brix/acid ratio, and vitamin C content. The pH was measured using a microcomputer pH meter (inoLab® Multi 9310 IDS pH, WTW, Weilheim, 370 Germany). The total titratable acidity of the samples was established using the recommended method by the Association of Official Agricultural Chemists (AOAC) [59]. The titratable acidity of juice was measured by titrating with standardized 0.1 N NaOH until reaching pH 8.2. Citric acid was calculated based on the titrable acidity, assuming that the acidity in beverages is usually calculated as g/L citric acid [60]. Total soluble solids (°Brix) were determined using an OPTi Digital Handheld Refractometer (Bellingham + Stanley Ltd., London, UK). Vitamin C was determined iodometrically using the procedure of Trifunschi et al. [61]. All the tests were performed nine times, and the data obtained in the present investigation were subjected to statistical analysis of variance (ANOVA).

3.5. Inductively Coupled Plasma—Optical Emission Spectrometry Analysis

The first step in the sample preparation was lyophilization, carried out immediately after delivery of the samples. The lyophilized preparations were stored in sealed plastic bags at −20 °C. Concentrations of twenty trace elements (Na; K; Fe; Ca; Pt; Zn; Cd; Mg; Pb; Cu; Co; Ni; Mo; Al; Mn; Sr; Cr; Ba; V; Hg) were analyzed in all samples. The determination process of nineteen of these, except for Hg, was performed directly using a lyophilized sample and preceded by microwave-assisted mineralization. Multiwave Go microwave mineralizer (Anton Paar, Graz, Austria) equipped with a rotor and high-performance reaction vessels with pressure-activated-venting made of polytetrafluoroethylene-trifluoroacetic acid (PTFE-TFA) was used for the closed vessel

microwave-assisted acid digestion of samples. About 0.5 g of lime samples were accurately weighted in the dried PTFE digestion vessels, and 8 mL of HNO_3 was immediately added. The tightly closed vessels were placed in the microwave oven to digest the samples. The process lasted 50 min and consisted of five stages:

- Stage I: 10 min.—temperature rise to 100 °C,
- Stage II: 10 min. at 100 °C,
- Stage III: 10 min.—temperature rise to 180 °C,
- Stage IV: 10 min. at 180 °C,
- Stage V: 10 min.—temperature reduction to 60 °C.

After digestion, the contents of the reaction vessels were quantitatively transferred to 25 mL volumetric flasks. Each volumetric flask was then refilled with deionized water to a nominal volume. All prepared and blank samples were transferred to the polypropylene autosampler tubes for MP-OES analysis. The 4210 MP-OES supplied by Agilent has been used to determine most elements. Mercury/MA-3000 supplied by Nippon Instruments Corporation (NIC, Tokyo, Japan) was used to analyze mercury by cold vapour technique, and purified dry air was used as the carrier gas. The validation parameters of the analytical procedure are shown in Table 10.

Table 10. Validation parameters of the procedure for determining selected elements in kaffir lime juice samples.

Analyte	Wavelength [nm]	LOD [mg/kg]	LOQ [mg/kg]	Linearity				
				Calibration Range [mg/kg]		Number of Meas. Points	Number of Repetitions	R^2
				min.	max			
Na	568.263	1.1	3.3	10	200	5	4	0.9998
K	766.491	0.16	0.48	2.5	20	4	4	0.9997
Fe	371.993	0.33	1.0	1.0	100	8	4	0.9997
Ca	430.253	2.0	6.0	10	250	6	4	0.9995
Pt	265.945	0.075	0.23	0.40	4.0	4	4	0.9994
Zn	213.857	0.19	0.58	0.58	10	9	4	0.9995
Cd	228.802	0.022	0.066	0.066	20	8	4	0.9998
Mg	279.553	0.40	1.2	1.2	40	6	4	0.9996
Pb	405.781	0.012	0.035	0.050	5.0	6	4	0.9999
Cu	327.395	0.026	0.077	0.30	20	6	4	0.9999
Co	345.351	0.012	0.035	0.050	1.0	5	4	0.9999
Ni	361.939	0.0070	0.021	0.10	20	7	4	0.9999
Mo	386.410	0.0060	0.018	0.018	20	9	4	0.9995
Al	396.152	0.088	0.26	1.0	100	8	4	0.9998
Mn	403.076	0.0064	0.019	0.019	1.0	5	4	0.9999
Sr	421.552	0.0045	0.013	0.013	40	6	4	1.0000
Cr	425.433	0.0027	0.0082	0.01	10	8	4	0.9999
Ba	493.408	0.21	0.63	0.63	3.0	4	4	0.9962
V	437.923	0.0057	0.017	0.017	20	9	4	0.9997
Hg	253.700	0.00096	0.0029	0.0029	0.10	10	3	0.9999

3.6. Three-Dimensional Fluorescence Analysis (3D-FL)

The properties of bioactive substances in kaffir lime juice were determined by using three-dimensional (3D-FL) fluorescence (model FP-6500, Jasco spectrofluorometer, serial N261332, Tokyo, Japan) using the method of Kim et al. [62]. The 3D-FL was measured at emission wavelengths between 200 and 795 nm, and the initial excitation wavelength was 200 nm. All solutions for protein interaction were prepared in 0.05 mol/L Tris-HCl buffer (pH 7.4) containing 0.1 mol/L NaCl. The initial fluorescence intensities of albumin were measured before their interactions with the investigated samples. The changes in the fluorescence intensities were used to estimate the binding activities. The determination of the binding properties was done five times with an average.

3.7. Data Processing and Presentation

Data processing of chromatographic analysis was performed using a chromatographic peak deconvolution algorithm implemented in the software ChromaTOF® (LECO Corp., version 4.44.0.0, St. Joseph, MI, USA). Microsoft® Excel® spreadsheet was used for data entry and calculations. Tables and charts were prepared using Microsoft Office Professional Plus 2016. GC chromatograms and 3D-FL images, and optimization plots were implemented from the software.

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6.4 Quality of limes juices based on the aroma and antioxidant properties

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Quality of limes juices based on the aroma and antioxidant properties

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ABSTRACT

Kaffir (*Citrus hystrix*) and Key (*Citrus aurantifolia*) limes juices were investigated and compared. Two dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOF-MS) was applied to assess the botanical origin of Kaffir and Key limes juices, based on volatile substances. The biggest differences in the contents of selected terpenes in Kaffir and Key limes occur in chemical compounds such as Limonene, Citral, Terpinen-4-ol. Limonene concentration is almost 8 times higher in the Key lime volatile fraction than in Kaffir lime. The difference in concentration of Citral in Kaffir lime is almost 20 mg/kg lower than in Key lime. Higher concentration of Terpinen-4-ol was noted in Kaffir lime samples and the content was almost 20 times higher. The concentrations of α-Pinene, Citronellal, Camphene, Nerol, *trans*-Geraniol and β-Pinene are at similar levels in the volatile fraction of both fruits. Bioactive substances (polyphenols, flavonoids, tannins and flavanols) and the values of antioxidant capacities by four radical scavenging assays (DPPH, CUPRAC FRAP, ABTS) were determined and compared in water and methanol extracts in Kaffir and Key limes juices. The bioactivity of Kaffir lime differ significantly in water extracts in comparison with Key lime juices. The ^{1}H NMR shifts in methanol and chloroform extracts showed some differences in aromatic region between the two varieties of lime juices. Terpinen-4-ol for Kaffir lime and Citral for Key lime were used as potential markers. The GC×GC-TOF-MS allows better separation of substances originating from complex matrices than one-dimensional chromatography, based on improved resolution, increased peak capacity and unique selectivity. The possible falsification of mentioned juices can be detected by the use of GC×GC-TOF-MS, antioxidant assays and NMR shifts.

1. Introduction

Kaffir lime (*Citrus hystrix*) is one of the most popular fruits in Thailand or Laos. Kaffir lime leaves are one of the most commonly used Thai spices. Despite the leaves, the skin is also used for culinary purposes, because of specific aroma. Both the leaves and the skin contain many chemical compounds with a healthy effect. Limonene, α-Terpineol, 2β-Pinene, Terpinen-4-ol, γ-Terpinene, α-Terpinene, and α-Terpinolene are common terpenes in leaves (Srisukh et al., 2012a,b). In turn, the content of the individual terpenes in the skin were estimated: Limonene 40.65%, Terpinen-4-ol 13.71%, α-Terpineol 13.20% (Srisukh et al., 2012a,b; Thanaboripat, Chareonsettasilp, & Pandee, 2006). Kaffir lime pulp and juice are not consumed directly (Waikedre et al., 2010). However, they also contain many bioactive substances. Kaffir limes do not grow in temperate climate, and these fruits are also not imported into European countries. In Europe, the most popular and available between lime varieties is Key lime (*Citrus aurantifolia*), which also contains many bioactive terpenes (Spadaro, Costa, Circosta, & Occhiuto, 2012).

The content of individual terpenes varies in the volatile fractions

Abbreviations: Polyph, polyphenols; GAE, gallic acid equivalent; CE, catechin equivalent; Flavan, flavanols; Flavon, flavonoids; Vit C, vitamin C; Anthoc, anthocyanins; CGE, cyanidin-3-glucoside equivalent; Chlor, chlorophyll; Xan+Carot, xanthophylls+carotenes; ABTS, 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; FRAP, Ferric-reducing/antioxidant power; CUPRAC, Cupric reducing antioxidant capacity; 1,1-diphenyl-2-picrylhydrazyl, DPPH; TE, trolox equivalent.

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of each above-mentioned fruits. It is extremely important to determine terpenes in fruit products, because of their health-promoting effect or on the other site their excess can cause health problems. One of the most popular food products made from limes is juice. Key lime juice is used as an additive to beverages or sauces, oppositely, Kaffir lime juice has sour and bitter taste and very often is classified as an industrial waste. In many countries for economic reasons adulteration investigations of products containing Key lime with Kaffir lime juice is provided. The major chemical compounds found in the Kaffir lime juices volatile fraction may have potential allergic effects (Rubel, Freeman, & Southwell, 1998) as well as a large number of antioxidants may induct allergic diseases (Allan, Kelly, & Devereux, 2010). Assessment of the authenticity of juices is also important for food industry. It prevents producers from material losses due to contamination of the production line. Therefore, it is extremely important to develop an analytical method to identify possible botanical origin of limes.

The applications of two-dimensional gas chromatography (GC×GC) and time of flight mass spectrometry (TOFMS) to analyze aroma of food products are shown in a number of reports (Bogusz Junior et al., 2015; Dymerski et al., 2015; 2016). Two-dimensional gas chromatography is useful tool to analyze fruit samples. Aroma profile of the volatile fraction of apples, pears, and quince fruit were performed (Schmarr & Bernhardt, 2010). In turn, 3-methylbutan-1-ol, 3-methylbutan-1-ol acetate, 2-phenylethyl acetate and phenylethyl alcohol were selected as compounds characteristic for banana smell (Capobiango et al., 2015). Using GC×GC-TOFMS technique it was also possible to quantify the volatile compounds of different kinds of berries (Dymerski et al., 2015). Untargeted analysis was also performed after the postharvest and the storage of apples (Risticevic, Deell, & Pawliszyn, 2012). It was also possible to indicate terpenes in the samples of grapes (Banerjee et al., 2008; Rocha, Coelho, Zrostlíková, Delgadillo, & Coimbra, 2007) and blueberries (Kupska, Chmiel, Jędrkiewicz, Wardencki, & Namieśnik, 2014). Strawberries growing in Australia have been distinguished due to their botanical origin (Samykanno, Pang, & Marriott, 2013) and different varieties of chili due were classified according to their species (Bogusz Junior et al., 2015). Strawberries were also examined in order to analyze profile of volatile fraction (Williams, Ryan, Olarte, Marriott, & Pang, 2005). Dymerski et al. (2016) classified samples of cranberries, blueberries and cranberries. It is also possible to determine the pesticide residues in fruit samples (Zrostlíková, Hajšlová, & Cajka, 2003).

The composition of the volatile fraction of essential oil of *C. aurantifolia* was analyzed using GC-MS by Spadaro et al. (2012). Analysis of volatile fraction of Kaffir lime was performed using GC-MS technique. It was possible to select 15 major chemical responsible for the flavor of Kaffir lime (Kasuan et al., 2013). Nevertheless, there are no literature reports about authenticity markers of abovementioned types of limes, including also the use of two-dimensional gas chromatography.

Similarly, the situation is revealed in case of studies concerning the comparison of antioxidant activities of Kaffir and Key fruit juices. There are only a few investigations, in which a total phenolic and flavonoid contents, ferric reducing antioxidant power (FRAP) and 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity were determined (Ghafar, Prasad, Weng, & Ismai, 2009). The characterization of lime juices from the point of their antioxidant status is important. Therefore, the aim of this study was to compare Kaffir and Key lime juices regarding to their aroma and antioxidant properties. For this reason, the advanced analytical methods were elaborated, with the use of two dimensional gas chromatography coupled with time-of-flight mass spectrometry, ^{1}H NMR spectroscopy and the investigation concerning antioxidant properties, using a number of radical scavenging assays were included. According to the best of our knowledge, there are no literature reports about the quantitative determination of selected terpenes of above-mentioned juices using spectrometric methods and there is a lack of information about comparison of these matrices in respect of their bioactivities and NMR shifts in the aromatic region. Such investigations are very important for food control of the prepared limes juices.

2. Materials and methods

2.1. Chemicals

Analytical terpene standards: α-Pinene, Limonene, Citronellal, Aromadendrene, Camphene, Linalool, Nerol, *trans*-Geraniol, β-Pinene, Terpinen-4-ol, Myrcene, γ-Terpinene, α-Terpineol, Citral (Sigma-Aldrich, St. Louis, MO, USA) were used to prepare standard solutions for calibration step. Methanol (Avantor Performance Materials Poland S.A) was used as a solvent of these solutions. Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid); 2,2′-azobis-2-methyl-propanimidamide; $FeCl_3x6H_2O$; Folin–Ciocalteu reagent (FCR); Tris, tris (hydroxymethy1)aminomethane; lanthanum (III) chloride heptahydrate; $CuCl_2\times 2H_2O$; and 2,9-dimethyl-1,10-phenanthroline (neocuproine), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium persulfate, deutered chloroform ($CDCl_3$), deuterated methanol-d4 (CH_3OH-d4), and deuterium oxide (D_2O) were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionized and distilled water were used throughout.

2.2. Sample preparation

The objects of study were the pulps of Kaffir lime (*Citrus hysteria, Citrus hystrix*) and Key lime (*Citrus aurantifolia*). The samples of Kaffir lime fruits were imported from Thailand where they had been bought on the floating market in Taling Chan, which is located in the western part of Bangkok. Samples were transported to Poland in sealed plastic bags in portable fridge maintained at between 10 and 15 °C. Key limes were bought in local distribution point in Poland. According to the seller's information, the country of origin of the fruit was Brazil.

In order to prepare for analysis, the fruits were washed with tap water and rinsed with distilled water. The fruit peel was then separated from the pulp and then squeezed to obtain the juices (Fig. 1). The next step was to weigh out 5.0 ± 0.1 g of sample unified composition in vials of 20 mL and then 1 mL of deionized water was added to the sample. The vials were closed with caps with silicone Teflon membrane. The procedure was repeated three times for each species of lime, each time using a new fruit.

2.3. Isolation and enrichment of analytes

Solid phase microextraction was used to carry out isolation and enrichment of analytes. The extraction was conducted using the divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber with thickness of 50/30 μm and length of 2 cm (Sigma-Aldrich, St. Louis, MO, USA). The extraction was carried out at 40 °C for 35 min. After this step the thermal desorption of the analytes at temperature of 250 °C for 5 min was provided. Between each analysis fiber was desorbed at 250 °C for 5 min. Extraction step was made using a MPS autosampler (Gerstel Co., Mülheim, Germany).

2.4. Instrumentation

Two-dimensional gas chromatograph Agilent 7980 (Agilent

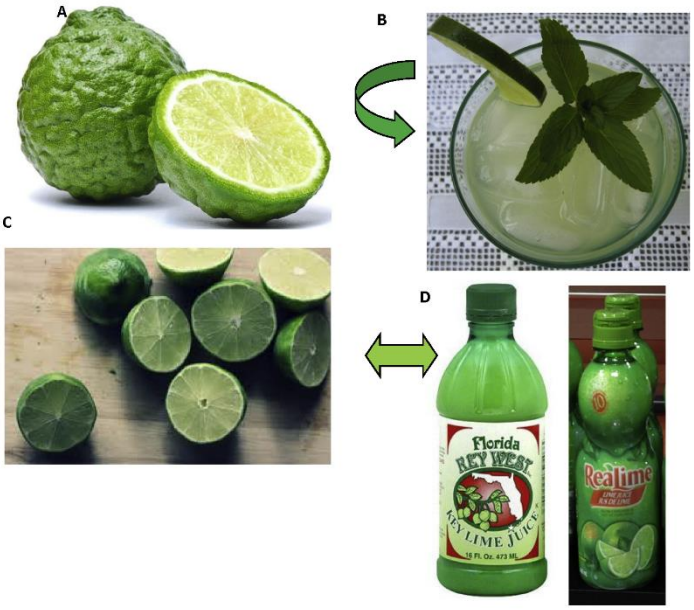

Fig. 1. A, B, C, D, Kaffir lime, Kaffir juice, Key lime, Key juice.

Technologies, Palo Alto, CA, USA) equipped with a liquid nitrogen cooled two-stage cryogenic modulator and the dispenser, working in spilt/splitless mode was used to carry out the analysis. Different types of chromatography columns were chosen to provide proper separation according to the rule of orthogonality. Table 1 shows the column parameters. Separation of substances was done by using the following temperature program: initial temperature of 40 °C was held for 3.5 min, then a linear increase of temperature to 250 °C at a rate of 5 °C/min was applied. The final temperature was held for 5 min. The temperature program applied in the secondary oven was set up with 5 °C shift. Modulation period was set up to 4 s. As the cooling medium, the liquid nitrogen was used, and hydrogen of purity N 6.0 was utilized as a carrier gas.

The volumetric flow rate was 1 mL/min. A single run time was 43.5 min. The injector worked in splitless mode at temperature of 250 °C. Temperature of transfer line and ion source was also 250 °C. The voltage of detector was set up at 1600 V. The data were collected over a mass range of m/z from 40 up to 500 with the acquisition rate of 125 spectra/s.

2.5. Data analysis

To identify the chemicals time of fight mass spectrometer Pegasus 4D produced by LECO (LECO Corp., St. Joseph, MI, USA) was used. Processing of data was done automatically using chromatographic peak deconvolution algorithm implemented in the software ChromaTOF (LECO Corp., version 4.44.0.0). Tentative identification of analytes was made by comparing experimental spectra with the spectra included in NIST 11 and Wiley libraries and by comparing calculated linear temperature-programmed retention indices (LTPRIs) with literature values. LTPRI values were calculated by performing analysis of C8–C20 n-alkanes. Positive identification was done using analytical terpenes standards.

2.6. Determination of bioactive compounds and total antioxidant capacities (TACs)

Polyphenols were extracted with methanol and water (concentration 20 mg/mL) during 1 h in a cooled ultrasonic bath. Total polyphenols (mg gallic acid equivalents (GAE)/g DW) were

Table 1
The parameters of chromatographic columns.

Parameters	I dimension column	II dimension column
Type:	capillary	capillary
Length:	30 m	1.6 m
Internal Diameter:	250 μm	100 μm
Maximum temperature:	325 °C	280 °C
Trade name of stationary phase:	Equity 1 (Supelco, Bellefonte, PA, USA)	SGWAX (SGE Analytical Science, Austin, TX, USA)
The film thickness of the stationary phase	0.25 μm	0.10 μm

determined by Folin-Ciocalteu method using spectrophotometer (Hewlett-Packard, model 8452A, Rockvile, USA) and measuring obtained absorbance after the complex reaction at wavelength of 750 nm (Singleton, Orthofer, & Lamuela-Raventos, 1999). Anthocyanins were determined by the measuring of absorbances of lime extracts (1 g of the defatted sample was extracted with 1 mL of acetonitrile containing 4% acetic acid) at 510 nm and 700 nm in buffers at pH 1.0 and 4.5, and calculated using following equation: $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$ with a molar extinction coefficient of cyaniding-3-glucoside of 29, 600. Results were expressed as milligrams of cyaniding-3-glucoside equivalent per 100 g dw (Cheng & Breen, 1991). Total carotenoids (xanthophylls+carotenes) were extracted with 100% acetone and determined spectrophotometrically at different absorbances (nm) such as at 661.6, 644.8, and 470, respectively (Boyer, 1990). Flavonoids, extracted with 5% $NaNO_2$, 10% $AlCl_3$ x H_2O and 1 M NaOH, were measured at 510 nm. Total flavanols were estimated using the *p*-dimethylaminocinnamaldehyde method, and the absorbance was measured at 640 nm (Feucht & Polster, 2001). The extracts of condensed tannins (procyanidins) with 4% vanillin solution in MeOH were measured at 500 nm. (+)Catechin served as a standard for flavonoids, flavanols and tannins as previously was described in details (Leontowicz et al., 2016). Total ascorbic acid was determined by CUPRAC assay in water extract (100 mg of lyophilized sample and 5 mL of water). The absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm (Ozyurek, Guclu, Bektasoglu, & Apak, 2007).

TACs were determined using the following methods:

2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) method. ABTS radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with methanol and the absorbance was measured at 734 nm (Re et al., 1999).

Ferric-reducing/antioxidant power (FRAP): FRAP reagent (2.5 mL of a 10 mmol ferric-tripiridyltriazine solution in 40 mmol HCl plus 2.5 mL of 20 mmol $FeCl_3xH_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6) of 900 μL was mixed with 90 μL of distilled water and 30 μL of asparagus extract samples as the appropriate reagent blank and absorbance was measured at 595 nm (Benzie & Strain, 1996).

1, 1-Diphenyl-2-picrylhydrazyl method (DPPH) solution (3.9 mL, 25 mg/L) in methanol was mixed with the samples extracts (0.1 mL). The reaction progress was monitored at 515 nm until the absorbance was stable. The scavenging rate on DPPH radicals was calculated (Brand-Williams, Cuvelier, & Berset, 1995).

Cupric reducing antioxidant capacity (CUPRAC): To the mixture of 1 mL of copper (II)-neocuproine and NH_4Ac buffer solution, acidified and non acidified methanol extracts of lime (or standard) solution (x, in mL) and H_2O [(1.1-x) mL] were added to make the final volume of 4.1 mL and the absorbance was measured at 450 nm (Apak, Guclu, Ozyurek, & Karademir, 2004).

2.7. Sample extraction and ^1H NMR analysis

Fine powder freeze dried material of 70 mg of each sample was added with either 700 μL of $CD_3OD + D_2O$ (ratio 1:1) or 700 μL of $CDCl_3$. The suspension (in a 1.5 mL Eppendorf tube) was ultrasonicated at room temperature for 30 min. And then, the suspension was centrifuged at 13.000 rpm for 10 min. The supernatant was transferred into 5 mL NMR tube and analyzed for its ^1H NMR. $CD_3OD + D_2O$ aimed to extracts polar metabolites, while $CDCl_3$ extracted non polar metabolites. All NMR experiments were recorded on Bruker 500 NMR spectrometer equipped with a 5-mm PABBO BB-probe head (499.953 for ^1H shifts) at 25 °C. NMR data processing was performed using MestReNova software (Abdul Hamid et al., 2017; Drzewiecki et al., 2016).

2.8. Statistical and classification analysis

The results of quantitative analysis were expressed as mean value and standard deviation (SD) of three measures of concentrations. Differences between groups were analyzed using two-way analysis of variance (ANOVA) followed by Duncan's new multiple range test with $\alpha = 0.05$. The analysis was carried out using STATISTICA 12 (StatSoft, Inc., Tulsa, Oklahoma, USA).

The peak areas obtained by GC×GC-TOF-MS analysis were used to sample classifications. Orange Canvas Data Mining (Bioinformatics Lab, University of Ljubljana, Slovenia) was used to perform Support Vector Machine (SVM), Tree Classification (TC), Naïve Bayes (NB) and Random Forest (RF) classifications with 2-fold cross-validation. The target class was the average over classes. All the classifiers were taken with their optimal settings.

3. Results and discussion

3.1. Composition of volatile substances

Detected chemical compounds were grouped according to their chemical classes (Fig. 2).

Comparing the volatile fractions of both species of fruits, it can be observed that the most numerous groups of chemical compounds presented in the volatile fraction, are terpenes. They represent nearly 88% of all volatile substances present in the Kaffir lime pulp, while in case of *Citrus aurantifolia* is about 53%. Kaffir lime pulp is therefore contains more aromatic compounds than Key lime. Additionally, high content of terpenes, which are considered as bioactive chemical compounds, makes this fruit as a rich source of prohealth constituents. In addition, 30% of difference in terpenes content explains the different odors of both fruits. It is well proven that the aroma of citrus fruits is composed of complex mixture of terpenes, which are chemical compounds whose main skeleton was formed by the combination of five-carbon isoprene units (Sharon-Asa et al., 2003). They are therefore the main group of Kaffir lime compounds and have a complex of bioactive properties such as antioxidant, antimicrobial or antiulcer effects (Al-Doghairi, El-Nadi, Elhag, & Al-Ayedh, 2004). Based on these properties, Kaffir limes can be classified as a superfruit, which were characterized by pro-health properties backed up by scientific research, contained bioactive compounds, stand out in exotic origin and taste. Alcohols, esters are the groups of chemical compounds with the smallest contribution in the composition of volatile fraction of *Citrus Hysteria*, which do not exceed 1%. In *Citrus aurantifolia* their content in volatile fraction is about 3%. Hydrocarbons are the next group and their percentage is more than 10%. Ketones represent 4% and aldehydes only 2% of the total content of the headspace of Kaffir lime. In case of *Citrus aurantifolia*, the content of aldehydes is similar. Such a distribution of all compounds is responsible for the smell of the fruits. Characteristic, intense scent is caused by the high content of terpenes compounds. Due to the very low content of carboxylic acids (<1%), the aroma of Kaffir lime has some irritating odor, which could cause some negative sensory perceptions during food control. To compare the content of selected terpenes, an analysis of *Citrus aurantifolia*, a fruit very popular and widely available in Europe, was also conducted. The object of the study was the juice of the above-mentioned lime fruits. The content of different terpenes for Kaffir and Key limes based on the results of Table 2 differ between them. In the volatile fraction of Kaffir lime, 119 terpenes were detected, and in case of Key lime - 87. Only chemical compounds with the mass spectral match factor, similarity >800 were considered.

The volatile fractions of both species of limes with the identified major chemical compounds are shown in Table 2. They were

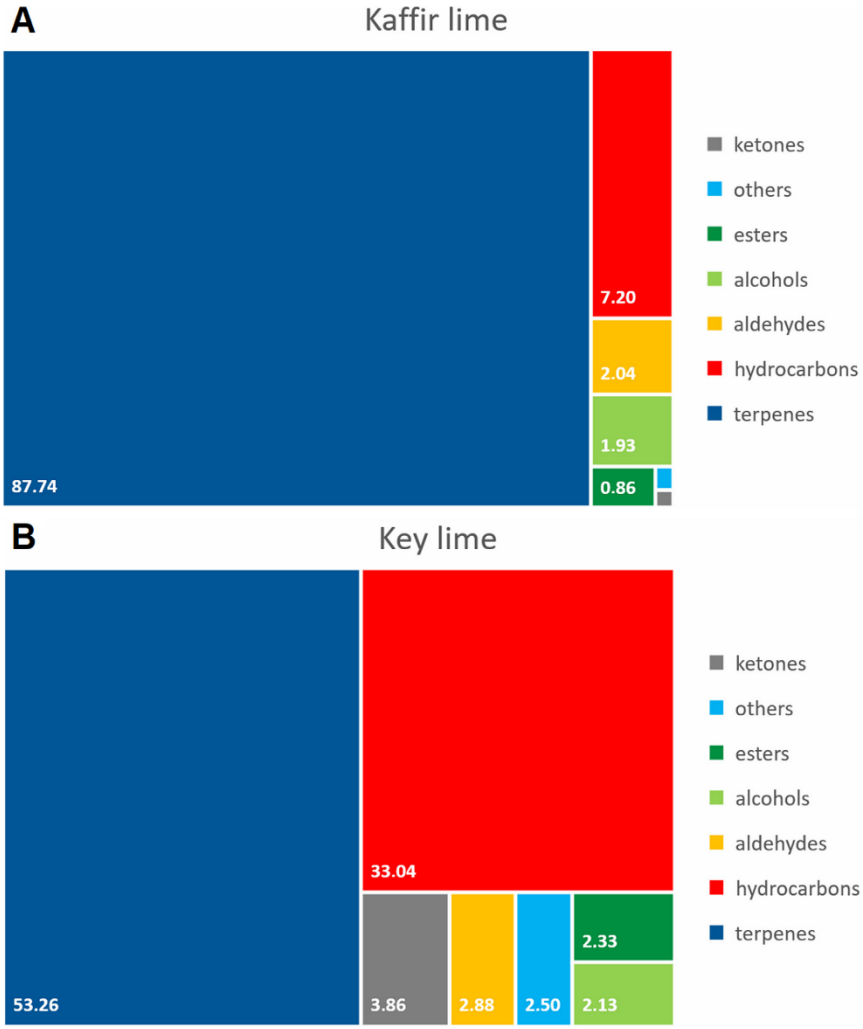

Fig. 2. Distribution of volatiles by chemical classes for: A. Kaffir lime, B. Key lime.

identified based on comparing spectra and LTPRI with literature data and the retention times were compared with retention time of internal standard. As it can be seen, all of the compounds belong to the terpenes family.

It can be observed, that the biggest differences in the content of selected terpenes in Kaffir and Key limes occur in case of chemical compounds such as Limonene, Citral, Terpinen-4-ol (Table 3). Limonene concentration is almost 8 times higher in the Key lime volatile fraction than in Kaffir lime. Extremely low content of Limonene compound in Kaffir lime was also found in citrus fruits (Waikedre et al., 2010). In the case of Citral, the difference in concentration of this compound in Kaffir lime is almost 20 mg/kg lower than in Key lime. Higher concentration of Terpinen-4-ol was noted in Kaffir lime samples and the content was almost 20 times higher. Terpinen-4-ol is the major chemical compound of volatile fraction of Kaffir lime. Terpinen-4-ol was selected as a major component of *Citrus hystrix* essential oil (Waikedre et al., 2010). For the other terpenes, differences in the contents are not statistically significant. The concentrations of α-Pinene, Citronellal, Camphene, Nerol, *trans*-Geraniol and β-Pinene are at similar levels in the volatile fraction of both fruits. In addition, in both cases the amount of the discussed analytes does not exceed 5 mg/kg. Terpenes, whose numbers in the fraction of fruits don't reach 10 ppm, are Myrcene and γ-Terpinene. The variation in the amount of the compounds explains the significant differences in taste and aroma of both fruit species. Among the determined terpenes, potential markers of Key

Table 2
The major compounds identified in the volatile fraction of Kaffir and Key limes using GC×GC-TOF-MS.

No.	Chemical compound	RT1 [s]	Average RT2 [s]	Similarity	Unique mass	$LTPRI_{lit}$	$LTPRI_{calc}$
1	β-Pinene	862	1.3	854	93	962	963
2	Sabinene	1386	2.3	939	93	958	959
3	Citronellal	1114	1.8	886	69	1132	1131
4	Linalool	1060	2.3	745	71	1082	1083
5	α-Terpineol	1294	1.4	810	59	1289	1289
6	β-Citronellol	1238	2.8	928	69	1211	1209
7	Citronellyl acetate	1402	1.7	916	69	1335	1337
8	α-Copaene	1757	1.4	937	161	1353	1354
10	α-Cubebene	1434	1.23	870	161	1345	1348
11	β-Caryophyllene	1580	1.7	917	93	1421	1420
12	Limonene	945	1.5	936	93	1022	1024
13	Germacrene D	1536	1.2	907	161	1486	1485
14	α-Pinene	796	1.3	861	93	949	950
15	Capmhene	812	1.3	951	91	953	956
16	Terpinen-4-ol	1170	2.3	864	71	1163	1060
17	α-Terpinene	930	1.4	939	93	1010	1008
18	Myrcene	857	1.4	808	93	994	993
19	α-Phellandrene	880	1.4	915	93	991	992
20	α-Thujene	1286	3.7	899	93	923	923
21	γ-Terpinene	1042	1.7	914	93	1050	1052
22	β -Phellandrene	850	1.3	836	93	1031	1030
23	Citral	1250	2.4	885	69	1240	1241
24	Nerol	1242	3.1	923	69	1228	1231
25	Geraniol	1318	3.9	890	69	1233	1232
26	Aromadendrene	1720	1.8	851	91	1455	1456

RT 1 – first dimension retention time, RT 2 – second dimension retention time, LRI_{calc} – Linear Retention Index calculated; LRI_{lit} – Linear Retention Index reported in the literature for DB 1 or equivalent stationary phase.

Table 3
Concentration of selected terpenes in the volatile fraction of Kaffir and Key limes.

No.	Chemical compound	R^2	LOQ	LOD	Concentration ± SD [mg/kg]	
					Kaffir lime	Key lime
1	α-Pinene	0.995	1.08	0.36	3.07 ± 0.03	1.04 ± 0.01
2	Limonene	0.994	1.22	0.40	10.78 ± 0.17	50.5 ± 2.1
3	Citronellal	0.992	0.25	0.49	<LOQ	0.55 ± 0.01
4	Aromadendrene	0.999	0.47	0.15	1.00 ± 0.07	<LOQ
5	Camphene	0.996	1.00	0,33	4.86 ± 0.67	3.38 ± 0.34
6	Linalool	0.990	1.69	0.56	20.13 ± 0.71	3.45 ± 0.09
7	Nerol	0.990	1.67	0.55	<LOQ	2.77 ± 0.10
8	*trans*-Geraniol	0.991	1.53	0.51	<LOQ	1.86 ± 0.10
9	β-Pinene	0.995	1.11	0.37	<LOQ	2.10 ± 0.11
10	Terpinen-4-ol	0.997	0.86	0.28	44.79 ± 1.09	1.96 ± 0.06
11	Myrcene	0.994	1.24	0.41	22.36 ± 0.95	24.89 ± 0.96
12	γ-Terpinene	0.993	1.32	0.44	25.01 ± 0.28	19.41 ± 0.69
13	α-Terpineol	0.996	0.98	0.32	1.50 ± 0.06[a]	0.68 ± 0.02[a]
14	Citral	0.990	1.64	0.54	<LOQ	20.91 ± 0.60

LOQ - limit of quantitation, LOD-limit od detection, SD - standard deviation, Mean ± SD of 3 measurements, Averages in rows marked with the same letters not differ significantly ($P > 0.05$), LOQ and LOD were calculated of the materiality level $\alpha = 0,05$.

and Kaffir limes aroma were selected. It was chosen that 20-fold difference in content was used as a criterion for qualifying a chemical compound to a group of potential indicators. In the case of Kaffir lime, Terpinen-4-ol was selected as a marker, with a content of 44.79 mg/kg. This is a chemical compound with a characteristic woody aroma. In the case of Key lime, Citral is an indicator with fresh citrus scent. The presence of the two chemical compounds mainly determines the smell of the fruits. Although Limonene's most important ingredient in Key Lime was not chosen as a flavor factor, because it is the most abundant chemical compound in many citrus juices (Moufida et al., 2003). Citral was repeatedly listed as one of the Key lime component (Cruz-Valenzuela, Tapia-Rodriguez, Vazquez-Armenta, Silva-Espinoza, & Ayala-Zavala, 2015). The content of Citral in lime up to 5% was found and this refers to essential oil (Costa et al., 2014; Spadaro et al., 2012). The results of quantitative determination of these chemical compounds in lime juices have not been done so far. A quantitative analysis of 5 citrus fruits shows that in each case Limonene is a major component, which confirms the correctness of the research. Key lime and Kaffir lime were not found among the fruits which were tested and Citral content was not determined (Moufida et al., 2003).

The chromatographic peak area was used as an input data for chemometric analysis. Data set was preprocessed before classification. Preprocess was based on center by mean and scaling using standard deviation SD. Orange Canvas was used for training of four classifiers and for performance cross validation evaluation. Table 4 shows the confusion matrices and evaluation results for used classification models, for results obtained by chromatographic technique. Proportion of predicted are presented in confusion matrices. All samples were correctly classified when 3 statistical models were used: SVM, NB and RF. CT and RF models are based on simple decision trees. SVM and NB models use more complex algorithms and therefore perform better. Based on results, the superiority of the RF model over CT can be observed. This may be due to the fact that CT is a single decision tree, while RF is a collection of decision trees. Table 4 with evaluated results contains information about area under curve (AUC), accuracy (CA), F1 score, precision and recall (sensitivity). When values of all described parameters are equal 1.00, the model is perfect. These results were achieved in three cases: SVM, NB and RF. In summary, the combination of GC×GC-TOF-MS technique with chemometric analysis may be a useful tool for classifying lime juice in terms of botanical origin. The most reliable results were achieved when 3 statistical models were used (SVM, NB and RF).

3.2. Antioxidant properties of juices

The results of bioactive compounds in the juices of two lime cultivars are presented in Table 5. In order to compare the changes in the amount of bioactive compounds, polyphenols and antioxidant capacities their values were determined in the pulp of the

Table 4
Data obtained by using GC×GC-TOF-MS and four statistical models.

Confusion matrix

SVM		Predicted		
		K	L	Σ
Actual	K	1.00	0.00	3
	L	0.00	1.00	3
	Σ	3	3	6

CT		Predicted		
		K	L	Σ
Actual	K	1.00	0.25	3
	L	0.00	0.75	3
	Σ	2	4	6

NB		Predicted		
		K	L	Σ
Actual	K	1.00	0.00	3
	L	0.00	1.00	3
	Σ	3	3	6

RF		Predicted		
		K	L	Σ
Actual	K	1.00	0.00	3
	L	0.00	1.00	3
	Σ	3	3	6

Evaluation results for classification models

Method	AUC	CA	F1	Precision	Recall
SVM	1.000	1.000	1.000	1.000	1.000
CT	0.875	0.833	0.857	0.875	0.833
NB	1.000	1.000	1.000	1.000	1.000
RF	1.000	1.000	1.000	1.000	1.000

K – Kaffir lime samples, L – Key lime samples, SVM – Support Vector Machine, CT – Classification Tree, NB – Naïve Bayes, RF – Random Forrest Classification, AUC – Area Under Curve, CA – Accuracy, F1 – F_1 score

Table 5
Bioactive substances per g dry weight (DW) in (A) water (W) and methanol (M) extracts and (B) vitamin C, anthocyanins, chlorophylls and carotenoids of Kaffir and Key limes juices.

Indices	Kaffir limeW	Kaffir limeM	Key limeW	Key limeM
Polyph,mgGAE	23.16 ± 2.18^{a}	15.79 ± 1.34^{b}	11.93 ± 7.65^{c}	12.78 ± 1.32^{bc}
Flavan, μgCE	91.48 ± 8.54^{a}	83.16 ± 6.54^{b}	64.44 ± 5.43^{bc}	51.18 ± 5.14^{c}
Flavon, mgCE	0.62 ± 0.05^{b}	1.41 ± 0.14^{a}	0.36 ± 0.05^{c}	0.65 ± 0.04^{b}
Tannins,mgCE	1.92 ± 0.19^{bc}	12.91 ± 2.11^{a}	0.78 ± 0.07^{c}	9.12 ± 0.85^{b}
ABTS,μMTE,	161.64 ± 8.54^{a}	75.43 ± 6.32^{bc}	57.49 ± 5.56^{c}	61.05 ± 7.65^{b}
FRAP, μMTE,	28.50 ± 2.43^{a}	20.01 ± 2.23^{b}	14.68 ± 1.32^{c}	16.20 ± 1.65^{bc}
CUPRAC,μMTE	124.28 ± 5.43^{a}	57.13 ± 5.21^{b}	27.96 ± 2.67^{c}	30.97 ± 2.45^{bc}
DPPH, μMTE	33.29 ± 3.21^{a}	14.83 ± 1.32^{b}	12.03 ± 1.18^{c}	17.15 ± 1.45^{ab}

Indices	Kaffir lime	Key lime
Vit C,mgAsc	1.74 ± 0.17^{a}	2.44 ± 0.11^{b}
Anthoc, mgCGE/kg	63.45 ± 5.15^{a}	47.32 ± 4.11^{b}
Chlor a, μg	468.50 ± 12.12^{a}	432.16 ± 10.98^{a}
Chlor b, μg	260.08 ± 7.65^{a}	245.76 ± 5.32^{a}
Chlor a+b,mg	729.00 ± 16.54^{a}	677.92 ± 12.54^{a}
Xan+Carot, μg	217.11 ± 5.32^{a}	195.43 ± 4.43^{a}

Mean ± SD (standard deviation) of 5 measurements. Averages in rows marked with different letters differ significantly ($P < 0.05$).

fruits and also in the fresh prepared juices. The differences were not significant and the decrease was about 1–2%. The polyphenols in water extract of Kaffir lime were twice higher than in Key lime, but in methanol extract were nearly equal. The bioactive compounds in Kaffir lime such as polyphenols, flavanols, flavonoids, and tannins were in water extract higher than in methanol of about 1.5 times. The antioxidant values were in similar correlation in 4 applied methods and the highest values showed ABTS and CUPRAC assays in water fraction (Table 5A). It means that most of bioactive substances were in the water extract. Key lime samples showed different picture. The amount of polyphenols, flavanols, flavonoids and tannins were slightly higher in methanol extract than in water. The corresponding values of antioxidants were in direct correlation with polyphenols. Anthocyanins, chlorophylls and carotenoids were similar in two limes, accept only the amount of vitamin C in Key lime (Table 5B). The results presented in Table 5 can be compared only with a few data from cited reports such as Damian-Reyna, Gonzalez-Hernandez, Maya-Yescas, de Jesus Cortes-Penagos, and Del Carmen Chavez-Parga (2017). Total phenolics, total flavonoids, and ascorbic acid contents in Mexican sweet lime (*Citrus limetta*) juice were determined at two commercial maturity stages. The results of the above shown indices differ from the presented in this report, but the lime variety was different with polyphenols (725 ± 9.14 mg/L), flavonoids (45.91 ± 1.00 mg/L), and ascorbic acid (222 ± 16 mg/L). Results indicated that Mexican citric fruits were good sources of antioxidant agents. The results were compared with Barros, Ferreira, and Genovese (2012). Four citrus species (*C. sinensis*, cvs. Pera and Lima; *C. latifolia* Tanaka cv. Tahiti; *C. limettioides* Tanaka cv. Sweet lime and *C. reticulate*, cv. Ponkan) were characterized in relation to pulps and peels contents of ascorbic acid, total polyphenols and antioxidant capacities. The antioxidant capacity of citrus was correlated both to vitamin C and phenolics. Aside from citrus pulps, the peels are also good sources of bioactive compounds and minerals, and can be explored for their health promoting values in food products.

In our previous report (Arancibia-Avila et al., 2012) was shown the influence of different time durations of thermal processing on berries quality.

The antioxidant activity only of berries subjected to thermal processing for 10 and 20 min did not differ from the non thermally processed, showing high correlation between the total polyphenols, flavanols and the antioxidant activities (Arancibia-Avila et al., 2012). In another report for preservation of bioactive compounds long cold storage of smoothie-type 'Hayward' kiwifruit beverages was suggested (Park et al., 2016). Both of the treatments as thermal and cold can be applied in the future research on the freshly prepared kiwifruit and berries juices, but also on limes.

3.3. NMR shifts in juices

NMR spectra of two limes cultivars are shown on Fig. 3. The assignments of ^{1}H spectrum of limes chloroform and methanol extracts were obtained, where only peaks of aromatic regions appeared between 6 and 10 ppm. The spectra were similar for two

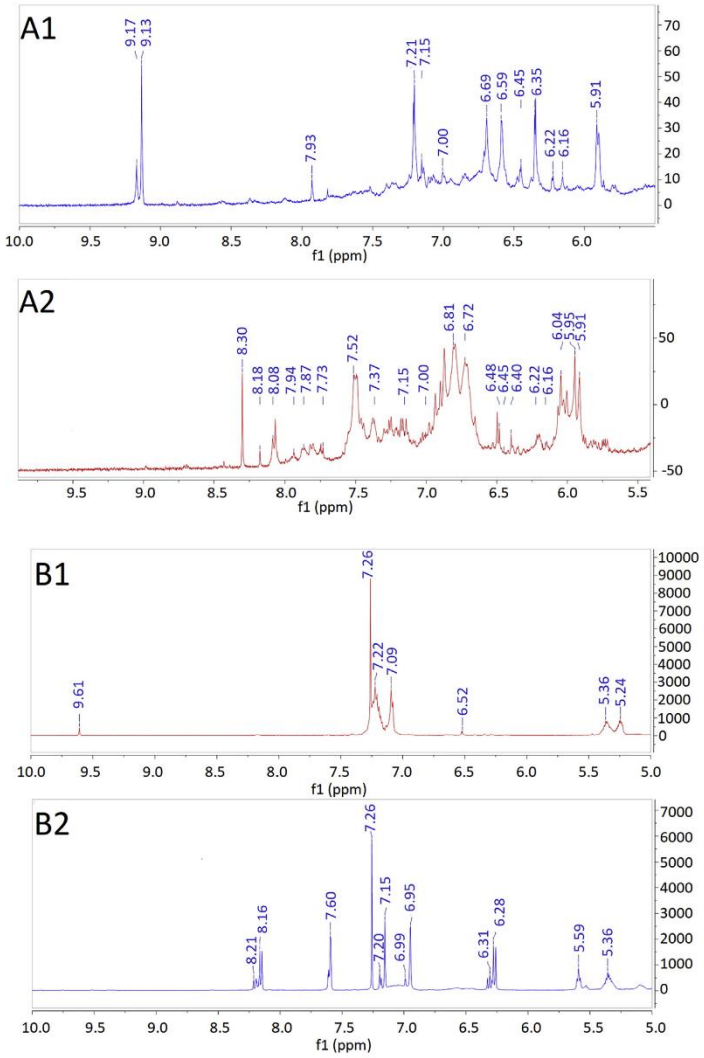

Fig. 3. Comparison of ^{1}H NMR spectra of limes juices in aromatic region: A1, A2, methanol extracts of Key and Kaffir limes; B1, B2, chloroform extracts of Key and Kaffir limes.

cultivars, but some slight differences were estimated. In the case of chloroform or methanol, mostly the sugars were the main compounds and the aromatic part was the minor (ppm). The mixture of water and methanol is usually used for extraction and subsequent NMR analysis (Fig. 3): for Key lime (Fig. 3A1: 9.17; 9.13; 7.93; 7.21; 7.15; 7.00; 6.69; 6.59; 6.45; 6.35; 6.22; 6.16; 5.91) and for Kaffir lime (Fig. 3A2: 8.30; 8.18; 8.08; 7.94; 7.87; 7.73; 7.52; 7.37; 7.15; 7.00; 6.81; 6.72; 6.48; 6.45; 6.40; 6.22; 6.16; 6.04; 5.95; 5.91) ppm. In two varieties of limes the common shifts appeared at 7.93; 7.15; 7.00; 6.45; 6.16 and 5.91 ppm. All cited literature of fruits was dealing only with aliphatic parts of juices. The aromatic range is shown in Balan, Nicolescu, Stavarache, Ciobanu, and Deleanu (2013). Most of the shifts were between 6.21 and 8.07 for orange juice: 6.65; 6.91; 7.07; 7.21; 7.32; 7.62; 7.72; and for grapefruit from 6.21 till 7.75 with the middle ones at 6.62; 6.92; 7.21; 7.38; 7.63 and 7.75 ppm. With orange and Key lime is a common shift in methanol fraction of 7.21 ppm, and for Kaffir lime at 8.08 ppm. For grapefruit juice the shifts are 6.21; 6.62; 6.91; 7.23; 7.38; 7.63; 7.75 ppm. Key lime showed a couple shifts common with grapefruit. In chloroform extract the following shift numbers appeared in Key lime (Fig. 3B1: 9.61; 7.22; 7.09; 6.52; 5.36; 5.24), Kaffir lime (Fig. 3B: 8.21; 8.16; 7.60; 7.20; 7.15; 6.99; 6.95; 6.31; 6.28; 5.59; 5.36). Common shifts

for two varieties were at 5.36 ppm. It is known that phenolic compounds are poorly soluble in water, but not for lime samples (Table 5, the amount of phenolic substances). Less peaks appeared in chloroform than in methanol. This result is obtained on the basis of the amount of polypenols extracted with chloroform is less than with methanol. Previously it was described how were characterized different kiwifruit cultivars. Using the NMR approach, it has been possible to identify primary as well as secondary metabolites of different fruits such as grape, orange, apple juice, mandarin, kiwifruits, mango, black raspberry, melon, watermelon, blueberry, and peaches (Abdul Hamid et al., 2017; Balan et al., 2013; Drzewiecki et al., 2016; Sobolev et al., 2015). In NMR-based analyses, the extraction procedure is probably the most critical step aimed to the quantitative transfer of the metabolites from the solid matrix. Peaks in the aromatic region indicate naringin and hesperidin, the main flavanone glycosides in citrus fruits and from 5.10 ppm showed glucose, sucrose, fructose, and formate (Maltese, Erkelens, van der Kooy, Choi, & Verpoorte, 2012; Rosa et al., 2015).

4. Conclusions

In this paper a usefulness of two dimensional gas chromatography coupled with time-of-flight mass spectrometry and NMR shifts were proven to assess the botanical origin of Kaffir lime and Key lime juices. Regarding to this, possible falsification of mentioned juices can be detected. GC×GC-TOF-MS allows for better separation of substances originating from complex matrices than in case of using one-dimensional chromatography, because of improved resolution, increased peak capacity and unique selectivity. On the basis of obtained results potential markers of botanical origin of limes were selected, namely Terpinen-4-ol for Kaffir lime and Citral for Key lime. Presented solution can be also treated as an alternative to other analytical techniques used for determining juice adulteration, such as HPLC or isotopic measurements. Furthermore, antioxidant property assessment of these juices shows that it can be a new natural source for everyday consumption.

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6.5 Human serum interactions with phenolic and aroma substances of Kaffir (*Citrus hystrix*) and Key lime (*Citrus aurantifolia*) juices

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Human serum interactions with phenolic and aroma substances of Kaffir (*Citrus hystrix*) and Key lime (*Citrus aurantifolia*) juices

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ABSTRACT

To understand the therapeutic application of polyphenols extracted from Kaffir (PolKaf) and Key (PolKey) limes different analytical methods were applied. Based on quantitative analysis by two dimensional gas chromatography (GC × GC) and time of flight mass spectrometry (TOFMS) it can be observed that the biggest differences in the contents of selected terpenes of Kaffir and Key limes occur in chemical compounds such as limonene, citral and terpinen-4-ol. Limonene concentration is almost 5 times higher in the volatile fraction of Key lime than in Kaffir lime. In the case of citral, the difference in concentration of this compound in Kaffir is 20 μg/g lower than in Key lime. Higher concentration of terpinen-4-ol was noted in Kaffir lime samples and the content was almost 23 times higher. Terpinen-4-ol is the major chemical compound of volatile fraction of Kaffir lime. Among the determined terpenes, potential markers of aroma were selected: terpinen-4-ol and citral for characterization of Kaffir and Key limes. Antioxidant assays revealed the highest bioactivity of Kaffir lime. Fluorescence studies between the interaction of polyphenols with human serum albumin (HSA) showed relatively high binding abilities in comparison with some antiplatelet drugs. The docking results showed that the hydrophobic residues are responsible for the interaction with the phyto-constituents. Citral is the best scored ADMET descriptor. The antioxidant strong affinity to HSA and synergism in bioactivity are the main indices in health application of citrus fruits.

1. Introduction

Juices or real pulps of some commercially grown citrus fruit (*Rutaceae*), grapefruit (*Citrus paradisi*), lemon (*Citrus limon*), lime (*Citrus x aurantiifolia*) and sweet orange (*Citrus sinensis*) were widely studied, where the phenolics and volatiles were the main antioxidant compounds found in all fruits [1,2]. Four citrus species (*C. sinensis*, *cvs.* Pera and Lima; *C. latifolia* Tanaka cv. Tahiti; *C. limettioides* Tanaka cv. Sweet lime and *C. reticulate*, cv. Ponkan) were characterized in relation to contents of ascorbic acid, total polyphenols and antioxidant capacity of pulps. The antioxidant capacity of citrus fruit was correlated both to vitamin C and phenolics. Aside from citrus pulps, the peels are also good sources of bioactive compounds and minerals, and can be explored for their health promoting values in food products [3]. There are a number of reports including Kaffir (*Citrus hystrix*) and Key (*Citrus aurantifolia*) limes. *Citrus aurantifolia* is mainly used in daily consumption and in juice production, based on antibacterial, anticancer, antidiabetic, antifungal, antihypertensive, anti-inflammatory, and antilipidemic properties. Antioxidants moreover can protect heart, liver, bone, and prevent urinary diseases. The secondary metabolites are alkaloids, carotenoids, coumarins, essential oils, flavonoids, phenolic acids, and triterpenoids. The other important constituents are apigenin, hesperetin, kaempferol, limonoids, quercetin, naringenin, nobiletin, and rutin, all of these contribute to its remedial properties [4]. The volatiles of limes have a wide application as essential oil microparticles. The use of prebiotic biopolymers can be a good option to add value to encapsulated products, thus promoting health benefits [5]. A combination of bioactive compounds makes all citrus fruits a useful source of everyday diet from a number of diseases [6–8]. As it was mentioned above there are some reports showing the composition of different kinds of limes [1,3,4], but it is a lack of research dealing with bioactive compounds and their interaction with serum proteins. The interactions between polyphenols, especially flavonoids and plasma proteins, have attracted great interest [9–13]. Few papers, however, have focused on

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the structure-affinity relationship of polyphenols and volatiles, on their affinities for plasma proteins, especially on human serum albumin with plant bioactive compounds [14,15] and with citrus fruits particularly [16,17]. The aim of this study was to determine the bioactive and aroma substances and their binding and antioxidant properties, using GC×GC-TOFMS, 2D- and 3D-fluorescence, spectroscopic antioxidant assays measurements and molecular docking.

2. Materials and methods

2.1. Materials

The following chemical compounds: Aromadendrene, Camphene, Citral, Citronellal, Limonene, Linalool, Myrcene, Nerol, Terpinen-4-ol, trans-Geraniol, α-Pinene, α-Terpineol, β-Pinene, γ-Terpinene, were used as standards (Sigma-Aldrich, St. Louis, MO, USA). Methanol (Avantor Performance Materials Poland S.A) was used to prepare the calibration solution mixtures. The mixture of *n*-alkanes from C8 to C20 (Sigma-Aldrich, St. Louis, MO, USA) was utilized for calculation of retention indexes. During the research deionized water of high purity from MilliQ A10 Gradient/ Elix System (Millipore, Bedford, MA, USA) was added to samples. Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid); Folin–Ciocalteu reagent (FCR); Tris, tris (hydroxymethy1)aminomethane; lanthanum (III) chloride heptahydrate; $CuCl_2 \times 2H_2O$; and 2,9-dimethyl-1,10-phenanthroline (neocuproine), 1,1-diphenyl-2-picrylhydrazyl (DPPH), were obtained from Sigma Chemical Co., St. Louis, MO, USA. All reagents were of analytical grade.

2.2. Sample preparation

The studies were performed on juices of two lime species: Kaffir and Key limes. Kaffir lime (*Citrus hysteria, Citrus hystrix*) was bought on the floating market in Taling Chan in Bangkok. Kaffir lime samples were transported to Poland in travel fridge and the temperature was between 10^0C and 15^0C. Key lime samples (*Citrus aurantifolia*) were purchased in local market in Gdansk, where they had been imported from Brazil. During the research for each sample, three repetitions were performed. Before analysis, the fruits were washed and rinsed with distilled water. Next step was to squeeze the juice from the fruit. Samples were prepared in the proportion of 5.0 + 0.1 g of fruit pulp and 1.0 mL of deionized water. Mixtures were then transferred into 20-mL vials. All samples were sealed with caps with 20 mm thick PTFE/silicone membrane [18].

2.3. Methods

2.3.1. Two-dimensional gas chromatography (GC×GC) and time of flight mass spectrometry (TOFMS)

HS-SPME (Headspace Solidphase Microextraction) extraction was done by the GC×GC-TOFMS procedure. A Gerstel autosampler (MPS autosampler, Gerstel, Mülheim, Germany) with agitator and SPME fiber conditioning station was used to isolate and to enrich the analytes from citrus samples. Before the extraction, the samples were kept at 40 °C for 5 min. Extraction was carried out at 40 °C for 35 min using a DVB/CAR/PDMS SPME fiber of 50/30-μm thickness and 2-cm length (Sigma-Aldrich, St. Louis, MO, USA). After the extraction, the fiber was removed from the vial and transferred to the injector of a gas chromatograph for thermal desorption of the analytes at 250 °C for 5 min. The GC×GC apparatus Agilent 7890 A (Agilent Technologies, Palo Alto, CA, USA) equipped with liquid nitrogen-based quad-jet cryogenic modulator and an injector in split/splitless mode, coupled with Pegasus 4D time-of-flight mass spectrometer (LECO Corp., St. Joseph, MI, USA), were used for the analysis. Two different capillary columns were used for the analysis. The first non-polar column was Equity-1 (30 m × 0.25 mm i.d. × 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA). The second column with polar stationary phase SolGel-Wax (2 m × 0.1 mm i.d. × 0.1 μm film thickness) was purchased from SGE Analytical Science (Austin, TX, USA). The chromatographic separation was performed using the following temperature program for the primary oven: Initial temperature 40 °C, kept for 3.5 min, ramped at 5 °C/min to 250 °C, and held for 5 min. The secondary oven temperature was programmed from 45 °C, kept for 3.5 min, ramped at 5 °C/min to 255 °C, and held for 5.83 min. The carrier gas was hydrogen (N6.0 class) at a constant flow rate at 1.0 mL/min. Temperature of the MS transfer line and the MS source was 250 °C. The modulation time was 4 s. The mass spectra data acquisition rate was 125 spectra/s. The data were collected over a mass range of 40–400 *m/z*. The voltage of detector was 1600 V. Analysis of the data obtained after the chromatographic analysis using GC×GC-TOFMS system was done using the algorithm for peak deconvolution implemented in the ChromaTOF software (LECO Corp., version 4.24). Analytes were tentatively identified by comparison of experimental spectra with the NIST 2011 mass spectral library. Analytes were also identified by comparing calculated linear temperature-programmed retention indices (LTPRIs) with literature values [18].

2.3.2. Determination of bioactive compounds and antioxidant activities

Polyphenols were extracted from lyophilized samples with water (concentration 20 mg/mL) during 1 h in a cooled ultrasonic bath. The extracts were filtered through the Buchner funnel. These extracts were submitted for determination of bioactive compounds. The polyphenols were determined by Folin-Ciocalteu method with measurement at 750 nm with spectrophotometer (Hewlett-Packard, model 8452 A, Rockvile, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g DW [19].

The total antioxidant capacity (TAC) was determined by the following assays:

Cupric reducing antioxidant capacity (CUPRAC): To the mixture of 1 mL of copper (II)-neocuproine and NH_4Ac buffer solution, acidified and non acidified ethanol extracts of berry (or standard) solution (x, in mL) and H_2O [(1.1 − x) mL] were added to make a final volume of 4.1 mL. The absorbance at 450 nm was recorded against a reagent blank [20].

1, 1-Diphenyl-2-picrylhydrazyl method (DPPH) solution (3.9 mL, 25 mg/L) in methanol was mixed with the samples extracts (0.1 mL). The reaction progress was monitored at 515 nm until the absorbance was stable. The scavenging rate on DPPH radicals was calculated [21].

2.3.3. Fluorometric measurements and binding properties

Fluorometric measurements were used for the evaluation of binding properties of citrus extracts to human serum albumin. Two dimensional (2D–FL) and three dimensional (3D-FL) fluorescence measurements were recorded on a model FP-6500, Jasco spectrofluorometer, serial N261332, Japan. The concentrations of citrus extracts were ranged from 0 to 1.5 mg/mL, and the total accumulated volume of citrus extracts was no greater than 150 μL. The corresponding fluorescence emission spectra were then recorded in the range of 300–500 nm upon excitation at 280 nm in each case. The emission wavelength was recorded between 200 and 795 nm for three-dimensional fluorescence spectra. All solutions for protein interaction were prepared in 0.05 mol/L Tris-HCl buffer (pH 7.4), containing 0.1 mol/L NaCl [14,18].

2.3.4. Molecular docking

The Ligand Fit module of Discovery Studio (DS Version 2.5) package was used for molecular docking. The X-ray crystallographic structures of HSA, 1h9z.pdb solved at 2.5 Å and complexed with ligands was retrieved from the protein data bank (PDB) and modified for docking calculations [14]. The co-crystallized ligands and the water molecules were removed from the protein structure. The H atoms were added and side chains were fixed using the protein preparation protocol. Optimization of the atomic charges and the structure minimization was

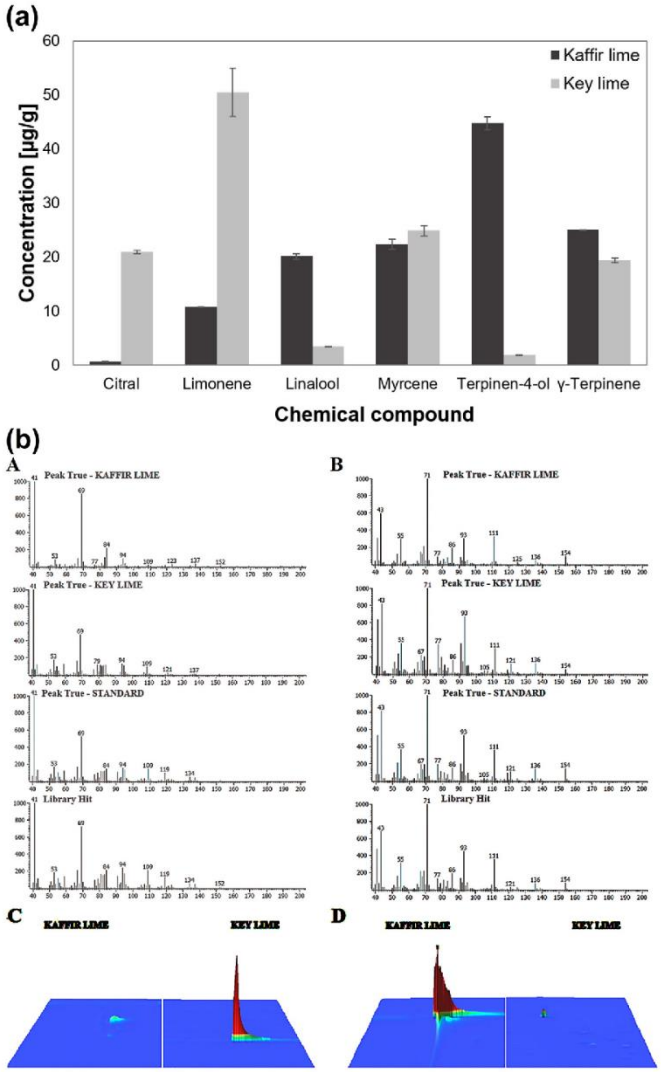

Fig. 1. **(a)** Concentrations of selected terpenes in Kaffir and Key limes juices. **(b)** Data obtained from GC×GC-TOF-MS analysis: **A**, citral mass spectrum, **B**, terpinen-4-ol mass spectrum, **C**, 3D chromatograms for citral (selected masses: 41, 69, 84, 94), **D**, 3D chromatograms for terpinen-4-ol (selected masses: 43, 77, 93, 111).

performed using CHARMM force field. To gain further insight in the interaction of Kaffir and Key lime extracts with HSA proteins, 14 active phyto-constituents detected in lime. Among which the 6 ligands based on the flavoring and aroma of the lime were selected for docking studies (Fig. 1). Two dimensional structures of these phyto-constituents were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The correct protonation states and partial charges were applied using the Ligand preparation module available with DS 2.5. Further the prepared ligands and protein structure were loaded to the docking protocol workspace. Best 10 poses were further processed and calculated [22].

2.3.4.1. In silico ADMET (absorption distribution metabolism excretion and toxicity) studies. Pharmacokinetic properties of the ligands were studied using SwissADME [23] and Molinspiration [24]. ADMET descriptor such as GI absorption, CYP1A2 inhibitor, PSA and MolLogP were predicted. The program requires the input information in SDF/MOL or SMILES file format.

2.3.5. Statistical analysis

To verify the statistical significance, means ± SD of five independent measurements were calculated. One-way analysis on variance (ANOVA) for statistical evaluation of results was used, following

by Duncan's new multiple range tests to assess differences between group's means. P values of < 0.05 were considered to be significant.

3. Results and discussion

3.1. Aroma, polyphenols and antioxidant activities in lime samples

Based on Fig. 1a, it can be observed that myrcene and γ-terpinene occur in similar amounts in both types of lime juices. However, the remaining four selected terpenes show more significant differences in concentrations in the tested samples. The essential oil of *Citrus aurantifolia* [25] possesses important spasmolytic properties, which are due to their major constituents, such as limonene (58.4%), δ-pinene (15.4%), γ-terpinene (8.5%), and citral (4.4%). Limonene and citral are chemical compounds characteristic of Key lime. The concentration of limonene in Key lime is estimated to 50.5 ± 2.1 μg/g and it is higher than in Kaffir lime. In the case of citral, the concentration of this substance is more than 30-fold higher in the volatile fraction in the Key lime than in the Kaffir lime. Terpinen-4-ol and linalool are the main terpenes of the Kaffir lime volatile fraction. The content of these substances was respectively more than 20- and 5-fold higher in Kaffir lime compared to the Key lime. Above-mentioned facts can explain the variation in flavour and aroma of tested fruit species. Among the determined substances, potential indicators of Kaffir and Key limes were selected. As a criterion for qualifying a chemical compound to a group of potential markers was 20-fold difference in concentration. For Kaffir lime, terpinen-4-ol which concentration was estimated at 44.8 ± 1.1 μg/g, was selected as the indicator. This compound is characterized by a woody aroma. In the case of Key lime, the substance with fresh and citrus scent, namely citral with estimated concentration at 20.91 ± 0.60 μg/g, was chosen as a marker (Fig. 1 b). The main constituent of the volatile fraction of Key lime is limonene. This substance was not selected as a potential flavor indicator, because its presence is observed in many citrus juices.

The content of citral in lime up to 5% was found and this refers to essential oil [25,26]. The results of phenolic acids and antioxidant activities are presented in the Table 1. The amount of polyphenols in water extract in Kaffir lime was as much as twice higher than in Key lime. Citral as an indicator of Key lime was 1.5 times higher than terpinen-4-ol for Kaffir lime. The antioxidant activities were in accordance with the amount of polyphenols. There is a lack of data to compare the present results with the refereed literature. Most recent reports are connected with citrus essential oils from peels. Between four different varieties of citrus species peels *Citrus aurantifolia* contained high amounts of bioactive substances such as phenolics and flavonoids [27]. For estimation of antioxidant activity in peels the same methods as in the present research were used and correlation of total phenolics in various extracts was found [28]. Recent reports were also based on the activity of leaves from limes [29]. As it was mentioned above, there are few reports dealing with antioxidants and terpenes in lime juice and pulp. The values of bioactivity obtained in this report are in line with the recent report [30], where was concluded that the health benefits of *citrus aurantifolia* are associated with its high amounts of photochemical and bioactive compounds such as flavonoids and phenols. The phenolic content and antioxidant properties of the manually squeezed lemon juice had higher total phenol content (64.5 GAE mg/L), while lime juice had higher total flavonoid content (29.5 QE mg/L). These results were slightly lower than the found in this research. Both juices exhibited antioxidant activities as typified by their ferric reducing power, and radicals (DPPH·, ABTS·, OH·, and NO·) scavenging abilities. Lime juices showed higher antioxidant activities than lemon. The inhibition of anti-angiotensin-1-converting enzyme (ACE) activity *in vitro* and *in vivo* hypocholesterolemic effect of the juices could explain the use of the juices in the management of cardiovascular diseases [31]. Comparison of antioxidant activities of water extracts [32] of different plants showed that *Phoenix dactylifera and Citrus aurantifolia* had a significantly higher total phenol and DPPH scavenging activities than other investigated plants. The water extracts of *Phoenix dactylifera* and *Citrus aurantifolia* had the highest protective ability and this probably due to its higher antioxidant activity, total phenol content, and DPPH scavenging activity [32]. Our results are in accordance with reported in [33], where all oils showed the effects on DPPH in the range of 17.7–64.0%. The oils of Ichang lemon (64.0%, 172.2 mg TE/mL), Tahiti lime (63.2%, 170.2 mg TE/mL), and Eureka lemon (61.8%, 166.2 mg TE/mL) showed stronger radical scavengers than other citrus oils. Citrus volatile components such as geraniol (87.7%, 235.9 mg TE/mL), terpinolene (87.4%, 235.2 mg TE/mL), and γ-terpinene (84.7%, 227.9 mg TE/mL) showed marked scavenging activities on DPPH ($p < 0.05$). These numbers are in accordance with the results presented in Table 1, where terpinen-4-ol and citral showed relatively high DPPH values. The results of antioxidant activity of citral was similar to [34], where citral isolated from sweet orange possesses antioxidant activity by DPPH radical scavenging activity and cytotoxic properties, and is a potential source of active ingredients for food and pharmaceutical industry. Limonene, linalool and citral are common non-phenolic terpenoid components of essential oils, with attributed controversial antioxidant properties. Results indicate that antioxidant behavior of limonene, linalool and citral occurs by co-oxidation with the substrate, due to very fast self-termination and cross-termination of the oxidative chain [35]. Individual substances such as citral and limonene had the minimum antioxidant activities, but the antioxidant activities of their mixture were higher. The synergistic effects in the antioxidant activity and stability of the main oil components were found [36]. In another report [37] as well were discussed the similarities and differences between the antioxidant activities of some essential oils and their main components such as thymol, estragole, menthol, eugenol, carvacrol, camphor and limonene. The comparison of antioxidant values of the oils and their components shows that the

Table 1
Antioxidant and binding properties of limes and monoterpenes in water extract.

Indices	Kaffir lime	Key lime	Terpinen-4-ol	Citral
Polyphenols, mgGAE/g DW	23.65 ± 2.5^{a}	12.13 ± 1.1^{ab}	5.84 ± 0.6^{c}	8.32 ± 0.8^{b}
CUPRAC, μMTE/g DW	123.45 ± 10.4^{a}	28.34 ± 2.3^{ab}	13.43 ± 1.3^{c}	19.54 ± 1.8^{b}
DPPH, μMTE/g DW	33.87 ± 3.6^{a}	17.21 ± 1.6^{ab}	8.54 ± 0.8^{c}	12.23 ± 1.7^{b}
FI (peak a), A.U.	450.69 ± 9.7^{b}	488.90 ± 7.7^{ab}	520.80 ± 11.6^{a}	164.15 ± 10.3^{c}
FI (peak b), A.U.	709.71 ± 12.4^{b}	730.85 ± 5.2^{ab}	784.42 ± 6.3^{a}	–
FI (peak c), A.U.	168.32 ± 6.3^{a}	83.64 ± 2.5^{b}	–	–
Binding to HSA, peak a %	20.96 ± 1.5^{b}	19.04 ± 4.6^{b}	8.67 ± 0.9^{c}	28.79 ± 2.8^{a}
Binding to HSA, peak b %	16.74 ± 1.2^{a}	14.26 ± 0.5^{ab}	7.66 ± 0.7^{b}	–

Values are means ± SD of 5 measurements; Means within a row with the different superscripts are statistically different ($p < 0.05$; Student's *t*-test). Abbreviations: GAE, gallic acid equivalent; CUPRAC, Cupric reducing antioxidant capacity; DPPH, 1, 1-Diphenyl-2-picrylhydrazyl method; TE, trolox equivalent; FI, fluorescence intensity; A. U., arbitral units; per g dry weight (DW); HSA, human serum albumin; FI of HSA in water according to peak **a** is equal to 570.21 ± 9.2; peak **b is** equal to 852.40 ± 11.3.

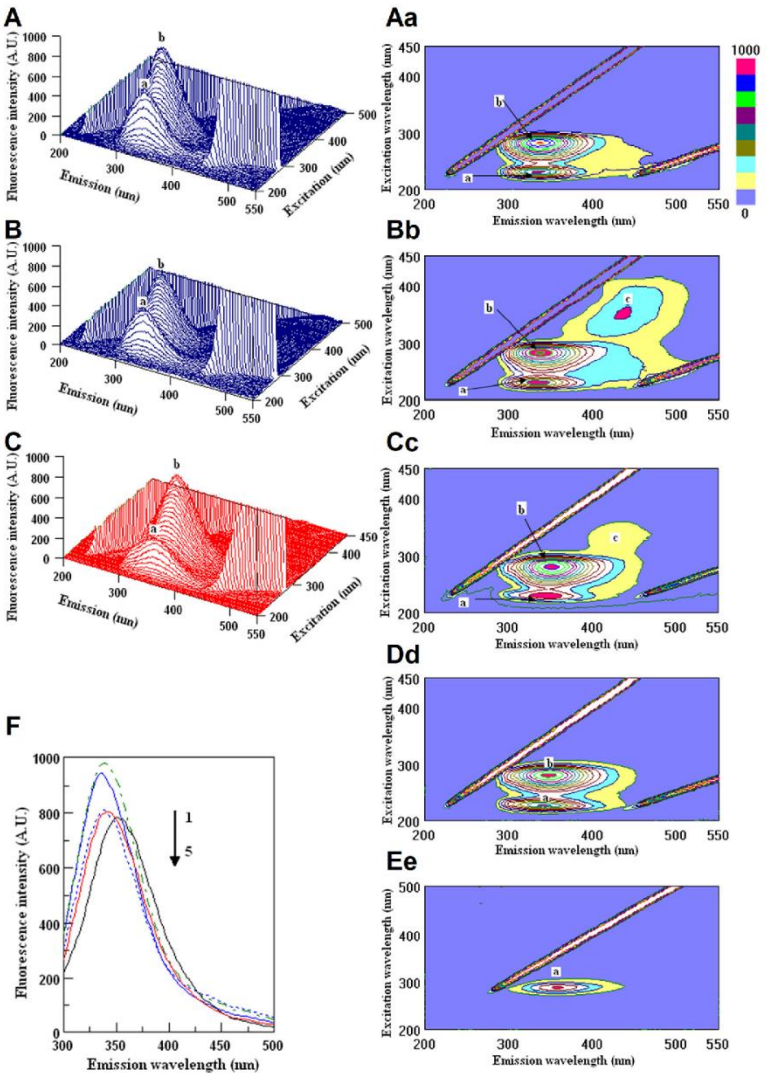

Fig. 2. 3D-fluorescence spectra of water extracts of polyphenols in interaction with HSA, **A–C**, HSA, Kaffir lime, Key lime; **Aa, Bb, Cc, Dd, Ee,** corresponding contour spectral images of HSA, Kaffir lime, Key lime, Terpinen-4-ol, Citral. The values of peaks **a, b** and **c** are given in Table 1. **F,** lines from the top: 1–5, HSA in buffer (FI = 980.87), HSA after interaction with water (FI = 945.87); HSA after interaction with catechin (FI = 821.00); HSA after interaction with Key lime (811.00); HSA after interaction with Kaffir lime (FI = 787.55). Measurements in 2D- FL were done in water extracts of citrus samples with concentration 0.83 mg/mL; Catechin was at concentration of 0.001 mM; λem 290 nm, λex 280 nm; HSA, human serum albumin; FI, fluorescence intensity in A.U.

antioxidant properties of essential oil do not always depend on the antioxidant activity of its main component, and that they can be modulated by their other components. The obtained results in this study support the conclusion that when comparing the antioxidant properties of essential oils and their main components, the concepts of synergism, antagonism and additivity are very relevant [34–37].

3.2. Fluorescence studies

The fluorescence properties of lime extracts and standards are shown in Table 1 and Fig. 2. The highest binding properties were in Kaffir lime (Table 1 and Fig. 2B, in two peaks). Lower binding properties in comparison with Kaffir were in Key lime (Table 1, Fig. 2C). The binding properties of the polyphenols extracted from limes were relatively high showing the correlation between the antioxidant and quenching properties of polyphenols towards human serum albumin (Table 1). Peak **b** mainly reveals the spectral behavior of Trp and Tyr residues. Peak **a** mainly exhibits the fluorescence spectral behavior of polypeptide back- bone structures, and its intensity relates to the secondary structure of the protein. Both fluorescence peaks of HSA had been quenched by limes, but to different extents (Table 1). Peak **c**, which was detected only after interaction of lime samples with HSA, did not influence the quenching and remained in the same position and with the same fluorescence intensities in two samples (Fig. 2B, C).

The results of 2D-fluorescence showed the changes in the fluorescence intensity (FI) according only to one peak, and the results of

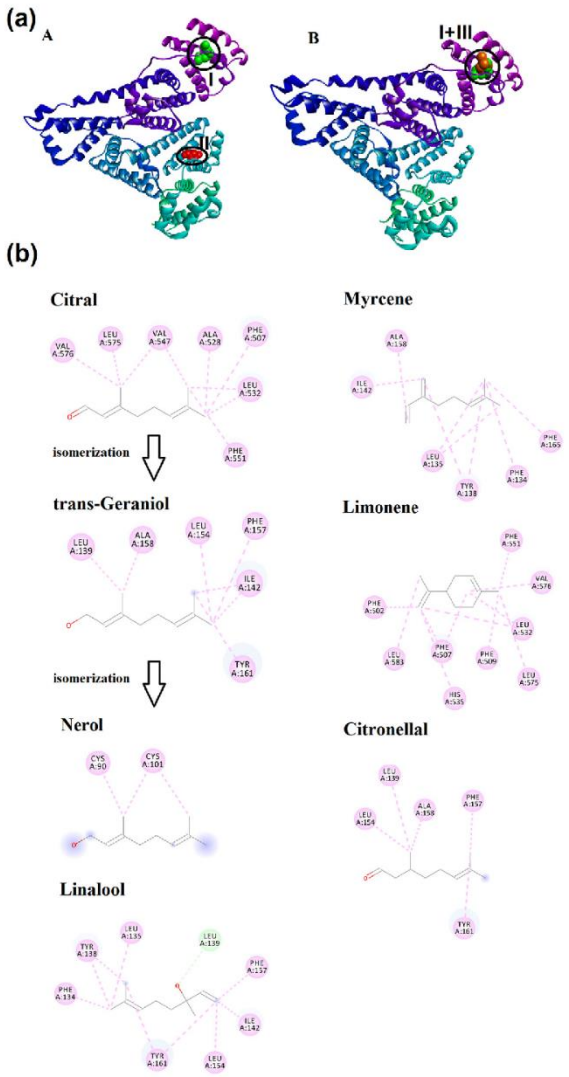

Fig. 3. **(a)** The docking images of the chemotypes. **A.** Binding ligands- citral/myrcene from the Chemotype I. **B.** Binding of the ligand - citral/limonene from chemotype II. Roman numerals represent: I-citral; II-myrcene; III-limonene. **(b)** 2D images showing the active residues in the binding site involved in the interaction of the ligands with the receptor protein (HSA).

binding (%) were the following: with catechin (13.21, Fig. 2F, line 3 from the top), Key lime (14.26, Fig. 2F, line 4 from the top) and Kaffir lime (16.74, Fig. 2F, line 5 from the top).

The present results are in agreement with [38], where the effects of three kinds of flavonoids, quercetin, rutin and baicalin, on the binding of ticagrelor to HSA were investigated using fluorescence. According to the data in [38] the binding properties of ticagrelor, a new antiplatelet drug, is about 28.8%. The addition of Kaffir lime to HSA showed increased binding of 37.7%, and with Key lime was slightly lower of 33.3%. As it was described above volatile and polyphenol substances have numerous pharmacological activities, including cardiovascular effects, antioxidant, anti-inflammatory, antiallergic, antimicrobial, antithrombotic, antiviral, antidiabetic, estrogenic and anticarcinogenic activities. Our results of interaction of volatile substances with human serum albumin are in line with other research reports. It was shown that the volatiles possess antioxidant activity similar to polyphenols, then their binding properties can be compared with polyphenols [39–41]. The present results showed that polyphenols extracted from limes quenched HSA (Table 1) similar to the drugs shown in the cited above reports.

Table 2a
Flavour profile of the phyto-constituents and interaction of the ligands with HSA.

S. No	Phyto-contituents of lime	Flavor profile	Docking Fitness	LOQ	Binding Energy (kcal/mol)	Interacting residues from HSA
1	Citral	Lemon	240.900	1.64	-89.92	VAL576;LEU575;VAL547; ALA528;PHE507;LEU532; PHE551
2	Nerol	Floral, Fruit	236.001	1.67	-9.36	CYS90; CYS101
3	Geraniol	Geranium, Lemon Peel, Passion Fruit, Peach, Rose	238.232	1.53	-66.26	LEU139; ALA158; LEU154; PHE157; ILE142; TYR161
4	Myrcene	Balsamic,Fruit, Geranium, Herb, Must	246.248	1.24	-53.75	ALA158; ILE142; LEU135;TYR138; PHE134;PHE165
5	Limonene	Citrus, orange	213.456	1.22	-56.22	VAL576;PHE551; LEU532; LEU575; PHE509;PHE507; HIS535;LEU583; PHE502
6	Citronellal	Citrus, Leaf	135.074	0.25	-64.92	LEU139; ALA158; LEU154; PHE157; TYR161
7	Linalool	Coriander, Floral, Lavender, Lemon, Rose	138.067	1.69	-61.94	TYR138;LEU135; LEU139:PHE157; ILE142;LEU154; TYR161;PHE134

Table 2b
ADMET properties of the compounds from lime.

S.no	Phyto-contituents of lime	TPSA	$LogP_{o/w}$	GI absorption	MolLogP > 4.15	Drug likeness (Lipinski)
1	Citral	17.07	2.35	High	3.49	− 1.08
2	Nerol	20.23	2.78	High	3.33	− 1.11
3	Geraniol	20.23	2.78	High	3.33	− 1.11
4	Myrcene	0.00	3.43	Low	3.91	− 1.30
5	Limonene	0.00	3.37	Low	3.83	− 1.51
6	Citronellal	17.07	2.94	High	3.37	− 0.98
7	Linalool	20.23	2.66	High	3.25	− 0.99

3.3. Molecular docking

Based on the flavoring profile, high concentration and LOQ the phyto-constituents from lime were selected and the ligands were subjected to docking with HSA. Citral, myrcene, linalool, nerol and trans-Geraniol showed good dock score compared to the other monoterpenes (Fig. 3a, b). The LOQ is also high for these compounds compared to the other volatiles. Citral at low pH and under oxidative stress can isomerize from geraniol to nerol that leads to the degradation of the citrus aroma [42]. The docking results have also shown that binding energy of the trans-geraniol and nerol is less compared to citral. The binding energy of citral was 1.44 times higher than for terpinen-4-ol [39]. This is in direct correlation with the binding properties determined by fluorescence (Table 1), because the binding of citral was 1.76 times higher than for terpinen-4-ol. This result substantiate that isomerisation of the citral from trans-geraniol to nerol can reduce the binding efficiency. Citral and limonene are major compounds available at high concentration in Key lime which gives the unique lemon odor and also makes the difference from the Kaffir lime. There are three different chemotypes reported with essential oil of lime such as Chemotype-I; citral/myrcene, Chemotype-II; citral/limonene and Chemotype-III; limonene/linalool/citronellal. Chemotypes I and II have been vastly studied as antitumor [43], antibacterial, antifungal [44] and antidepressant [45]. Citral, limonene and myrcene are the main chemical constituents of the three chemotypes. From the Ligandfit module, 24 binding sites were predicted in HSA. Citral and myrcene from chemotype-I have similar dock score and binding energy. Interestingly, citral showed interaction with amino acid residues in binding site 20 of HSA and myrcene interacts with binding site 2 of HSA. Citral/limonene under the chemotype II demonstrates interaction with the same amino acids in the binding site 2 of HSA. However, in chemotype III citronellal and linalool showed interaction with binding site 2 and limonene have interaction with residues from binding site 20 (Table 2a). Therefore from the docking results substantiate that site 2 and 20 as the active sites of HSA which is exclusively exposed with the hydrophobic residues responsible for interaction with the phyto-constituents.

3.3.1. In silico ADMET studies

In ADMET descriptors, total polar surface area (TPSA) is one of the most widely-used descriptors for predicting membrane permeability. The ligands myrcene and limonene the membrane permeability was poor compared to the other flavoring compounds of lime. Citral was detected with TPSA of 17.07. The GI absorption is used to reveal the absorption of the drug across the intestine. Intestine has a surface rich in microvilli and covers about 1000-fold of the stomach thus GI absorption of the drugs is efficient pharmacokinetic properties. Therefore among the top scoring compounds (citral, limonene and myrcene) it was observed that citral is the best scored ADMET descriptors (Table 2(b)).

4. Conclusions

Citral, myrcene and limonene are well known as plant-derived natural products finding their use in therapeutic applications in recent decades. The present investigation provides an insight into the binding properties of HSA with these pharmacologically important molecules. Chemotype II showed similar binding characteristics, with the latter have a stronger affinity to HSA. Both the compounds bind to site-2 with hydrophobic residues enclosed in the binding pocket of HSA. The data from present study can be useful in the establishment of their pharmacokinetic profiles in the process of future health food development. The binding properties of polyphenols from citrus fruits to HSA were relatively high in comparison with other plants, and it was a correlation between the binding properties and their bioactivities. This study gives evaluation of the bioactive interaction with human physiological system since HSA is the most important serum protein.

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6.6 Comparison of the Physical and Sensory Properties of Hybrid Citrus Fruit Jaffa® Sweetie in Relation to the Parent Fruits

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Comparison of the Physical and Sensory Properties of Hybrid Citrus Fruit Jaffa® Sweetie in Relation to the Parent Fruits

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Abstract: In the presented study, an overall Jaffa sweetie evaluation was made to find a correlation between *Citrus grandis* Osbeck × *Citrus paradisi* Macf. and its parent fruits' (*Citrus grandis* Osbeck, *Citrus paradisi* Macf.) properties. Based on the sensory analysis, it was found that the taste and aroma of the new hybrid fruit are close to pummelo. By the use of chromatographic analysis, the selected monoterpenes present in the fruits were quantified. α-terpineol was typed as the main monoterpene compound in the headspace of sweetie and grapefruit, with the concentrations: 20.96 and 87.9 μg/g, respectively. In turn, γ-terpinene was chosen as the most important monoterpene determining the flavor of sweetie fruit. Based on two-dimensional gas chromatography (GC × GC-TOF-MS) and principal component analysis (PCA) of the data, several volatile compounds were associated with analyzed fruits' aroma. Jaffa Sweetie is the hybrid fruit with sensory properties similar to pummelo with a higher content of monoterpenes, which improves its health benefits compared to the parent fruit. The research presents an instrumental method for assessing the aroma properties of the fruit as a reference method for sensory analysis, commonly used in the industry.

Keywords: flavoromics; fruit hybridization; gas chromatography; sensory analysis; terpenes

1. Introduction

Fruits are important elements of the human diet. According to the latest recommendations of dietitians, several portions of fruit should be consumed every day. Due to the consumers' willingness for the consumption of fruits with the greatest health benefits, scientists and fruit farmers are still looking for new plant varieties that meet the expectations related to the content of vitamins or other substances with health-promoting effects. From the point of view of fruit farmers and producers, the new fruits should represent some specific functional properties, such as higher yields, greater resistance to climatic factors, or lack of seeds [1]. One of the popular solutions, used for many years and gaining more and more popularity, is the production of hybrid plants. Cross-breeding, also called fruit hybridization, is the botanical mating of two different plant species or varieties to create the hybrids that have all the best qualities of parent plants and none of their defects [2]. Hybrid plants can be created by cross-breeding individual varieties or species. Hybridization within one species can lead to a phenomenon known as heterosis. Heterotic individuals are characterized by higher fertility, better lifespan, and higher fruitfulness. The newly-created fruits, despite functional properties and health properties, also have new organoleptic attributes [3].

One of the popular hybrid fruit in recent years is sweetie (*Citrus grandis* Osbeck × *Citrus paradisi* Macf.), also called oroblanco, a hybrid between the giant orange, called pummelo (*Citrus grandis* Osbeck) and grapefruit (*Citrus paradisi* Macf.). The fruit was patented in 1981 by scientists at the University of California at Riverside [4]. This hybrid was created to improve the taste qualities of grapefruit and functional properties of pummelo while maintaining the nutritional properties. According to the reports, in 2019 sweeties market outlook in China was very promising in the long run [5]. Gorinstein et al. provided a complementary characteristic of bioactive properties of sweetie, by the determination of total phenol content and the antioxidant activity which was higher in oroblancos than in grapefruits [6].

The taste and the aroma of the fruit are very important factors determining the consumption of fruit by the consumers. It is also essential to determine the content of chemical compounds with pro-health effects, such as terpenes, polyphenols, anthocyanins, and flavonoids [7,8]. Determining the physical characteristics of the fruit is also useful when planning technological or logistic processes. In addition, based on the determination of the color of the fruit's peel, the preliminary evaluation of fruit's quality or freshness is possible. As there are four main characteristics that impart distinctive quality to the fruits: (1) color and appearance, (2) flavor (taste and aroma), (3) texture, and (4) nutritional value [9], the goal of the research was to show the new approaches in the evaluation of visual and flavor properties of Jaffa sweetie and comparing with its parent fruits to provide a full characteristic of the fruit. The second objective was the assessment of the effectiveness of the hybridization process of the oroblanco fruit from the consumers' point of view. For this purpose, the basic physical parameters of hybrid fruits were evaluated and the sensory analysis was carried out. The comparison of monoterpenes content was provided for sweetie, grapefruit, and pummelo. Determination of this group of compounds is important not only due to their sensory attributes but also because of their bioactive properties which complements the research previously conducted in terms of nutritional values. All chromatographic analyses were conducted using two-dimensional gas chromatography with mass spectrometry (GC × GC-MS) which has been widely used for the analysis of a variety of complex food samples. According to the best of our knowledge, no work has been performed so far to evaluate the monoterpenes content in Jaffa sweetie fruit and its parents' fruits using GC × GC-MS. The provided analysis of sweetie supplements the research on the bioactive properties of sweetie [6] in the full characteristics of this fruit. The results ensure a background for further industrial purposes.

2. Results and Discussion

Quality and consumers' acceptance of fruits depends on several attributes, such as color, appearance, flavor, texture, and nutritional properties. First of all, the investigation strategy involves determining its nutritional properties, as well as the chemical compounds responsible for flavor and aroma, and defining the degree of acceptance of the fruit by consumers in organoleptic tests. Moreover, by comparing the attributes of hybrid fruits with their parent fruits, the effectiveness of the crossbreeding process can be evaluated.

2.1. Overall Visual Fruit Evaluation

The first stage of the analysis was the overall visual assessment of the fruits, as well as the measurement of the fruit's weight and the outer diameter of the fruit at the widest point. The physical properties of the fruit are determined mainly as the quality of the fruit. The knowledge of some important physical properties is essential for the design of the storage structures, processing equipment, and processes [10]. The individual physical characteristics of the sweetie and its parent fruits are presented in Table 1.

of sweetness, the pummelo fruit was chosen. The bitterness (2.2 and 2.3 points on a 10-point scale) and tartness (1.9 and 1.0 points) of oroblanco are similar to pummelo. The sweetie hybrid fruit is as sour as pummelo, according to the respondents' assessment. However, in the case of grapefruit, it was found that it is the most bitter and acidic of the fruits under evaluation. These are undesirable flavor properties in the fruit, therefore it can be concluded that this is the least tasty fruit. This is confirmed by the overall taste rating. Over half of the respondents chose this fruit as the least tasty. Grapefruit is the juiciest of the three analyzed citruses. Oroblanco got a slightly lower rating, in this category. It can be concluded that this property was originating from a grapefruit. Based on the research, it can be stated that the sweetie fruit is more similar in taste to pummelo. The new hybrid also managed to get rid of the bitter taste of grapefruit, which is an advantage when choosen by consumers.

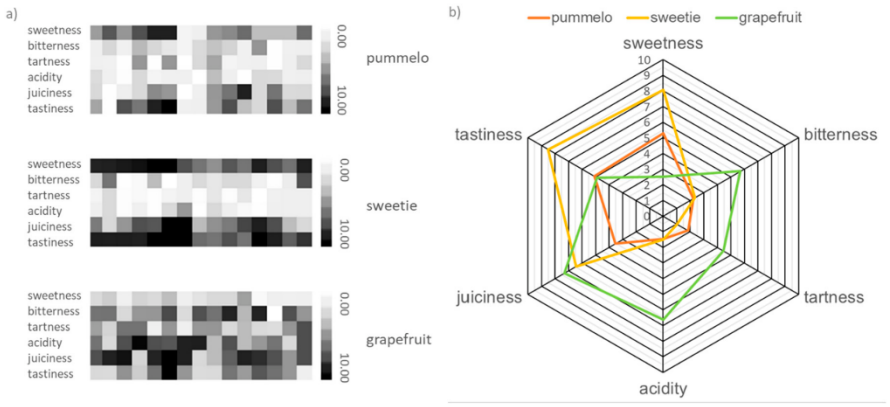

Figure 2. Results of sensory analysis of pummelo, sweetie, and grapefruit: (**a**) heat map of panelists' choices and (**b**) radar plot of average values of main sensory properties.

2.3. Chromatographic Analysis

Despite the tart and bitter taste, grapefruits are still very popular and more eagerly consumed then other fruit tested. This can be associated with the lower availability of sweetie compared to grapefruits, but above all, from the general opinion about the health-promoting properties of grapefruit. Several studies have been carried out on the analysis of chemical compounds with health effects in grapefruit samples [6,14,15], pummelo [17–19], and sweetie [6,15,20,21]. Many of these compounds, including terpenes, flavonoids, and polyphenols can be determined using gas chromatography. For determination of compounds from the terpenes group in a complex matrix, which is food, it is reasonable to use the technique of multidimensional gas chromatography [22]. The peak capacity in GC × GC is much higher comparing the one dimensional GC, which results in a significantly improved separation of individual analytes, and their separation from interfering matrix compounds. There is a lack of scientific reports about utilizing GC × GC for volatile organic compounds (VOCs) determination of pummelo, white grapefruit and sweetie samples. Total ion chromatogram contour plots of the citrus fruits VOCs using GC × GC analysis are presented in Figure 3.

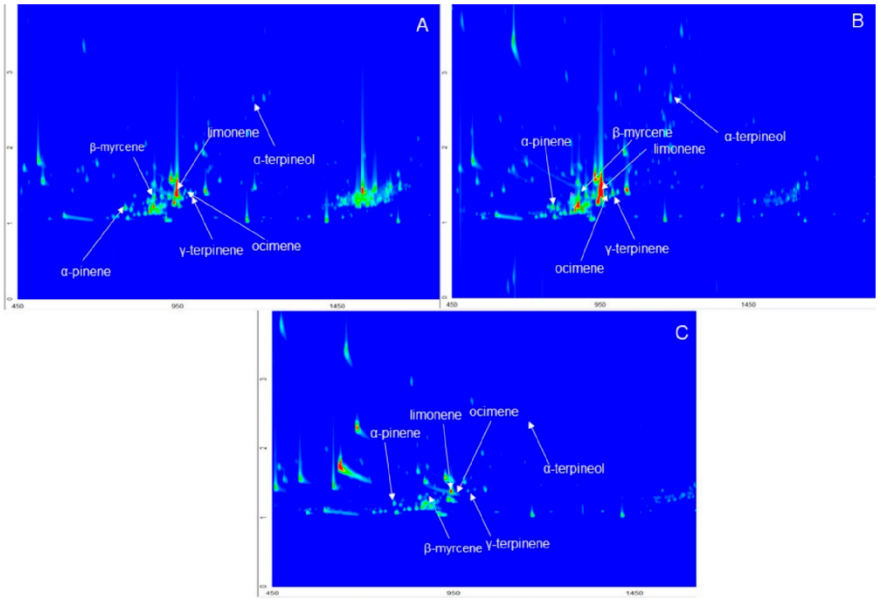

Figure 3. Two-dimensional gas chromatography (GC × GC) contour plots in total ion current (TIC) mode of (**A**) grapefruit, (**B**) sweetie, and (**C**) pummelo samples.

2.3.1. Qualitative Analysis

Based on the provided analysis, it was possible to detect about 600 chemical compounds in samples of pummelo, grapefruit and sweetie. The main chemical compounds identified in the samples of sweetie, grapefruit, and pummelo are presented in Table 2.

Table 2. Identification of selected volatiles compounds in sweetie, pummelo and grapefruit samples using HS-GC × GC-TOFMS technique.

Chemical Compound	CAS Number	Average		ID	S	G	P	Odor Type	Flavor Type
		RT1 [s]	RT2 [s]						
Terpenes									
p-Menthane	99-82-1	862	1.16	MS	+	+	+	pine	n.d.
p-Cymene	99-87-6	938	1.58	MS	+	+	+	terpenic	terpenic
Ocimene	6874-44-8	970	1.41	MS, RT	+	+	+	fruity	n.d.
γ-Terpinene	99-85-4	926	1.36	MS, RT	+	+	+	terpenic	terpenic
β-Myrcene	123-35-3	878	1.38	MS, RT	+	+	+	spicy	woody
Limonene	138-86-3	950	1.38	MS, RT	+	+	+	citrus	citrus
α-Pinene	80-56-8	790	1.22	MS, RT	+	+	+	herbal	woody
Citronellene	2436-90-0	806	1.20	MS	+	+	+	floral	n.d.
β-Pinene	127-91-3	858	1.30	MS, RT	+	+	+	herbal	pine
α-Terpineol	98-55-5	1186	2.67	MS, RT	+	-	+	terpenic	citrus
Alcohols									
Hexanol	111-27-3	654	3.47	MS	+	+	+	herbal	green
Pentanol	71-41-0	486	3.66	MS	+	+	+	fermented	fusel
3-Hexenol	928-97-2	638	0.15	MS	+	+	+	green	green
2-Hexenol	2305-21-7	658	0.27	MS	+	+	+	fruity	fruity
Octanol	111-87-5	1002	2.69	MS	+	+	+	waxy	waxy

Table 2. *Cont.*

Chemical Compound	CAS Number	Average		ID	S	G	P	Odor Type	Flavor Type
		RT1 [s]	RT2 [s]						
Aldehydes									
Hexanal	66-25-1	518	1.84	MS	+	+	+	green	green
Heptanal	111-71-7	702	1.79	MS	+	+	+	green	solvent
Nonanal	124-19-6	1046	1.70	MS	+	+	+	aldehydic	aldehydic
Octanal	124-13-0	878	1.76	MS	+	+	+	aldehydic	aldehydic
Esters									
Ethyl 2-methylbutyrate	7452-79-1	626	1.44	MS	+	+	+	fruity	fruity
Ethyl butanoate	105-54-4	534	1.56	MS	+	+	+	fruity	fruity
Ethyl hexanoate	123-66-0	882	1.50	MS	+	+	+	fruity	fruity
Ethyl isobutyrate	97-62-1	470	1.43	MS	+	+	+	fruity	ethereal
Ethyl octanoate	106-32-1	1190	1.49	MS	+	+	+	waxy	waxy
Hydrocarbons									
2,6-Dimethyl-2,6-octadiene	2792-39-4	902	1.23	MS	+	+	+	n.d.	n.d.
Octane	111-65-9	554	1.08	MS	+	+	+	gasoline	n.d.
Nonane	111-84-2	734	1.08	MS	+	+	+	gasoline	n.d.
4-Decene	19689-18-0	766	1.10	MS	+	+	+	n.d.	n.d.
Tetradecane	629-59-4	1362	1.10	MS	+	+	+	n.d.	n.d.
Ketones									
3-Octanone	106-68-3	854	1.68	MS	+	+	+	herbal	mushroom
6-Methyl-5-hepten-2-one	110-93-0	850	2.06	MS	+	+	+	citrus	n.d.
2-Heptanone	110-43-0	686	1.82	MS	+	+	+	cheesy	cheesy
4-Nonanone	4485-09-0	998	1.58	MS	+	–	+	n.d.	n.d.
6-Dodecanone	6064-27-3	1482	1.56	MS	+	–	+	n.d.	n.d.
Others									
2-Pentylfuran	3777-69-3	874	1.55	MS	+	+	+	fruity	green

ID—Method of identification: MS—identification by comparison with National Institute of Standards and Technology (NIST) mass spectral libraries; RT—identification by comparison with the retention time of analytical standard compound; Odor and flavor types were taken from The Good Scents Company; S—Sweetie, P—Pummelo, and G—Grapefruit, +/- — detected/not detected.

The compounds were divided into seven chemical classes (Figure 4). Terpenes are the dominant class in the fruits' headspace. Terpenes are secondary metabolites of many plants, produced to meet specific biological functions, such as hormone biosynthesis, but also protects against UV radiation and photooxidative stress, including pest and toxin repellents, growth regulators, pollinator attractors, photosynthetic dyes, and electron acceptor. They are the main ingredients of citrus essential oils accumulated in flavedo [23]. Citrus fruits are the main source of terpenes, especially limonene, in the human diet [24].

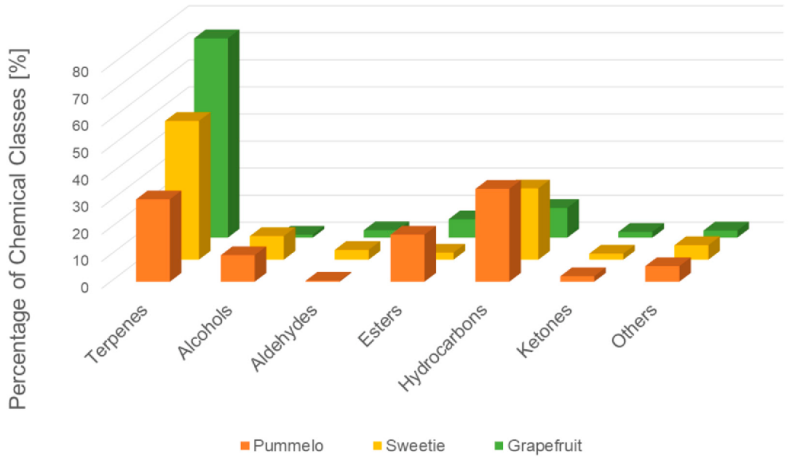

Figure 4. Distribution of volatiles by chemical classes.

Human thresholds of the selected terpenes are low and, thus, they have a big influence on creating citrus aroma even though they do not constitute the largest content in citruses. Therefore, highly sensitive and selective methods for the quantification of these compounds are needed [25]. The high content of terpenes in the citrus peels can determine the bitter taste of citrus [26]. The groups of esters, alcohols, aldehydes, and ketones were significant chemical classes, regarding the amount of identified substances. These chemical substances are characterized by a specific, often intense odors. Their presence in the headspace and synergistic interactions influence the intense aroma of fruits. Among the identified chemical compounds, limonene, citronellene, and γ-terpinene are characterized by a pleasant citrus aroma [27]. The high content of terpenes in sweetie fruit makes it a valuable component of the human diet. This is in agreement with the previous results [6].

2.3.2. Quantitative Analysis

Quantitative analysis is an essential step during the analysis of the influence of each volatile on the aroma of the sample. In the case of food samples, terpenes quantitation is also important for the understanding of the pro-health properties. For the quantitative analysis, the class of terpenes was chosen because of its greatest percentage distribution in the analyzed samples. The monoterpenes with the largest peak area were selected. The results of the quantitation of selected monoterpenes are presented in Table 3.

Table 3. Quantitation of selected monoterpenes present in the volatile fraction of sweetie, pummelo, and grapefruit (μg/g).

Monoterpene	R^2	S	P	G	LOQ	LOD
α-Pinene	0.999	0.8241 ± 0.0096	<LOQ	2.851 ± 0.015	0.657	0.219
Limonene	0.996	5.298 ± 0.058	2.75 ± 0.54	15.79 ± 0.30	1.416	0.472
Ocimene	0.995	1.600 ± 0.097	<LOQ	2.057 ± 0.078	1.504	0.501
β-Myrcene	0.991	4.1 ± 0.14	<LOQ	3.22 ± 0.029	2.077	0.692
γ-Terpinene	0.997	7.27 ± 0.34	<LOQ	2.566 ± 0.026	1.152	0.384
α-Terpineol	0.992	20.96 ± 0.70	<LOQ	87.9 ± 2.0	1.928	0.643

LOQ—Limit of quantitation, LOD—Limit of detection, S—Sweetie, P—Pummelo, and G—Grapefruit.

Based on the quantitative determination, the aroma properties of the fruits can be explained. As the terpenes are the main group of chemical compounds in the three tested fruits, according to quantitative analysis, it can be stated that pummelo is the least aromatic of mentioned fruits. Despite the use of the two-dimensional technique, in the pummelo fruits' flesh, it was possible to quantitatively determine only one volatile, namely limonene. The reason for this fact can be explained by the low interchange of chemicals between flesh and volatile fraction. Until now, the use of chromatographic techniques allows to determine the content of terpenes only in pummelo juices or extracts [13,28]. In contrast, in the sweetie and grapefruit volatile fraction, six terpenes were determined. In both cases, α-terpineol was the compound with the highest content. Its content was more than four-times higher in grapefruit samples. The earthy odor description of α-terpineol can be one of the reasons for the bitter flavor of grapefruit fruit. Limonene was identified and determined in all fruits, which is consistent with literature reports of Rodríguez et al. in which this volatile is described as a characteristic for citrus [29]. The highest content of limonene was determined in the grapefruit headspace, namely 15.79 ± 0.30 μg/g. The obtained results are correlated with the report of Zhang H et al. [28]. Twelve terpenes in oroblanco and three in pummelo juice samples were determined. However, it was not possible to identify α-terpineol in oroblanco samples. Nevertheless, α-terpineol may be formed in citrus fruit from limonene during biochemical processes [30]. Hence, many factors, such as growing and storage conditions, can affect this difference. In turn, Buettner et al. determined five terpenes in the samples of the yellow grapefruit juice, and the concentration of limonene was 2308 μg/kg [14]. The sources of uncertainty associated with the determination of terpenes in citrus fruit samples are presented graphically using the Ishikawa diagram (Figure 5). On the basis of the content of individual

monoterpenes in the volatile fraction of the sweetie and its parent fruits flesh, it can be stated that the hybrid fruit is more aromatic than pummelo.

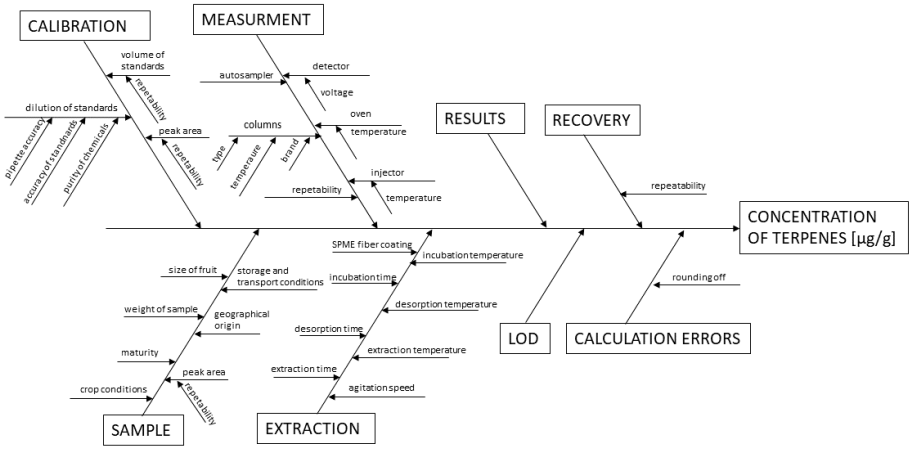

Figure 5. Ishikawa diagram presenting the influence of parameters on the analytical process for the determination of monoterpenes in citrus fruit samples.

Based on the quantitative analysis, the odor parameters of the selected compounds were calculated (Table 4). Almost all of the determined volatile components presented odor activity values (OAVs) greater than 1, which means that they are odor active compounds (OAC) and have a greater potential to influence samples' aroma [31]. Ocimene (OAV—47.1 ± 2.9), limonene (OAV—26.49 ± 029), and β-myrcene (41 ± 1.4) with pleasant fruity and citrus odors are the key aroma compounds of sweetie. In the case of grapefruit, OAV values are also high for α-pinene and α-terpineol, 15.01 ± 0.08 and 17.58 ± 0.40, respectively. These volatiles with herbal and terpenic odor contribute to the aroma of grapefruit.

Table 4. Selected volatile compounds determined in sweetie, grapefruit, and pummelo with their respective odor threshold and odor active compounds (OAC).

Chemical Compound	OT [ppm]	Sweetie	Pummelo	Grapefruit
		OAV + SD [-]		
α-Pinene	0.19	4.335 ± 0.051	-	15.01 ± 0.080
Limonene	0.2	26.49 ± 0.29	13.8 ± 2.7	78.95 ± 1.5
Ocimene	0.034	47.1 ± 2.9	-	60.5 ± 2.3
β-Myrcene	0.1	41 ± 1.4	-	32.20 ± 0.29
γ-Terpinene	0.26	28.0 ± 1.3	-	9.86 ± 0.10
α-Terpineol	5	4.2 ± 0.14	-	17.58 ± 0.40

OT—odor threshold values were taken from the literature [32].

2.4. Multivariate Analysis

The results of the principal component analysis (PCA) that was made to distinguish between citrus fruits based on the main monoterpenes content fruits are shown in Figure 6. In the case of the dataset obtained during the analysis of citrus fruits, the first two components explained 96% of the total variance (axis 1 (63.5%) and axis 2 (32.5%)). It can be observed, that the total separation of citrus samples along the two first main components was obtained. The PCA biplot grouped samples in a distinct cluster, showing that their properties are different. However, based on the analysis and the

distance between data points in multidimensional space it can be stated that sweetie is more similar to grapefruit in the context of the content of monoterpenes. Moreover, PCA-biplot allows correlating between the selected monoterpenes and the group of citrus fruits. Γ-terpinene was positively correlated with the samples of sweetie, while α-terpineol, α-pinene, and limonene were positively correlated with grapefruit. Herbal and terpenic flavor description of these compounds may explain the bitterness of grapefruit samples, which is in agreement with sensory analysis.

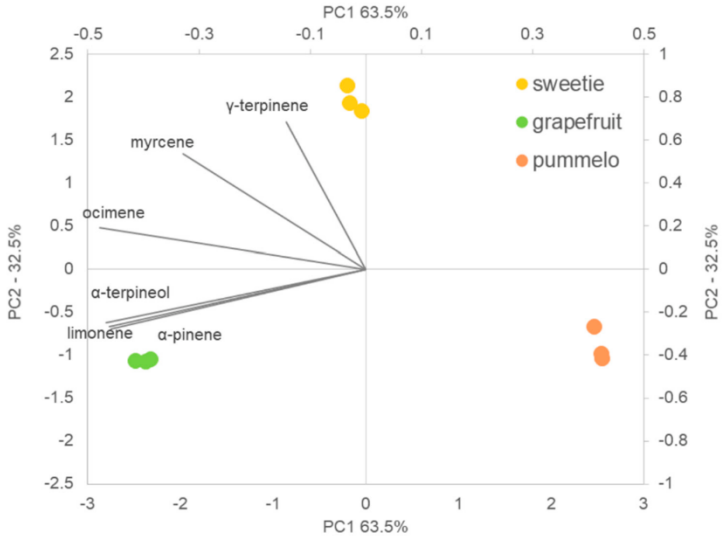

Figure 6. Principal component analysis (PCA) biplots of the assignments of sweetie, grapefruit and pummelo fruits in respect of individual volatile organic compounds (VOCs).

3. Materials and Methods

3.1. Sensory Analysis

Sensory analysis was carried out by the fifteen-member panel with the use of profiling method [33]. The fleshes of grapefruit, sweetie and pummelo were the subjects of the test. The evaluation of the samples was performed with the use of six descriptors which are the most common in the case of fruits analysis: sweetness, bitterness, tartness, acidity, juiciness, and tastiness. Before the test, fruits were washes with diluted water and peeled. The flesh was manually separated from the feel and from membranes which bitter taste could falsify the results. Fruit samples were coded by the person carrying out the analysis. Panelists graded the perceived hedonic quality on a 10-mm-long axis, with 10 and 0 denoting the most and least desirable qualities, respectively. The final assessment was based on the average of values set by the panelists. Section lengths were measured by caliper.

3.2. Sample Preparation for Chromatographic Analysis

The fruits for analysis were purchased at local distribution points in the Pomeranian Voivodship. From the information provided by the supplier, it appeared that the fruit was harvested in a similar degree of maturity and that the time since harvest was the same. Samples were analyzed immediately after purchasing. Solid phase microextraction was used for the isolation and enrichment of analytes. Analysis of each fruit was conducted in triplication, each time using the new fruit from each variety from a different supplier. The scheme of sample preparation is shown in Figure 7.

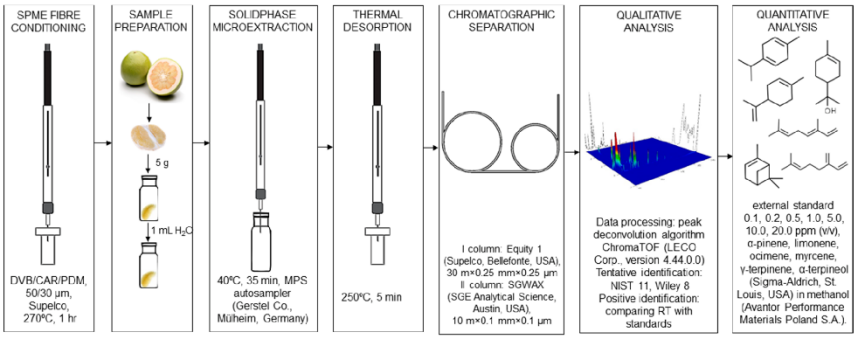

Figure 7. The process of sample preparation for analysis.

3.3. GC × GC-TOF-MS Analysis

Samples of citrus fruits were analyzed using two-dimensional gas chromatography. The utilized apparatus consists of Agilent 7980A chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a dual-stage cryogenic modulator which was coupled with a time-of-flight mass spectrometer (LECO Corp., St. Joseph, MI, USA). The parameters and conditions of chromatographic analysis are shown in Table 5.

Table 5. Parameters and conditions of GC × GC-TOF-MS analysis.

Element	Parameter	Value
Carrier gas	Hydrogen	1 mL/min
Front inlet	Temperature	250 °C
Temperature program	Initial temperature	I. column 40 °C II column 45 °C
		Modulator 60 °C
	Time to maintain the initial temperature	I, II column, modulator 210 s
	Temperature rate	I, II. column, modulator 6 °C/min
	Final temperature	I column 250 °C II. column 255 °C Modulator 265 °C
	Time to maintain the set temperature	I column 300 s II column, modulator 350 s
Modulation	Modulation period	4 s
Modulation	Hot pulse time	0.80 s
Modulation	Cool time between stages	1.20 s
Cooling medium	Type of medium	Liquid nitrogen
Detector	Mass range	40–400 u
Detector	Voltage	1600 V
Detector	Acquisition rate	125 spectra/s
Detector	Electron Energy	−70 V

3.4. Data Processing and Statistical Analysis

Data processing was performed using chromatographic peak deconvolution algorithm implemented in the software ChromaTOF (LECO Corp., version 4.44.0.0, St. Joseph, MI, USA). Tentative identification of analytes was made by comparing experimental spectra with the spectra included in National Institute of Standards and Technology (NIST) 11 and Wiley libraries. Microsoft®

Excel® spreadsheet was used for data entry and calculations. LOQ and LOD were calculated at the materiality level $\alpha = 0.05$. The LOD values were calculated based on the residual standard errors of the calibration curve (SE) and slope of the curve (a): LOD = 3.3 SD/a. LOQs values were calculated as three LOD. Odor activity values (OAV) were calculated as a ratio of the mean concentration of selected compounds and their odor threshold values taken from literature. Chromatographic peak areas for 6 selected chemical compounds were used as input data for Unsupervised Principal Component Analysis (PCA). Statistical analysis was performed using Orange v.3.8.0 software ((Bioinformatics Lab, University of Ljubljana, Slovenia).

4. Conclusions

In the present work, GC × GC-TOF-MS and sensory analysis were used to evaluate the flavor properties of Jaffa Sweetie and its parents' fruits. Moreover, the physical characteristics were assessed. The main attributes of *Citrus grandis* Osbeck × *Citrus paradisi* Macf., *Citrus paradisi* Macf., and *Citrus grandis* Osbeck were compared. It was shown that the visual properties of sweetie were originating from the grapefruit. Based on the obtained results of the sensory panel, it can be concluded that the taste and aroma of sweetie are most desirable by consumers and closer to pummelo. Considering the concentration of individual monoterpenes, it was proved that sweetie is a hybrid in which the health-promoting properties of the grapefruit were preserved. The content of individual monoterpenes is higher than in pummelo. α-Terpineol with the concentration of 20.96 ± 0.70 μg/g is the most abundant monoterpene in the volatile fraction of sweetie, notwithstanding ocimene with pleasant fruity odor is the monoterpene with the greatest influence on the sweetie aroma because of the high value of odor activity value. In addition, the functional properties of the hybrid fruit were improved in contrast to pummelo. Sweetie has a thinner peel, is juicier, and contains no seeds. All these properties make the fruit a rich source of health-promoting compounds with a pleasant taste. This study has proven the purpose and effectiveness of cross-breeding of sweetie fruit. With the reports of the results of bioactive compounds obtained previously, the conducted research will provide the complex characteristics of the fruit, proving a background for application and trade of sweetie fruit. Nevertheless, due to the numerous biochemical changes that occur in the fruit over time, which may affect the change in monoterpenes' content, further research on citrus fruit should focus on monitoring the changes in monoterpenes concentration over time. Moreover, the conducted research focused on commercially available fruits, which is a certain limitation due to the lack of complete certainty as to the degree of fruit maturity, therefore further research including fruit analysis immediately after harvest taking into account post-harvest conditions and degree of maturity should be carried out.

Author Contributions: Conceptualization, M.L.-S.; methodology, M.L.-S. and T.D.; formal analysis, M.L.-S.; investigation, M.L.-S.; resources, M.L.-S. and T.D.; writing—original draft preparation, M.L.-S.; writing—review and editing, M.L.-S. Ż.P., and S.G.; visualization, M.L.-S.; supervision, Ż.P. and S.G.; project administration, M.L.-S. and Ż.P.; funding acquisition, M.L.-S. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples are not available from the authors.

6.7 Influence of steam cooking on pro-health properties of Small and Large variety of *Momordica charantia*

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Influence of steam cooking on pro-health properties of Small and Large variety of *Momordica charantia*

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Cooking

ABSTRACT

Steam cooking is one of the most common preparations of *Momordica charantia* dishes. This method improved the tastiness of the fruits but at the same time, some changes occurred in the volatile and non-volatile parts of their food matrices. In this study, for the first time these properties were correlated with the found substances affecting the bioactivity of this fruit. Two varieties of *Momordica charantia* were analysed and compared. It was possible to differentiate both types of fruits using two-dimensional gas chromatography and time-of-flight mass spectrometry (GC×GC-TOF-MS) as well as to assess botanical and geographical origin. In the case of volatiles, 212 chemical compounds were tentatively identified, which can be classified into seven chemical classes, such as aldehydes, alcohols, esters, ketones, terpenes, hydrocarbons. Furthermore, 16 of them were quantified and calculated in terms of OAV and ROC values. Bioactive substances (polyphenols, flavonoids, tannins and flavanols) and the values of antioxidant capacities by four radical scavenging assays (DPPH, CUPRAC FRAP, ABTS) were determined and compared in water and methanol extracts of Chinese and Indian varieties. It was proven that steam cooked Chinese variety has greater value due to its flavour than Indian variety and consists more volatile, non-volatile and bioactive constituents with high antioxidant effect. The binding properties of polyphenols to HSA were relatively high in comparison with other plants. A strong positive correlation of binding properties and bioactivity of *Momordica charantia* was estimated. One of the volatiles, namely citronellol, has key importance in respect of antidiabetic effect of *Momordica charantia* Chinese variety. This study indicates pro-health preponderance of Chinese variety over Indian variety and confirms that steam cooking is in lines with the canons of safe food preparation.

1. Introduction

Bitter melon (*Momordica charantia*) is a tropical and subtropical species of the *cucurbits* family. This fruit probably originates from southern China or eastern India, currently its occurrence mainly includes tropical and subtropical regions of Asia, Amazonia, East Africa and the Caribbean, but it began to be grown around the world, due to its culinary use and healing properties.

Information about its cultivation is contained in Ayurvedic books from 2000 BCE. (Kwatra, Dandawate, Padhye, & Anant, 2016). Numerous scientific studies indicate that *Momordica charantia* has the highest nutritional value among other fruits of the *cucurbits* family (Kwatra et al., 2016). In addition, the bitter melon contains numerous bioactive chemical compounds, which include glycosides, saponins,

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alkaloids, terpenes, proteins and steroids. The presence of these compounds makes the plant used in Eastern folk medicine, as a helpful agent in the treatment of diabetes, colic, locally for wound healing and internally for the treatment of ulcers and fight against parasites. In India, it is used to treat jaundice, leprosy, kidney stones, gout, eczema, pneumonia, rheumatism and psoriasis (Grover & Yadav, 2004). *Momordica charantia*, depending on the geographical origin, differs in size with shape, colour, or the content of particular chemicals. The typical Chinese phenotype is 20–30 cm long, it is oblong with conical and blunt ends, pale green in colour, with a slightly wavy and crinkly surface. Bitter cucumber typical in India has a narrower shape with sharper ends and a surface covered with jagged, triangular furrows and edges. The colour scheme varies depending on the variety, it may be green or white. One of the main pharmacological properties attributed to bitter melon is the antidiabetic effect. The chemical compounds in the fruit that have hypoglycaemic effect are a mixture of steroidal saponins (charanthin), insulin-like peptides and alkaloids (Raman & Lau, 1996). These compounds regulate the level of sugar in the body and also increase tolerance to hyperglycaemia. They accelerate the elimination of glucose inside the cells, acting similarly to insulin. They also increase the release and activity of insulin. Bitter melon is a popular ingredient of Eastern cuisine. In India, the fruit is eaten raw or cooked as an addition to dishes and sauces. One of the most popular ways to prepare dishes from bitter melons is steam cooking. Numerous scientific studies prove that it is one of the healthiest ways to prepare foodstuffs, protecting against the loss of nutrients and taste attributes (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008). Small loss of health-promoting compounds makes it possible to consider dishes with bitter melons as functional foods with strong antidiabetic activity. *Momordica charantia* is characterized by a pleasant smell. Previous studies of the volatile fraction showed that *trans*-nerolidol constitutes over 60% of volatiles of *Momordica charantia* seeds (Braca, Siciliano, D'Arrigo, & Germanò, 2008). In turn, for the bitter gourd flower, the most abundant compound was identified as linalool (5% of total headspace). A characteristic feature of bitter melon is its pleasant aroma. Composition of *Momordica charantia* fruit volatile fraction was analysed using gas chromatography coupled with a mass spectrometer (SPME-GC-MS) (Sahu, Jain, & Nayak, 2011). On the basis of the conducted research, it was possible to identify only 11 volatile compounds in the bitter melon methanolic extract (Bhavani, 2017). A similar amount of chemical compounds was identified by other investigators also with the use of GC-MS (Singh, Kumar, Giri, Bhuvaneshwari, & Pandey, 2012).

A wider range of chemical compounds has been identified in volatile oils (Moronkola et al., 2009). The solution, which could provide detection and determination of more chemicals in bitter melon samples, concerns the application of SPME-GC×GC-TOF-MS technique, which ensures increased peak capacity, resolution and detectability (Lubinska-Szczygieł et al., 2018; Majchrzak et al., 2018). There is a lack of scientific reports about using this approach. Aroma is a mixture of many chemical compounds and depends on the interaction of these compounds with each other and the matrix. Smell sensation also depends on odour threshold, which is why the selection of major aroma compounds is an insufficient approach to characterizing an aroma. The current state of knowledge about the contribution of a given compound in creating the aroma of *Momordica charantia* fruit is still inexhaustible. In this article, the content of aromatic compounds of two varieties of bitter melon, namely Small and Large one, from different parts of Thailand were compared to indicate key aroma compounds occurring in each of them. The differences in the composition of the *Momordica charantia* volatile fraction depending on the geographical origin were considered. Attempts have also been made to determine the effect on the bioactivity and antidiabetic properties of this fruit, regarding its volatile and non-volatile substances contents. It was previously reported that many of the volatiles present in the fruits have bioactive activity (Lubinska-Szczygeł et al., 2018). The compositional profiles of *Momordica charantia* were evaluated using antioxidant scavenging assays and fluorescence spectroscopy. The quenching properties of polyphenols were determined by the interaction of the main drug career in blood human serum albumin (HSA). Furthermore, the activities have been taken to reach the main goal of this investigation, namely to determine the influence of steam cooking process on aromatic and antioxidant properties of both varieties of bitter melon. The correlation between the results of investigation of fresh fruit and steamed cooked samples was also performed.

2. Materials and methods

2.1. Chemicals

Analytical grade standards of 2-Hexenal, Hexanal, 1-Hexanol, 3-Hexen-1-ol, 2-Hexen-1-ol, 2-Ethylhexanol, 1-Heptanol, Hexyl acetate, 3-Pentanone, Bornylene, α-Limonene, γ-Terpinene, Citronellal, Citronellol, Linalool, α-Terpineol were used as well as standard of n-alkanes from C6 to C20 (Sigma-Aldrich, St. Louis, MO, USA) utilized for calculation of linear retention indexes (LRI). The theoretical LRI value was obtained from the LRI and Odour Database (www.odour.org.uk). All analytical grade standard substances were used to prepare calibration solutions. Solution of calibration curves in methanol (Avantor Performance Materials Poland S.A., Gliwice, Poland) was prepared with different concentrations of each substance in the range of 1–200 ppm (v/v). Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid); 2,2′-azobis-2-methyl-propanimidamide; $FeCl_3x6H_2O$; Folin–Ciocalteu reagent (FCR); Tris, tris (hydroxymethy1)aminomethane; lanthanum (III) chloride heptahydrate; $CuCl_2x2H_2O$; and 2,9-dimethyl-1,10-phenanthroline (neocuproine), potassium persulfate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), caffeic acid and human serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionised and distilled water were used throughout.

2.2. Samples

Two species of *Momordica charantia* were used for the study. Both of them were imported from Thailand. The Larger fruit, Chinese variety, was bought in Bangkok, while the Smaller one was purchased in the north of Thailand in the city of Chiang Rai. The Larger fruit is from hybrid variety seed, its common name is Chinese Mara, its variety named Sorn Daeng no.16 from East-West Seed Company. The Smaller one is local variety, the common name is Mara Kee Nok, and gardener collects the seed from one generation to other generations. It is opened pollinate variety. These are the two most popular varieties of *Momordica Charantia* fruit. Fruit, despite belonging to the same species, differed in shape and taste, resulting from different places of ripening. Although both fruits were collected in similar climatic conditions (same country, similar time), the fruit from Chang Rai was significantly smaller than the classic variety of this fruit. The samples were transported to the laboratory immediately in sealed plastic bags in a container keeping the temperature low. Before analysis, the fruits were washed and then rinsed with distilled water. Cut pieces of both kinds of *Momordica Charantia* were steamed for 20 min, separately. This is the most common method of preparing dishes from bitter melon. Next step was homogenization of the fresh and steamed pulps in agate mortar.

2.3. Solid phase microextraction

The MultiPurpose Sampler (MPS) (Gerstel, Mülheim, Germany) was used for the automation of the isolation and enrichment analytes procedure. DVB/CAR/PDMS fibres (50/30 μm thickness and 2 cm length) were purchased from Sigma-Aldrich (St. Louis, MO, USA) before used, fibres were conditioned into GC injector port at 260 °C for 45 min. Before the extraction, the samples were kept at 40 °C for 15 min. For extraction, the fibre was directly exposed to the sample for 30 min at

40 °C, with stirring at a rotational speed of 550 rpm. After the extraction, the fibre was removed from the vial and transferred to the injector of a gas chromatograph for thermal desorption of the analytes at 250 °C for 3 min.

2.4. Chromatographic analysis

The Pegasus 4D (LECO, St. Joseph, MI, USA) system was used during the study. It consists of the Agilent 7890A gas chromatograph and the LECO mass spectrometer with a time-of-flight analyser. Modulation was carried out using a liquid nitrogen-based quad-jet cryogenic modulator. In order to ensure the separation of the analytes, the following set of capillary columns was used: in the first dimension, the column filled with non-polar stationary phase Equity-1 (30 m × 0.25 mm i.d. × 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA) and in the second dimension, the column filled with a polar stationary phase SolGel-Wax (2 m × 0.1 mm i.d. × 0.1 μm film thickness) was purchased from SGE Analytical Science (Austin, TX, USA). The chromatographic separation was performed using the following temperature program for the primary oven: initial temperature 40 °C, kept for 5 min, ramped at 6 °C/min to 260 °C, and held for 5 min. The secondary oven temperature was programmed from 45 °C, kept for 5 min, ramped at 6 °C/min to 265 °C, and held for 5.83 min. The carrier gas was hydrogen (N6.0 class) at a constant flow rate at 1.0 mL/min. The modulation time was 5 s (1.0 s hot pulse time and 1.5 s cool pulse time). Temperature of the MS transfer line and the MS source was 250 °C. The mass spectra data acquisition rate was 125 spectra/s. The data were collected over a mass range of 30–450 m/z. The voltage of detector was 1700 V.

2.5. Plant extracts preparation

Two species of *Momordica charantia* samples were weighed, chopped and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was lyophilized for 48 h (Virtis model 10–324), and the dry weight (DW) was determined. The samples were ground to pass through a 0.5 mm sieve and stored at −80 °C (Leontowicz et al., 2016). Polyphenols were extracted with methanol at room temperature and water (concentration 20 mg/mL) during 1 h in a cooled ultrasonic bath.

2.6. Determination of bioactive compounds

All the applied methods previously were described in details (Leontowicz et al., 2016). Total polyphenols (mg gallic acid equivalents (GAE)/g DW) were determined by Folin-Ciocalteu method using spectrophotometer (Hewlett-Packard, model 8452A, Rockvile, USA) and measuring obtained absorbance after the complex reaction at wavelength of 750 nm (Singleton, Orthofer, & Lamuela-Raventos, 1999). Flavonoids, extracted with 5% $NaNO_2$, 10% $AlCl_3xH_2O$ and 1 M NaOH, were measured at 510 nm. Total flavanols were estimated using the *p*-dimethylaminocinnamaldehyde method, and the absorbance was measured at 640 nm (Feucht & Polster, 2001). The extracts of condensed tannins (procyanidins) with 4% vanillin solution in MeOH were measured at 500 nm. (+)Catechin served as a standard for flavonoids, flavanols and tannins. Anthocyanins were determined by the measuring of *Momordica charantia* samples absorbances of extracts (1 g of the defatted sample was extracted with 1 mL of acetonitrile containing 4% acetic acid) at 510 nm and 700 nm in buffers at pH 1.0 and 4.5, and calculated using following equation: $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$ with a molar extinction coefficient of cyaniding-3-glucoside of 29 600. Results were expressed as milligrams of cyaniding-3-glucoside equivalent per 100 g DW (Cheng & Breen, 1991). Chlorophylls *a* and *b*, and total carotenoids (xanthophylls + carotenes) were extracted with 100% acetone and determined spectrophotometrically at different absorbances (nm) such as at 661.6, 644.8, and 470, respectively (Boyer, 1990). Total ascorbic acid was determined by CUPRAC assay in water extract (100 mg of lyophilized sample and 5 mL of water). The absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm (Ozyurek, Guclu, Bektasoglu, & Apak, 2007).

2.7. Total antioxidant capacities (TACs)

2,2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) method. ABTS radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with methanol and the absorbance was measured at 734 nm (Re et al., 1999).

Ferric-reducing/antioxidant power (FRAP): FRAP reagent (2.5 mL of a 10 mmol ferric-tripiridyltriazine solution in 40 mmol HCl plus 2.5 mL of 20 mmol $FeCl_3xH_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6) of 900 μL was mixed with 90 μL of distilled water and 30 μL of *Momordica charantia* extract samples as the appropriate reagent blank and absorbance was measured at 595 nm (Benzie & Strain, 1996).

Cupric reducing antioxidant capacity (CUPRAC): To the mixture of 1 mL of copper (II)-neocuproine and NH_4Ac buffer solution, acidified and non acidified methanol extracts of *Momordica charantia* samples (or standard) solution (x, in mL) and H_2O [(1.1-x) mL] were added ribbed to make the final volume of 4.1 mL, and the absorbance was measured at 450 nm (Apak, Guclu, Ozyurek, & Karademir, 2004).

1, 1-Diphenyl-2-picrylhydrazyl method (DPPH) solution (3.9 mL, 25 mg/L) in methanol was mixed with the samples extracts (0.1 mL). The reaction progress was monitored at 515 nm until the absorbance was stable. The scavenging rate on DPPH radicals was calculated (Brand-Williams, Cuvelier, & Berset, 1995).

2.8. Fluorometric measurements

Two (2D-FL) and three dimensional (3D-FL) fluorescence measurements for *Momordica charantia* samples extracts at a concentration of 0.01 mg/mL were recorded on a model FP-6500, Jasco spectrofluorometer, serial N261332, Japan. The 2D-FL was taken at emission wavelengths from 310 to 500 nm and an excitation of 295 nm. The 3D-FL spectra were collected with subsequent scanning emission spectra from 250 to 500 nm at 1.0 nm increments by varying the excitation wavelength from 200 to 350 nm at 10 nm increments. Caffeic acid was used as standard. All solutions for protein interaction were prepared in 0.05 mol/l Tris–HCl buffer (pH 7.4), containing 0.1 mol/l NaCl (Leontowicz et al., 2016; Sinisi, Forzato, Cefarin, Navarini, & Berti, 2015).

2.9. Data processing and statistical analysis

To verify the statistical significance of the quantitative analysis results, means of the of three measures of concentrations were calculated. In addition, for each average concentration of chemical compound, standard error and standard deviation were determined. Microsoft Excel software was used for LOD and LOQ calculations. LOQ and LOD were calculated of the materiality level $\alpha = 0.05$. The LOD values were calculated based on the residual standard errors of the calibration curve (SE) and slope of the curve (a): LOD = 3.3SD/a. LOQs values were calculated as three LOD. All determined chemical compounds meet the requirement that c > LOD. OAV was calculated by dividing the mean concentration of compound by its odour threshold value taken from literature. The relative odour contribution (ROC) is the ratio of OAV of each compound and the sum of the OAV of compounds that showed OAV > 1. Concentrations for 16 selected chemical compounds were used as input data for Unsupervised Principal Component Analysis (PCA) in order to distinguish raw and steamed Chinese and Indian varieties of *Momordica charantia* samples. Statistical analysis was performed using Orange v.3.14 software. Results presented in this study

Table 1
Tentative identification of volatile compounds in *Momordica charantia* using HS-SPME-GC × GC/TOFMS.

No.	Compound	RT1	RT2	UM	Large	Small
					Relative content (%)	
Aldehydes						
1	2-Methyl-2-butenal	586	2.656	55	0.138	< LOD
2	2-Pentenal	602	2.944	55	0.049	0.040
3	3-Hexenal	682	2.968	55	1.983	0.013
4	Hexanal	686	2.272	56	0.452	0.192
5	2-Hexenal	782	2.368	55	2.209	4.215
6	Heptanal	882	2.552	70	0.005	< LOD
7	2.4-Hexadienal	890	3.496	81	0.016	< LOD
8	Benzaldehyde	978	3.384	77	0.198	0.175
9	2-Ethyl-hexanal	986	2.392	57	0.005	< LOD
10	2.4-Heptadienal	1046	2.976	81	0.027	0.132
11	Octanal	1066	2.36	84	0.011	< LOD
12	Benzeneacetaldehyde	1118	3.52	91	0.002	0.012
13	2-Octenal	1134	2.648	70	0.004	< LOD
14	Nonanal	1234	2.240	57	0.003	0.072
15	2.6-Nonadienal	1306	2.472	70	0.056	0.006
16	4-Ethylbenzaldehyde	1350	2.184	134	0.020	0.015
17	Decanal	1390	2.208	57	< LOD	0.017
18	Dodecanal	1674	2.176	57	0.007	0.004
19	1.3-Dimethylcyclohex-2-ene-1.2-dicarbaldehyde-1-ethylene-acetal	1882	2.040	138	0.007	0.002
20	Pentadecanal	2034	2.096	57	0.006	0.008
Alcohols						
21	1-Penten-3-ol	498	2.744	57	0.889	2.019
22	3-Pentanol	526	2.872	59	0.008	0.086
23	3-Methyl-1-butanol	586	955.000	55	0.018	0.243
24	2-methyl-1-butanol	594	3.048	57	0.018	0.065
25	1-Pentanol	642	2.744	55	0.112	1.017
26	2-Penten-1-ol	646	2.936	57	1.083	0.833
27	3-Hexen-1-ol	794	3.008	67	22.235	9.140
28	2-Hexen-1-ol	826	2.648	57	3.908	7.193
29	1-Hexanol	838	2.784	56	26.896	28.851
30	2-Heptanol	894	2.896	45	0.091	0.029
31	4-Hexen-1-ol	922	2.688	67	< LOD	0.018
32	2-Ethylphenol	926	2.400	107	< LOD	0.011
33	2.2-Dimethyl-1-pentanol	950	2.824	43	0.011	< LOD
34	1-Hexyn-3-ol	990	2.936	55	< LOD	0.010
35	4-Hepten-1-ol	1002	2.720	81	0.127	0.054
36	1-Heptanol	1014	2.592	70	0.098	0.106
37	1-Octen-3-ol	1030	2.632	57	< LOD	0.088
38	6-Methyl-5-hepten-2-ol	1054	2.504	95	0.080	0.015
39	3-Octanol	1062	2.584	59	0.107	0.088
40	2-Octanol	1070	2.440	45	0.229	0.030
41	2.5-Heptadien-1-ol	1094	3.352	79	0.006	0.036
42	2-Ethyl-1-hexanol	1114	2.560	57	2.685	0.301
43	Benzyl alcohol	1118	0.840	79	0.045	0.023
44	2-Octen-1-ol	1182	2.792	57	0.107	0.016
45	5-Octen-1-ol	1182	2.648	81	0.047	0.021
46	1-Octanol	1186	2.456	56	0.199	0.133
47	6-Methyl-1-heptanol	1210	2.472	69	0.050	< LOD
48	1-Nonen-4-ol	1214	2.568	55	< LOD	0.219
49	2-Butyl-1-octanol	1234	2.000	57	0.020	0.005
50	Phenylethyl alcohol	1246	3.920	91	0.008	0.083
51	1-Amino-2-propanol	1270	2.056	30	< LOD	0.017
52	4-Methyloctan-1-ol	1286	2.352	69	0.201	< LOD
53	6-Methyl-1-octanol	1302	2.36	55	0.269	< LOD
54	3.6-Nonadien-1-ol	1322	2.864	67	0.075	0.006
55	3-Nonen-1-ol	1322	2.568	55	0.031	0.009
56	6-Nonenol	1342	2.472	82	0.106	0.099
57	1-Nonanol	1346	2.368	56	0.121	0.125
58	2-Undecanol	1346	2.008	45	0.150	0.013
59	3-Decanol	1366	2.264	69	0.006	0.001
60	3-(Cyclopentyl)-2-methylpropanol	1430	2.184	251	0.006	< LOD
61	4-Decen-1-ol	1474	2.432	68	0.269	0.003
62	1-Decanol	1494	2.344	56	0.010	0.004
63	1-Dodecanol	1762	2.248	70	0.005	0.004
Esters						
64	Ethyl propanoate	542	2.528	57	< LOD	0.033
65	Propyl acetate	546	2.296	43	< LOD	0.180
66	Isoamyl acetoacetate	586	2.952	70	0.027	0.008
67	2-Methyl-ethyl butanoate	794	2.28	74	< LOD	0.018
68	3-Methyl-1-butanol acetate	842	2.392	70	0.004	0.058
69	2-Penten-1-ol acetate	906	2.520	43	0.034	0.027

(*continued on next page*)

Table 1 (*continued*)

No.	Compound	RT1	RT2	UM	Large	Small
					Relative content (%)	
70	Pentyl acetate	910	2.480	43	0.012	0.401
71	3-Hexen-1-ol formate	918	2.664	67	0.021	0.022
72	Methyl hexanoate	930	2.288	74	< LOD	0.333
73	Hexyl formate	934	2.320	56	0.004	0.354
74	Methyl 2-hexenoate	998	2.512	55	< LOD	0.100
75	Ethyl hexanoate	1062	2.160	88	< LOD	0.211
76	3-Hexen-1-ol Acetate	1070	2.184	67	1.336	1.941
77	2-Hexen-1-ol Acetate	1082	2.176	43	0.005	0.048
78	Hexyl acetate	1086	2.184	43	0.687	2.596
79	Methyl heptanoate	1106	2.376	74	< LOD	0.005
80	Ethyl 2-hexenoate	1130	2.248	97	0.019	0.116
81	3-Hexen-1-ol propanoate	1226	2.224	57	0.023	0.014
82	Ethyl heptanoate	1226	2.208	88	< LOD	0.003
83	Hexyl propanoate	1238	2.168	57	< LOD	0.036
84	Methyl octanoate	1270	2.200	74	0.016	0.139
85	Glycidyl methacrylate	1282	2.392	69	0.343	0.003
86	3-Hexenyl butanoate	1298	2.200	67	0.073	0.017
87	Ethyl benzoate	1338	2.688	105	< LOD	0.005
88	Hex-3-enyl-2-methyl but-2-enoate	1362	2.192	83	0.282	0.002
89	3-hexenyl hexanoate	1362	2.192	82	0.019	0.002
90	Hexyl butanoate	1374	2.136	71	0.005	0.017
91	Ethyl octanoate	1378	2.136	88	0.003	0.046
92	Methyl nonanoate	1422	2.176	74	< LOD	0.017
93	3-Hexenyl pentanoate	1434	2.128	82	0.326	0.034
94	Hexyl-2-methyl butyrate	1442	2.064	103	0.043	0.165
95	2-Phenylethyl acetate	1454	2.768	104	< LOD	0.007
96	4-*tert*-Butylcyclohexyl acetate	1534	2.096	56	0.039	0.006
97	Methyl decanoate	1562	2.096	74	0.002	0.010
98	2-methyl-2-propenyl 2-butenoate	1570	2.112	57	0.095	< LOD
99	Myrtenyl acetate	1574	2.184	91	0.067	0.008
100	Hexyl hexanoate	1646	2.088	43	0.001	0.006
101	Ethyl decanoate	1658	2.096	88	0.002	0.003
102	Methyl dodecanoate	1818	2.088	87	0.010	0.023
103	3-Hexen-1-ol benzoate	1874	2.368	105	0.008	< LOD
104	Nonyl-2-methyl propanoate	2026	2.016	57	0.010	0.003
105	Methyl hexadecanoate	2246	2.056	74	< LOD	0.042
106	Ethyl hexadecanoate	2310	2.072	57	0.002	0.018
Ketones						
107	1-Penten-3-one	494	2.528	55	1.181	0.055
108	2-Pentanone	494	2.440	43	0.011	1.326
109	3-Pentanone	510	2.232	57	4.522	5.503
110	2-Heptanone	866	2.696	58	0.043	0.402
111	6-Methyl-2-heptanone	982	2.504	58	0.014	0.016
112	2.3-Octanedione	1026	2.368	43	0.002	0.431
113	6-Octen-2-one	1034	2.424	68	0.029	0.993
114	6-Methyl-5-hepten-2-one	1034	2.504	69	0.029	0.011
115	3-Octanone	1038	2.288	57	0.015	0.215
116	2-Octanone	1042	2.400	58	0.034	0.197
a117	6-Nonen-2-one	1078	2.440	67	0.011	< LOD
118	3-Hepten-2-One	1102	2.544	55	0.144	0.184
119	2.2.6-Trimethylcyclohexanone	1126	2.272	82	0.019	0.020
120	1-Phenylethanone	1166	3.712	105	0.011	0.046
121	3.5-Octadien-2-one	1170	2.888	95	0.016	0.026
122	2-Nonanone	1214	2.328	44	0.049	0.076
123	2-Nonanone	1214	2.24	57	0.044	0.076
124	2-Methyl-3-octanone	1218	2.752	71	0.012	0.374
125	Nonafluorobutyl-but-3-enyl ketone	1278	2.504	55	0.013	0.010
126	5-Methyl-2-Hexanone	1282	2.224	58	0.027	< LOD
127	1-(2.5-Dimethylphenyl)-ethanone	1474	2.824	122	0.005	0.004
128	2-Undecanone	1518	2.256	58	0.068	0.014
129	6.10-dimethyl-2-undecanone	1674	2.136	58	0.004	0.003
130	6.10-Dimethyl-5.9-undecadien-2-one	1726	2.208	69	0.069	0.035
Terpenes						
131	α-Thujene	962	2.024	93	0.257	0.285
132	α-Pinene	978	2	93	0.172	0.120
133	Camphene	1002	2	93	0.030	0.017
134	Sabinene	1042	2.048	93	0.583	0.824
135	β-Pinene	1050	2.016	93	1.490	1.301
136	β-Myrcene	1062	2.088	93	0.136	0.156
137	*o*-Cymene	1082	2.128	119	0.013	0.019
138	*p*-mentha-1.8-dien-7-ol	1090	2.176	93	0.018	0.019
139	α-Phellandrene	1094	2.136	93	0.081	0.062
140	*m*-Cymene	1118	2.160	119	0.691	0.628

(*continued on next page*)

Table 1 (*continued*)

No.	Compound	RT1	RT2	UM	Large	Small
					Relative content (%)	
141	*p*-Cymene	1130	2.176	119	0.270	0.075
142	α-Limonene	1138	2.080	68	1.725	2.019
143	Bornylene	1138	2.056	68	2.995	3.418
144	Ocimene	1158	2.192	93	0.013	0.007
145	Isophorone	1166	2.44	82	0.036	0.020
146	γ-Terpinene	1182	2.080	93	0.590	0.354
147	dihydromyrcenol	1190	2.408	59	0.055	0.022
148	Sabinene hydrate	1194	2.360	93	0.029	0.003
149	Fenchone	1222	2.248	81	0.013	0.073
150	Terpinolene	1234	2.088	93	0.070	0.021
151	Linalool	1238	2.432	71	0.325	0.051
152	Rose Oxide A	1258	2.208	139	0.057	0.004
153	α-Thujone	1266	2.288	81	0.006	0.021
154	Camphor	1306	2.312	95	0.029	0.006
155	Citronellal	1310	2.432	69	0.118	0.008
156	Pinocarvone	1334	2.336	101	0.066	0.057
157	Sabinol	1342	2.720	91	0.023	0.020
158	1-Borneol	1350	2.432	95	0.015	0.007
159	Menthol	1358	2.360	71	0.088	0.101
160	Estragole	1382	2.480	148	0.003	0.014
161	Myrtenal	1382	2.312	79	0.033	0.301
162	Dihydrocarvone	1386	2.624	67	0.015	0.001
163	Myrtenol	1394	2.392	79	1.843	1.888
164	*p*-Menth-2-en-7-ol	1406	2.512	93	0.016	0.018
165	Isogeraniol	1414	2.576	81	0.016	0.026
166	β-Cyclocitral	1422	2.304	137	0.016	0.017
167	Citronellol	1426	2.432	69	3.598	0.050
168	Carvone	1446	2.648	82	0.038	0.011
169	Citral	1478	2.528	69	0.088	0.028
170	*p*-Cymen-7-ol	1514	3.088	135	0.001	0.054
171	α-Cubebene	1634	1.992	119	0.013	0.003
172	α-Copaene	1670	2.008	119	0.035	0.003
173	α-Ionone	1706	2.216	121	0.053	0.059
174	Caryophyllene	1730	2.032	93	0.019	0.002
175	Humulene	1770	2.056	93	0.012	0.002
176	α-Ionone	1778	2.248	177	0.031	0.072
177	α-Muurolene	1818	2.040	68	0.008	0.003
178	Calamene	1838	2.104	159	0.016	0.044
179	γ-Cadinene	1842	2.024	161	0.012	0.003
Hydrocarbons						
180	1.3.5-Cycloheptatriene	646	2.456	91	0.154	0.005
181	Octane	726	2.064	43	0.028	0.004
182	o-Xylene	838	2.256	91	0.044	0.042
183	Nonane	914	2.048	57	0.015	0.005
184	1.3-hexadiene	918	2.632	79	0.022	0.022
185	3-Ethyl-1.5-octadiene	1082	2.024	69	0.583	1.474
186	Decane	1102	2.032	57	0.036	0.022
187	2.6.7-Trimethyldecane	1150	1.976	57	0.047	0.006
188	*N,N*-diethyl-*N'*-methyl-*N'*-nitroso-urea	1378	2.368	72	0.018	0.049
189	1-Decene	1394	2.016	70	0.014	0.002
190	Dodecane	1410	1.976	57	0.004	0.072
191	Tridecane	1410	1.976	57	0.016	0.031
192	2-methylundecane	1534	1.952	57	0.004	0.001
193	Tetradecane	1554	1.960	57	0.083	0.194
194	3-Tetradecene	1670	1.976	69	0.016	0.011
195	Pentadecane	1766	1.952	57	0.008	0.003
196	Heptadecane	1810	1.952	57	0.015	0.049
197	Octadecane	2038	1.96	57	0.007	0.017
Others						
198	Tetrahydrofuran	510	2.104	42	0.114	0.103
199	2-Ethylfuran	526	2.248	81	0.735	0.519
200	tert-Butyl-isopropylidenecyclopropylether	642	2.240	59	0.011	0.009
201	1-methyl-2-(δ-hydroxy)propylacetylene	686	2.480	83	0.096	0.003
202	Ethylbenzene	822	2.312	91	0.021	< LOD
203	2-Ethylthiophene	830	2.336	97	0.001	0.011
204	Methoxy-phenyloxime	910	1.720	133	0.822	0.252
205	2-Pentylfuran	1058	2.160	81	0.388	0.706
206	4-Methoxymethoxybut-1-yne	1066	2.208	45	0.019	0.003
207	2-(2-Pentenyl)furan	1074	2.216	94	0.030	0.031
208	2-Ethyl-1.6-dioxaspiro[4.4]nonane	1214	2.160	127	0.022	0.084
209	2-Methoxy-3-(2-methylpropyl)-pyrazine	1358	2.200	124	0.007	0.050
210	Naphtalene	1370	3.016	128	0.012	0.021
211	1-Methoxy-4-(2-propenyl)-benzene	1382	2.408	148	0.015	0.043
212	*N,N*-Dibutylformamide	1526	2.624	72	0.012	0.017

represent means ± SD of three independent measurements. One-way analysis of variance (ANOVA) was used to test the statistical significance (at $P < 0.05$, representing 95% interval o reliability) of the results employing the Duncan's multiple range test.

3. Results and discussion

3.1. Volatile substances

On the basis of the conducted research, it was possible to detect more than 300 chemical compounds in samples of both fruits, of which over 200 were identified on the basis of the obtained MS spectrum by comparison with the spectrum from the library (Table 1). The correctness of identification of quantified compounds has been further confirmed by comparing the retention time with the retention time of the reference substance. The greatest group by number of identified chemical compounds was being esters and terpenes. Both groups of compounds are characterized by characteristic scent properties that can condition the aroma of the fruit. Identified chemical compounds were divided into seven groups based on their belonging to a selected chemical class. It has been shown that hexyl acetate and 3-hexene-1-ol acetate were found in the biggest proportion among all esters identified. Among all identified chemical compounds, lipid derivatives - 2-hexenal, 3-hexen-1-ol, 2-hexen-1-ol, 1-hexanol, 1-hexanol, 2-ethyl are a large group, which is consistent with earlier literature reports (Agrawal & Tyagi, 2015). During researches of aerial shrubs of *Momordica charantia L.* these chemicals were selected as the major compound (Moronkola et al., 2009). All of these chemical compounds are characterized by a fresh, green or grassy scent.

Results of quantitative analysis of major chemical compounds are presented in Table 2. Among all the chemical compounds quantified in the samples of two varieties of bitter melon, in the case of the Chinese variety the highest content was noted in the case of β-citronellol (raw: 96.6 μg/g and steamed: 126.7 μg/g), while in the Indian variety 1-hexanol, 85.8 μg/g in raw fruit and 2-hexenal, 49.2 μg/g in steamed fruit. In addition, in the Indian variety five chemical compounds were below the limit of quantification. Whereas, in the processed fruit of Chinese variety two selected substances could not be quantified.

Odour activity values (OAVs) for 16 selected volatile compounds were shown in Table 3. The volatile compound will contribute to the final aroma of the samples if its concentration is above its threshold value. To some extent, the OAV may reflect the proportion of each aromatic compound in the characteristic odour of the sample. The threshold value for bornylene remained unknown. For fresh and steamed Large bitter melon samples, OAVs for 15 and 12 volatile compounds were calculated respectively, and for Small bitter melon samples OAVs only 10 and 9 substances were obtained. The compound should present an odour aromatic activity value > 1 to be a perceived human nose and have an influence on the aroma perception (Nuzzi, Lo Scalzo, Testoni, & Rizzolo, 2008). All selected volatiles were characterized by OAV > 1, as shown in Table 3. The highest OAV values for fresh fruit of both varieties were obtained for the representative of the ester group - hexyl acetate (Large fruit OAV = 3600, Small fruit OAV = 16030). Esters can be formed by the degradation of fatty acids during fermentation and storage processes (Shalit et al., 2001).

Hexyl acetate contributed to the composition of fruity and sweet fragrance notes in the tested samples (Flath, Black, Guadagni, Mcfadden, & Schultz, 1967). During thermal processing, the content of esters was reduced, but the aldehydes content in the volatile fraction of the studied samples was increased (Fig. 1). For this reason, the chemical compound with the high OAV value for heat-treated samples of both varieties was a substance from the aldehyde class, i.e. 2-hexenal (Large fruit OAV = 2260, Small fruit OAV = 2896). Aldehydes can be formed by reduction of unsaturated fatty acids or partial degradation of amino acids (Phillips & Galliard, 1978). The high content of 2-hexenal caused green aroma of samples (Flath et al., 1967). The volatile substance characterized by high OAV was also α-terpineol (Large fruit OAV = 1519 and 3308, Small fruit OAV = 901 and 2273, for raw and steamed fruit, respectively). Terpenes are substances, found in many exotic fruits, and their presence affects pronounced terpenic and citrus scent notes (Buttery, Seifert, Guadagni, & Ling, 1971). These results may indicate large similarities in the aroma of the bitter melon fruit. The substance differentiating two varieties of *Momordica charantia* was a compound from the terpenes group - citronellol, which was characterized by high OAV in the Large variety (raw: OAV = 3864 and steamed: OAV = 5066), while in the Small variety it was impossible to calculate OAV, because the concentration of this substance was lower than LOQ or the OAV was very low. This chemical compound is characterized by a strong floral and citrus aroma (Lubinska-Szczygeł et al., 2018).

The Relative Odour Contribution (ROC), which represents the percentage of each of the quantified volatile compounds in the aroma, is also shown in Table 3. Hexyl acetate is the compound that showed the highest contribution in the aroma of the fresh Small bitter melon (ROC = 78.6%), followed by 2-hexenal (ROC = 11.4%). Hexyl acetate and 2-hexenal are also compounds with a distinctive contribution to the

Table 2
Numbers of merit of the HS-SPME-GC × GC/TOFMS method used for the determination of volatile compounds in *Momordica charantia*.

No.	Compound	LRI_{calc}	LRI_{lit}	R2	Cal. curve equation	LOQ	LOD	Raw		Steamed	
								Large	Small	Large	Small
								Conc. + SD (ppm)			
1	2-Hexenal	824	825	0.9998	y = 54536.4x + 92043.5	0.491	0.162	24.62 ± 0.83	39.5 ± 2.1	38.7 ± 1.1	49.2 ± 1.2
2	Hexanal	772	772	0.9997	y = 769918.7x + 24685.7	0.698	0.230	3.81 ± 0.14	1.407 ± 0.068	5.60 ± 0.23	3.04 ± 0.12
3	1-Hexanol	855	850	0.9984	y = 1611636.7x-414903.5	1.954	0.645	23.22 ± 0.48	85.8 ± 8.3	1.97 ± 0.10	3.47 ± 0.22
4	3-Hexen-1-ol	837	834	0.9992	y = 1823290.9x-413645.0	1.684	0.556	35.7 ± 2.3	23.8 ± 1.2	< LOQ	< LOQ
5	1-Heptanol	953	950	0.9993	y = 900013.1x-178860.8	0.601	0.198	0.838 ± 0.046	0.793 ± 0.036	< LOQ	< LOQ
6	2-Ethylhexanol	1013	1015	0.9986	y = 1956636.6x-618103.5	2.563	0.769	13.38 ± 0.65	< LOQ	8.91 ± 0.69	< LOQ
7	2-Hexen-1-ol	848	844	0.9990	y = 1810612.7x-390962.7	0.708	0.234	11.13 ± 0.67	19.25 ± 0.94	0.831 ± 0.029	4.16 ± 0.16
8	Hexyl acetate	991	995	0.9984	y = 317256.2x-14342.4	3.312	1.093	7.21 ± 0.29	32.1 ± 1.4	< LOQ	10.61 ± 0.71
9	3-Pentanone	696	643	0.9977	y = 2775491.4x-1224804.4	4.032	1.331	12.1 ± 1.8	13.41 ± 1.22	19.7 ± 1.7	25.1 ± 1.1
10	Bornylene	1026	nd	0.9965	y = 1754877.3x-592397.5	2.307	0.761	10.80 ± 0.37	9.49 ± 0.15	16.87 ± 0.54	13.58 ± 0.39
11	α-Limonene	1026	1024	0.9957	y = 1495377.1x-928700.5	2.550	0.842	6.65 ± 0.13	5.74 ± 0.12	11.07 ± 0.22	8.75 ± 0.28
12	γ-Terpinene	1054	1059	0.9971	y = 1581015.3x-507593.4	2.073	0.684	2.098 ± 0.028	< LOQ	5.18 ± 0.10	< LOQ
13	Citronellal	1139	1137	0.9996	y = 231449.5x-40007.6	3.462	1.142	12.66 ± 0.66	< LOQ	28.7 ± 1.7	< LOQ
14	Citronellol	1223	1215	0.9978	y = 266564.1X-88746.7	1.807	0.596	96.6 ± 1.3	< LOQ	126.7 ± 1.6	1.977 ± 0.020
15	Linalool	1090	1090	0.9900	y = 37965.3x-191056.9	3.888	1.283	5.697 ± 0.089	< LOQ	9.65 ± 0.16	3.907 ± 0.095
16	α-Terpineol	1263	1289	0.9920	y = 39007.3x-10116.5	3.471	1.146	9.116 ± 0.089	5.41 ± 0.22	19.85 ± 0.19	13.64 ± 0.34

Table 3
Selected volatile compounds determined in *Momordica charantia* with their respective odour threshold, OAV and ROC.

No.	Compound	Odour threshold (ppm)	Ref.	OAV+SD (-)				ROC (%)				Odour descriptor
				Raw large	Raw small	Steamed large	Steamed small	Raw large	Raw small	Steamed large	Steamed small	
1	2-Hexenal	0.017	(Flath et al., 1967)	1448 ± 49	$23.3 \cdot 10^2 \pm 1.2 \cdot 10^2$	2260 ± 64	2896 ± 73	11.20	11.40	16.29	47.926	fresh, leafy, green, clean, fruity, herbal, spicy
2	Hexanal	0.005	(Flath et al., 1967)	761 ± 28	281 ± 14	1119 ± 46	607 ± 24	5.89	1.38	8.07	10.05	green, fatty, leafy, fruity
3	1-Hexanol	0.5	(Flath et al., 1967)	46.44 ± 0.96	172 ± 17	3.94 ± 0.19	6.94 ± 0.43	0.36	0.84	0.03	0.11	green, fruity, apple skin, oily
4	3-Hexen-1-ol	0.07	(Buttery et al., 1971)	309 ± 33	339 ± 17	-	-	2.39	1.66	-	-	green, leafy
5	1-Heptanol	0.003	(Guadagni et al., 1963)	279 ± 15	264 ± 12	-	-	2.16	1.30	-	-	musty, pungent, leafy, green with vegetative
6	2-Ethylhexanol	0.07	(Eastman Chemical Company, 2017)	159.0 ± 9.2	-	127.3 ± 9.8	-	1.23	-	0.92	-	citrus, fresh, floral, oily, sweet
7	2-Hexen-1-ol	0.4	(Tao & Zhang, 2010)	34.2 ± 1.7	48.1 ± 2.3	2.077 ± 0.072	10.40 ± 0.41	0.26	0.24	0.01	0.17	fruity, green, leafy
8	Hexyl acetate	0.002	(Flath et al., 1967)	$36.0 \cdot 10^2 \pm 1.8 \cdot 10^2$	$160.3 \cdot 10^2 \pm 8.8 \cdot 10^2$	-	-	27.87	78.55	-	-	green, fruity, sweet, fatty, fresh, apple, pear
9	3-Pentanone	0.85	(Dravnieks, 1974)	14.2 ± 2.2	15.51 ± 0.64	23.1 ± 2.0	29.6 ± 1.3	0.11	0.08	0.17	0.49	ethereal, acetone
10	Bornylene	nd	-	-	-	-	-	-	-	-	-	woody, spicy, citrus, terpenic, green, oily
11	α-Limonene	0.2	(Rychlik, Schieberle, & Grosch, 1998)	33.23 ± 0.66	28.67 ± 0.58	55.3 ± 1.1	43.7 ± 1.4	0.26	0.14	0.40	0.72	citrus, orange, fresh, sweet
12	γ-Terpinene	0.01	(Buttery et al., 1971)	209.8 ± 2.8	-	518 ± 10	-	1.62	-	3.74	-	terpenic, sweet, citrus, tropical lime
13	Citronellal	0.025	(Ahmed, Dennison, Dougherty, & Shaw, 1978)	507 ± 27	-	1147 ± 68	-	3.92	-	8.26	-	sweet, floral, rose, waxy, citrus, green
14	Citronellol	0.025	(Lubińska-Szczygeł et al., 2018)	3864 ± 51	-	5066 ± 63	79.07 ± 0.78	29.88	-	36.52	1.31	floral, rose, citrus, terpenic
15	Linalool	0.04	(Plotto, Margaría, Goodner, Goodrich, & Baldwin, 2004)	142.4 ± 2.2	-	241.3 ± 4.0	97.7 ± 2.4	1.10	-	1.74	1.62	citrus, orange, floral, terpenic, waxy, rose
16	α-Terpineol	0.006	(Buttery et al., 1971)	1519 ± 15	901 ± 37	3308 ± 31	2273 ± 56	11.75	4.42	23.85	37.60	pine, terpenic, lilac, citrus, woody, floral

Fig. 1. The appearance of *Momordica charantia* before and after steam cooking.

aroma of Large bitter melon (ROC = 27.9% and ROC = 11.2%, respectively), however, the α-terpineol (ROC = 11.8%) and citronellol (ROC = 29.9%) have a significant effect on the aroma of this fruit variety. For this reason, the fruit of the Chinese variety has a more complex aroma, while the Indian fruit has a sweeter and fruitier aroma and flavour. Heat treatment caused a change in the flavour of the fruit samples. The chemical compound with the high ROC for steamed fruit of both cultivars was 2-hexenal (Large fruit ROC = 16.3%, Small fruit ROC = 47.9%), followed by α-terpineol (Large fruit ROC = 23.9%, Small fruit ROC = 37.6%). The differentiating compound of the Chinese and Indian varieties of steamed samples was citronellol (Large fruit ROC = 36.5%, Small fruit ROC = 1.3%).

3.2. Antidiabetic effects of volatile chemicals

Numerous scientific studies show that one of the main chemicals of bitter melon - hexanol - exhibits insulin secretion, increasing the antidiabetic properties resulting from the content of a mixture of steroidal saponins (charanthin), insulin-like peptides and alkaloids (Bharti, Krishnan, Kumar, & Kumar, 2018). In addition, high antidiabetic effects have been reported during β-citronellol studies (Srinivasan & Muruganathan, 2016). It is the main chemical compound of the Chinese variety of *Momordica Charantia*, whose concentration is 96.6 μg/g. In melon of Indian variety was below the limit of quantification. This can testify to the greater suitability of a Larger variety of melon to be consumed by diabetics. According to the scientific reports, however, Indian variety contains more total phenolic contents (Horax, Hettiarachchy, & Islam, 2006). In addition, as shown by studies, monoterpenoids such as limonene, cymene, menthol, borneol, citronellol, geraniol, carvone, thujone and myrtenal, detected in samples of bitter melons, are strong antidiabetic agents (Habtemariam, 2018). Research conducted using the diabetic animal model showed the antidiabetic effect of triterpenoids isolated from *Momordica Charantia* (Tan et al., 2008).

3.3. Chromatographic fingerprinting and multivariate data analysis

In order to distinguish between raw and steamed bitter melon fruit samples from two botanical varieties, a principal component analysis (PCA) was performed. In Fig. 2A, the PCA biplot was shown, on the basis of which it can be determined which chemical compounds have the greatest impact on the aroma of the tested fruit samples. Fig. 2 is a projection of the first two principal components (PCs) constituting over

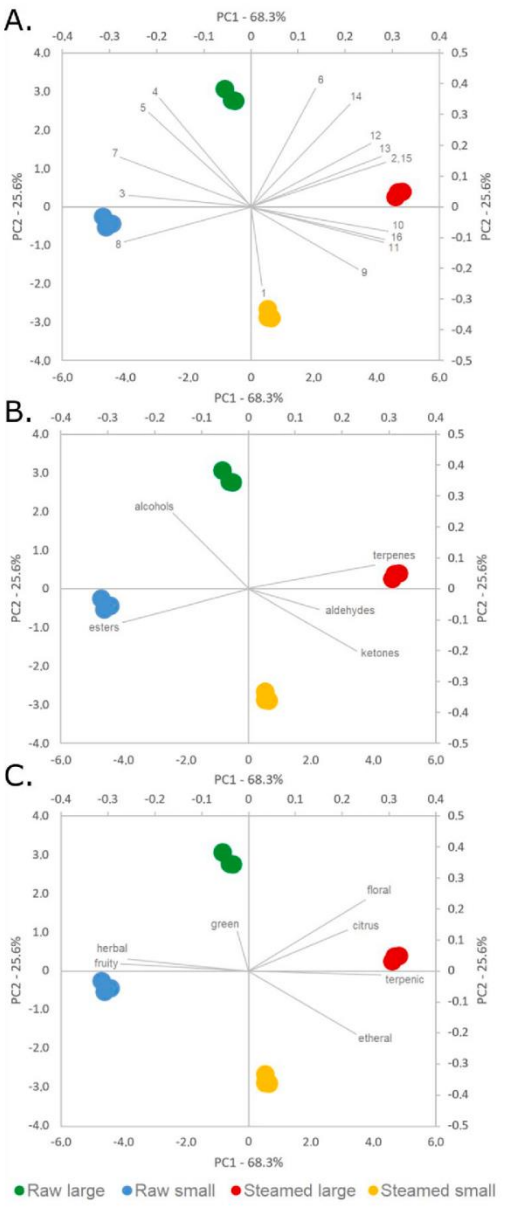


Fig. 2. PCA biplots of the assignments of raw and steam cooked Chinese and Indian variety of *Momordica charantia* in respect of (A) individual VOCs, (B) chemical classes, (C) aroma descriptors.

90% of the variance in the data set (PC1- 68.3%, PC2- 25.6%). It can be observed, that the total separation of *Momordica charantia* samples along the two first main components was obtained. In addition, correlations between the fruit samples and the chemical compounds

characteristic for them can be noticed. For example, 2-hexenol and 1-hexanol were positively correlated with samples of Large raw bitter melon while hexyl acetate was positively correlated with samples of Small raw bitter melon. Whereas, 2-hexenal was associated with samples of steamed Small bitter melon. In contrast, the following substances: citronellal, linalool, γ-terpinene, α-terpineol, bornylene, limonene and hexanal were characteristic for samples of steamed Large *Momordica charantia* fruit. Moreover, the higher content of citronellol in Large variety, especially after steam cooking, is another argument that this certain type of *Momordica charantia* is more healthily because it increases antidiabetic effect of this fruit, as well as this specific thermal treatment of food enriches positive influence on pro-health properties.

In the second biplot where chemical classes were used as loadings (Fig. 2B), an east/west pattern can be observed, that distinguish raw and steamed fruit, and the north/south pattern distinguishing the botanical varieties of the samples. Raw *Momordica charantia* fruit has higher abundance of esters and alcohols, while steamed *Momordica charantia* fruit has higher abundance of aldehydes, ketones and terpenes. Alcohols constitute the important chemical class of bitter melon raw fruit. Steaming led to large loss of alcohol content in the studied samples. This may be due to the high solubility of alcohols in water. In fruit, esters are formed mainly by the esterification of alcohols and carboxylic acids (Bhandari, D'Arcy, & Young, 2001). The major ester identified in raw fruit samples was hexyl acetate. Substances belonging to this chemical class are subject to thermal degradation during heat treatment, which causes a reduction in esters content in steamed fruit. Heat treatment contributes to the formation of aldehydes with a linear structure as a result of oxidative degradation of unsaturated fatty acids and branched-chain aldehydes via the Strecker degradation (Kebede et al., 2015). In Fig. 2B, it can be observed that aldehydes are positively correlated with samples of steamed fruits. Ketones, such as 3-pentanone, may be derived from unsaturated fatty acids (Köckritz & Martin, 2008). The ketone content increased during steam cooking, therefore ketones were associated with thermally treated samples (De Rodríguez-Bernaldo Quirós, López-Hernández, González-Castro, De La Cruz-García, & Simal-Lozano, 2001). Terpenes were detected in both raw and steamed fruit samples. During the steaming process, the content of terpenes in *Momordica charantia* increased. Despite the fact that more volatile terpenes can evaporate more during the heat treatment, the amount of lower boiling point terpenes can be higher in respect of appearing biological processes. Nevertheless, the exact metabolic pathways of these compounds were not recognized yet. In Fig. 2C, loadings were assigned to the certain types of odours. The information about odour descriptors was provided from The Good Scent Company (www.thegoodscentscompany.com). Many of these volatiles have favourable odour type, which can be recognized as citrus, floral or green. On the other hand, a few volatiles have unfavourable odour type such as etheral or terpenic. Volatiles corresponding with raw bitter melon have lower aroma volatile content but with favourable notes such as fruity, green or herbal. These odours are characteristic for green fruit and vegetables. Samples subjected to steam cooking were characterized by unpleasant odour notes, like an etheral scent. However, they can be obscured by the less-undesirable terpenic odour. It was found that the samples of Chinese variety (Large one) of bitter melon, both raw and steamed, have higher citrus and floral aroma than Indian variety (Small fruit). It can be noticed that volatiles have partial influence of summary tastiness of these fruits. These results are not representative for the actual reception of the taste by the consumer. Non-volatile constituents of this matrix have a dominant role in this case. Steam cooking improves tastiness of *Momordica charantia* in both varieties, because of changes occurring in non-volatile fraction of fruit (Fig. 3).

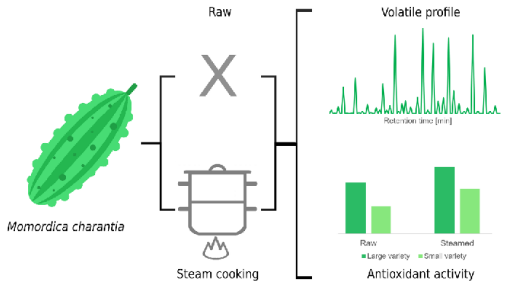


Fig. 3. Graphical characterization of the volatile substances of *Momordica charantia*.

3.4. Effect of different Momordica charantia fruit cultivars on bioactive compounds

Phenolic profiles and antioxidant activities of *Momordica charantia* fruit cultivars (Table 4) were established using a rich variety of spectrophotometric methods such as Folin, ABTS, CUPRAC, DPPH and FRAP, which are known to be electron transfer-based antioxidant capacity measurement methods finding a wide use in scientific reports, together with flavonoid- and tannin-specific colorimetric tests (Ghous, Aziz, Mehmood, & Andleeb, 2015; Horax, Hettiarachchy, & Chen, 2010; Leontowicz et al., 2016; Singh et al., 2012). The FRAP method produced the lowest antioxidant capacity results (Table 4) in comparison with ABTS and CUPRAC, as this method has been established not to respond to certain phenolics within the protocol time of the assay. As regards to polyphenols, their concentrations were slightly higher in Large *Momordica charantia* fruit than in Small. The comparison indicates the differences in polyphenols in Large fruit of *M. charantia* in water and methanol extracts from 12.76 to 7.05.08 mg GAE/g and in Small from 11.58 to 5.08 mg GAE/g. Other vegetables showed similar results (Im et al., 2011 and 2012). The obtained values by Horax et al. (2006 and 2010) in regards to polyphenols were partly in accordance with the results in this report. Phenolics were extracted from pericarp (fleshy portion) and seeds of bitter melons harvested at three maturation stages (immature, mature and ripe) using ethanol and water solvent systems. In the present research, all bioactive substances were extracted from ripe bitter melons, using water and methanol (Table 4). The solvents used for the extraction influenced the amount of obtained substances. Horax et al. (2010) showed that total phenolic assessment demonstrated 80% of ethanol to be the optimal solvent level to extract phenolics either from pericarp or seed. Flavonoids concentration was the highest in Large *Momordica charantia* only in methanol extract (Table 4). Evaluation of Large and Small *Momordica charantia* fruit extracts in antioxidant activity indicated flavonoids and phenolics, which were dominant bioactive compounds such as ferulic acid, kaempferol, quercetin, rutin and isorhamnetin (Horax et al., 2010). The lowest values between the four antioxidant assays were in DPPH (Table 4). Horax et al. (2010) showed that free radical scavenging assay by DPPH showed the bitter melon extracts as slow rate free radical scavenging agents. In most cases it was a correlation between the antioxidant assays and the polyphenols in contrast that there were low correlations between the total phenolic contents and antiradical power values of the extracts, suggesting a possible interaction among the phenolic constituents occurred. Bitter melon phenolic extracts contain natural antioxidant substances, and could be used as antioxidant agents in suitable food products (Horax et al., 2010). The present results are in accordance with Ghous et al. (2015), where the antioxidant, metal chelating and antiglycation activities of aqueous extracts of *M. charantia* fruit flesh (MCF) and fruit pulp (MCP) fractions were compared. The results show that MCP has pronounced DPPH and ABTS radical scavenging potential compared to MCF. The results shown in Table 4, especially of ABTS, were similar. The antioxidant assays with regression values of MCP (0.981 and 0.991) and MCF (0.967 and 0.999) were also recorded, which is in line with our results. Padmashree, Sharma,

Table 4
Bioactive substances of water (W) and methanol (M) *Momordica charantia* extracts per g dry weight (DW).

Indices	Large W	Large M	Small W	Small M	Large WS	Large MS	Small WS	Small MS
Polyph, mgGAE	12.76 ± 1.21^{a}	7.05 ± 0.57^{c}	11.58 ± 1.11^{ab}	5.08 ± 0.41^{cd}	11.89 ± 1.25 ^{ab}	6.61 ± 0.87^{d}	10.88 ± 0.97^{b}	4.74 ± 0.97^{d}
Flavan, µgCE	49.90 ± 4.19^{b}	108.94 ± 9.6^{a}	0.093 ± 0.01^{d}	29.94 ± 2.87^{c}	46.40 ± 4.65^{b}	101.21 ± 10.11^{a}	0.085 ± 0.01^{d}	27.82 ± 2.87 ^{c}
Flavon, mgCE	NF	1.80 ± 0.18 ^{a}	NF	1.16 ± 0.11^{b}	NF	1.66 ± 0.17^{a}	NF	1.07 ± 0.09 ^{b}
Tannins, mgCE	6.80 ± 0.65^{b}	9.95 ± 0.83^{a}	2.96 ± 0.29^{c}	6.80 ± 0.62^{b}	6.32 ± 0.13^{b}	9.23 ± 0.15^{a}	2.74 ± 0.18^{c}	6.28 ± 0.18^{b}
Vit C, mgAsc	1.81 ± 0.18^{a}	NF	1.15 ± 0.11^{b}	NF	1.68 ± 1.69^{a}	NF	1.05 ± 0.17^{b}	NF
Anth, mgCGE/kg	577.78 ± 41.3 ^{a}	NF	170.33 ± 14.18 ^{b}	NF	531.43 ± 52.2 ^{a}	NF	156.62 ± 15.62 ^{b}	NF
Chlor *a*, µg	624.00 ± 43.07^{a}	NF	350.00 ± 31.05 ^{b}	NF	587.43 ± 57.71^{ab}	NF	322.65 ± 31.61^{b}	NF
Chlor *b*, µg	519.50 ± 42.12^{a}	NF	229.00 ± 21.12^{b}	NF	477.48 ± 0.01^{ab}	NF	210.68 ± 0.01^{b}	NF
Xan + Carot, µg	229.50 ± 22.37^{a}	NF	206.50 ± 21.54^{ab}	NF	200.42 ± 0.56^{ab}	NF	186.66 ± 0.19^{b}	NF
ABTS, µMTE	39.60 ± 3.76^{a}	20.38 ± 2.76^{bc}	35.54 ± 3.12^{ab}	14.76 ± 1.28^{c}	36.05 ± 3.12^{ab}	18.54 ± 1.76^{bc}	32.31 ± 3.16^{b}	13.54 ± 1.16^{c}
FRAP, µMTE	25.90 ± 2.34^{a}	14.42 ± 1.12^{bc}	23.48 ± 2.56^{a}	10.29 ± 1.23^{cd}	23.58 ± 2.34^{a}	13.12 ± 1.27^{c}	21.38 ± 1.95^{b}	9.38 ± 0.87^{d}
CUPRAC, µMTE	52.36 ± 5.32^{a}	28.95 ± 2.32^{bc}	47.67 ± 4.23^{ab}	20.81 ± 2.65^{bc}	47.12 ± 4.12^{ab}	26.16 ± 2.33^{bc}	42.87 ± 3.45^{b}	18.79 ± 1.45^{c}
DPPH, µMTE	26.04 ± 2.43 ^{a}	14.34 ± 1.32 ^{bc}	23.35 ± 2.2 ^{ab}	10.36 ± 1.23 ^{cd}	23.43 ± 2.18 ^{ab}	12.98 ± 1.21 ^{c}	21.21 ± 2.08 ^{b}	9.32 ± 0.87^{d}

Mean ± SD (standard deviation) of 5 measurements. Average in rows marked with different letters differ significantly ($P < 0.05$).
Abbreviations: Large, *Momordica charantia* Chinese cultivar; Small, *Momordica charantia* Indian cultivar; S, steamed cooking; Polyph, polyphenols; GAE, gallic acid equivalent; CE, catechin equivalent; Flavan, flavanols; Flavon, flavonoids; VitC, vitamin C; Anth, anthocyanins; NF, not found; CGE, cyanidin-3-glucoside equivalent; Chlor, chlorophyll; Xan + Carot, xanthophylls + carotenes; ABTS, 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; FRAP, Ferric-reducing/antioxidant power; CUPRAC, Cupric reducing antioxidant capacity; 1,1-diphenyl-2-picrylhydrazyl, DPPH; TE, trolox equivalent.

Semwal, and Bawa (2011) showed that bitter gourd pulp and seed powders and their ethanol/water extracts exhibited stronger anti-oxygenic activity than other solvent extracts. This may be attributed to the presence of higher amounts of phenolics and flavonoids, which have been reported as potential antioxidants (Aljohi, Matou-Nasri, & Ahmed, 2016; Dandawate, Subramaniam, Padhye, & Anant, 2016). The carotenoid content was 229.5 µg/g. The differences among the profiles of seven carotenoids identified in the fruit at several maturation stages. Riper fruits had higher carotenoid concentrations than less ripe fruits (Tuan, Kim, Park, Lee, & Park, 2011). The chlorophyll *a* was 624.0 µg/g and chlorophyll *b* was 519.5 µg/g, ascorbic acid concentration reached 1.81 mg/g and anthocyanins were found as 577.8 mg CGE/kg in Large variety of *Momordica charantia* extracts (Table 4). These results were in accordance with Bahrami, Heidari, and Ghorbani (2016), where chlorophyll *a* and *b* and anthocyanin content were determined on increasing salinity during growing.

3.5. Effect of cooking conditions on antioxidant capacities and their correlations with bioactive compounds

The antioxidant, antiinflammatory and anticancer activities of *Momordica charantia* with important chemical constituents are the basis for establishing biological activities of the plant and developing novel drug molecules based on the active chemical constituents. All parts of the plant, including the fruit, are commonly consumed and cooked with different vegetables, stir-fried, stuffed or used in small quantities in soups or beans to give a slightly bitter flavour and taste. It is well known that a heat process significantly influences the antioxidant activity of fresh fruits. However, the steaming process of bitter gourd has not been studied so far. *Momordica charantia* fruits showed slight changes after cooking. In *Momordica charantia* samples after cooking determined decrease in all bioactive substances between 6 and 12% (Table 4). The changes in the bioactivity of different plants during cooking was studied on berries (Arancibia-Avila et al., 2012), but mostly on different vegetables, including lotus, onions, garlic (Im et al., 2011, 2012; Gorinstein et al., 2009). The obtained results on steaming of *Momordica charantia* are in line with previous research which was performed on raw and boiled for 10, 20, 40 and 60 min Korean lotus roots (KLR) and Polish white onion (PWO) and their evaluation of the contents of bioactive compounds, antioxidant activity and thermostability. Small and Large *Momordica charantia* samples had similar changes in polyphenols, flavanols, flavonoids, anthocyanins, tannins and other bioactive compounds as lotus roots. The antioxidant activity of raw KLR water extract for DPPH, FRAP, CUPRAC and ABTS was significantly higher than in white onion, and in the present results were higher in Large *Momordica charantia*. The thermostability of the water extract of polyphenols, flavanols, flavonoids, anthocyanins and tannins was high in Large *Momordica charantia* and slightly lower in Small sample after 20 min of steaming of 20 min and was equal in the changes to the KLR sample after 10 min boiling (Im et al., 2011, 2012; Gorinstein et al., 2009). The results are in line with the report of Myojin et al. (2008), proving that the bioactive compounds (polyphenols, flavonoids, flavanols, tannins, carotenoids and ascorbic acid) and the levels of antioxidant activities assessed by four assays in methanol and water extracts of *Momordica charantia* fruits significantly differed in the steamed investigated samples and were the highest in Large fruit. In Myojin et al. (2008) slightly different cooking was applied, and blanching of sliced bitter gourd resulted in considerable losses of radical-scavenging activity (RSA), and total phenolics, and most extensively, of ascorbic acid. The positive effect of bitter gourd on diabetes has been attributed in part to the remarkable free radical scavenging activity of its boiled water extract from sun-dried fruits. It is well known that a heat process significantly influences the antioxidant activity of fresh fruits. Free radical scavenging capability of bitter gourd extract significantly increased after the heat drying process, while the content of flavonoids and phenols, which are the main antioxidant components, remained unaffected. Therefore Maillard reaction products may be the main contributors to the increase in antioxidant capability. These results suggest in comparison with our obtained data (Table 4) that controllable conditions in the heat-drying processing of fresh bitter gourd fruit is of significance for enhancing the total free radical scavenging capacity (Wei et al., 2013). The water-soluble phenolic content (WPC) and total antioxidant activity was increased in cooked water convolvulus, broccoli and bitter gourd, estimated based on FRAP and ABTS. Pressure cooking did not cause any significant decline in the antioxidant property. Boiling generally improved the overall antioxidant activity in all the vegetables. Correlation analysis suggests that WPC contributed to significant antioxidant activities in these vegetables what is in line with obtained results. Our results correspond with recent reports on different ways of cooking. Cooking methods (steaming, boiling, microwave heating for different durations and pressure cooking) enhanced the antioxidant activity, water–soluble phenolic and vitamin C contents. Thus, the findings of this study show that cooking did not reduce the nutritional value of the vegetables and also suggested that appropriate cooking method and duration for different vegetables could be selected to improve or preserve their nutritional value (Ng et al., 2011; Subramaniam, Rosdi, & Kuppusamy, 2017; Xie, Luo, Hao, & Li, 2015).

Table 5
Fluorescence properties in 2 D- (**A**) and 3D-measurements (**B**) of water extracts and standards in interaction with HSA.

A

No of lines	Samples	λ Em	FI
1F	HSA	355	953.22
2F	HSA + MeOH	351	902.40
3F	SmallS + HSA	339	845.78
4F	Small + HSA	353	822.50
5F	Large + HSA	338	811.04
6F	CA + HSA	354	777.68

B

Peaks	Indices	Sample				
		HSA	CA	Small	SmallS	Large
a	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	227/349	228/354	231/332	229/337	233/334
	Int F_0	765.90	249.70	564.56	591.42	478.38
a1	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	–	–	231/632	230/627	231/630
	Int F_0	–	–	108.40	106.35	79.07
b	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	279/353	278/357	282/339	280/340	280/339
	Int F_0	875.01	758.11	850.44	863.51	786.12
b1	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	–	–	282/644	279/645	282/642
	Int F_0	–	–	164.47	152.63	143.51
c	$\lambda_{ex}/\lambda_{em}$ (nm/nm)	–	–	368/449	–	356/436
	Int F_0	–	–	209.64	–	159.33
BPa/b,%		–	67.4/13.4	26.3/2.8	22.9/1.3	37.5/10.2

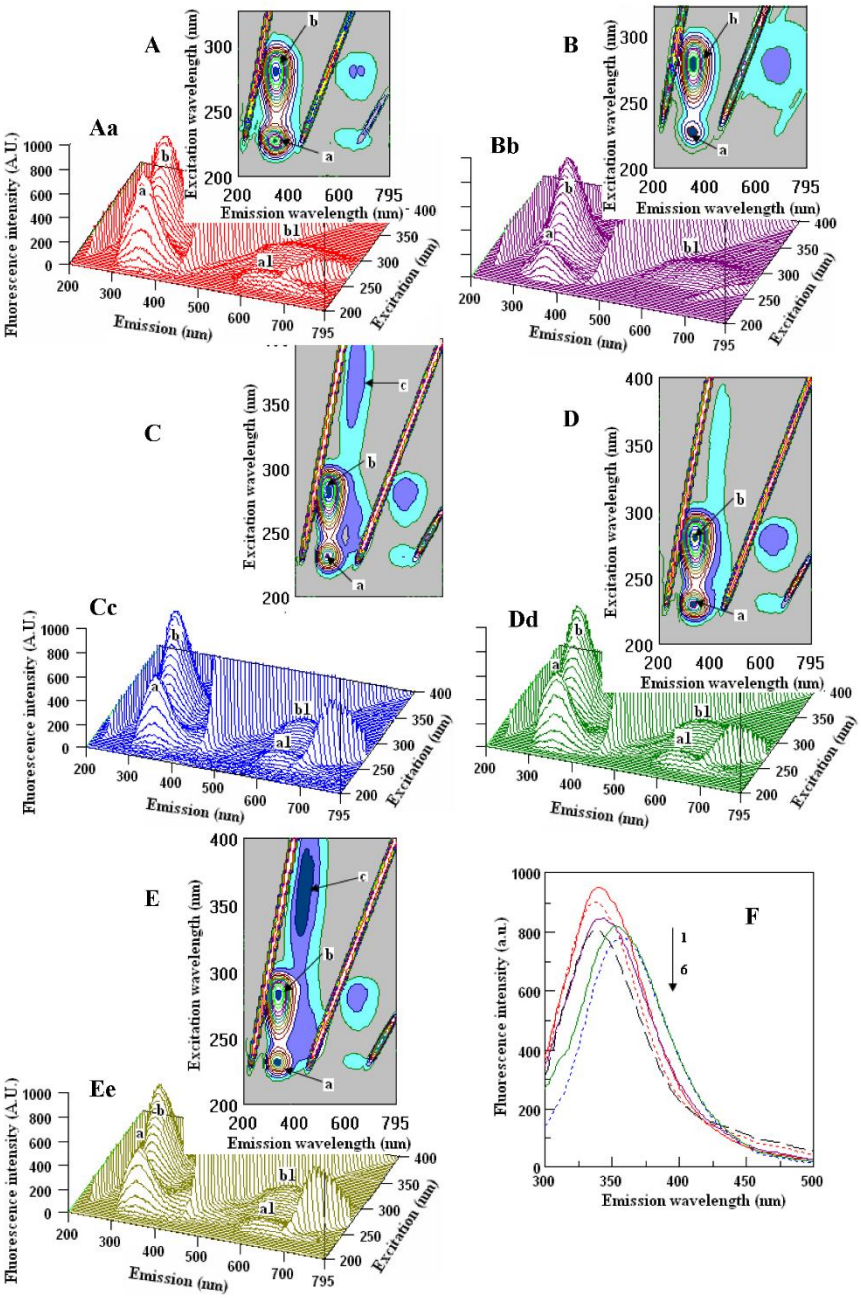
Measurements were done in water *Momordica charantia* extracts with concentration 0.17 mg/mL, caffeic acid concentration 0.028 mM; HSA concentration 2 mM × 10^{-5}; λ_{em} 300 nm/λ_{em} 280 nm for 2D-fluorescence. Abbreviations: Small, SmallS Large, *Momordica charantia* Small, Small steamed, Large; FI, AU, fluorescence intensity in arbitral units; $\lambda_{ex}/\lambda_{em}$, nm/nm. HSA, human serum albumin; BP, binding properties, calculated for peaks **a** and **b**. Numbers of lines in 5A correspond with Fig. 4F.

3.6. Fluorescence studies

The fluorescence properties of methanol extracts are shown in Table 5 and Fig. 4. 2D –measurements showed that the fluorescence intensities (FIs) correspond with the amount of polyphenols in the majority of investigated samples (Table 5A, B, Fig. 4). The maxima wavelengths in all samples varied from 338 to 355 nm. Caffeic acid as a standard showed one peak at 354 nm. The fluorescence intensities of the found peak in Large sample was slightly different than in Small. Peaks **a, b** corresponded with the peaks of caffeic acid **a** (228/354 nm) and with **b** (278/357 nm, Table 5B, Fig. 4C and E). The highest binding properties were in Large sample (BPa/b,% = 37.5/10.2; Table 5B and Fig. 4E, in two peaks). The lowest binding properties were in Small steamed sample (Fig. 4D, BP a/b = 22.9/1.3). Peak **c** was found only in Small and Large samples, and the lowest fluorescence intensity was in Large one (Fig. 4C and E, Table 5B). It was direct coordination between the polyphenols, antioxidant activities and binding properties. The binding properties of the polyphenols extracted from *Momordica charantia* were relatively high showing the correlation between the antioxidant and quenching properties of polyphenols towards human serum albumin. This also supports the statement that *Momordica charantia* has important health properties and can be a part of human diet in comparison with other studies vegetables, because the infusions obtained from the fruit and leaves presented antimicrobial activity (Brandao et al., 2016) and the extract was observed to be beneficial for diseases that are associated with free radical-induced oxidative damage (Choo, Waisundara, & Hoon, 2014). Recent reports have shown also that *in vivo* studies have demonstrated the relatively low toxicity of all parts of the bitter gourd plant when ingested orally (Raghavan, Kumar, & Ilaiyaraja, 2015). According to Bharti et al. (2018) due to the antioxidant activity, *Momordica charantia* is a potential candidate as antidiabetic and antiglycation agents. This conclusion can be supported by other reports that flavonoids and polyphenols have been shown to exhibit many human health benefits due to their antioxidant activity. The present results are in accordance with Dandawate et al. (2016), Ghous et al. (2015) and Grover and Yadav (2004), showing that *Momordica charantia* possesses bioactive constituents, especially polyphenols and flavonoids that improves the plasma lipid profile and prevents hepatic oxidative damage. Our results are in line with Sinisi et al. (2015), where HSA interacted with coffee polyphenols, four acids and four lactones. Dissociation constants of the complexes were comparable with the most powerful binders of albumin and more favourable than the values obtained for the majority of drugs.

4. Conclusions

In the present study, the influence of steam cooking on *Momordica charantia* was investigated. Most of the studies on this topic concern the research devoted to non-volatile constituents of this fruit. Our results for the first time demonstrated correlation between volatiles and non-volatile substances present in *Momordica charantia* regarding their impact on bioactivity. It was proven that steam cooking has positive effect on bioactive properties due to the increased amount of bioactive compounds characterized by a wide range of volatility. This method of heat treatment enriches not only the content of bioactive compounds but at the same time improves the taste of bitter melon. It can be explained by the increased amount of some volatiles, especially terpenes. The higher content of the one of volatiles, namely citronellol, explains the antidiabetic effect of this fruit, which is convergent with the results of non-volatile content of *Momordica charantia.* The certain amount of citronellol is the result of transformation of other terpenes to this specific terpene under the influence of steam cooking. Regarding the applied analytical technique to the above-described research objectives, GC×GC-TOFMS also allows for botanical and geographical determination of Small and Large variety of *Momordica charantia.* It was possible to define that the Large type of this fruit has greater value in case of flavour. The polyphenols and antioxidant capacities in Large variety were slightly higher than the Small sample. 2D-measurements showed



(caption on next page)

Fig. 4. Corresponding contour maps and three-dimensional fluorescence spectra of water extracts of *Momordica charantia* samples after interaction with HSA: **A, B, C, D, E**, HSA, CA, Small, SmallS, Large. **F,** Spectral data of two dimensional fluorescence measurements (2D-FL) of water extracts and standard from the top: 1, 2, 3, 4, 5, 6, HSA, HSA + buffer; SmallS + HSA; Small + HSA; Large + HSA; CA + HSA.
Line 6 in **F** is caffeic acid at concentration of 0.0091 mM; Measurements in 2D- FL were done in water extracts with concentration 0.17 mg/mL, λ Em 300 nm, λ Exc 280 nm; Count peaks of water simple extracts + HSA; $2\,M \times 10^{-5}$.
Abbreviations; HSA, human serum albumin; CA, caffeic acid; Small, cultivar; SmallS, steamed cultivar; Large, cultivar. The fluorescence intensities of the investigated samples are given in Table 5A and **B, C, D, E**, 3D-fluorescence spectra and **Bb**, **Cc, Dd, Ee**, corresponding cross spectral images. The values of peaks **a, a1, b, b1, c** are given in Table 5B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

that the fluorescence intensities (FIs) correspond with the amounts of polyphenols in the majority of investigated samples. The binding properties of polyphenols to HSA were relatively high in comparison with other plants and it was a correlation between the binding properties and their bioactivities. Summarizing up, one of the most popular method of bitter melon preparation was proven to be a safe and adequate one due to changes in the composition of the volatile fraction of bitter melon as well as on the basis of the changes in its non-volatile content and antioxidants. Our research may indicate that insufficient growing conditions in northern Thailand caused that Small type of *Momordica charantia* has less pro-health properties than Large one produced in all other regions in Thailand. We conclude that both extracts possess high antioxidant activities and are equally good sources of antioxidants. The future prospects in monitoring oxidative stability of foods and edible plants are based on the developed protein-polyphenol interactions, especially with human serum albumin in order to evaluate their binding properties.

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6.8 Bioactivities of Phenolic Compounds from Kiwifruit and Persimmon

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MDPI

Article

Bioactivities of Phenolic Compounds from Kiwifruit and Persimmon

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Abstract: Fruit used in the common human diet in general, and kiwifruit and persimmon particularly, displays health properties in the prevention of heart disease. This study describes a combination of bioactivity, multivariate data analyses and fluorescence measurements for the differentiating of kiwifruit and persimmon, their quenching and antioxidant properties. The metabolic differences are shown, as well in the results of bioactivities and antioxidant capacities determined by ABTS, FRAP, CUPRAC and DPPH assays. To complement the bioactivity of these fruits, the quenching properties between extracted polyphenols and human serum proteins were determined by 3D-fluorescence spectroscopy studies. These properties of the extracted polyphenols in interaction with the main serum proteins in the human metabolism (human serum albumin (HSA), α-β-globulin (α-β G) and fibrinogen (Fgn)), showed that kiwifruit was more reactive than persimmon. There was a direct correlation between the quenching properties of the polyphenols of the investigated fruits with serum human proteins, their relative quantification and bioactivity. The results of metabolites and fluorescence quenching show that these fruits possess multiple properties that have a great potential to be used in industry with emphasis on the formulation of functional foods and in the pharmaceutical industry. Based on the quenching properties of human serum proteins with polyphenols and recent reports in vivo on human studies, we hypothesize that HSA, α-β G and Fgn will be predictors of coronary artery disease (CAD).

Keywords: kiwifruit; persimmon; polyphenols; human serum proteins; quenching properties; biomarkers

1. Introduction

Antioxidant activities of bioactive compounds for human nutrition and health are directly connected with different fruits, such as exotic and traditional, which are commonly used in daily consumption [1,2]. Many tropical fruits are known, but only a small number is widely consumed [3]. Persimmons and kiwifruits are on the list of the most-used tropical fruits, together with banana, mango, and avocado. Consumption of fruit and its biomarkers of intake are the main indices of a healthy diet [4]. Persimmons (*Diospyros kaki*) are recognized as an outstanding source of biologically active components that

exhibit many health benefits, such as antioxidant behavior, radical scavenging activity, antihypertensive and antiatherosclerosis activities [5]. The metabolite profiles related to triglyceride/cholesterol metabolism treated with persimmon were studied [6]. The persistent calyx of *Diospyros kaki* is reported to contain phenolic compounds, including condensed tannins together with phenolic components, estimated in 70% acetone extract [7]. Kiwifruits also exhibit antioxidative, antiproliferative, anti-inflammatory, antimicrobial, antihypertensive, antihypercholesterolemic, neuroprotective and antiobese properties and promote gut health [8]. Nuclear magnetic resonance (NMR) has increasingly become an attractive tool in metabolomics analysis and has been combined with multivariate data analysis such as principal component analysis (PCA) and partial least-squares discriminate analysis (PLS-DA) in order to evaluate the properties of the bioactivity of compounds, especially phenolic ones with antioxidant properties. This approach has been used to identify differences among varieties of foods, the quality of cultivar selection, and taste evaluation [9–12]. The metabolic profiling of three kiwifruit varieties, including the most consumed Hayward cultivar (*Actinidia deliciosa*), the mini kiwi (*Actinidia arguta*), and the less-known Bidan (*Actinidia eriantha*), and their nutritional component analyses in different development stages were investigated in recent studies [13,14]. In spite of the frequent consumption of kiwifruit (*Actinidia*) and persimmon (*Diospyros kaki*), and also of their belonging to the same category as tropical fruits [3] such as mango (*Mangifera indica*), avocado (*Persea Americana*), dragon fruit (*Hylocereus undatus*), and others, information about metabolites in human studies is unavailable [4,15]. The bioactivity of fruit polyphenols in interaction with human serum proteins is an important factor to characterize their health and nutritional properties [16]. Recent reports conducted preclinical and clinical studies on the polyphenol bioavailability and health benefits of aronia berry consumption [17]. While reports on polyphenol bioavailability have increased, there is still limited knowledge about the dynamics of polyphenol metabolism [18–20].

Polyphenols and flavonoids are plant metabolites that interfere with different biological processes in the human metabolism. After absorption, in our case through fruits, they bind to human serum albumin (HSA), the most abundant carrier protein in the blood, which also binds various drugs. The binding of flavonoids to HSA may impact their distribution, influencing the active concentration in the blood. Other human proteins in addition to HSA, such as globulins and fibrinogen, also react with polyphenols. Human proteins, including albumin, α-acid glycoprotein, lipoproteins, fibrinogen and α, β, and γ globulins play an important role in the pharmacokinetic and pharmacodynamic properties of food diets. The polyphenols–protein interaction is reversible in that the polyphenols–protein complex can dissociate and release free polyphenols [21,22]. For the first time, our findings indicated that one of the positive benefits of fruit consumption in patients with coronary artery disease (CAD) was diminishing the production of fibrinogen and its stability, which reduces the potential risk exerted by this protein [23]. Serum albumin is a powerful prognostic marker in patients with cardiovascular diseases [24]. The albumin-to-globulin ratio (AGR) is used as a prognostic marker in acute ischemic cardiovascular events. Investigations were directed to determine whether serum AGR, fibrinogen, and the fibrinogen-to-albumin ratio (FAR) are related to the presence and severity of coronary artery disease [25,26].

Using animal models and humans in vitro and in vivo, it was estimated that various fruits, including persimmon and kiwifruit, contained a high number of potential antioxidants and improved the lipid and antioxidant status. Kiwifruit consumption changes plasma lipids, fibrinogen and insulin resistance in the context of a normal diet [27,28]. Pretreatment of platelets with tannic acid led to significant reductions in soluble fibrinogen binding, supporting the inhibition of diverse platelet activation pathways [29]. Some phenolic acid derivatives displayed anticoagulant activities [30].

In spite of wide information on the properties of fruits, the additional information provided in this study on the in vitro interaction with some metabolites is important to understand the health properties of fruit consumption. Based on our recent results and

information, in vitro studies will be performed on persimmons and kiwifruits. The contents of the bioactive compounds in these fruits and their influence on the quenching properties of the main proteins in human metabolism such as HSA, globulins and fibrinogen will be studied using advanced analytical methods, including NMR, fluorescence, and different antioxidant assays.

2. Results and Discussion

2.1. Identification of Bioactive Compounds in Fruit Extracts

In this study, three different varieties of kiwifruits, such as *Actinidia* (A.) *deliciosa* cv. Hayward (KH), *A. eriantha* cv. Bidan (BC), *A. arguta* Cheongsan (AM) and one cultivar of *Diospyros kaki* Thunb. cv. Fuyu (PF), were subjected to NMR analysis, and the obtained spectra were further evaluated using multivariate data analysis (MVDA). The identified metabolites and their NMR characteristics, as well as their presence in each sample, are listed in Table 1 and Figure 1.

Table 1. The identified bioactive compounds and their characteristics.

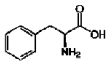
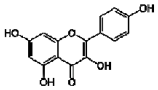
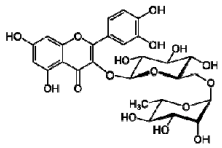
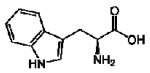
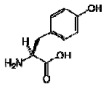
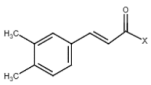
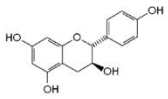
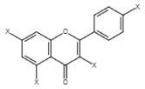
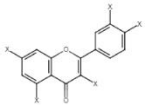
No.	Compound	CAS	Structure	δH(ppm), Multiplicity, J Value (Hz)
1	Phenylalanine	63-91-2		7.40, m (2H) 7.35, m 7.30, d, 7.4 (2H)
2	Kaempferol	520-18-3		8.01, d, 8.0 6.95, d, 8.0 6.32, br d (small d) 6.10, br d (small d)
3	Rutin	153-18-4		7.65, d, 2.0 7.60, dd, 6.82, d, 8.5 6.38, d, 6.19, d, 1.05, d, 7.0 4.51, br s (small d) 5.05, d, 8.0
4	Tryptophan	73-22-3		7.70, d, 8.0 7.54, d, 8.0 7.20, t, 7.0
5	Tyrosine	60-18-4		3.94, m 7.15, d, 8.0 6.82, d, 8.0
6	Caffeic acid derivatives			7.57, d, 13.0 7.28, br s (small d) 7.22, d, 8.0 6.95, d, 8.0 6.55, d, 13.0
7	Protocatechuic acid	99-50-3		7.39, br s (small d) 7.35, br d (dd), 8.0 6.92, d, 8.0
8	Catechol	120-80-9		6.77–6.84, m 4.52, d, 7.20 2.94, dd, 15.7, 6.2 2.47, dd, 15.0, 8.0
9	Syringic acid	530-57-4		7.26, s, 2H 3.89, s

Table 1. *Cont.*

No.	Compound	CAS	Structure	δH(ppm), Multiplicity, J Value (Hz)
10	Afzelechin	2545-00-8		2.83; 2.80; 2.79, dd,15.6, 4.8 2.68, dd 6.85, d, 8.0 (2H) 7.17, d, 8.0 (2H)
11	Kaempferol derivatives			6.97, d, 2.7 6.46, d, 2.7
12	Quercetin derivatives			7.52, d, 3.5 6.66, d, 3.5
13	Gallic acid	149-91-7		7.01 (s)

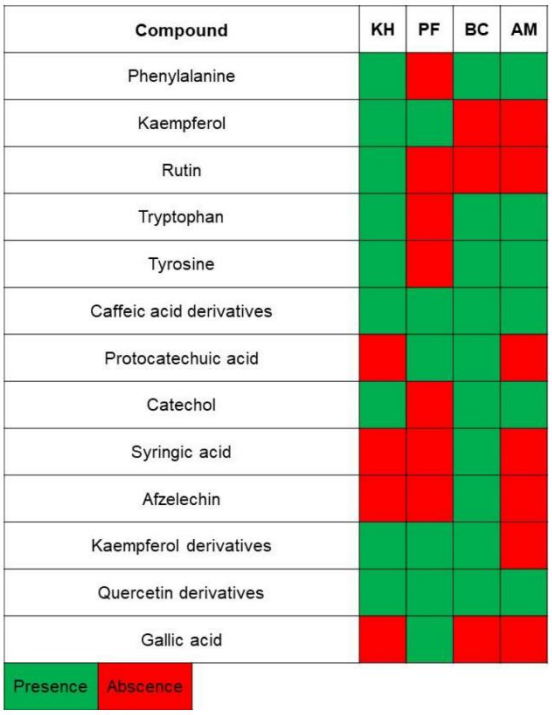


Figure 1. The presence of bioactive compounds in fruit samples. Abbreviations: KH, *Actinidia (A.) deliciosa* cv. Hayward; BC, *A. eriantha* cv. Bidan; AM, *A. arguta* cv. Cheongsan; PF, *Diospyros kaki* Thunb. cv. Fuyu.

There are differences in some metabolites of the different fruit samples, which can be detected from the ^{1}H-NMR spectra. The principal peak areas of the spectra impart, as an immediate measure of metabolite concentration, permitting whole metabolites to be quantified based on a single internal standard. Different peak intensities can be observed at δ 5.20 (α glucose) and δ 5.40 (sucrose), where persimmon Fuyu showed the lowest concentration of sucrose as compared with the other kiwifruit samples (Figure 2A). It is also worth noting that different levels of peaks can be seen in the aliphatic region (δ 0.50–δ 3.00, Figure 2B). The same trend of intensities was observed in persimmons with the lowest signal compared with the tested kiwifruits. However, visual inspection of ^{1}D-NMR does not permit any detection of compounds in the aromatic region (δ 6.00–δ 8.50). The ^{1}D-NMR spectra of three different kiwifruits and persimmon, focusing on the region of δ 0.02 to 3.00 and different levels of signal intensities, can be observed in the aliphatic region (Figure 2B). Persimmons demonstrated the lowest intensities compared with kiwifruits.

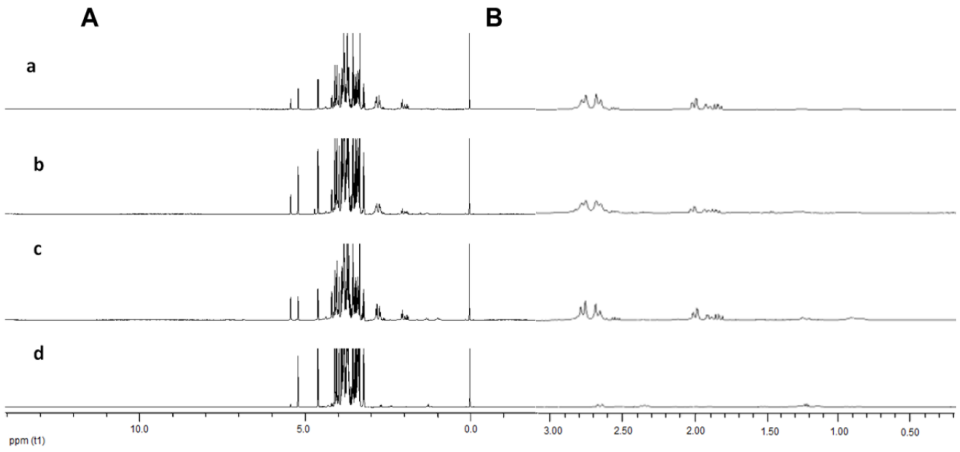


Figure 2. (**A**) The representative ^{1}H-NMR spectra in the δ 3.00 to 5.00 range of kiwifruits (**a**) Hayward, (**b**) Bidan, (**c**) *A. arguta* and (**d**) persimmon Fuyu; (**B**) the representative expanded ^{1}D-NMR spectra in the δ 0.02 to 3.00 range from top to bottom: Hayward, Bidan, *A. arguta* and persimmon Fuyu.

2.2. *Principal Component Analysis (PCA) and Multivariate Data Analysis (MVDA)*

PCA, as an unsupervised classification method, can be performed without prior knowledge of the data set, simplifying the dimensionality of the numerous variables while virtually sustaining the variances. The outcome of the PCA examination comprised of score plots, which signified the variation of the classes based on the metabolomics similarity, and loading plots, which offers information as to which NMR spectral regions were contributing with respect to the grouping attained in the score plots. Three different clusters were formed between the studied fruit samples (Figure 3A). The persimmon can be discriminated from kiwifruit samples by PC1. Meanwhile, BC can be distinguished from the other two kiwifruit varieties by PC2. However, no separation can be observed between KH and AM. The results (Figure 2A) prompted us to proceed with MVDA by excluding the sugar regions from the ^{1}D-NMR spectrum (Figure 3B).

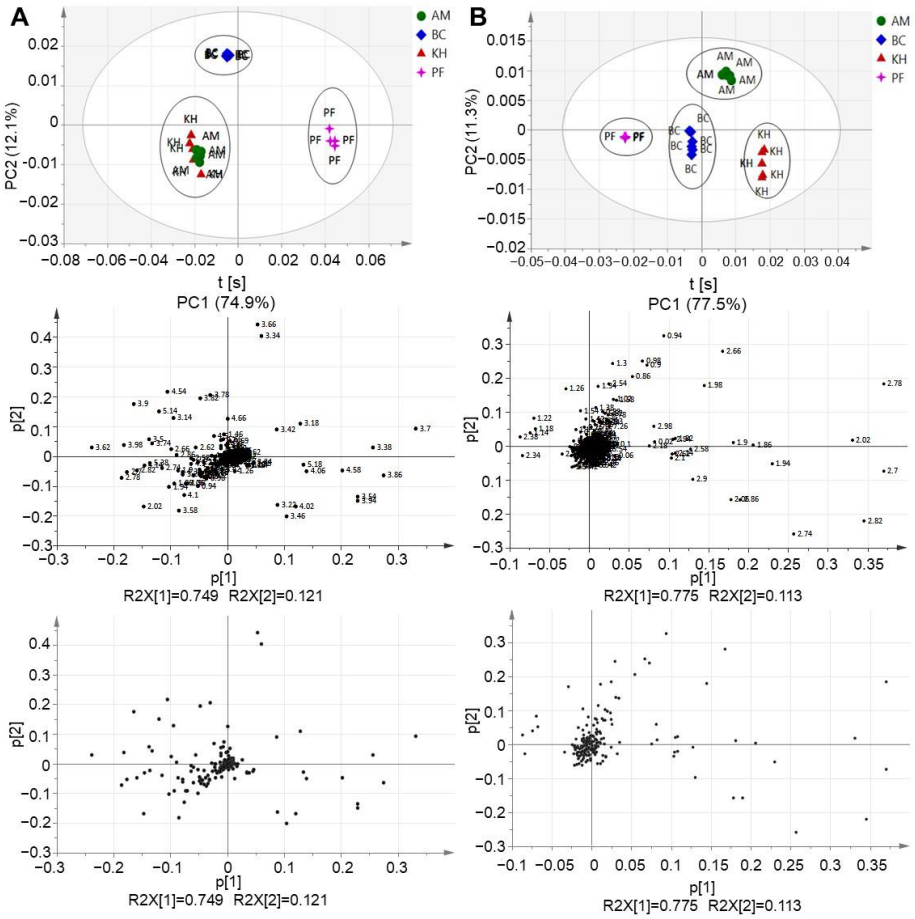


Figure 3. (**A**) Principal Component Analysis (PCA) was performed on the NMR chemical shifts and revealed a significant separation among the samples. PCA score plot of three different kiwifruits: Hayward (KH), Bidan (BC), *A. Arguta* (AM) and persimmon Fuyu (PF). (**B**) PCA score plot after excluding the sugar region of KH, BC, AM and PF.

Therefore, as the next step, the sugar region (δ 3.00–δ 5.50) was removed from the binned excel file, and the unsupervised MVDA analysis was then repeated. This was carried out to observe if there was any clustering that could be formed without the interference of sugars in the samples. It is interesting to note that the removal of the sugar region managed to distinguish the KH from AM. Four distinct clusters can be observed where PF and BC are discriminated from AM and KH by PC1. Meanwhile, AM can be separated from the rest of the samples by PC2. Thus, it can be suggested that KH and AM are discriminated from each other without the masking effects of high sugar components (Figure 3B).

The upper regions of the PCA loading column plot, which corresponded to the persimmon, showed a lower intensity for most of the signals in the aliphatic regions, δ 0.50 to δ 3.00, compared with the lower regions of the model (Figure 4A). This was consistent with the trend demonstrated in the ^{1}D-NMR spectra (Figure 2B). The relationship between the three varieties of kiwifruits and the persimmon was further evaluated in the first level

of our hierarchical model (Figure 4B). PF was discernible from the other kiwifruit samples, based on different metabolite constitutions. Different results appeared after removing the sugar region (Figure 4C,D).

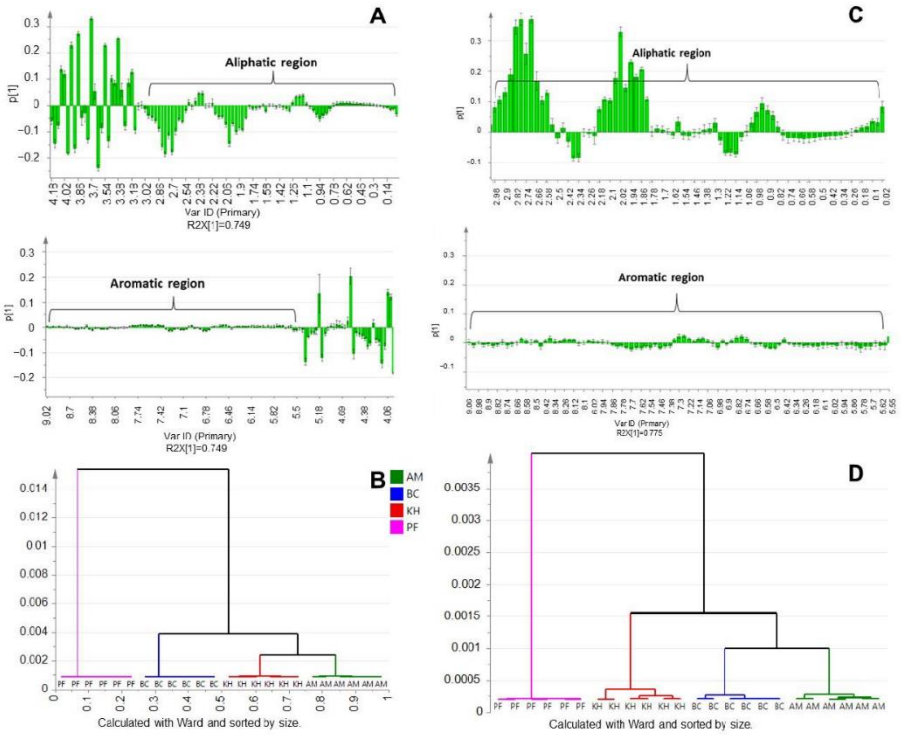


Figure 4. (**A**) PCA loading column plot of three different kiwifruits: Hayward (KH), Bidan (BC), *A. arguta* (AM) and persimmon Fuyu (PF). (**B**) Hierarchical cluster analysis (HCA) for KH, BC, AM and PF, based on group average cluster analysis of the different metabolite components. (**C**) PCA loading column plot for KH, BC, AM and PF after removing sugar regions. (**D**) HCA for KH, BC, AM and PF, based on group average cluster analysis of the different metabolite components after removing sugar regions.

The obtained results can be compared with our recent data [13], where, in the same three varieties of kiwifruit, phenylalanine, tyrosine, arginine, citric acid, glutamine-hydroxy-L-proline, 4-aminobutyrate (GABA), glutamate, glutamine, quinic acid, actinic acid, shikimate, mannose, syringic acid and afzelechin were detected. The present results on kiwifruit metabolites can be compared with other reports, but there aliphatic part was most prominent. Some investigations were also carried out on parts of fresh kiwifruit or on fruit juices obtained with specific food processing procedures [31]. Similar results for the metabolites in persimmons were obtained in our report [32]. The differences in metabolites among five major Japanese persimmon cultivars were investigated using a nuclear magnetic resonance (NMR)-based metabolomics approach. Among the non-astringent cultivars analyzed, the Matsumotowase-Fuyu cultivar contains ethyl-beta-glycosides, as characteristic components, which may relate to fruit softening. The quantitative metabolomics approach based on broadband WET NMR spectra was mostly discussed in the aliphatic region as well [11,12].

2.3. Determination of Bioactive Compounds

The number of bioactive compounds in kiwifruits and persimmon showed the following ranges (Figure 5).

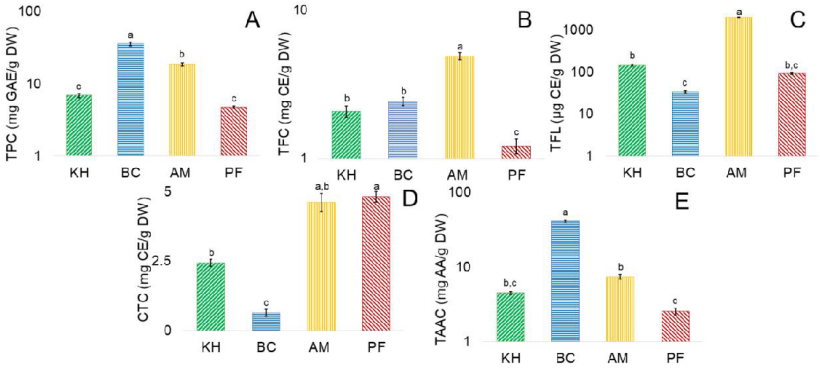


Figure 5. The content of bioactive compounds in investigated fruits. (**A**) Total phenolic content (TPC, mg GAE/g DW); (**B**) total flavonoid content (TFC, mg CE/g DW); (**C**) total flavanols content (TFL, µg CE/g DW); (**D**) condensed tannin content (CTC, mg CE/g DW); (**E**) total ascorbic acid content (TAAC, mg AA/g DW). In figures (**A**–**E**) values are ± SD per gram dry weight (DW); n = 5 samples per cultivar, each subsampled and analyzed five times. Values in bars with different superscript letters are significantly different between the groups of investigated fruit samples in each independent analysis of their quality ($p < 0.05$). Abbreviations: KH, *Actinidia (A.) deliciosa* cv. Hayward; BC, *A. eriantha* cv. Bidan; AM, *A. arguta* cv. Cheongsan; PF, *Diospyros kaki* Thunb. cv. Fuyu; GAE, gallic acid equivalent; CE, catechin equivalent; AA, ascorbic acid; DW, dry weight.

From the presented results, nearly all bioactive compounds were the highest in BC, following by AM, and similar for KH and PF. Total phenolic content (TPC, mg GAE/g DW) from PF to BC was in the range of 4.74 ± 0.18 to 36.61 ± 2.15, respectively (Figure 5A). Total flavonoid content (TFC, mg CE/g DW) showed the lowest value of 1.21 ± 0.14 for PF and the highest for AM of 4.91 ± 0.28 (Figure 5B). Estimation of total flavanols content (TFL, µg CE/g DW) showed the highest for AM of 1969.81 ± 12.41 and the lowest for BC of 33.63 ± 2.43 (Figure 5C). Condensed tannin content (CTC, mg CE/g DW) changed in the investigated samples from 0.64 ± 0.13 for BC and the highest was for PF of 4.81 ± 0.19 (Figure 5D). Total ascorbic acid content (TAAC, mg AA/g DW) was expressed in PF as the lowest of 2.52 ± 0.27 and with the highest of 40.89 ± 1.18 for BC (Figure 5E). These results are in agreement with some recent reports. *A. eriantha* 'Bidan' peeled fruit methanol extracts had a higher TPC of 57.4 in comparison with 12.9 and 6.4 mg GAE/g DW for *A. arguta*, and *A. deliciosa* 'Hayward' extracts, respectively [13]. The TPC of six cultivars was in a range of 4.2–14.5 for ethanol extracts and 5.3–16.3 mg GAE/g for water extracts [33]. TPC of three *A. deliciosa* cultivars ranged from 5.3 to 6.6 mg GAE/g DW. *A. arguta*, *A. macrosperma*, and *A. polygama* had TPC of 8.15, 5.57, and 4.71 mg GAE/g DW, respectively [34]. Extracts (70% aqueous acetone) of *A. deliciosa* 'Hayward' varied in TPC (479 µg/g DW) [35]. Fresh Korean 'Hayward' had a TPC of 8.19 mg GAE/g [36]. TPC and TFC of pulp were 12.21 mg GAE/g DW and 5.92 mg CE/g DW in *Actinidia arguta*. High antioxidant activity was observed (FRAP: 151.41 µmol ferrous sulphate equivalents (FSE)/g DW; DPPH: 12.17 mg TE/g DW). These results emphasize the richness of *A. arguta* fruit pulp to be used in different food products [37]. The TPC of *A. arguta* 'Chiak' and 'Darae No. 2' ranged from 88 to 113 mg GAE/100 g FW. Hayward cultivar, grown in China, showed 78 mg GAE/100 g FW of TPC; the TFC was evaluated as 10.25 mg CE/100 g FW and DPPH and FRAP were 4.87 and 7.08 µmol TE/g FW [38]. Ethanol and water extracts of the TFC ranged as 1.2–4.3 and 0.6–1.8 mg CE/g DW, respectively [33]. The amount

of bioactive compounds varied: total phenolics (mg GAE/g DW) 3.75–8.192–16.52; total flavonoids (mg CE/g DW) 2.11–2.472 and total flavanols (µg CE/g DW) 0.14–0.162 were reported in some recent reports [39,40]. Total flavan-3-ols contents in fourteen kiwifruit cultivars ranged from 96 to 824 µg/g DW [41]. Extraction solvents possibly influenced the solubility of kiwifruit flavonoids [42]. *Actinidia eriantha* [43] is a precious material to study the metabolism and regulation of ascorbic acid (AsA) because of its high content. The other cultivars have relatively high amounts of ascorbic acid, which is shown in Figure 5E and in the published reports [44,45].

2.4. Antioxidant Capacities of Investigated Samples

The antioxidant capacities of investigated samples are shown in Figure 6.

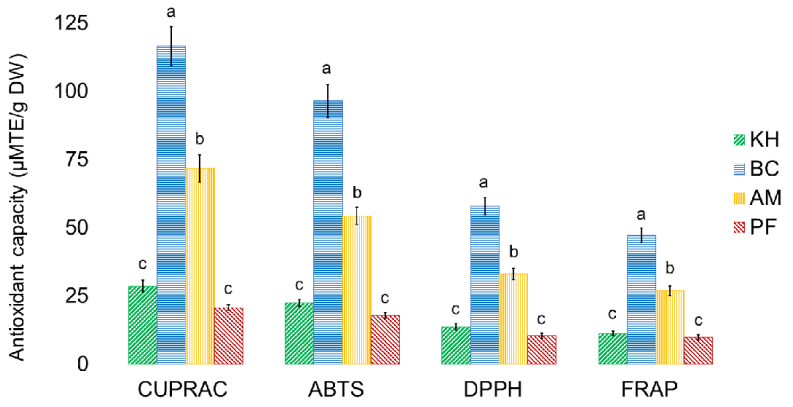


Figure 6. Antioxidant capacities in kiwifruits and persimmons. Values are ± SD per gram of dry weight (DW); $n = 5$ samples per cultivar, each subsampled and analyzed 5 times. Values in bars with different superscript letters are different between the groups of investigated fruit samples in each independent analysis of their quality ($p < 0.05$). Abbreviations: KH, *Actinidia (A.) deliciosa* cv. Hayward; BC, *A. eriantha* cv. Bidan; AM, *A. arguta* cv. Cheongsan; PF, *Diospyros kaki* Thunb. cv. Fuyu; CUPRAC, Cupric reducing antioxidant capacity; ABTS, 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; DPPH, 1, 1-Diphenyl-2-picrylhydrazyl method; FRAP, Ferric-reducing/antioxidant power; TE, Trolox equivalent.

The lowest values of CUPRAC, ABTS, DPPH, FRAP (µM TE/g DW) were 20.72 ± 1.23, 17.96 ± 1.02, 10.45 ± 0.35, 9.86 ± 0.61 for PF, and the highest values were 116.63 ± 7.21, 96.48 ± 6.14, 57.87 ± 3.12, 47.37 ± 2.05 (Figure 6). These results are similar to some reports. 'Hayward' water extracts had higher FRAP, ABTS, CUPRAC and DPPH values than the investigated methanol extracts [33]. *A. eriantha* 'Bidan' extracts had higher DPPH, ABTS, FRAP and CUPRAC values than *A. arguta* and *A. deliciosa* 'Hayward' [13,46]. TPC was the greatest antioxidant contributor in the DPPH and FRAP assays, which is in line with other findings [38], as well as with ABTS and CUPRAC (Figure 6). Diversity in the results of bioactive metabolites depends on the varieties and plant parts, extraction, analytical and processing methods, and this affects the physicochemical and biological properties of kiwifruit-derived ingredients [47,48]. The results obtained in this research can be compared with recent reports. Apart from the treatment used, changes in the content of metabolites are also affected markedly by the persimmon variety. Soluble tannins were 23.8 ± 4.3 and 14.3 ± 1.6; soluble non-tannins were 17.4 ± 2.4 and 15.9 ± 0.5; and total phenolic compounds were estimated as 89.1 ± 5.8 and 78.6 ± 4.5, expressed as g of epicatechin equivalents (EE)/kg DW for Rojo Brillante and Kaki Tipo, respectively. Gallic acid was the predominant phenolic compound found in the Rojo Brillante variety (0.953 mg/100 g), whereas the concentration of *p*-hydroxybenzoic acid was higher in the Triumph variety (0.119 mg/100 g). The antioxidant activity values ranged from 1.280 ± 0.069 to 8.865 ± 0.056 µmol TE/g when

measured by ABTS, 0.458 ± 0.05 to 2.633 ± 0.03 µmol TE/g when measured by DPPH, and 0.206 ± 0.01 to 0.965 ± 0.005 µmol TE/g when the FRAP method was used. The greatest ABTS scavenging capacity was detected in Rojo Brillante (6.572 µmol TE/g), while the lowest was found in the non-astringent variety Triumph (41.484 µmol TE/g). The antioxidant activities of all extracts determined as DPPH radical scavenging ability ranged from 2.633 to 0.458 µmol TE/g. The ferric-reducing antioxidant power of Rojo Brillante and Triumph extracts was similar to the DPPH scavenging activity and ABTS scavenging capacity, with higher values for the astringent variety (0.965 ± 0.005 µmol TE/g). In general, the astringent variety (Rojo Brillante) showed much higher antioxidant activity than the non-astringent variety (Triumph) for both ABTS (6.572 µmol TE/g and 1.484 µmol TE/g, respectively), DPPH (2.417 µmol TE/g and 0.492 µmol TE/G, respectively), and FRAP assay (0.731 µmol TE/g and 0.242 µmol TE/g, respectively). The Rojo Brillante variety also had the highest values of total phenol content, as measured by the Folin method (380.786 µg GAE/g and 81.568 µg GAE/g, respectively). For persimmon samples, the results were similar to those of other authors, which ranged from 1027.03 to 1667.65 µmol/kg, and the concentrations very close to those reported in our study, although very few papers discussed antioxidants in the fruits considered in this study. Our results are similar and consistent with the data from other research reports, which investigated diverse persimmon genotypes [48–51].

2.5. *Quenching Properties of Phenolic Compounds of Investigated Fruits with Human Serum Proteins*

The interactions of fruit extracts with human serum albumin (HSA), fibrinogen (Fgn) and α, β-globulin (α, β-G) are shown in Figures 7–9.

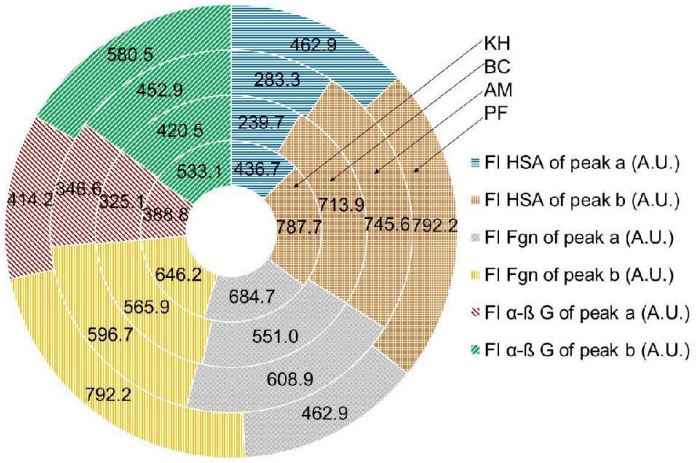


Figure 7. Fluorometric measurements in three-dimensional fluorescence analysis (3D-FL) of kiwifruit and persimmon extracts after interaction with HSA, Fng and α-β-G. Abbreviations: KH, *Actinidia (A.) deliciosa* cv. Hayward; BC, *A. eriantha* cv. Bidan; AM, *A. arguta* cv. Cheongsan; PF, *Diospyros kaki* Thunb. cv. Fuyu; HSA, human serum albumin, α-β G, α-β-globulin; Fgn, fibrinogen, FI, fluorescence intensity, A.U, arbitral units. The values of fluorescence intensity for used human serum proteins before interaction with extracted proteins were the following: FI HSA of peak **a** (A.U.) = 545.9; FI HSA of peak **b** (A.U.) = 814.9; FI Fgn of peak **a** (A.U.) = 877.4; FI Fgn of peak **b** (A.U.) = 809.6; FI α-β G of peak **a** (A.U.) = 457.3; FI α-β G of peak **b** (A.U.) = 661.1. The locations of peaks **a** and **b** are shown in Figures 8 and 9 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

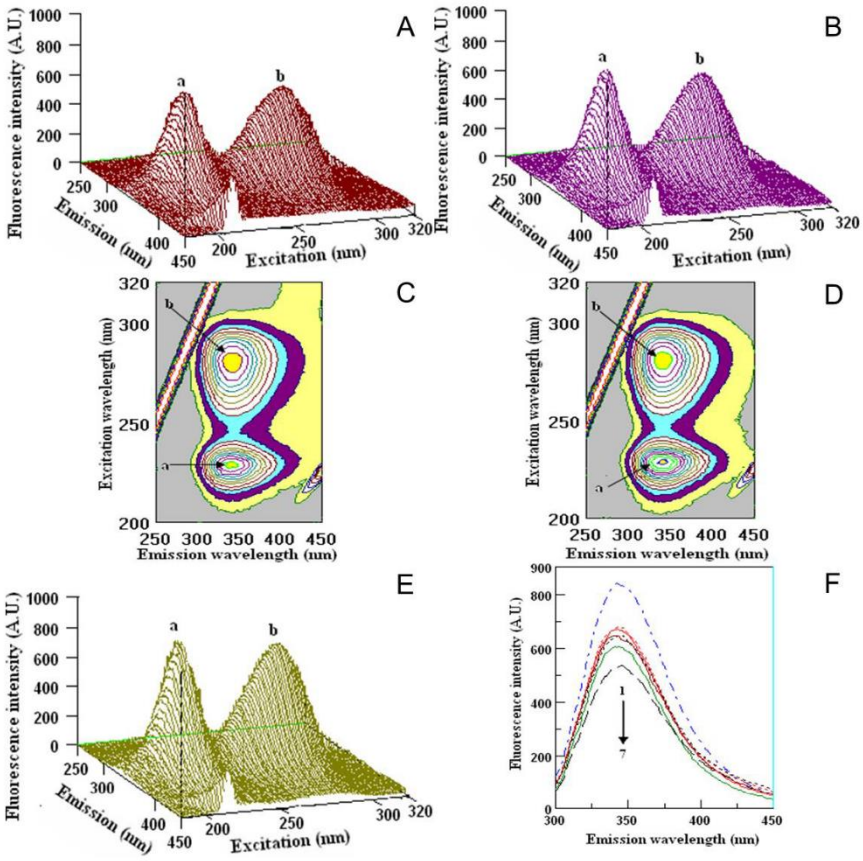


Figure 8. Fluorometric measurements in three-dimensional fluorescence analysis (3D-FL) of kiwifruit and persimmon extracts after interaction with fibrinogen. (**A**,**B**) 3D-FL of KH and PF after interaction with Fng; and (**C**,**D**) their cross images. (**E**) Fibrinogen. (**F**) Spectral data of two-dimensional fluorescence measurements (2D-FL) of fruit extracts and Fng from the top: 1, 2, 3, 4, 5, 6, 7, Fgn (FI = 834.71 A.U.), Fgn + quercetin (FI = 680.42 A.U.), Fgn + caffeic acid (FI = 671.79 A.U.), Fgn + KH (FI = 649.13 A.U.), Fgn + PF (FI = 648.29 A.U.), Fgn + catechin (FI = 606.17 A.U.), Fgn + tannic acid (FI = 538.23 A.U.). Abbreviations: KH, *Actinidia (A.) deliciosa* cv. Hayward; PF, *Diospyros kaki* Thunb. cv. Fuyu; Fgn, fibrinogen, FI, fluorescence intensity, A.U, arbitral units. The locations of peaks **a** and **b** are shown in Figures 7 and 9 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

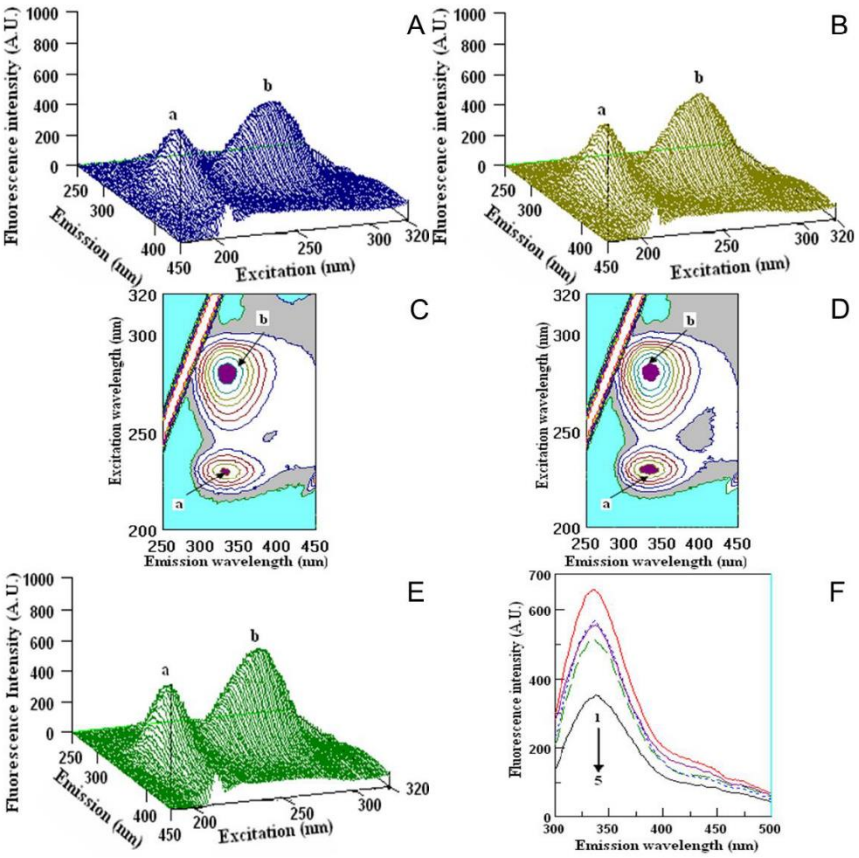


Figure 9. Fluorometric measurements in three-dimensional fluorescence analysis (3D-FL) of kiwifruit and persimmon extracts after interaction with globulin. (**A**,**B**) 3D-FL of KH and PF after interaction with α-β G, and (**C**,**D**) their cross images. (E) α-β G; (**F**) spectral data of two-dimensional fluorescence measurements (2D-FL) of fruit extracts and α-β G from the top: 1, 2, 3, 4, 5, α-β G (FI = 658.57.71 A.U.), α-β G + PF (FI = 567.49 A.U.); α-β G + quercetin (FI = 554.04 A.U.); α-β G + KH (FI = 515.68 A.U.), α-β G + tannic acid (FI = 353.19 A.U.). Abbreviations: KH, *Actinidia (A.) deliciosa* cv. Hayward; PF, *Diospyros kaki* Thunb. cv. Fuyu; α-β G, α-β-globulin; FI, fluorescence intensity; A.U, arbitral units. The locations of peaks **a** and **b** are shown in Figures 7 and 9 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

The interaction with the above serum proteins and extracted fruit polyphenols is evaluated by the changes in the fluorescence intensity of the proteins. The changes appeared mostly in the position and value of peak **a**. The values in the fluorescence intensity (F.I.) of peak **a** in HSA with fruit extracts were the highest for PF (462.9 ± 6.9 A.U.) and the lowest for BC (239.7 ± 5.1). Small changes appeared in the position and value of peak **b**. The fluorescence intensity of HSA with fruit extracts was the highest for PF (792.2 ± 9.3 A.U.) and the lowest for BC (713.9 ± 9.9 A.U.), in comparison with the initial ones (Figure 7). The FI of peak **a** of Fgn after interaction with fruit extracts was the highest for PF (775.2 ± 8.2 A.U.) and the lowest for BC (551.0 ± 6.9). Lower changes in comparison with peak **a** appeared in the position of peak **b**: the highest peak was measured for PF (700.2 ± 7.5 A.U.) and the

lowest one was measured for BC (565.9 ± 5.9 A.U.). The exact locations of peaks **a** and **b** during the interaction of KH and PF are presented in Figures 7 and 8.

The images of the interaction of Fgn with KH and PF (Figure 8A–D) and the Fgn (Figure 8F) showed the maximum peaks **a** and **b** and their locations. The comparison of the values of fluorescence intensity of the native Fgn (Figure 8F, line 1 from the top) showed that the lowest value was obtained by its interaction with tannic acid (Figure 8F, line 7). In 2D-FL, the values of fluorescence intensities with extracts of KH and PF were nearly similar (Figure 8F, lines 4 and 5).

The fluorescence measurements with serum globulin and fruit extracts are presented in Figures 7 and 9. Peak **a** of α-β-globulin (α-β G) after interaction with fruit extracts was the highest for PF (414.2 ± 7.4 A.U.) and the lowest for BC (325.1 ± 3.9). Lower changes appeared in the position of peak **b**: the highest peak was measured for PF (580.5 ± 7.5 A.U.) and the lowest was measured for BC (420.5 ± 3.3 A.U.). The exact locations of peaks **a** and **b** during the interaction with KH and PF are presented in Figures 7 and 9.

The images of the interaction of α-β G with KH and PF (Figure 9A–D) and the α-β G (Figure 9E) showed the maximum of peaks **a** and **b** and their locations. The comparison of the values of fluorescence intensity of the native α-β G (Figure 9F, line 1 from the top) showed that the lowest value was obtained by its interaction with tannic acid (Figure 9F, line 5). In 2D-FL, the changes in fluorescence intensities with extracts of KH and PF were almost similar to Fgn, but showed slightly different values in reaction with globulin, and KH was more reactive than PF (Figure 9F, lines 4 and 2, respectively).

Albumin, fibrinogen, lipoproteins and α, β, and γ globulins play an important role in the pharmacokinetic properties of food nutriments. Globulins make up 35% of plasma proteins and are used in the transport of ions, drugs and lipids [21,22]. It was important to compare the standard metabolites with the polyphenol extracts. The polyphenols-protein interaction is reversible in that the polyphenols–protein complex can dissociate and release free polyphenols. Polyphenols and their metabolites rapidly exchange between free and bound forms within the circulation. Reversible binding to plasma proteins may have consequences for the delivery of the polyphenols and their metabolites to cells and tissues [21,22].

The quenching properties (%) between HSA and polyphenols for PF and BC were in the range of 15.2 ± 0.9 to 56.15.8, calculated by peak **a** and 2.8 ± 0.1 to 12.4 ± 1.1, according to peak **b**, respectively. Different values of quenching properties (%) were calculated with fibrinogen interaction: PF and BC were in the range of 11.6 ± 1.1 to 37.8 ± 2.4, calculated by peak **a**, and 13.5 ± 1.1 to 28.9 ± 1.2, according to peak **b**, respectively, and globulin showed 9.4 ± 0.9 and 37.8 ± 2.4, calculated by peak **a**, and 12.2 ± 1.1 and 36.4 ± 2.4, according to peak **b**, respectively (Figure 10).

The presented data varied between the varieties and the used serum proteins, but showed the same correlation between samples, where BC was the strongest and KH and PF were in the same range of their bioactivities (Figures 7–10).

In the present report, we have used a simplified measure to show only the decrease in fluorescence emission after the addition of a single concentration of ligands. This can be regarded as a relative measure of quenching, providing that the inner filter is similarly negligible within the series of ligands. Thus, the % decrease of fluorescence represents the fraction of the binding sites of the protein occupied by the ligand, rather than the fraction of the total ligand bound to the protein [52].

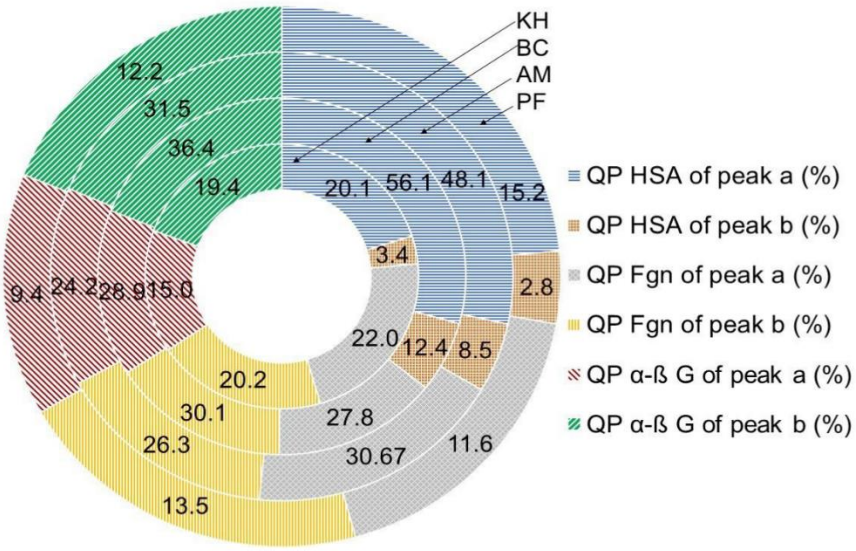


Figure 10. The quenching properties (QP, %) of fruit-extracted polyphenols based on interaction with human serum proteins and fluorometric measurements. Abbreviations: KH, *Actinidia (A.) deliciosa* cv. Hayward; BC, *A. eriantha* cv. Bidan; AM, *A. arguta* cv. Cheongsan; PF, *Diospyros kaki* Thunb. cv. Fuyu; HSA, human serum albumin; α-β G, α-β-globulin; Fgn, fibrinogen. The locations and values of peaks **a** and **b** are shown in Figures 7–9 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

The obtained metabolite results of persimmon samples showed relatively high amounts of tannins in comparison with the investigated three samples of kiwifruits and are in line with several reports [5,6,11,12]. The estimation of quenching of serum proteins such as fibrinogen (Figure 8F, line 8) and globulin with tannic acid (Figure 9, line 5) are in full agreement with the amount of tannic acid in persimmon samples. Based on these results, PF showed nearly the same quenching properties as KH. The presently determined high quenching properties of persimmon in vitro with relatively new metabolite indices such as fibrinogen and globulin, showing protective action and preventing CAD, are in line with some reported in vitro and in vivo studies. Evaluation of the prognostic significance of changes in serum albumin levels among patients that underwent percutaneous coronary intervention (PCI) showed that a decrease in albumin levels following PCI is an independent prognostic marker of worse long-term outcomes [24]. It was found that, similar to traditional risk factors, plasma fibrinogen and albumin levels showed a close relation with the presence and severity of CAD [25]. The fibrinogen-to-albumin ratio index is a valuable biomarker associated with ST- elevation myocardial infarction and may be useful in the prediction of the long-term prognosis of patients with such diseases [26]. So, following the recent reports on humans discussed above and the results of the supplementation of fruits, it was shown that the triglyceride (TG)/cholesterol profile depended on the treatment of persimmon water extracts, and tannin-enriched persimmon concentrate stimulated hypocholesterolemic actions [6,53,54]. Similar action was obtained by a combination of *Diospyros kaki* fruit and *Citrus unshiu* peel mixture as a potential therapeutic agent for treating nonalcoholic fatty liver disease with the remarkable growth of obesity [55]. Variation in tannin amount depends on the cultivars of persimmon, even in co-products from cvs. Rojo Brillante' and Triumph' persimmon juice extraction processed to obtain flours rich in the main metabolites, such as sugars, organic acids, tannins, and bioactive compounds, suggesting their use as a functional ingredient with antioxidant properties in different

food products [56]. Similar results for the quenching properties of kiwifruit polyphenols were obtained in the present study. As such, the health properties of kiwifruit polyphenols results shown in Figures 7–10 are in compliance with previous studies, where the intervention of green kiwifruit effectively lowered the total cholesterol (TC) and increased the high-density lipoprotein cholesterol (HDL) concentration in hypercholesterolaemic and healthy individuals. It was proven that the bioavailability of polyphenols depends on physicochemical stability, complex formation, food interaction, gastrointestinal absorption, and hepatic and gut metabolism [19]. Consumption of fruits influences the total cholesterol, LDL, HDL, proteins, lipid peroxidation and oxidative stress biomarkers [17,18,20]. Consumption of at least one kiwi/week is associated with lower plasma concentrations of fibrinogen and improved plasma lipid profile in the context of a normal diet and regular exercise [28,57,58]. Similar results were reported on antihypercholesterolemia Male Wistar rats when 1% cholesterol-enriched diet induced-hypercholesterolemia improved liver somatic index and lipid profiles after supplementing with 5% lyophilized Polish grown kiwifruit. *A. arguta* 'Geneva', 'Anna', and 'Weiki' showed the most significant results [46,59]. Cellular antioxidant activity (CAA) assays, combined with clinical trials, will more effectively identify antioxidant phytochemicals in fruits that can be used as dietary additives or drugs for human health. In spite of advanced methods in the determination of antioxidant activities, in vitro studies of the interaction of polyphenols with human serum proteins, in vivo experiments, or clinical trials are still required to verify the efficacious activity when fruit polyphenols are used as dietary supplements or drugs to combat oxidative stress [60].

3. Materials and Methods

3.1. Chemicals and Materials

The chemicals 2,4,6,-tripyridyl-s-triazine (TPTZ), 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH), lanthanum(III) chloride heptahydrate, $CuCl_2 \cdot 2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine), 2,2-azino-bis(3-ethylbenzothiazloine-6-sulphonic acid) (ABTS) radical cation, ferric chloride, caffeic acid, quercetin, tannic acid, catechin, human serum albumin (HSA), fibrinogen, globulin, phosphate buffer and Folin-Ciocalteu reagent (FCR) were purchased from Sigma (St. Louis, MO, USA) and Fluka Chemie Gm bH, Buchs, Switzerland. All NMR chemicals, including 3-trimethylsilylpropanoic acid (TSP), potassium phosphate monobasic (KH_2PO_4), methanol-d4 (CD_3OD, 99.8%), sodium deuterium oxide (NaOD), and deuterium oxide (D_2O, 99.9%), were purchased from Merck (Darmstadt, Germany).

3.2. Sampling and NMR Metabolomics

Three batches of organic kiwifruits, including *Actinidia (A.) deliciosa* cv. Hayward (KH), *A. eriantha* cv. Bidan (BC), *A. arguta* Cheongsan (AM) and one batch of *Diospyros kaki* Thunb. cv. Fuyu (PF), were collected in different commercial orchards from Boseong and Muan counties, Jeonnam and Wonju-si, Gangwon-do provinces, South Korea [61]. Each batch was composed of 25 fruits, about two kg in weight. The cultivars reached the commercial maturity stage. The samples were washed with tap water and dried. The fruits were fractionated into an edible fraction (pulps), peels and seeds. Only for PF, 5–8 seeds were separated from pulps. Their edible parts were prepared manually without using steel knives. The peeled fruits (pulps) were weighed, chopped and homogenized in liquid nitrogen in a high-speed blender (Silex professional model, Hamilton Beach, Virginia, USA). A weighed portion (50–100 g) was then lyophilized for 48 h (Virtis model 10–324, Midland, ON, Canada), and the dry weight was determined. The samples were ground to pass through a 60-mesh sieve and stored at −20 °C until the bioactive substances were analyzed.

The proton (^1H) and two-dimensional (2D) J-resolved NMR procedure was carried out according to the previously reported protocols with small modifications. The extraction of samples was carried out by transferring 100 mg of each sample into a 2 mL Eppendorf tube, followed by the addition of 375 µL of both CD_3OD solvent and KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% TSP. The solution was then vortexed for 1 min before being

subjected to sonication for 15 min at a controlled temperature. To get a clear supernatant, the mixture was afterward centrifuged at rpm for 10 min, and 600 µL of it was pipetted to a NMR tube prior to analysis. The ^{1}H-NMR analysis was performed at 25 °C on an INOVA 500 MHz spectrometer (Varian Inc., Palo Alto, CA, USA). For each sample, the required time was 3.53 min, recording 64 scans with an acquisition time, a pulse width, and a relaxation delay of 220 s, 3.75 ms, and 1.0 s, respectively. These settings were for presaturation prior to ^{1}H-NMR, which is required to suppress the water signal using low power selective irradiation. In addition, the spectral width of the recorded spectra was 20 ppm. The processing for all spectra, including phasing and baseline corrections, was performed manually with Chenomx software (Version 6.2, Edmonton, AB, Canada). Moreover, the 2D J-resolved was conducted to endorse metabolite identification [13,32,62].

3.3. Determination of Bioactive Compounds

The detailed procedures of the extraction, determination of bioactive compounds and their antioxidant capacities were described in our very recent reports [52,63,64]. For polyphenol extraction, the freeze-dried powders of investigated samples were immersed in absolute methanol (1/10 *w*/*v*). The filtrate was collected three times with constant stirring of the mixture at every 24 h interval of a 72 h total collection period at room temperature. The extract was then concentrated under reduced pressure at 45 °C using a vacuum rotary evaporator.

A Folin–Ciocalteu assay was used for the determination of total polyphenol content (TPC) in methanol fruit extracts of 0.25 mL with 1 mL of Folin–Ciocalteu reagent (Sigma, St. Louis, MO, USA). Then, 0.75 mL of 1% sodium carbonate was added. Absorbance of the mixture was measured on a Hewlett-Packard model 8452A spectrophotometer (Hewlett-Packard, Rockville, MD, USA) at 750 nm. The results were calculated in mg gallic acid equivalents (GAE) per g DW [65]. Total flavonoid content (TFC, mg catechin equivalents (CE) per g DW) was measured at 510 nm after extraction with 5% $NaNO_2$, 10% $AlCl_3xH_2O$ and 1 M of NaOH [66]. The absorbance of total flavanols (TFL, µg CE per g DW) was measured at 640 nm following the *p*-dimethylaminocinnamaldehyde (DMACA) method: 1 mL of DMACA solution was added to 0.2 mL of fruit extracts [67]. Condensed tannin content (CTC, mg CE per g DW) was estimated by spectrophotometric measurements in the mixture of methanol fruit extracts and the addition of 4% methanol vanillin solution. Absorbance was measured at 500 nm after the end of the reaction [68]. Total ascorbic acid content (TAAC, mg ascorbic acid (AA) per g DW) was evaluated in water fruit extracts, where 100 mg of the freeze-dried sample was extracted with 5 mL water. Then, CUPRAC method was conducted, and formed bis (Nc)-copper (I) chelate was determined spectrophotometrically at 450 nm [69].

3.4. Determination of Antioxidant Capacities

Total antioxidant capacity was determined by the following assays, which are also described in our recent reports [52,63,64].

Cupric reducing antioxidant (CUPRAC) assay is based on utilizing the copper (II)−neocuproine reagent as the chromogenic oxidizing agent. Absorbance at 450 nm was measured in a mixture of [Cu (II)-Nc] and NH_4Ac buffer solution and fruit methanol extracts [70].

2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS•+) was generated by the interaction of ABTS (7 mM) and $K_2S_2O_8$ (2.45 mM). This solution was diluted with methanol and measured at 734 nm [71]. Scavenging free radical potentials were tested in a methanolic solution (3.9 mL) of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) with the sample extracts in methanol (0.1 mL) [72]. Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) [73]. All values of antioxidant capacities were expressed in µM trolox equivalent (TE)/g DW.

3.5. Fluorometric Studies

The profiles and properties of polyphenols in methanol extracts were determined by two (2D-FL) and three-dimensional (3D-FL) fluorescence (model FP-6500, Jasco spectrofluorometer, serialN261332, Tokyo, Japan). The 2D-FL measurements were taken at emission wavelengths from 310 to 500 nm and at excitation of 295 nm. The 3D-FL was measured at emission wavelengths between 200 and 795 nm and the initial excitation wavelength at 200 nm. For comparison of the obtained results, caffeic acid, quercetin, tannic acid and catechin were used [36]. The quenching properties of phenolic compounds in kiwifruit and persimmon extracts to human serum albumin (HSA), fibrinogen and globulin were evaluated by 2D and 3D-FL. For the fluorescence measurements, 3.0 mL of 1.0×10^{-5} mol/L HSA were prepared in 0.05 mol/L Tris–HCl buffer (pH 7.4), containing 0.1 mol/L NaCl. Fibrinogen and globulin stock solutions were made by dissolving in phosphate buffer (10 mM, pH 7.4) to obtain a concentration of 20 µM. Standards phenolic solutions, such as tannic acid, quercetin, catechin, and caffeic acid stock solution, were prepared daily by dissolving at a concentration of 10 mM in methanol and then diluting with 10 mM phosphate buffer at pH 7.4. Samples were prepared by mixing fibrinogen, fruit extracts and standards of phenolic compound solutions in varying proportions. The highest resulting methanol concentration was about 1%, which had no appreciable effect on protein structure. All samples were kept at 4 °C prior to the analysis. The initial fluorescence intensities of HSA, globulin and Fgn were measured before interaction with the investigated samples and pure substances and after interaction with the samples (quenching of the fluorescence emission of proteins, in our case of HSA, globulin and fibrinogen and polyphenols of fruits). As mentioned above, changes in the fluorescence intensities were used in the estimation of quenching activities. [52,63,64].

3.6. Data Analysis

NMR data analysis followed the reported procedure [74]. The conversion of ^{1}H-NMR spectra to an ASCII file using Chenomx software was carried out prior to multivariate data analysis (MVDA) and performed using SIMCA-P+ version 13.0 (Umetrics AB, Umeå, Sweden). This analysis consists of the exclusion of the residual water (4.70–4.90 ppm) and methanol (3.23–3.34 ppm) signals range. Next, all spectra were scaled to TSP and bucketed to bins with a width of 0.04 ppm, forming a spectral region of 0.52–9.99 ppm. The binned integral of ^{1}H-NMR data were then subjected to principal component analysis (PCA), which was applied to clearly differentiate the ^{1}H-NMR spectra of the kiwifruit and persimmon samples. The Pareto method was also used for scaling purposes to ensure the same importance was given to all x variables in the analyses. All obtained data were calculated on the basis of statistical analysis of Duncan's multiple range test. Values are means ± SD per gram of dry weight (DW) of 25 measurements, representing the commercial maturity status of fruits and their replicates. Five biological replications of five extracts from each cultivar were performed. To determine the statistical significance as a 95% interval of reliability, one-way analysis of variance (ANOVA), was used.

4. Conclusions

We obtained relatively high amounts of antioxidants in the raw pulp of investigated fruits and high quenching properties of fruit extracts in comparison with pure metabolites. The addition of such fruits to generally accepted diets could be beneficial for hyperlipidemic, especially hypertriglyceridemic, patients suffering from coronary atherosclerosis. We expect that HSA, Fgn and α-β G will serve as predictors of cardiovascular events.

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Sample Availability: Samples of the compounds are not available from the authors.

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6.9 Properties of Different Varieties of Durian

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Properties of Different Varieties of Durian

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Abstract: Durian (*Durio zibethinus* Murr.), like many other exotic, tropical, and conventional fruits, is important in the prevention of different diseases. In this study, the characterization of the main bioactive compounds of the most popular cultivars of durian and their properties are described. The changes in the quality indices of the antioxidant status were determined by CUPRAC, ABTS, FRAP, DPPH, and ORAC assays. The profiling of phytochemicals was carried out by Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). For the first time, in vitro studies were performed by the interaction of extracted durian polyphenols with human serum proteins (HSP) such as human serum albumin (HSAlb), fibrinogen (HSFib) and globulin (HSGlo) as novel biomarkers of coronary artery disease (CAD). The fluorescence measurements of the resulting intensity and calculated binding properties of the interaction of polyphenols with proteins showed that the most reactive was Monthong durian cultivar. This study suggests that durian cultivars have relatively strong antioxidant, binding, and health potentials and could be a significant source of natural antioxidants used in daily fresh consumption and for functional foods.

Keywords: durian; polyphenols; serum; albumin; globulin; fibrinogen; binding properties; health biomarkers

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1. Introduction

Fruits are one of the important parts of human consumption. All fruits have a rich composition as natural antioxidants. Durian has been recognized as an important fruit, especially for the underlying nutritional attributes of the fruit [1]. Durian belongs to the category of exotic fruits, having specific taste as a mixture of mango and avocado [2]. Durian is also rich in polyphenols such as flavonoids (flavanones, flavonols, flavones, flavanols, anthocyanins), phenolic acids (cinnamic and hydroxybenzoic acids), tannins, and other bioactive components, such as carotenoids and ascorbic acid [3–7]. Durian fruit possessed an acute effect on the blood pressure of hypertensive rats, but heart rate was unaffected [8]. The present studies showed that polyphenols decrease the risk of chronic diseases, such as heart diseases, diabetes, and others [9,10]. Potential health benefits with special regards to cholesterol-lowering effects were described in durian and its products [11]. The ripe and overripe fruits increased the expression of hepatic HK2 and PFKFB4 glycolytic genes and stimulated glucose utilization in HepG2 cells [12]. Although

in our previous studies different durian cultivars were used [13], mostly the research was concentrated on the main Monthong cultivar [14]. Metabolic variations in the pulps of two durian cultivars (Thai Chanee and Monthong) identified cultivar-dependent metabolite markers [15], related to durian fruit quality traits, such as nutritional value (pyridoxamine), odor (cysteine, leucine), and ripening process (aminocyclopropane carboxylic acid). As discussed above, durian in vitro studies by antioxidant assays [3], in vivo on the animal model [9,10] and cells experiments [12,14] contained relatively high amounts of potential antioxidants, improved the lipid and serum antioxidant status in diets high in cholesterol and possessed antiproliferative activities and proapoptotic potential in relation to the total content of bioactive compounds. Our findings for the first time indicated that one of the positive benefits of fruit consumption in patients with coronary artery disease (CAD) was diminishing the production of plasma circulation fibrinogen and its stability, which reduced the potential risk exerted by this protein [16], decreasing the triglycerides, total and low-density cholesterol. Phenolic compounds in general, under non-oxidative conditions, form reversible complexes with plasma proteins, involving hydrogen bonds, electrostatic interactions, hydrophobic effects and van der Waals forces. The distribution of drugs and dietary phenolic compounds in the human metabolism depends also on binding to plasma proteins, because of their biological activity, forming phenol-human serum protein complexes [16–18]. Despite the significant progress in the investigation of this special fruit, there is a lack of the comparison of durian cultivars, their properties, especially in relation to Monthong, which is the most popular and commonly consumed. The research area of the bioactivity of extracted polyphenols with the main human serum proteins such as albumin, globulin, and fibrinogen, which are the biomarkers of health properties, has not been exhausted yet. In this research, local Thai durian cultivars will be compared on the basis of their physicochemical and antioxidant characterization. The binding properties of the main serum proteins with the extracted polyphenols will be determined by fluorescence studies and antioxidant assays. In addition, the Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) measurements will be employed in order to characterize the bioactivity of durian cultivars. Therefore, the objectives of this report were the following: determination of physicochemical properties of local Thai durian cultivars as an additional index and fingerprint of the ripening stage; quantitative antioxidant status by phenolic compounds and their antioxidant capacities; qualitative estimation of antioxidant profiles by FTIR and DSC spectra; fluorometric and binding properties of extracted fruit polyphenols in interaction with main serum human proteins as an indicator of health properties of durian; correlation between the activity of new biological markers (albumin, globulin and fibrinogen) in interaction with standards and fruit extracted polyphenols.

2. Materials and Methods

2.1. Chemicals and Materials

The chemicals 2,4,6,-tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH), lanthanum(III) chloride heptahydrate, $CuCl_2x2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine), 2,2-azino-bis (3-ethylbenzothiazloine-6-sulphonic acid) (ABTS) radical cation, ferric chloride, caffeic acid, quercetin, tannic acid, catechin, human serum albumin (HSAlb), fibrinogen (HSFib), globulin (HSGlob), phosphate buffer and Folin-Ciocalteu reagent (FCR) were purchased from were from Sigma (St. Louis, MO, USA) and Fluka Chemie GmbH, Buchs, Switzerland. 2,2′-azobis (2-methylpropanimidamide dihydrochloride) (AAPH) and fluorescein were from Merck Eurolab GmbH (Darmstadt, Germany).

2.2. Sampling

Durian fruits (*Durio zibethinus* Murr.) of three cultivars Monthong, Chanee and Puangmanee were obtained from the orchard, in Chanthaburi province, eastern Thailand in 2018. All samples were in the ripe stage, according to the definition of ripe durian flesh (DR): harvested and left to soften (the stage when the fruit is ready for consuming)

and it normally takes 3–5 days. Five fruits were used for each cultivar. The peeled fruits (pulps) were weighed, chopped and homogenized in liquid nitrogen in a high-speed blender (Silex professional model, Hamilton Beach, VI, USA). A weighed portion (50–100 g) was then lyophilized for 48 h (Virtis model 10–324, Midland, ON, Canada), and the dry weight was determined. The samples were ground to pass through a 60-mesh sieve and stored at −20 °C until the bioactive substances were analyzed [12]. Then, 1 g of each lyophilized sample was extracted with 10 mL of methanol (3 replications). The methanol was evaporated, and the extracts were kept in an aluminum bag at −20 °C before analysis. For a comparison of the maturity levels, local durian cultivars Monthong, Chanee, and Kanyao were obtained from Chanthaburi province in June 2019, and the physicochemical properties assessed.

2.3. Determination of Physicochemical Properties

The immature (young) durians were kept at room temperature until the overripe stage. The maturity of durian was characterized using the following criteria: Immature (young) durian was harvested before mature level 3–5 days, but at this level durian is not consumed, having a hard texture and without any smell. At the mature level durian can be consumed with a firm texture and desirable smell. When mature durian is left for 1–2 days, then it becomes ripe. At this level, durian has a soft texture and a desirable smell. The levels between ripe and overripe (Ripe+) durians are achieved when the ripe durian is left for 1–2 days. This stage of durian had a softer texture and stronger smell than ripe level. In some cases, ripe durian is left for 3–5 days. Durian at this time has a sloppy, mushy texture and odoriferous. This is a standard procedure for durian ripening, taking about 3–5 days from stage to stage, but the duration of the fruit is variable depending on the cultivar and the size of the fruit.

According to Table 1, the first day of each cultivar from the orchard was in the young stage at 0 day. The mature stage began from Kanyao to Monthong (3–5 days). The ripe stage started from Kanyao to Monthong (5–7 days). The ripe + 1 stage began from Kanyao to Monthong (7–9 days). The overripe stage was found in Kanyao (9 days) and in two other investigated cultivars Chanee and Monthong at 10 days (10 days).

Table 1. The duration (days) of durian ripening at different maturity levels and cultivars.

Cultivars	Maturity Levels (Day)				
	Young	Mature	Ripe	Ripe + 1	Overripe
Monthong	0	5	7	9	10
Chanee	0	4	6	8	10
Kanyao	0	3	5	7	9

The pH, acidity, °Brix and total soluble solids of durian in the ripe stage of three cultivars (Monthong, Chanee and Puangmanee) were determined. The pH, acidity, °Brix, and total soluble solids of durian in different maturity levels of three slightly different cultivars (Monthong, Chanee and Kanyao) were determined as well in order to recheck the ripe stage of durian harvested in 2018. The pH of the durian was determined by the following method: minced durian pulp (10 g) was mixed with 90 mL of distilled water. The mixture was filtered, and the pH was measured using a digital pH meter [19]. The acidity was measured by titrating the samples of durian with 0.1 N NaOH solution, according to AOAC Method 942.15 [20]. Ten grams of sample were diluted with 75 mL of distilled water. Then, the mixture was homogenized and 2–3 drops of phenolphthalein were added. The mixture was titrated with 0.1 N NaOH until the color of the solution turned pink and remained stable for 30 s. The value of titratable acidity was expressed as g malic acid/100 g wet weight, using the following formula: % acid = [NaOH (mL) × molarity of NaOH × (0.067) × 100]/sample (g), where a milliequivalent factor of 0.067 was used, with the assumption that malic acid is the predominant acid [21]. Total soluble solids (°Brix) of

fresh durian pulps were determined by the method of Tan et al. [21]. Minced durian pulp (10.0 g) was mixed with 20 mL of distilled water and filtered. The total soluble solids were measured in the mixture, using a digital refractometer. The total soluble solids (%) value was obtained by multiplying °Brix values by 3.

2.4. Determination of Bioactive Compounds

The detailed procedures of bioactive compounds and their antioxidant capacities determinations were described in our very recent reports [22,23]. Folin–Ciocalteu assay was used for the determination of total polyphenol content (TPC) in methanol durian extracts of 0.25 mL with 1 mL of Folin–Ciocalteu reagent. Then, 0.75 mL of 1% sodium carbonate was added. The absorbance of the mixture was measured on Hewlett-Packard, model 8452A spectrophotometer at 750 nm. The results were calculated in mg gallic acid equivalents (GAE) per g of dry weight (DW) [24]. Total flavonoid contents (TFC) were measured at 510 nm after extraction of durian samples with 5% $NaNO_2$, 10% $AlCl_3xH_2O$ and 1 M NaOH and expressed as mg catechin equivalent (CE) per g DW [25].

2.5. Determination of Antioxidant Capacities

Cupric reducing antioxidant (CUPRAC) assay is based on utilizing the copper (II) − neocuproine reagent as the chromogenic oxidizing agent. The absorbance at 450 nm was measured in a mixture of [Cu(II)-Nc] and NH_4Ac buffer solution and fruit methanol extracts [26]. 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS•+) was generated by the interaction of ABTS (7 mM) and $K_2S_2O_8$ (2.45 mM). This solution was diluted with methanol and measured at 734 nm [27]. Scavenging free radical potentials were tested in a methanolic solution (3.9 mL) of 1,1-diphenyl-2-picrylhydrazyl (DPPH) with the samples extracts in methanol (0.1 mL) [28]. Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) [29]. Oxygen radical absorbance capacity (ORAC) assay was carried out using 50 g of the pulp extracted with water and dimethyl sulfoxide (DMSO). The solutions were combined and subjected to ORAC assay [30] with minor modifications [31] on a fluorescent plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA). The results of all antioxidant assays are expressed as micromoles of Trolox equivalent (TE) per g DW. Radical scavenging activities using DPPH and ABTS were also determined and the results are expressed as inhibition percentage: % radical scavenging activity) (control OD − sample OD/control OD) × 100, where OD is optical density. Changes in the absorbance of the samples were measured at 517 nm for DPPH and for ABTS at 734 nm [32,33].

2.6. Fourier Transform Infrared Spectrometry (FTIR) and Differential Scanning Calorimetry (DSC) Measurements

Spectra profiles of durian extracts were determined by Fourier transform infrared spectrometer (Thermo Scientific, model Nicolet 6700, Dreieich, Germany). The durian extracts were applied to a diamond cell and measured from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1} and 100 scans [34,35]. The 5 mg of durian extracts were weighted in a sealed type aluminum crucible. Then, the samples were analyzed DSC (NETZSCH, model DSC 204 F1, Selb, Germany), using a modification of the procedure. The temperature program started at 0 to 300 °C at a heating rate of 10 °C/min [34].

2.7. Fluorometric Studies

Profiles and properties of polyphenols in methanol extracts were determined by two (2D-FL) and three-dimensional (3D-FL) fluorescence (model FP-6500, Jasco spectrofluorometer, serial N261332, Tokyo, Japan). The 2D-FL measurements were taken at emission wavelengths from 310 to 500 nm and at excitation of 295 nm. The 3D-FL was measured at the emission wavelengths between 200 and 795 nm, and the initial excitation wavelength at 200 nm. For comparison of the obtained results caffeic acid, quercetin, tannic acid and catechin were used [22,23]. Binding properties of durian extracts to human serum albumin

(HSAlb), fibrinogen and globulin were evaluated by 2D and 3D-FL. For the fluorescence measurements, 3.0 mL of 1.0 × 10−5 mol/L HSA were prepared in 0.05 mol/L Tris–HCl buffer (pH 7.4), containing 0.1 mol/L NaCl. Fibrinogen and globulin stock solution was made by dissolving in phosphate buffer (10 mM, pH 7.4) to obtain a concentration of 20 µM. Standards phenolic solutions such as tannic acid, quercetin, catechin, caffeic acid stock solution was prepared daily by dissolving at a concentration of 10 mM in methanol and then diluting with 10 mM phosphate buffer at pH 7.4. Samples were prepared by mixing albumin, globulin fibrinogen, durian extracts, and standards of phenolic compounds solutions in varying proportions. The highest resulting methanol concentration was about 1%, which had no appreciable effect on protein structure. All samples were kept at 4 °C before the analysis. The initial fluorescence intensities of albumin, globulin, and fibrinogen were measured before the interaction with the investigated samples and pure substances and after interaction with the samples (quenching of fluorescence emission of proteins in our case of albumin, globulin and fibrinogen and polyphenols of durian). As it was mentioned above, the changes in the fluorescence intensities were used in the estimation of binding activities [22,23,36].

2.8. Data Analysis

All obtained data were calculated on the basis of statistical analysis of Duncan's multiple range test. Values are means ± SD per gram dry weight (DW) of 25 measurements, representing the commercial maturity status of fruits and their replicates. Five replications of five extracts from each cultivar were performed. To determine the statistical significance as 95% interval of reliability, ANOVA, one-way analysis of variance, was used.

3. Results and Discussion

3.1. Physicochemical Properties

Four local Thai durian cultivars were investigated in the present study. Durian fruits (*Durio zibethinus* Murr.) of three cultivars Monthong, Chanee, and Puangmanee (Figure 1) were obtained in the ripe stage, according to the definition of ripe durian flesh (DR): harvested and left to soften for several days (Table 1). In these fruits, which were accepted as ripe ones, the amount of total soluble solids (TSS, %) showed the following values: 25.21 ± 0.43; 25.17 ± 0.41; 25.48 ± 0.39; pH was in the order of 6.81 ± 0.01, 6.68 ± 0.04, 6.79 ± 0.02; acidity (% of malic acid): 0.17 ± 0.01, 0.17 ± 0.01 and 0.15 ± 0.01.

In order to be sure that all investigated methods were applied to the samples in the correct stage of ripening, in the next 2019 harvest year, three similar local cultivars (Monthong, Chanee and Kanyao) were collected (Figure 1) during different stages of ripening, and the results are presented in Table 1. As it was mentioned in Materials and Methods, the samples were collected in different stages of ripening: young, mature, ripe, ripe + 1 and overripe. All the analyses were performed on durian harvested in 2018, except for the physicochemical properties. Most of the results (Figures 2–8) were conducted on durian harvested in 2018 and only at the ripe stage (the stage when the fruit is ready for consumption). However, there was no information about the physicochemical properties of durian in different maturity levels which could affect the antioxidant and binding properties and bioactive compounds. Therefore, the physicochemical properties of durian in different maturity levels and cultivars (Table 2) were assessed with the durian harvested in 2019, to confirm the physicochemical properties of the ripe stage with other maturity levels and cultivars. Moreover, this information provides general information about the physicochemical properties of durian in different maturity levels and cultivars for the reader. The physicochemical properties in this study could be used for the maturity levels determination as a fingerprint of local cultivars, because normally and practically the levels of maturity are always done by experts, who are able to characterize the differences of appearance, smell, texture, and maturity levels of durian by their experiences. The pH, acidity, °Brix, and total soluble solids of three slightly different cultivars compared to the harvest of 2018 were determined. Accordingly, the Puangmanee cultivar was unavailable

in the local orchard in 2019, so the Kanyao cultivar was provided instead, which is one of the most well-known cultivars obtained from the orchard in Chanthaburi province.

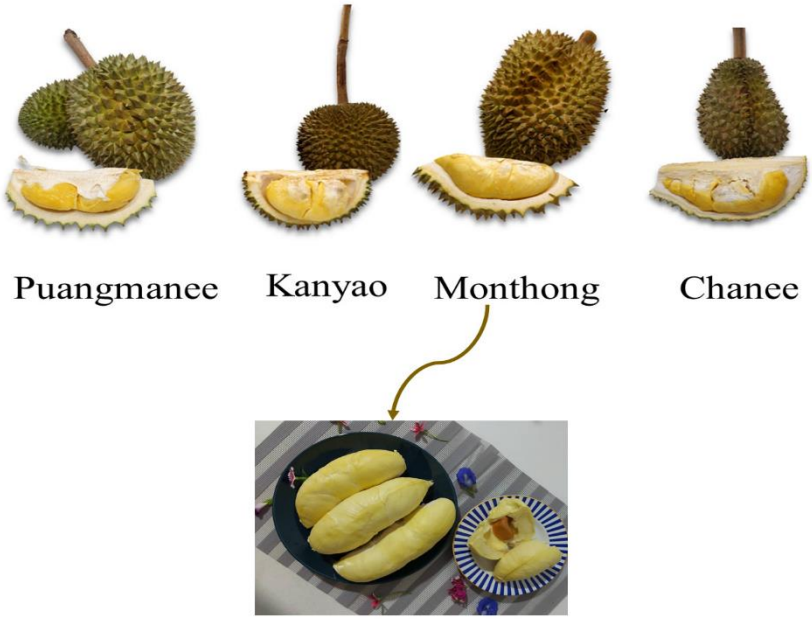

Figure 1. Local cultivars of Thai durian: Monthong (MT), Chanee (CN), Puangmanee (PM) and Kanyao (KN).

Table 2. The pH, acidity, °Brix and total soluble solid of durian in different maturity levels and cultivars.

Cultivars	Maturity Levels	pH	Acidity (%)	(°Brix)	Total Soluble Solids (%)
Monthong	Young	7.08 ± 0.02^{a}	0.07 ± 0.00^{h}	2.93 ± 0.15^{f}	8.80 ± 0.46^{f}
	Mature	6.74 ± 0.03^{d}	0.15 ± 0.01^{e}	6.77 ± 0.15^{d}	20.30 ± 0.46^{d}
	Ripe	6.76 ± 0.01^{d}	$0.18 \pm 0.01^{c,d}$	8.33 ± 0.15^{c}	25.00 ± 0.46^{c}
	Ripe + 1	6.68 ± 0.02^{e}	$0.20 \pm 0.00^{a,b}$	8.53 ± 0.15^{c}	25.60 ± 0.46^{c}
	Overripe	6.62 ± 0.02^{f}	0.21 ± 0.01a	9.53 ± 0.25^{b}	28.60 ± 0.75^{b}
Chanee	Young	6.83 ± 0.02^{b}	0.13 ± 0.01^{f}	5.30 ± 0.10^{e}	15.90 ± 0.30^{e}
	Mature	6.67 ± 0.05^{e}	$0.17 \pm 0.01^{d,e}$	6.80 ± 0.10^{d}	20.40 ± 0.30^{d}
	Ripe	$6.61 \pm 0.04^{f,g}$	$0.18 \pm 0.01^{b,c,d}$	8.37 ± 0.15^{c}	25.10 ± 0.46^{c}
	Ripe + 1	6.57 ± 0.03^{g}	$0.18 \pm 0.01^{b,c}$	8.63 ± 0.15^{c}	25.90 ± 0.46^{c}
	Overripe	6.45 ± 0.01^{h}	$0.19 \pm 0.00^{a,b,c}$	9.37 ± 0.12^{b}	28.10 ± 0.35^{b}
Kanyao	Young	$6.82 \pm 0.02^{b,c}$	0.11 ± 0.01^{g}	5.13 ± 0.12^{e}	15.40 ± 0.35^{e}
	Mature	$6.77 \pm 0.01^{c,d}$	0.13 ± 0.01^{f}	6.93 ± 0.25^{d}	20.80 ± 0.75^{d}
	Ripe	6.74 ± 0.02^{d}	0.14 ± 0.01^{f}	8.47 ± 0.12^{c}	25.40 ± 0.35^{c}
	Ripe + 1	6.83 ± 0.03^{b}	$0.18 \pm 0.01^{b,c,d}$	9.63 ± 0.23^{b}	28.90 ± 0.69^{b}
	Overripe	6.84 ± 0.01^{b}	$0.18 \pm 0.01^{b,c,d}$	10.63 ± 0.25^{a}	31.90 ± 0.75^{a}

Values are means ± SD; Different letters in the same column represent significant differences ($p < 0.05$).

From young to overripe stages (Table 2), the pH for Monthong decreased (7.08–6.62), Chanee (6.83–6.45) and Kanyao (6.82–6.84); acidity (%) increased for Monthong (0.07–0.21), Chanee (0.13–0.19) and Kanyao (0.11–0.18); °Brix increased for Monthong (2.93 to 9.53), Chanee (5.30–9.37), and Kanyao (5.13—10.63); TSS (%) increased for Monthong (8.80 to 28.60), Chanee (15.90–28.10), and Kanyao (15.40—31.90). The results of physicochemical

properties are in line with the investigations on varieties of fruits, but some show different dynamics. Biochemical changes in wood apple fruit (*Feronia elephantum Corr.*) were studied at three different stages (unripe, semi-ripe and ripe). Like most of the ripening fruits, the major observed changes were in a decrease in acidity from 3.5 to 3.2 g/100g. Unlike various other fruits, the total soluble solids were reduced upon ripening from 20.4 to 14.0 °Brix. Unlike most of the fruits, a decrease in TSS from 20.38 to 13.96 °Brix was observed in wood apple fruit during ripening [37]. A similar decrease in TSS has been reported in papaya [38]. The pH of the fruit pulp increased from 3.62 to 3.84 during ripening, while the titratable acidity decreased from 3.53% at the unripe stage to 3.20% at the ripe stage [39]. A significant increase in TSS was observed in all five tropical fruit extract species as the fruits mature. Meanwhile, a significant increase of TSS value ($p < 0.05$) of *Mangifera indica* fruit was observed from the mature to the ripe stage. From the young to ripe stages of the fruits, a significant decrease in total acidity (TA) (expressed as the concentration of oxalic acid) was observed in all fruit extracts tested. The value of pH was the lowest in young fruit and increased significantly ($p < 0.05$) during the early stage of ripening [40]. A very recent report showed the dynamics of six papaya cultivars, obtained from the seed, cultivated in a Mediterranean climate in Sicily in greenhouse conditions and harvested at late stages, where the physicochemical traits were measured in terms of the titratable acidity and soluble content [41].

3.2. Determination of Bioactive Compounds

Figure 2 presents the bioactive substances in three durian cultivars. Total polyphenol compounds (TPC, mg GAE/g DW) were in the range from 4.99 ± 0.45 to 3.17 ± 0.20 with the average values for Chanee (CN) and the lowest for Puangmanee (PM). Total flavonoid compounds (TFC, mg CE/g DW) were estimated as the highest for Monthong (MT) of 2.10 ± 0.12 and 1.33 ± 0.07 for PM. The two phenolic substances were the highest in the MT cultivar.

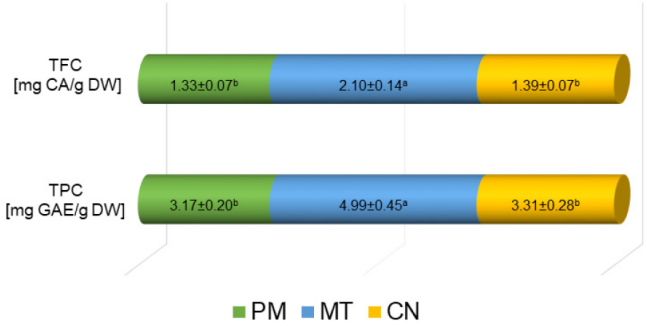

Figure 2. Total polyphenol compounds (TPC, mg GAE/g DW) and total flavonoid compounds (TFC, mg CE/g DW) in local cultivars of Thai durians: Monthong (MT), Chanee (CN) and Puangmanee (PM). Values are means ± SD per g dry weight (DW); n = 5 samples per cultivar, each subsampled and analyzed 5 times. Values with different superscript letters are significantly different ($p < 0.05$). Abbreviations: GAE, gallic acid equivalent; CE, catechin equivalent.

The obtained results of bioactive substances are in agreement with other recent reports and as well with our previous data. The TPC (mg GAE/g FW) and TFC (mg CE/g FW) in Chanee (CN) were in the range of 0.21–3.21 and 0.02–0.82; for Puangmanee (PM) estimated of 3.11 and 0.03–0.18 and for Monthong (MT) showed 0.56–3.74 and 0.04–0.94, respectively [3,4,6,10,14,42]. Unknown variety of durian [5] showed 0.99 mg GAE/g FW, which was similar to the present data of MT. Similar total polyphenol data were obtained from an unknown variety in another report [43] as 0.79 mg GAE/g FW. It was

interesting the comparison of Monthong, Kradum, and Kobtakam varieties which had higher phenolic content of 2.69–2.89 mg GAE/g DW [4] than that of the Chanee variety (0.67 GAE/g DW), which was lower than the level reported from Malaysia (2.54 mg GAE/g DW) [5]. The comparison of total polyphenol content (6.96–9.30 mg GAE/g DW) of five varieties of durian (Monthong, Chanee, Kradum, Kanyao, and Puangmanee) showed that the Monthong variety had the highest TP content, whereas the Kradum variety had the lowest [13]. In the report of four locally available varieties of durian fruit [44], where the total phenolics and flavonoids were extracted using dichloromethane: pentane (1:1 *v*/*v*) the results differed from the presented and total phenolics were found in the range of 690.62–998.29 mg/L, showing the significant inter-varietals variations. The total flavonoids were in the range of 211.36–220.34 mg/L. Caffeic acid and quercetin were the dominant antioxidant substances found in durian, therefore for fluorescence studies, these phenolics were used as standards. The bioactivity of ripe durian was high, and the total polyphenols were the main contributors to the overall antioxidant capacity [13,42]. The differences in the results of bioactive metabolites depend on durian investigated varieties, such as Monthong (MT), Chanee (CN), and Puangmanee (PM), extraction time and solvent used, and analytical methods. This affects the physicochemical and biological properties of the plant as a food ingredient.

3.3. *Antioxidant Properties of Durian Cultivars*

The values of five different antioxidant assays are presented in Figure 3. The results of the DPPH assay were the lowest and of ORAC are the highest. The results of antioxidant activities were expressed in μM TE/g DW and showed the following numbers: DPPH—from 4.58 ± 0.43 to 7.05 ± 0.22; FRAP—from 6.82 ± 0.29 to 11.69 ± 0.31; ABTS—from 11.37 ± 0.15 to 15.88 ± 0.37; CUPRAC—from 39.76 ± 7.21 to 56.18 ± 5.89 and ORAC—from 360.87 ± 14.38 to 377.64 ± 18.11.

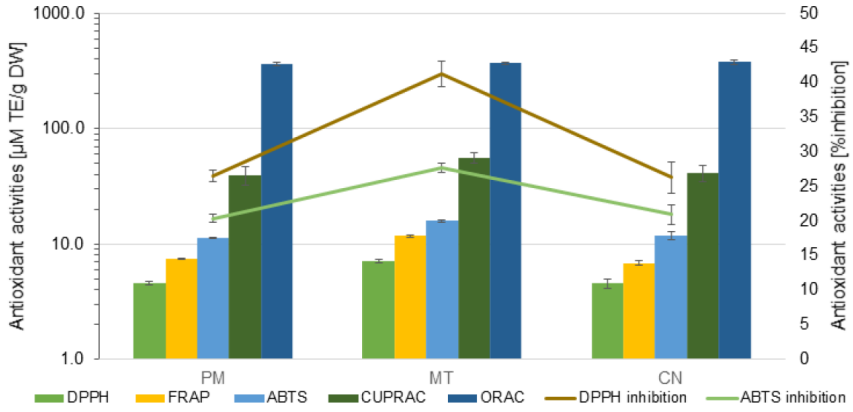

Figure 3. Antioxidant activities values (μM TE/g DW) of DPPH (1,1-Diphenyl-2-picrylhydrazyl method); FRAP (Ferric-reducing/antioxidant power); ABTS [(2,2-Azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)diammonium salt]; CUPRAC (Cupric reducing antioxidant capacity); ORAC (Oxygen radical absorbance capacity); antioxidant activities by DPPH and ABTS assays (% of inhibition) in durian cultivars. Values are means ± SD per g dry weight (DW); n = 5 samples per cultivar, each subsampled and analyzed 5 times. Abbreviations: TE, Trolox equivalent; PM, Puangmanee; MT, Monthong; CN, Chanee.

In DPPH and ABTS assays, where the antioxidant activity was determined as percentages of inhibition, showed the same relationships in the investigated durian cultivars as using the estimation of the antioxidant activities of these assays in μM TE/g DW. All used methods estimated that the Monthong cultivar was the strongest between investigated

cultivars, showing in most cases significant differences (Figure 3). As mentioned previously, there are many methods for total antioxidant potential determination, and each has its limitations [45]. As can be concluded from the present results that some of these antioxidant assays give different antioxidant activity trends [45], but all presented methods showed that Monthong variety was higher than others. The obtained results are in line with other investigations [14,42,46–48]. So, DPPH (μM TE/g FW) showed 0.98—13.66 and 1.28–2.46 for MT and CN, respectively. FRAP estimation (μM TE/g FW) was 0.72–7.49, 2.32–4.57 and 2.45 for MT, CN and PM, respectively. ORAC assay (μM TE/g FW) for Thai durian cultivars is not applied widely, then for MT is 19.03 and for CN is 23.04, respectively [4]. CUPRAC (μM TE/g FW) varied between the varieties and showed the following numbers: 4.28–10.76, 9.55 and 9.45 for MT, CN and PM, respectively. The obtained results of ABTS (μM TE/g FW) are in agreement with most of the reports and show 2.66–23.53, 20.91, and 20.20 for MT, CN and PM, respectively. Some results were obtained from unknown varieties of durian fruit, as FRAP values of 7.41 μM Fe^{2+}/g FW and TEAC values of 4.98 μM TE/g FW [42,44]. Durian varieties [47] showed antioxidant activities measured by DPPH, FRAP, and ORAC as followed: (4–8, 11–16 and 62–73 μM TE/g DW, respectively). These values were in the same range as the ripe Monthong variety measured by DPPH and FRAP which were proved by present results and reported [14,48]. The ORAC values shown in this report were lower than those found in the present study. The present results of durian extracts (361–378 μM TE/g by ORAC assay) can be compared to other fruits such as *Phyllanthus emblica* L. (455 μmol TE/g by ORAC assay) and *Spondias pinnata* (L.f.) Kurz (241 μmol TE/g by ORAC assay). Durian extract was prepared using the mixture of ethanol and 0.2 M HCl (1:1, *v*/*v*) exhibited the highest total phenolics value of 116.55 ± 1.51 mg GAE/g extract and the highest total flavonoid value of 92.37 ± 9.27 mg rutin equivalent (RE)/g extract. In antioxidant studies, the ABTS assay of durian fruit extract showed a greater value in antioxidant activity against control [33]. The results derived from DPPH, FRAP, and other assays showed lower antioxidant potential compared to the standard. In DPPH radical scavenging activity, the maximum radical scavenging activity was found (49.11 ± 2.55) at 1000 μg/mL of the sample concentration in comparison with the present results of MT (41.28 ± 1.81).

3.4. Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) Studies

The FTIR spectra and DSC peaks are presented in Figure 4. The main FTIR bands of three cultivars of durian polyphenol extracts were from wavelength numbers from 3200 to 500 cm^{-1} (3258, 2922, 1741, 1593, 1406, 1039, 987, 923, 828, and 523 cm^{-1}). Three durian cultivars showed similar spectra profile (Figure 4A). The wavelength numbers of FTIR spectra at 828 and 1039 cm^{-1} were assigned to –C–H alkenes and –C–O alcohols of catechin [3].

A band at a wavelength number of 1617 cm^{-1} was responsible for gallic acid. Additionally, a band at 923 cm^{-1} of FTIR spectra was derived from polyphenols in durian extracts [3]. FTIR spectra of Monthong and Chanee cultivars presented a band at 1741 cm^{-1}, which corresponds to the –CO (stretching) and the band at 2922 cm^{-1} was related to the C–H bond of saturated carbons [9]. Some bands corresponded with the measurements of pineapple fruit [49]. The band presented at 3600–3000 cm^{-1}, with a maximum value close to 3300 cm^{-1} was associated with the stretching vibration of O–H groups. Similar bands were shown in all durian cultivars at 3258 cm^{-1}. The peak at 1719 cm^{-1} was estimated for carbonyl group C=O stretching [49]. According to the present procedure of preparation, the samples were lyophilized, and then the O–H stretching band has to show carboxylic acids, alcohols, and phenols, which are important for the bioactivity of the fruits. In the pineapple, the bands between 1800 and 1500 cm^{-1} have been related to proteins as amide I and amide II ((1700–1600 cm^{-1} and 1565–1520 cm^{-1}), respectively, and fats (1745–1725 cm^{-1}) [50]. This region was found in durian samples between 1741 and 1593 cm^{-1}. Several peaks were found in the spectral range of 1400–800 cm^{-1} in pineapple and 1406–523 cm^{-1} in durian fruits. These bands can be assigned to with the stretching and bending of carbohydrates,

organic acids and colour pigments [49,50]. The obtained results of FTIR spectra, showing similar patterns for all durian cultivars can be compared with some standards found in durian and used as well for fluorescence studies. In tannic acid, one peak was found between 3495 and 3280 cm^{-1}, which shows the stretching vibration for O–H group. For durian extracts, one peak at 3258 cm^{-1} was assigned. The peaks (cm^{-1}) were found for tannic acid and durians at 2929 and 2922, respectively, indicating CH_2 asymmetric and symmetric stretching of the compound. The peak at 1703 cm^{-1} represents the stretching of the carbonyl (C=O) group in the compound. Such a peak does not appear at this number in durian extracts. The peak at 1500–1450 cm^{-1} allocated the carboxylic acid (O–C–O) and at 1314–1187 cm^{-1} suggested the bending vibration of the O-H group. In addition, a sharp absorption band at 1021, which assigned to the C-O group of molecules, corresponds with the durian extracts [35,51]. In quercetin, the peak obtained in the range of 3398–3314 cm^{-1} represented the O-H stretching vibration due to the intramolecular hydrogen bonding. The peak obtained at 1449–1400 cm^{-1} is indicated of the C-O bond [52]. As it was shown in our previous results, FTIR was widely used for avocado, durian, mango [3], apple, strawberry, red grapefruit, and for the determination of different stages of ripening as well, different kiwifruit cultivars. [36]. Thus, FTIR was used for the determination of functional groups present in different phytochemicals in the plant samples.

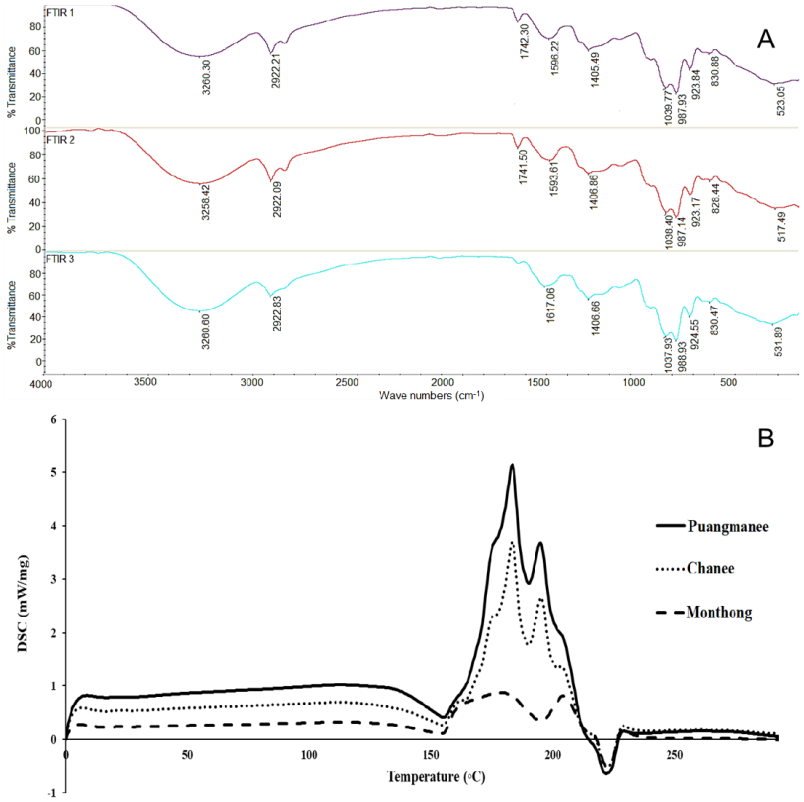

Figure 4. (**A**), Fourier transform infrared (FTIR) spectra of polyphenols of durian extracts from the top: Monthong, Chanee and Puangmanee. (**B**), Differential scanning calorimetry (DSC) measurements of Puangmanee, Chanee and Monthong.

DSC profiles of three durian extracts are shown in Figure 4B. The peaks of Puangmanee and Chanee occurred at slightly higher temperatures than Monthong. Monthong showed a broader peak compared to Puangmanee and Chanee extracts. Park et al. [53] reported that a broader peak is an indication of a broader distribution of phenolic molecules having different thermal stabilities. This is in correspondence with the study of antioxidant and nutritional properties, phenolic contents, and proteins of five durian cultivars. The significantly higher amounts for total polyphenols and flavonoids were detected in Monthong [13].

3.5. Binding Properties of Phenolic Properties of Durian Cultivars with Human Serum Proteins

The interaction with the above serum proteins and extracted durian polyphenols are evaluated by the changes in the fluorescence intensity of the proteins. The changes appeared mostly in the positions and values of peaks. The interactions of durian polyphenol extracts with human serum albumin (HSAlb), fibrinogen (HSFib), and globulin (HSGlo) are shown in Figures 5–7.

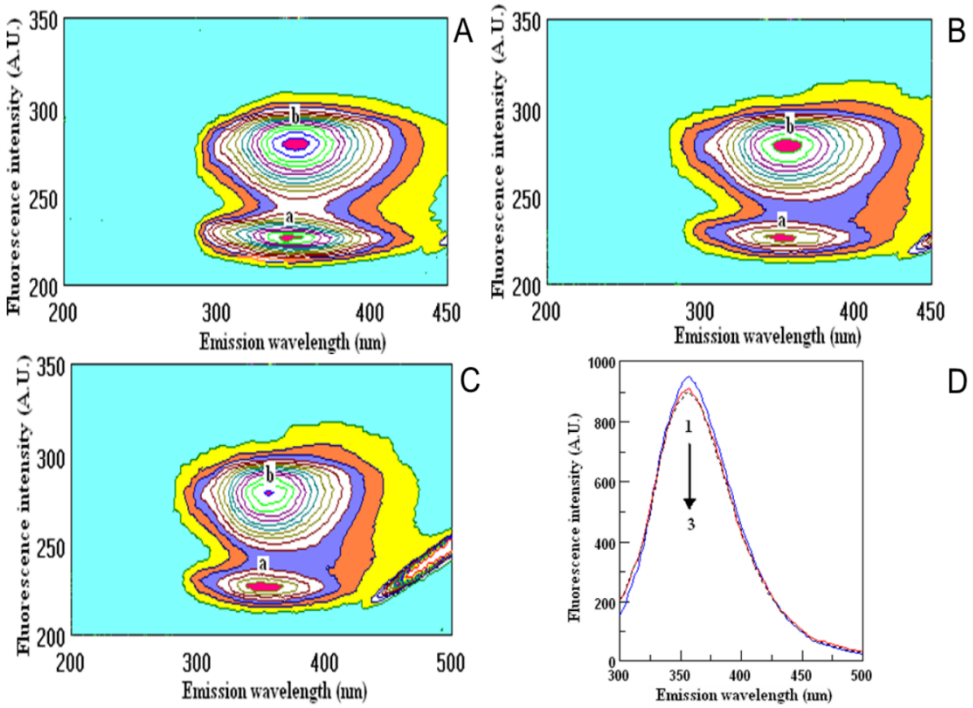

Figure 5. Fluorometric measurements in three-dimensional fluorescence analysis (3D-FL) of durian polyphenol extracts after interaction with human serum albumin (HSAlb). (**A**–**C**) cross images HSAlb in methanol, HSA + Monthong; HSA + Puangmanee; (**D**), Spectral data of two-dimensional fluorescence measurements (2D-FL) of durian extracts and HSAlb from the top: 1, 2, 3, HSAlb (FI = 954.29 A.U.); HSA+ Puangmanee (FI = 911.86 A.U.); HSA+ Monthong (FI = 897.13 A.U.). The location of peaks **a** and **b** are shown in Figures 5–7 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

The solution of HSAlb (Figure 5A) in methanol before interaction with polyphenols from three durian cultivars showed the fluorescence intensity (FI) of peak **a** FI_a =727.09 A.U. and of peak **b** FI_b =853.41 A.U. The interaction of HSA with extracted polyphenols from

Puangmanee (Figure 5C) showed peak **a** with FI_a =413.04 A.U. and FI of peak **b** =784.61 A.U. Interaction with Chanee showed similar results as FI_a = 412.87 A.U. and FI_b = 774.72 A.U. and with Monthong were the lowest in two peaks as FI_a = 410.22 A.U. and FI_b = 758.24 A.U. The calculated changes in the fluorescence intensity showed the binding properties (%) 51.25, 52.44 and 54.71%, for Puangmanee, Chanee, and Monthong, respectively, by 2 D-fluorescence measurements (Figure 5D). The fluorescence measurements by 3 D-FL showed more precise data, based on the difference in the intensity of two peaks. As it was shown in Figure 5, the bioactivity of the Monthong cultivar was higher than the two others. Therefore, in this report, only interactions of Monthong durian are presented on the Figures 6–9 and other samples were omitted. The interaction of human serum globulin (HSGlo) with durian polyphenol extracts and standard solutions is shown in Figure 6. HSGlo fluorescence intensity decreased and was the lowest for tannic acid of 353.2 A.U. and approximately 1.6 times higher than with durian of 587.3 A.U. (Figure 6C).

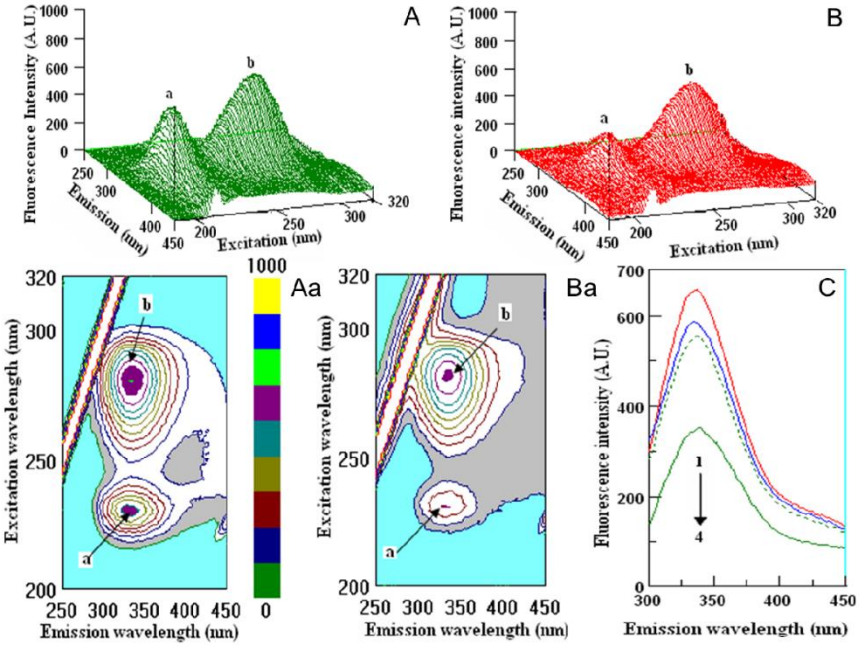

Figure 6. Fluorometric measurements in three-dimensional fluorescence analysis (3D-FL) of durian extracts after interaction with globulin (**A**,**B**), globulin (HSGlo) in buffer, Glo + Monthong; (**Aa**) and (**Ba**), their cross images. (**C**), Spectral data of two-dimensional fluorescence measurements (2D-FL) of durian extract, standards and Glo from the top: 1, 2, 3, 4, Glo (FI = 658.57 A.U.), Glo + durian (FI = 587.25 A.U.); Glo + quercetin (FI = 554.04 A.U.); Glo + tannic acid (FI = 353.19 A.U.); Abbreviations: Glo, globulin; FI, fluorescence intensity, A.U., arbitral units; The location of peaks **a** and **b** are shown in Figures 5–7 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Lower changes in comparison with the fluorescence intensities of peak a appeared in the position of peak b: the highest peak for durian extract (611.8 A.U.) and the lowest was measured for tannic acid (229.1 A.U.). The images of the interaction of HSGlob with standards and durian showed the maximum peaks **a** and **b** and their locations (Figure 6Aa,Ba). The comparison of the values of fluorescence intensity of the native HSGlo (Figure 6C, line 1 from the top) showed that the lowest value was obtained by its

interaction with tannic acid (Figure 6C, line 4). Quercetin showed lower bioactivity than tannic acid.

The interaction of investigated samples with human serum fibrinogen (HSFib) is shown in Figure 7. The FI of peak **a** of HSFib after interaction with durian extracts was 731.9 A.U. (Figure 7B,D) and in comparison with the native HSFib of 883.6 A.U. (Figure 7A,C) and the lowest of tannic acid of 657.4 A.U. The low changes appeared in the position of peak b: for durian of 645.6 A.U., native HSFib of 811.7 and the lowest was measured for tannic acid (595.9 A.U.).

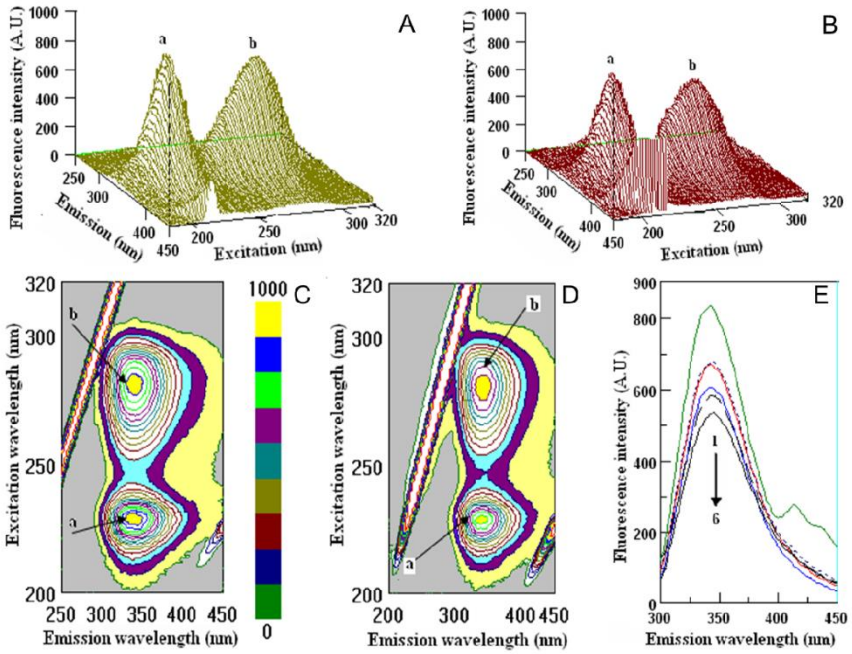

Figure 7. Fluorometric measurements in three-dimensional fluorescence analysis (3D-FL) of durian extracts after interaction with fibrinogen. (**A**,**B**), 3D-FL of Fib in buffer, Fib+ durian Monthong; (**C**,**D**), their cross images. (**E**), Spectral data of two dimensional fluorescence measurements (2D-FL) of durian extracts and Fib from the top: 1, 2, 3, 4, 5, 6, Fib (FI = 834.71 A.U.), Fib + quercetin (FI = 680.42 A.U.); Fib + caffeic acid (FI = 671.79 A.U.); Fib + catechin (FI = 606.17 A.U.); Fib + MT (FI = 584.43 A.U.); Fib + tannic acid (FI = 538.23 A.U.) Abbreviations: Fib, fibrinogen, FI, fluorescence intensity, A.U, arbitral units; The location of peaks **a** and **b** are shown in Figures 5–7 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

The comparison of the values of fluorescence intensity of the native HSFib (Figure 7E, line 1 from the top) showed that the lowest value was obtained by its interaction with tannic acid (Figure 7E, line 6).

The changes in the fluorescence intensities of peaks **a** and **b** in interaction with durian and one of the standards are shown in Figure 8.

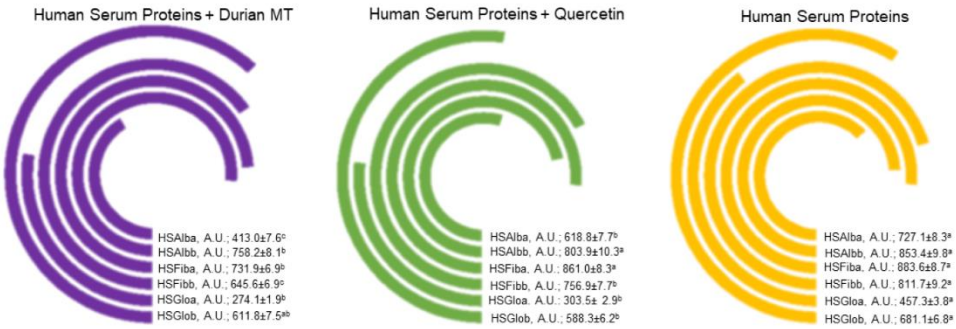

Figure 8. The changes in fluorescence intensity of peaks **a** and **b** of human serum albumin, globulin and fibrinogen (HSAlb, HSGlo, HSFib) with durian and quercetin.

The binding properties (%) between human serum proteins and polyphenols extracted from durian pulp are shown in Figure 9.

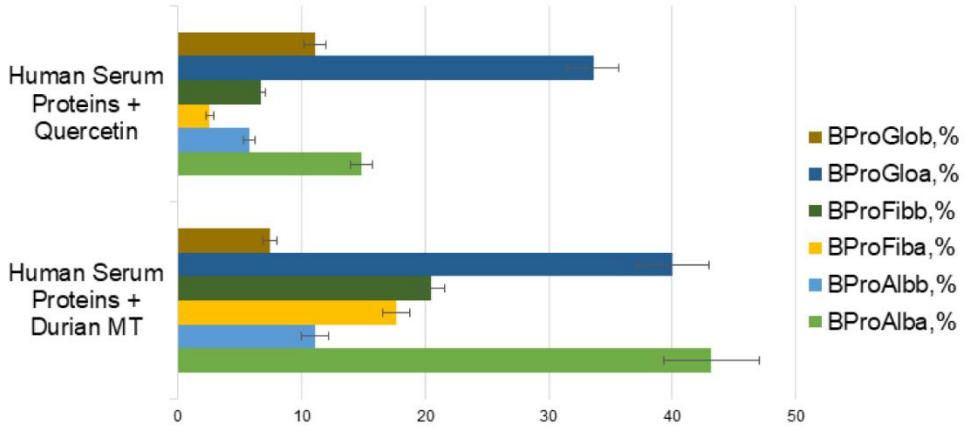

Figure 9. The binding properties (%) of durian extracted polyphenols based on fluorometric measurements interaction with human serum proteins. Abbreviations: MT, Monthong; BProGlo**b**, binding properties of human serum globulin by interaction with quercetin and durian, measured by changes in the fluorescence intensity of peak **b**; BProGlo**a**, binding properties of human serum globulin by interaction with quercetin and durian, measured by changes in the fluorescence intensity of peak **a**; BProFi**bb**, binding properties of human serum fibrinogen by interaction with quercetin and durian, measured by changes in the fluorescence intensity of peak **b**; BProFi**ba**, binding properties of human serum fibrinogen by interaction with quercetin and durian, measured by changes in the fluorescence intensity of peak **a**; BProAl**bb**, binding properties of human serum albumin by interaction with quercetin and durian, measured by changes in the fluorescence intensity of peak **b**; BProAl**ba**, binding properties of human serum albumin by interaction with quercetin and durian, measured by changes in the fluorescence intensity of peak **a**. The locations and the values of peaks **a** and **b** are shown in Figures 5–7 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Binding properties of HSAlb, for polyphenols from durian and quercetin, were 43.2 ± 3.9 and 11.9 ± 0.9, calculated on the basis of fluorescence decrease of peak **a** and 11.2 ± 1.1 and 5.8 ± 0.5, according to peak **b**, respectively. Different values were calculated with fibrinogen interaction: for polyphenols from durian and quercetin were in the range

of 17.7.6 ± 1.1 and 2.6 ± 0.2, calculated by peak a and 20.5 ± 1.1 and 6.8 ± 0.3, according to peak b, respectively, and with globulin estimated as 40.1 ± 2.9 and 33.6 ± 2.1, calculated by peak **a** and 7.5 ± 0.6 and 11.8 ± 0.9, according to peak **b**, respectively (Figure 9).

As it was mentioned previously, the decrease in the fluorescence intensity of native proteins was used for the calculation of the binding properties [22,23]. The comparison of total binding properties of durian polyphenols with main serum proteins showed the following data (%) of durian vs quercetin: HSAlb: 54.4/17.7; HSGlo: 47.6/45.4; HSFib:38.2/9.4. As can be seen from the presented evaluation that quercetin shows lower numbers than durian polyphenols and this can be explained by the synergism of the substances [1,10,18,52]. As it was mentioned, polyphenols might act synergistically with other phytochemicals in different fruits [54–57]. Our recent studies on the quenching of polyphenols with human serum proteins [22,23] and few reports were focused on the synergism of polyphenols as well as on their affinities for the proteins [58–60]. It was also shown that antioxidants may induce conformational and microenvironmental changes in interaction with human serum proteins, acting differently depending on the structure and bioactivity of the substances [61]. It was mentioned previously that the interaction of polyphenols with human serum proteins occurs through hydrophobic interaction. There are many studies explaining the essential role of polyphenolics derived from fruits in the regulation of epigenetic modifications, resulting in antiproliferative protection [42,62]. The doses of polyphenol durian extracts during interaction with human serum proteins showed relatively high bioactivity and binding properties at 0.65 µg/mL. The antiproliferative activity of durian using a breast cancer cell line MCF-7 showed that durian fruit can be considered as a potential source of polyphenols with protective effects against the nitric oxide-induced proliferation of MCF-7 cells. At the concentration of 600 µg/mL, durian fruit extracts inhibited MCF-7 cell growth by 40% [42,63]. Pre-treatment of non-differentiated U937 cells with 40 mg/mL and 20 mg/ mL extract from the pulp of the Monthong cultivar of durian reduced H_2O_2-induced ROS formation by 30% and 18%, respectively, and with 40 mg/mL of extract of the pulp from Chanee decreased H_2O_2-induced production of reactive oxygen species (ROS) by 21% while the lower concentrations of this extract failed to significantly alter the generation of ROS. These results suggested that pulp from the Monthong cultivar of durian contained a greater concentration of antioxidant compounds than that of the Chanee cultivar what is exactly in accordance with the present results [18]. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide) assay was used for the cytotoxicity of durian extract against Chang Liver cell lines [33]. The percentage of cytotoxicity increases with the higher concentrations of durian extract. The maximum cytotoxicity (74%) of fruit extract was recorded at 100 µg/mL. All durian fruit extracts at their higher concentrations (1–5 mg/mL) caused a significant decrease in HepG2 cancer cells viability. The cytotoxic effect (1 mg/mL) of immature and young durian fruits was significantly stronger (29.7 ± 2.5; 27.3 ± 2.1% of alive cells respectively) in comparison to the mature, ripe and overripe durians (51.3 ± 3.1%; 51.3 ± 3.2%; 53.3 ± 4.0% of alive cells, respectively) [33]. The cytotoxic activity of methanol extracts of Monthong durian at different stages of ripening on human pulmonary carcinoma cells Calu-6 and human gastric carcinoma cells SNU-601 of immature, mature, ripe, and overripe durian samples were low, >85% of the cells were alive, even at the highest concentration (2 mg/mL) used in this experiment. The cell survival rate (%) for mature durian fruits was 86.8 ± 1.5 and 88.5 ± 2.5%, on Calu-6 and on SNU-601, respectively, showing the highest activity among the investigated fruits at different ripening state, as opposed to our results, where immature fruits exhibited significantly higher cytotoxic activity in comparison to mature, ripe or overripe durians [14,33]. However, an in vivo study is needed to confirm this effect, based on the interaction of polyphenols with serum proteins in vitro and on cells results obtained. The physiological function of the polyphenols was estimated in the stability of the human proteins and protein-polyphenol system [17,64]. An example of an interaction was reported [62] whereby human serum albumin contributes to the stabilization of (−)-epigallocatechin gallate in serum, showing the participation of reversible covalent binding

for interaction and stabilization [65]. These data are in line with other reports, where it was shown that albumin, globulin, and fibrinogen are the main and the most important human serum proteins in metabolism and function, as well in the immune system [66,67]. All these proteins have the capacity to bind metabolites, drugs, organic compounds, and relevant antigens [59,60]. The determined in vitro health properties of durian are in line with some reports [68,69] and our previous studies in vivo [16,31]. The previous studies showed the prevention of coronary artery disease (CAD) by interaction with HSAlb, and in this study, relatively new indicators as globulin and fibrinogen were investigated. In was shown the positive effects of durian fruit at different stages of ripening on the hearts and livers of rats fed diets high in cholesterol [9,46]. The intervention of durian effectively lowered the total cholesterol (TC) and increased the high-density lipoprotein cholesterol (HDL) concentration in hypercholesterolaemic individuals. A reduction in the levels of plasma TC (12.1%), LDL-C (13.3%), and triglycerides (TG) (14.1%) compared with the control group was estimated when durian was added to the diet [10]. Such results were characteristic for a number of fruits [16,31]. The results were consistent when tested with another durian from Thailand varieties (Chanee and Kan Yao) compared with the control. Rats supplemented with ripe durian had significantly lowered TG (26.3%), but not significant in TC (4.8%) and LDL-C (6.3%). Histological analysis demonstrated that ripe durian protected the liver and aorta from exogenous cholesterol loading and protected the intimal surface area of the aorta [9]. The antioxidant potential of durian fruit is relatively high according to its functionality in vitro and in vivo studies and shows against lipid peroxidation metabolism by the production of reactive oxygen species (ROS). The antioxidant capacity of fruit extracts was measured from the suppressive effect on ROS formation. Durian extracts were more potent at suppressing ROS formation and decreasing the secretion of tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) than rambutan extracts [18]. The addition of such fruits to generally accepted diets could be beneficial for hyperlipidemic, especially hypertriglyceridemic patients, suffering from coronary atherosclerosis. We expect that HSAlb, Fib, and Glo will serve as predictors of cardiovascular events.

4. Conclusions

Currently, there are limited studies exploring the health benefits of bioactive components in durian. Hence, we studied the nutritional and bioactive compounds present in durian varieties from Thailand, in comparison with the same fruit grown in similar climate conditions in Indonesia, and mostly in Malaysia. The potential health benefits of durian were carried out by in vitro reactivity with the main human serum proteins, such as albumin, globulin, and fibrinogen. It was shown that durian polyphenols have relatively high binding properties in comparison with other fruits. To the best of our knowledge, this is the first report on the characterization and quantification of phenols and flavonoids, as well as the first investigation of the health properties, including the interaction with the main serum proteins. This study suggests that the durian extracts have strong antioxidant potential and could be a significant source of natural antioxidants and ingredients for functional foods formulation.

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6.10 Metabolomic and antioxidant properties of different varieties and origins of Dragon fruit

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Metabolomic and antioxidant properties of different varieties and origins of Dragon fruit

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ABSTRACT

Dragon fruit has appealed much concern from consumers as a novelty fruit with potent nutritional and medicinal benefits. Dragon fruit quality warrants comprehensive evaluation, based on the contents of pigments and health-promoting natural compounds in different varieties. This study was aimed to evaluate the differences among dragon fruit varieties extracted with methanol–water (CD_3OD-D_2O) and methanol (CD_3OD) by proton nuclear magnetic resonance (^{1}H NMR)-based metabolomics approach. The variation features of the metabolite profiles were studied between varieties and origins of dragon fruit, considering the differences in principal component analysis (PCA). The hierarchical clustering analysis (HCA) based on score values of PCA model was also performed to analyze the distance between samples based on metabolites contents. The results of ^{1}H NMR spectra showed that the CD_3OD-D_2O extracts quantitatively differ from CD_3OD ones. In dragon fruit extracts, 36 metabolites were identified. The results demonstrated that the methanol and methanol/water extracted similar compounds with higher intensity in methanol. The metabolic differences among varieties were also shown for CD_3OD extracts by comparing both Pareto and UV scaling methods. The big size red fleshed dragon fruit (samples 2 and 3), growing in Israel were clustered similar to that growing in Thailand with the abundance of phenolic compounds. Glucose and fructose were more prominent in the yellow and white fleshed fruit (samples 4 and 5) growing in Israel. To support the obtained results two dimensional ^{1}H–^{1}H J-resolved and UHPLC-MS measurements were carried out. This research gain novel insights into the field as the first NMR metabolites fingerprinting of the major dragon fruit varieties. The correlations between DPPH, CUPRAC, antioxidant and metabolomic properties were also evaluated. The chemical markers associated with varieties of dragon fruit quality and their appearances were identified and can be utilized for the basis of authentication purpose of this fruit.

1. Introduction

The intake of fruits and vegetables has been recommended to enrich human diets with beneficial components for preventing numerous chronic diseases. There are many evidences supporting this recommendation based on previous studies [1]. High fruits and vegetables intake was also considered for the treatment of chronic diseases. Based on World Health Organization, the recommended intake of fruits and vegetables is 400 g/day [2]. Hypothetically, all dietary constituents have ability to affect health status, treating or preventing diseases. In

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developing countries the consumption of the fruits are relatively low. Recent research was dedicated to the role of diet in managing health and decreasing the risk of chronic diseases [3]. Dragon fruit (Cactaceae) is known as Pitaya, which is specie belonging to *Hylocereus* genus. It is originated from Latin America and West Indies [4], which belongs to the exotic fruit group, little explored to date by the food and pharmaceutical industries. The high-efficient regeneration system and domestication transplanting technology for red pitaya (*Hylocereus polyrhizus*) was established by the systematical optimization on explants sterilizing, induction and multiplication of adventitious shoots, rooting and seedling transplanting [5]. There are 14 known species of genus *Hylocereus*, which categorized by triangular fruits with wide scales [6]. Currently, various *Hylocereus* species as significant economically fruit crops, are planted in several tropical and subtropical regions [7]. Seven common species of wild Cactaceae from different areas of Baja Califoornia, Mexico, were previously investigated [8], including two species of *Stenocereus thurberi* (Pitaya dulce) and *Machaerocereus gummosus* (Pitaya agria). The dragon fruit production has attracted interest in United States, Australia, Southeast Asia, Israel and other regions [9]. Dragon fruit was first produced in America and introduced to Israel in 1984, and since that various research studies have been attempted to improve the fruit quality and yield [10]. This fruit has gained considerable attention from consumers as it is a uniqueness fruit, which can tolerate with drought stress and contains considerable amount of nutrients. Recent research was focused on extraction polyphenols also from peel, an agro-industrial byproduct in pitaya juice processing [11], then in red pitaya (*Hylocereus undatus*) peel and pulp were intensively used. The peel and pulp colors of the fruit are associated with stable pigments [4], especially with nitrogen-containing betalains (betacyanins and betaxanthin), which are water soluble. Betalain pigments are characterized as noticeable aspects for quality of dragon fruit because they are prominent health-promoting natural constituents. In addition, this fruit has potent health properties and prominent nutritional value due to its richness in polyphenols, vitamins, sugars, amino acids and betalains pigments [12–14]. Furthermore, dragon fruit contains substantial amounts of unsaturated fatty acids (linoleic and linolenic) with wide applications in therapeutic and cosmetic preparations [4]. The dragon fruit has many potential biological activities due to the presence of bioactive components [4]. Pitaya or dragon fruits are becoming more popular due to their nutritional benefits. These exotic fruits can be utilized in production of functional products, including fruit juice [11]. Yet, there is still gap of information on the physicochemical alterations occurring during dragon fruit growth, which is important to cultivate superior. In this study, the problem was addressed by comparing seven dragon fruit varieties from different origins with various pulp colors that can be used for the quality control of this crop.

Metabolomics fingerprinting is increasingly advantageous approach that is used for the evaluation of plant varieties and accessions [15]. Metabolomics can be targeted by coupling analytical tools for the separation of compounds via gas chromatography (GC) or liquid chromatography (LC) with mass spectrometry (MS) for identification [16]. Another technique is direct spectral measurement, which is untargeted method using nuclear magnetic resonance spectroscopy (NMR) to evaluate compound mixtures, without the need of separation [17]. There are some limitations using MS and NMR as single methods for analysis, because the obtained results are insufficient. Although, the less sensitive of NMR spectroscopy, it is prevalent and provides comprehensively data about compounds in a short time with simple and non-destructive preparation steps [16]. Multivariate data analysis is importantly needed to identify the dominant differences between fruit varieties and to provide clues for the potent chemical markers, since the metabolomics data is complex.

The GC–MS analysis was previously used to discern three dragon fruit varieties with red-skinned cultivars with different pulp colors, nevertheless, no marker was formally recommended [4]. In addition, previous study has shown the discrimination of dragon fruits during maturation based on their phytochemical constituents using LC-MS metabolomics analysis [18]. There are studies showing the antioxidant activities of 33 fruits, where pitaya was investigated as well [19].

However, there is no study dealing with NMR metabolomics for dragon fruits extracts. Therefore, this study was aimed to assess the variation among of various cultivars of dragon fruit extracts obtained from different origins using ^{1}H NMR-based metabolomics approach, to provide understanding for differentiating dragon fruits varieties and origins. For clarification, seven dragon fruit cultivars from two different locations originated from Israel and Thailand were analyzed. The extraction of dragon fruit samples were performed using polar solvents, including CD_3OD and $CD_3OD + D_2O$ extracts. To support the obtained results two dimensional ^{1}H–^{1}H J-resolved and UHPLC-MS measurements were carried out. The PCA model and HCA were used to classify the cultivars and origins and to develop clusters of discrimination. The polyphenol and antioxidant profiles were determined by spectroscopic measurements and redox assays. The chemical markers in dragon fruit cultivars originated from different geographical location are also discussed. Hence, this study provides a comprehensive profile for the chemical markers distribution in dragon fruit extracts and their related products for food and pharmaceutical applications.

2. Materials and methods

2.1. Chemicals

The deuterated chloroform ($CDCl_3$), deuterium oxide (D_2O), deuterated methanol-d_4 (CD_3OD), nondeuterated potassium phosphate (KH_2PO_4), sodium deuterium oxide (NaOD), tetramethylsilane (TMS), trimethylsilylpropionic acid-d_4 sodium salt (TSP) and were purchased from Merck (Darmstadt, Germany).

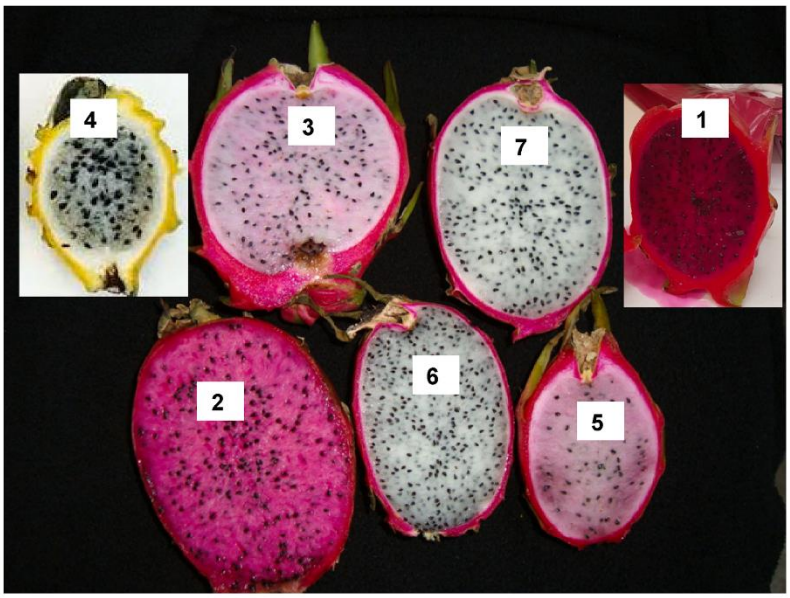
2.2. Sample materials

Each cultivar was composed of 20 fruits, about two kg of weight. The cultivars that reached commercial maturity stage were harvested in 2018. Samples 1–6 were purchased from Israeli market and sample 7 was obtained from Thai market. Samples 1, 2, 3 and 6 known as *Hylocereus costaricensis* or *H. polyrhizus* (*Pitaya roja* or red-fleshed pitaya) have red-skinned fruit with red flesh with greenish scales. However, the differences among the three samples are that sample 1 has average size of the fruit, where fruit size of sample 2 is big. Sample 3 has also big size of the fruit with very short leafy bracts, where the fruit of sample 6 is very small with short leafy bracts. Sample 4 belongs to *H. megalanthus* (*Pitaya amarilla* or yellow pitaya) is also known as *Selenicereus megalanthus*, which has yellow-skinned fruit with white flesh. Sample 5 is white-fleshed pitaya (*Pitaya blanca) from* the species of *H. undatus*, which is recognized by the pink-skinned fruit with white flesh, and it is the most commonly seen dragon fruit. On the other hand, sample 7 is the typical white Thai pitaya. All the samples were cleaned and pilled before removing the seeds. Then, they were frozen at −80 °C and freeze dried prior to extraction (Fig. 1A).

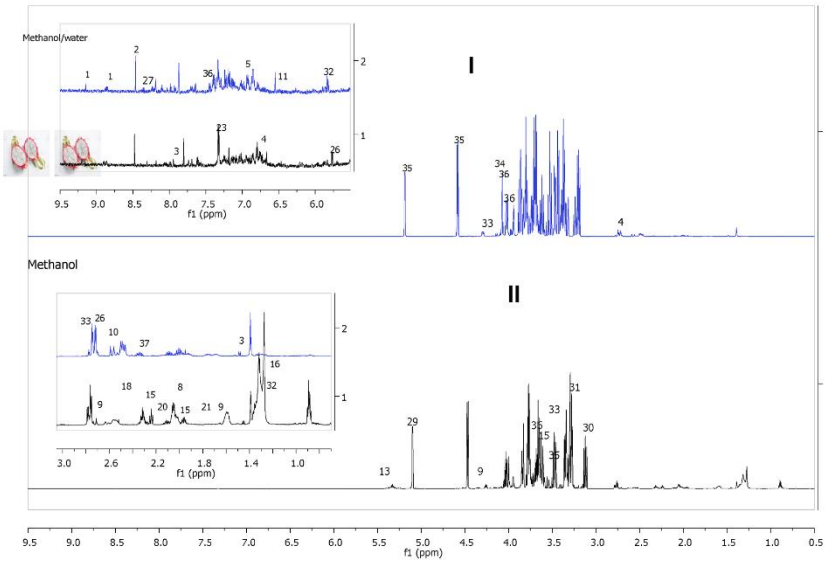
2.3. Extraction for NMR

The ground freeze dried portions of each sample (n = 7) were sieved and 50 mg was transferred to 1.5 mL Eppendorf tube. The samples were immersed in 700 μL of $CD_3OD + D_2O$ (ratio 1:1), and CD_3OD for polar extraction. The same weight of samples was also mixed with 700 μL of $CDCl_3$ for non-polar extraction. The $CDCl_3$ solvent contains tetramethylsilane (TMS) as an internal standard with concentration of 0.05%. However, the other internal standard 3-Trimethylsilylpropanoic acid (TSP) was added to solvents for polar extracts, concerning $CD_3OD + D_2O$ (0.1% TSP in D_2O) and CD_3OD (0.05% TSP). The mixtures were sonicated at room temperature for 30 min and centrifuged at 13,000 rpm for 10 min. Then, 650 μL of the supernatant was transferred into 5 mL NMR

A



B



(caption on next page)

Fig. 1. Appearance of lengthwise sliced pitaya fruits, used in this study. Samples 1, 2, 3 and 6 are Hylocereus *costaricensis* or *H. polyrhizus* (*Pitaya roja* or red-fleshed pitaya). Sample 1 has average size, sample 2 is big. Sample 3 has big size of the fruit with very short leafy bracts, Sample 6 is very small with short leafy bracts. Sample 4 belongs to *H. megalanthus* (*Pitaya amarilla* or yellow pitaya) is also known as *Selenicereus megalanthus*. Sample 5 is white-fleshed pitaya or *Pitaya blanca* from the species of *H. undatus*. sample 7 is the typical white Thai pitaya. b. Representative ^{1}H NMR spectra of (I) methanol–water (CD_3OD-D_2O) and (II) methanol (CD_3OD) extracts of dragon fruit varieties. Assignments: 1; Trigonelline, 2; Formic acid, 3; Alanine, 4; Shikimate, 5; Rutin, 6; Catechin, 7; Quercetin, 8; GABA, 9; Malic acid, 10; Citric acid, 11; Fumaric acid, 12; Succinic acid, 13; Sucrose, 14; Oleic acid, 15; Valine, 16; Fatty acids, 17; Choline, 18; Azelaic acid, 19; Glutamate, 20; Glutamine, 21; Arginine, 22; Leucine/ Isoleucine, 23; Caffeic acid, 24; Ascorbic acid, 25; Aspartic acid, 26; Betalamic acid, 27; Betanin, 28; 2′-*O*-glucosylbetanin, 29; Phyllocactin, 30; Hylocerenin, 31; 2′-*O*-apiosylbetanin, 32, 2′-*O*-Apiosyl-phyllocactin, 33; 2′-(5″-*O*-E-Sinapoylapiosyl)betanin, 34; Inositol, 35; Glucose, 36; Fructose.

tube and subjected to ^{1}H NMR measurements.

2.4. NMR measurements

The ^{1}H NMR analysis was employed using a 600 MHz Bruker Ascend 600 spectrometer (Bruker BioSpin, Rheinstetten, Germany), operating at frequency of 600.30 MHz at temperature of 299 K. For each sample, 64 scans were recorded using an acquisition time, a pulse width, and a relaxation delay. In the case of all $CD_3OD + D_2O$ extracts, the presaturation method was applied after acquiring the ^{1}H NMR in order to suppress the residual water signal with low power selective irradiation [1]. Special impulse sequences (presaturation and more complex multipulse sequences) were used for water signal suppression, which includes the NOESY-presaturation pulse sequence (Bruker 1D noesygppr1d pulse sequence) with irradiation during the recycle and mixing time delays. The noesygppr 1D-NMR experiment was set to the following parameters: 64 scans and 4 prior dummy scans of 4 k points were acquired with a spectral width of 11.0320 ppm, a receiver gain of 203, and an acquisition time of 4.9480 s. For all samples, the FIDs were Fourier transformed with line broadening (LB) = 0.3 Hz and the spectra were zerofilled to 32 K points. The data were acquired automatically under the control of ICON-NMR (Bruker BioSpin, Rheinstetten, Germany), requiring about 10.11 min acquisition time. The resulting spectra were manually phased, baseline corrected, and calibrated to TSP or TMS at 0.0 ppm.

2.5. J-resolved

To solve the overlapping problems of some compounds signals, further assistance for their identifications was accomplished by two dimensional ^{1}H–^{1}H J-resolved.

2.6. UHPLC-MS measurements

The UHPLC-MS/MS analysis was performed in ThermoFisher Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer (San Jose, CA, USA) using an ESI source, following the previous report [15]. The 2 mg of analyzed samples were dissolved in 2 mL of LCMS grade methanol, sonicated for 15 min and filtrated through a 0.22 μm PTFE membrane into a 2-mL screw-capped amber vial. The injection volume was set to 2 μL and an ACQUITY UPLC HSS T3 column (1.8 μm × 2.1 mm × 150 mm) was used to carry out the analysis. The gradient mobile phase consisted of 0.1% formic acid in both LCMS grade water (solvent A) and LCMS grade acetonitrile (solvent B). The gradient program proceeded with 5–100% solvent B from 0 to 30 min with a flow rate of 1.0 mL/min. All chromatographic techniques were achieved at room temperature. The range of *m/z* 150–1500 was used to record total ion chromatograms (TIC). Both positive and negative ionization modes were used for full scan data acquired at a mass resolution of 70 000 FWHM. The MS/MS analysis was based on the most abundant ions in each scan. The MS^n analysis was established as follows: spray voltpressure 4.0 kV, equipment capillary temperature 350 °C, sheath gas with flow rate 80 arbitrary units and auxilliary gas nitrogen flow at 40 units. The collision-induced dissociation energy was attained at 30%. The LC–MS/MS system was coupled with quadruple precursor ion selection with high resolution accurate mass (HRAMS) Orbitrap detection. It is convenient for targeted and untargeted selection of compounds to significantly identify unknown ones. The Thermo Xcalibur Qual Browser software 4.0 (ThermoFisher Scientific Inc., Waltham, MA, USA) was performed for data processing and evaluation

2.7. Polyphenol determination and antioxidant assays

2.7.1. Preparation of the extracts

Polyphenols were extracted twice from lyophilized fruits with absolute methanol at 20 °C (concentration 25 mg/mL). The proportion of sample to the solvent was 1/10 w/v. Exactly the same conditions were used for the extraction of polyphenols with water, but the temperature was kept at 4 °C. These extracts were used for polyphenol and antioxidant assays as previously was described [20,21].

2.7.2. Polyphenol determination

Folin–Ciocalteu assay was used for determination of total polyphenols in methanol and water fruit extracts of 0.25 mL with 1 mL of Folin–Ciocalteu reagent. In the next step, 0.75 mL of 1% sodium carbonate was added. The absorbance of the resulted mixture was measured on Hewlett-Packard, model 8452A spectrophotometer at 750 nm. The results were calculated in mg gallic acid equivalents (GAE) per g DW [22].

2.7.3. Antioxidant capacities

DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, is based on the reaction, when its radical form disappears after reduction by antiradical compounds, which was formed when 3.9 mL, 25 mg/L DPPH were mixed with 0.1 mL of methanol or water sample extract. The reaction progress was monitored at 515 nm until the absorbance was stable. The scavenging rate on DPPH radicals was calculated as μM TE (Trolox equivalent) per g DW [23].

CUPRAC, Cupric reducing antioxidant capacity, was carried out by a mixture of 1 mL of copper (II)-neocuproine and NH_4Ac buffer solution, acidified and non acidified methanol extracts of pitaya (or standard) solution (x, in mL) and H_2O [(1.1-x) mL] were added ribed to make the final volume of 4.1 mL, and the absorbance was measured at 450 nm [24].

2.8. Statistical analysis

The spectra were treated using processor and profiler in Chenomx software (v. 8.4, Alberta, Canada) with standard setting for each spectrum. The phasing and baseline corrections of each ^{1}H NMR spectrum were automatically performed and the bin size was 0.04 ppm for the spectral region 0.52–10.00 ppm. The residual signals of water and methanol were excluded from the data analysis at δ 4.60–4.90 and δ 3.20–3.36, respectively. The total of 239 bins was obtained for each spectrum in CSV file. The data in Excel file was statistically analyzed by multivariate data analysis with Pareto scaling method using SIMCA version 14.0 (Umetrics AB, Umea, Sweden). Based on PCA, the HCA was performed to visualize the chemotaxonomy relationship among dragon fruit varieties. The relative quantification of the significant compounds was also conducted using MetaboAnalyst 4.0, a free metabolomics data analytical tool, which is available online (http://www.metaboanalyst.ca) to reveal variations of metabolite among dragon fruit varieties. All obtained data for antioxidant status were calculated on the basis of statistical analysis of Duncan's multiple range test. Each analysis was

done five times with means ± SD. To determine the statistical significance as 95% interval of reliability ANOVA, one way analysis on variance, was used.

3. Results and discussion

3.1. Fruit description and weight

The sizes and flesh colors of fresh fruits are relevant in marketing as product and appearance for consumers. The grading of dragon fruits exported from Israel is by the fruits number per weigh [4]. Variances in Asian countries, the fruit size is graded differently as extra-large, large, regular, medium and small as > 500 g, 380 to 500 g, 300 to 380 g, 260 to 300 g, <260 g, respectively [14,25]. In overall shape and size of dragon fruit varieties were clearly observed to be different between genotypes. The fruits from red-fleshed pitaya (1, 2, 3 and 6) exhibited to have different scales. Accordingly, the samples 2 and 3 are big in size comparable to sample 7, as typical Thai pitaya, where sample 1 has averaged and sample 6 is small in size fruit. Notably, the size of samples 4 and 5 are medium.

3.2. ^{1}H NMR spectra inspection and identification of metabolites

Dragon fruit possesses several health and nutritional benefits. However, limited information is available on the variation of metabolites at diverse cultivars and from different locations.

Thus, a metabolomics analysis was performed to study the metabolite changes in the selected cultivars from Israel and Thailand. A combination of ^{1}H NMR spectra and LC-MS/MS was used to identify the variation of metabolites. In overall, ^{1}H NMR spectra of methanol and methanol–water extracts of all samples exhibited the existence of diverse classes of compounds. In the ^{1}H NMR spectra of all extracts, the compounds detected include organic acids, phenolics, betacyanins, flavonoids, sugars, fatty acids, and amino acids. Visually inspecting the spectra of each cultivar, no differences were observed in terms of compounds, except their intensities. The identified compounds cover a wide range with 36 constituents as presented in Table 1. A critical inspection of the ^{1}H NMR spectra of the different dragon fruit samples based on the characteristic signals helped in the assignment of these compounds. They were identified based on their ^{1}H NMR spectral data, the literature and the UPLC–MS/MS as well as 2D J-resolved spectra. The primary metabolites signals were matched with those in Chenomx database. The representative spectra of dragon fruit extracts is presented in Fig. 1B. The δ 3.00–5.00 region has the most intense signals, showing the sugars. These intense signals at this region revealed that the carbohydrates are the major compounds in dragon fruit extracts, where glucose, fructose, and sucrose are the dominated sugars identified in all samples. There are clear differences in the features of some compounds of the different fruit varieties, which can be visually observed from the ^{1}H NMR spectra. The peak intensity can refer to the concentration of compounds, which is possible to be quantified as relative to internal standard. The peak intensities can be clearly observed at δ 5.20 (α-glucose) and δ 5.40 (sucrose). In the aliphatic region (δ 0.50–3.00), the signals of amino acids were identified in all extracts, including alanine, arginine, valine, leucine, and GABA. In addition, fatty acids and some vitamins were identified. In the aromatic region (δ 5.5–9.0), there were signals assigned for betacyanins, rutin, quercetin, and catechin. However, the CD_3OD and CD_3OD-D_2O extracts of all sample spectra revealed noticeable difference in the compound signals intensity, especially in the aromatic region (Fig. 1B).

The ^{1}H NMR spectra allow to identify the betanin as betanidin system with sugar moiety and assign the individual coupled 1H-spin systems of the aglycone (betanidine) at H-2, H3a/b; H-11, H-12; H-14a/b, H-15 and of (Table 1). In addition, the H4 and H7 of betanidine were assigned at 7.06 and 6.98 ppm, respectively, as well as hexose moieties signals. A three-bond vicinal coupling constant $^3J_{H\text{-}1',\ H\text{-}2'} \approx 6.9$ Hz,

Table 1
Identified compounds in dragon fruit varieties from ^{1}H NMR spectra of CD_3OD-D_2O and CD_3OD extract.

N°	Tentative compound	^{1}H NMR characteristic signals
1	Trigonelline	9.14 (s), 8.83 (m), 8.07 (m), 4.44 (br s)
2	Formic acid	8.45 (s)
3	Alanine	1.48 (d, J = 7.0 Hz)
4	Shikimate	6.70, (m), 2.19 (dd, J = 17.0, 7.0), 4.43 (t, 4.0) 4.01 (m)
5	Rutin	6.51 (d, J = 2.0 Hz), 7.59 (dd, J = 8.5, 2.0 Hz), 7.67 (d, J = 2.0 Hz). Anomeric protons (glucosyl 4.97 (d, J = 8.0 Hz), rhamnosyl 4.54 (d, J = 1.0 Hz)
6	Catechin	2.50 (dd, J = 16.07, 8.14 Hz), 2.84 (dd, J = 16.12, 5.39 Hz), 3.97 (td), 4.56 (d, J = 7.54 Hz), 5.85 (d, J = 2.26 Hz), 5.92 (d, J = 2.26 Hz), 6.71 (dd), 6.76 (d), 6.83 (d, J = 1.98 Hz)
7	Quercetin derivatives	7.52 (d, J = 3.5) 6.66 (d, J = 3.5)
8	GABA	3.04 (t, J = 8.0 Hz), 2.29 (t, J = 7.0 Hz), 1.89 (m)
9	Malic acid	4.30 (m), 2.67 (dd, J = 15.5, 3.0 Hz), 2.36 (dd, J = 9.5, 15.0 Hz)
10	Citric acid	2.69 (d, J = 15.0 Hz), 2.52 (d, J = 15.0 Hz)
11	Fumaric acid	6.51 (s)
12	Succinic acid	2.42 (s)
13	Sucrose	5.39 (d, J = 3.5 Hz), 4.16 (d, J = 8.5 Hz), 4.07 (t, overlapped), 3.87–3.67 (m), 3.53–3.42 (m)
14	Oleic acid	5.34 (m), 2.35 (t, J = 7.5 Hz), 2.01 (m), 1.63 (m), 1.2 – 1.4 (20H), 0.88 (t, J = 7.0 Hz)
15	Valine	3.60 (m), 2.22 (m), 1.05 (d, J = 7.5 Hz), 0.99 (d, overlapped)
16	Fatty acids	1.28–1.32 (m)
17	Choline	3.21 (s)
18	Azelaic acid	2.35 (t, J = 7.2 Hz), 2.34 (t, J = 7.6 Hz), 1.30 and 1.80 (each 4H and 6H, broad s), 5.32 (OH)
19	Glutamate	2.24–2.34, m
20	Glutamine	3.77 (m), 2.48 (m), 2.14 (m)
21	Arginine	1.92 (m), 1.66 (m), 1.59 (m)
22	Leucine/ Isoleucine	3.73 (m), 1.74 (m), 0.96 (m)
23	Caffeic acid	7.33 (d, J = 16.0 Hz), 7.13 (d, J = 1.9 Hz), 7.00 (dd, J = 8.0, 2.0 Hz), 6.86 (d, J = 8.0 Hz), 6.35 (d, J = 16.0 Hz)
24	Ascorbic acid	4.51 (d, J = 2.0 Hz), 4.01 (m), 3.75 (m)
25	Aspartic acid	3.89 (m), 2.80 (dd, J = 17.5, 3.5 Hz), 2.68 (dd, overlapped)
26	Betalamic acid	2.76 (dd, J = 13.8, 1.9 Hz), 2.79 (dd, J = 13.8, 3.8 Hz), 4.49 (dd, J = 3.9, 1.9 Hz), 5.83 (d, J = 10.3 Hz), 6.96 (s), 9.55 (d, J = 10.3 Hz)
27	Betanin	5.23 (dd, J = 2.0, 10.2 Hz), 3.66 (dd, J = 10.4, 16.7 Hz), 7.13 (s) 7.12 (bs), 8.38 (bd, J = 11 Hz), 6.04 (bd, J = 11 Hz), 3.41–3.59 (overlap) 6.34 (bs), 5.00, (d, J = 6.9 Hz), 3.41–3.59 (overlap), 3.41–3.59 (overlap), 3.41–3.59 (overlap), 3.41–3.59 (overlap), 3.86 (dd, J = 1.4, 12.6 Hz), 3.70 (dd, J = 5.5, 12.6 Hz).
28	2′-*O*-glucosylbetanin	4.90 (dd, J = 3.5, 10.5 Hz), 3.62 (dd, J = 17.2, 10.2 Hz), 3.14 (dd, J = 3.5, 17.1 Hz), 7.15 (s), 7.28 (s), 8.18 (bs), 5.82 (bs), 3.18 (bm), 4.29 (bt, J = 7.7 Hz), 6.24 (bs), 5.13 (d, J = 7.3 Hz), 3.65 (overlap), 3.93 (dd, J = 12.1, 2.0 Hz), 3.67 (dd, J = 12.3, 1.6 Hz), 5.02 (d, J = 7.3 Hz), 3.84 (dd, J = 2.2, 12.5 Hz)
29	Phyllocactin	5.26, (bd, J = 10.6 Hz), 3.67 (dd, J = 10.6, 17.1 Hz), 7.09 (s) 7.13 (bs) 8.38 (bd, J = 10.0), 6.06 (bd, J = 10.0), 3.21 (dd, J = 7.2, 17.2), 3.44–3.60 (overlap), 6.3 (bs), 5.01 (d, J = 7.1), 3.44–3.60 (overlap.), 3.76 (ddd, J = 1.7, 6.1, 9.6), 4.49 (dd, J = 1.7, 12.3) 4.32 (dd, J = 6.0, 12.3), 3.28 (s)
30	Hylocerenin	3.56 (dd, J = 11.6, 16.5 Hz), 3.11 (dd, J = 4.3, 16.5 Hz), 7.00 (s), 6.94 (bs), 8.14 (bd, J = 11.8 Hz), 6.06 (bd, J = 12.6 Hz), 3.14 (bm), 4.33 (bt, J = 7.2 Hz), 6.20 (bs), 4.99 (d, J = 7.2 Hz), 3.56 (overlap), 3.56 (overlap), 3.47 (overlap), 3.47 (ddd, J = 1.9, 6.3, 9.4 Hz), 4.45 (dd, J = 1.6, 12.1 Hz), 4.23 (dd, J = 6.4, 12.2 Hz), 2.68 (d, J = 14.4 Hz), 2.64 (d, J = 14.4 Hz), 1.24 (s) 2.56 (d, J = 15.0 Hz), 2.52 (d, J = 15.0 Hz)

(continued on next page)

Table 1 (*continued*)

N°	Tentative compound	^{1}H NMR characteristic signals
31	2′-*O*-apiosylbetanin	4.92 (dd, J = 3.4, 10.2 Hz), 3.64 (dd, J = 16.6, 9.6 Hz), 3.17 (dd, J = 3.5, 16.9 Hz), 7.18 (s), 7.08 (s), 8,26 (bs), 5.88 (bs), 3.32 (bs), 3.29 (bdd), 4.35 (bt, J = 7.7 Hz), 6.29 (bs), 5.10 (d, J = 7.0 Hz), 3,75 (overlap), 3.70 (overlap), 3.58 (overlap), 3.93 (dd, J = 13.2, 1.6 Hz), 3.78 (dd, J = 12.6, 2.0 Hz), 5.42 (d, J = 3.6 Hz), 4.05 (d, J = 3.6 Hz), 4.09 (d, J = 10.0 Hz), 3.87 (d, J = 10.3 Hz), 3.63 (overlap)
32	2′-*O*-Apiosyl-phyllocactin	3.59 (dd, J = 16.8, 10.0 Hz), 3.33 (dd, J = 3.1, 16.8 Hz), 7.08 (s), 7.0 (s) 8.19 (bs), 5.81 (bs), 3.27 (bdd), 3.15 (bs), 4.28 (bt), 6.21 (bs), 5.04 (d, J = 7.6 Hz), 3.69 (overlap), 3.66 (overlap), 3.59 (overlap), 3.73 (overlap), 4.40 (dd, J = 12.6, 2.5 Hz), 4.36 (dd, J = 12.7, 4.4 Hz), 5.39 (d, J = 3.4 Hz), 3.99 (d, J = 3.4 Hz), 4.02 (d, J = 10.1 Hz), 3.80 (d, J = 10.1 Hz), 3.55 (overlap)
33	2′-(5″-*O*-E-Feruloylapiosyl)betanin	5.27 (dd, J = 2, 10 Hz), 3.65 (dd, J = 2, 10 Hz), 3.35 (dd, J = 2,10 Hz), 7.23 (s), 7.20 (s), 8.43 (d, J = 12.3 Hz), 6.09 (d, J = 12.3 Hz), 3.47 (bm), 3.30 (bm), 4.75 overlapped by D2O, 6.44 (bs), 5.01 (d, J = 7.7 Hz), 3.72 (d, J = 7.7 Hz), 3.77–3.40 (dd, J = 2, 12 Hz), 3.93 (dd, J = 2, 12 Hz), 5.46 (d, J = 2.3) 4.03 (d, J = 2.3), 4.27 (d, J = 9.7), 3.88 (d, J = 3.88) 4.31 (d, J = 11.3 Hz), 4.20 (d, J = 11.3 Hz), 7.08, (d, J = 1.6 Hz), 6.79 (d, J = 8.1 Hz) (d, J = 8.1 Hz), 7.05 (dd, J = 1.6, 8.1), 7.54 (d, J = 15.9 Hz), 6.28 (d, J = 15.9 Hz) 3.89 (OMe)
34	Inositol	3.53 (dd, J = 3.0, 10.1), 4.06 (t, J = 3.0), 3.59 (t, J = 9.4)
35	Glucose	5.19 (d, J = 3.5 Hz), 4.59 (d, J = 8.0 Hz)
36	Fructose	4.08 (m), 4.00 (dd), 3.93 (m), 3.79–3.85 (m), 3.69 (m), 3.51–3.57 (m)

indicated the β-linkage between the aglycone and glucopyranosyl moiety [26]. The 2′-*O*-glucosylbetanin was also tentatively identified as betanidin 5-*O*-β-sophoroside. Our study confirmed their structure by the NMR experiments and LC–MS/MS fragmentation of pseudomolecular ion at *m/z* 713 to ions at *m/z* 551 and 389, suggesting the presence of a second hexose moiety (713-551-162).

Phyllocactin was identified and confirmed by LC-MS and ^{1}H NMR spectroscopy. The presence of the betanin and malonyl moieties signals appear as the AB quartet of H-2′'a/H-2′'b at δ = 3.46 ppm for a malonate moiety. The low field chemical shift of H-6′a/ H-6′b (4.59 and 4.35) provides definitive evidence that the malonate moiety clearly revealed the presence of 6′-*O*-malonyl-betanin (phyllocactin) [27]. Hylocerenin had ^{1}H NMR spectra which were virtually identical to those of phyllocactin. Instead, an extra singlet of methyl group (CH_3-3′′) at δ = 1.57 ppm and singlet methylene groups at δ 2.89 of H-4′′ confirming the hylocerenin structure [28]. These data are in accordance with the MS data and are indicative for the presence of a 3-hydroxy-3-methylglutaryl moiety (HMG) at C-6′ of betanin. The characteristic signals of 2′-*O*-apiosylbetanin in ^{1}H NMR were similar to the signals of betanin (Table 1). In addition, the ^{1}H NMR spectra indicated a apiofuranosyl moiety by the presence of another anomeric proton with a small coupling constant (d 5.42, 1H, d, J = 3.6 Hz, H-1′′′) as well as by the presence of AB quartet methylene signals (d 3.80 and 4.02, 1H each, d, J = 10.1 Hz each, H-4′′′a and H-4′′′b, respectively) and a singlet (δ 3.55, 2H, s, H-5′′′) a β-configuration of the apiofuranosyl anomeric proton based on the coupling magnitude 3 J 1′′′–2′′′ at 3.9 Hz [28].

The characteristic signals of 2′-*O*-Apiosyl-phyllocactin were virtually the same as of 2′-*O*-apiosylbetanin (Table 1), except of the signals confirming the presence of the apiosyl moiety by the presence of another anomeric proton with a small coupling constant (d 5.42, 1H, d, J = 3.6 Hz, H-1′′′) as well as by the presence of AB quartet methylene signals (d 3.80 and 4.02, 1H each, d, J = 10.1 Hz each, H-4′′′a and H-4′′′b, respectively) and a singlet (δ 3.55, 2H, s, H-5′′′). In addition, the glucose moiety is indicated by the downfield shift proton signal H-2′ at 3.69 ppm. The presence of the apiofuranosyl moiety was further supported by LC-MS. Recent study on apiose-derived betacyanin suggested a β-configuration of the apiofuranosyl anomeric proton based on the coupling constant 3J1′′′–2′′′ (3.9 Hz) [27,29,30]. The magnitude of 3J1′′′–2′′′ found in our study (3.4 Hz) also suggested the β -configuration of the glycosidic linkage completing the structure elucidation of 3 betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl)-β-D-glucopyranoside (2′-*O*-Apiosyl-phyllocactin) (Fig. 1B) [31,32].

The compounds 2′-(5″-*O*-E-Feruloylapiosyl) betanin had ^{1}H NMR spectra which were virtually identical to those of 2′-*O*-Apiosylbetanin. Furthermore, an extra signal of sinapoyl moiety at δ = 3.89 due to the presence of methoxyl groups. Moreover, signals at aromatic region indicated the presence of four protons at 6.33 and 7.60 with coupling constant of 16 Hz (*E*-configuration) for the olefinic resonances H2′ and H3′ respectively, and singlet at 7.32 ppm to H5′′′ and H9′′′, which are the characteristic of a sinapoyl moiety.

The characteristic signals of 9 in the ^{1}H NMR spectra were virtually the same as of 2′-*O*-Apiosyl-phyllocactin (Table 1), except the signals confirming the presence of the feruloyl group, which is confirmed by the appearance of ABX-type aromatic proton signals at δ 6.75 (1H, d, J = 8.0 Hz), δ 7.04 (IH, dd. J = 2.0. 8.0 Hz) and δ 7.25 (IH. d. J = 2.0 Hz), with the singlet at δ 3.78 for the methoxyl group [29].

3.3. *J-resolved*

The inclusive information delivered by the two-dimensional J-resolved analysis on coupling constant and splitting patterns of overlapping signals aid in the assignment and endorsement of the compounds identified by 1D-NMR (Fig. S1). Generally, there were many overlapping signals in the aromatic and sugar regions (δ 3.50–9.0).

3.4. *UHPLC-MS identification*

A tentative identification of the 9 metabolites in dragon fruit extracts was achieved based on their *m/z* values and fragmentation patterns (in both negative and positive ions modes) from the literatures and online databases. The MS/MS revealed useful selectivity for the identification of isomers, solving co-eluting constituents and assignment of minor compounds. The TIC was determined by summing up the intensities of all the mass spectral peaks belonging to the same scan. Table S1 presents the tentative identification of assigned compounds, including their retention time, molecular ion and the fragmentation of each compound. The assignment of these metabolites was based on the UPLCMS/MS data (Table S1, [33–36]). These metabolites were previously reported in dragon fruit species and their fragmentation patterns were compared with literature data. The inositol (I, II and III) was tentatively identified based on the collision-induced dissociation fragmentation of the myo- and chiro-inositol precursor ion (*m/z* 179, $[M-H]^-$) that produced clear product ions *m/z* 161 and *m/z* 87. In addition to these precursor-to-transition reactions, we suggest that *m/z* 179 to *m/z* 161 appears as ring opening and water loss reactions, and the *m/z* 161 to *m/z* 87 transition is because of the change, exclusion and ring closing reactions [33].

3.5. *Metabolite profiling of dragon fruit samples by ^{1}H NMR*

Different dragon fruit cultivars were examined using ^{1}H NMR coupled with multivariate data analysis and profiled with UPLC–OBITRAP–MS. Fig. 3 presents the PCA of the variation based on their metabolic characteristics of dragon fruit varieties extracted with CD_3OD and CD_3OD-D_2O. According to ^{1}H NMR, the PCA score plot showed the separation of each sample (Fig. 2A). The samples based on solvents are separated by PC 1 (82.0%), and varieties are clustered differently by PC 2 (7.22%). Different metabolites were identified based on their NMR spectroscopic characteristics (Table 1). A total of 36

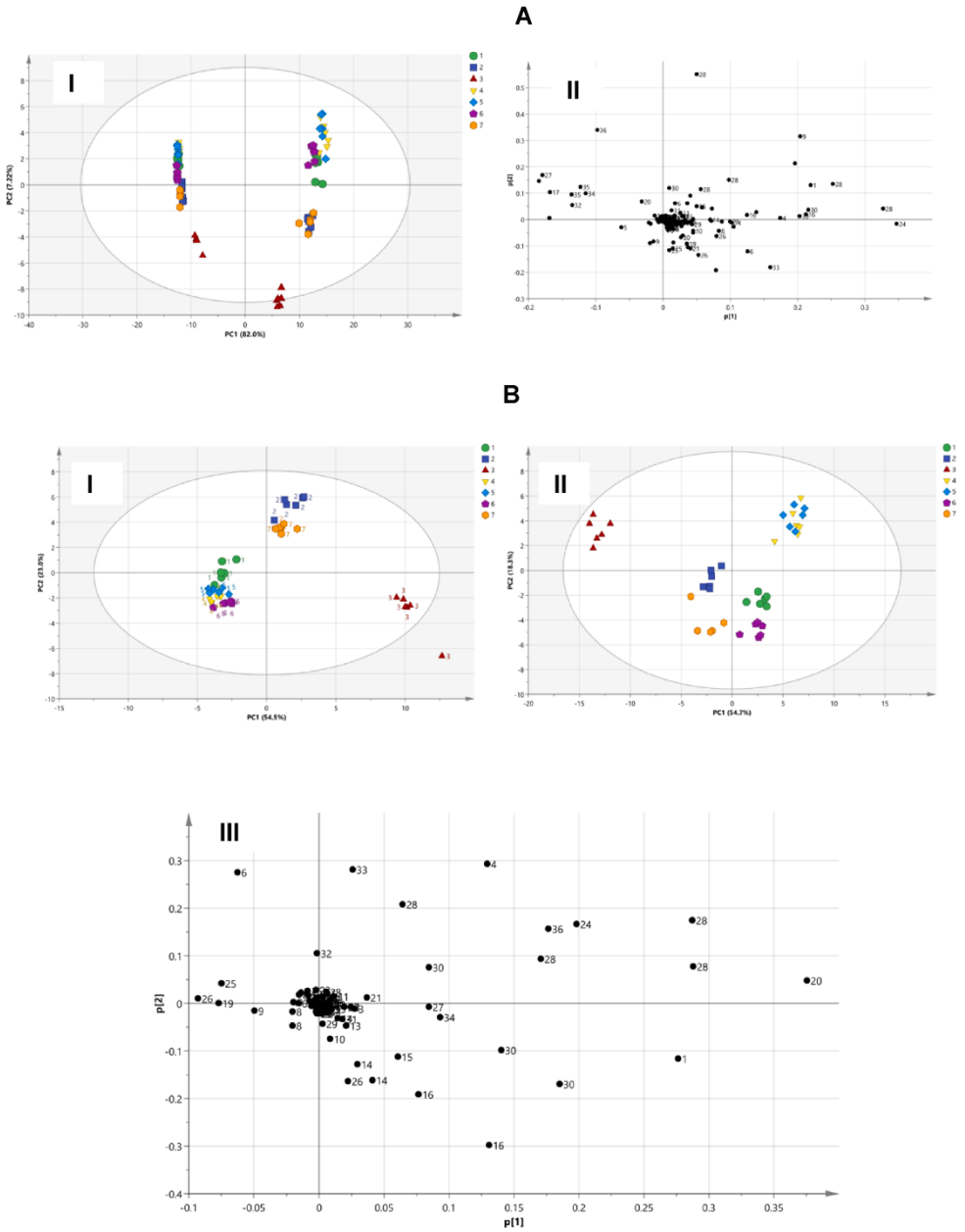


Fig. 2. PCA results in term of loading plots for ^{1}H NMR spectra from (I) methanol–water (CD_3OD-D_2O) and (II) methanol (CD_3OD) of dragon fruit varieties extracts 2b PCA score plots of for ^{1}H NMR spectra from (I) methanol–water (CD_3OD-D_2O) and (II) methanol (CD_3OD) and (III) loading plot based on Pareto scaling method of dragon fruit varieties extracts.

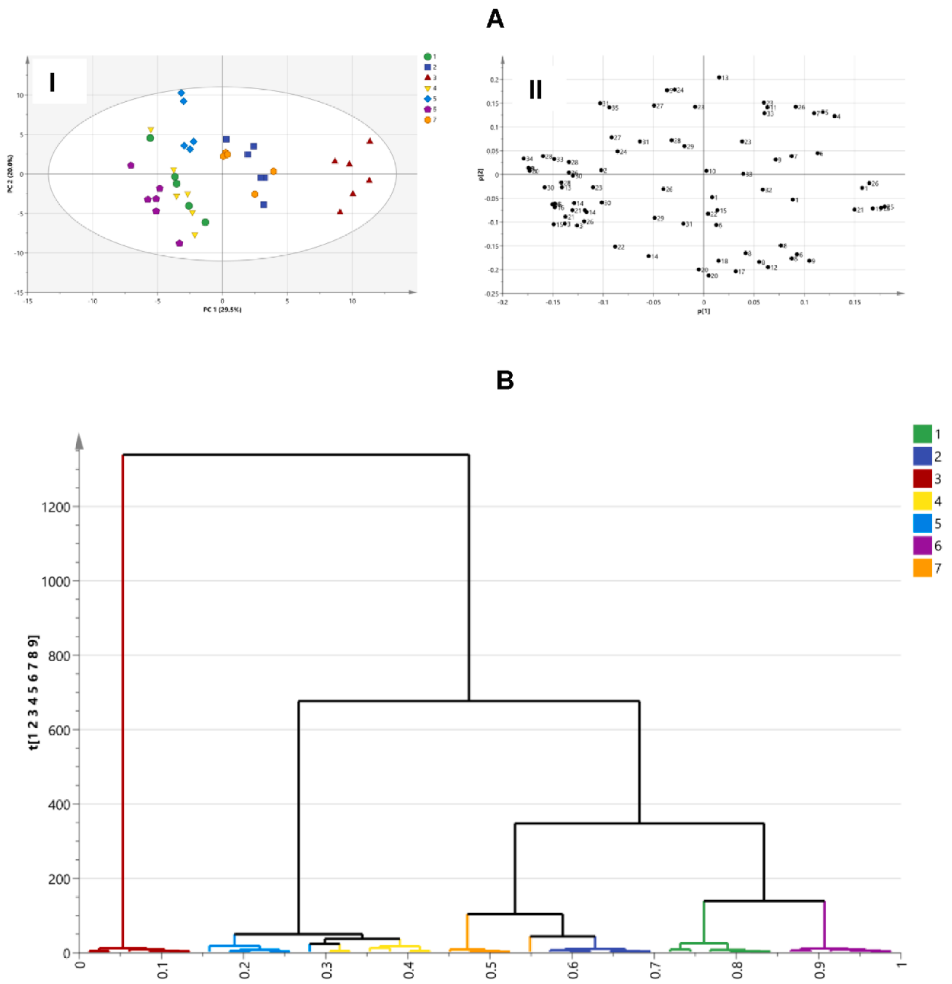


Fig. 3. PCA (I) score and (II) loading plots of ^{1}H NMR spectra from methanol (CD_3OD) extracts of dragon fruit varieties based on UV scaling method. 3b. Dendrogram of the hierarchical cluster analysis (HCA) of ^{1}H NMR spectra from dragon fruit methanol (CD_3OD) based on score values of PCA.

compounds were identified, including amino acids and amines, organic acids, sugars and sugar alcohols, and betacyanins. The solvent has significant effect compared to varieties. From the finding, CD_3OD solvent showed more intense signals of compounds compared to CD_3OD-D_2O. To recommend the metabolites contributing to the separation among different dragon fruit cultivars, variables were identified and presented in loading plots (Fig. 2B). The loading plots show the compounds, which caused the clustering of samples (Fig. 2B). Fructose and glucose were the main sugars and their levels were higher in the methanol/water extracts compared to methanol ones. Most compounds were responsible for the separation of methanol extracts from others. Methanol as organic solvent is preferably used in extracting bioactive compounds of plant sample.

To further show the differences between solvents, two models were analyzed on the data scaled using Pareto scaling method. Pareto and Unit Variance (UV) scaling techniques are the most regularly used for NMR based metabolomics dataset [37]. However, both methods have advantages and limitations. The severe over fitting occurred in PCA model of data scaled with UV revealed obvious advantage of Pareto because of the decrease of spectral noise. In Pareto, the scaling factor is the square root of the standard deviation which is good for the reduction of noise effect. However, it might cause the loss of importance of some signals with the possibility of low peaks suppression by the high ones [38]. In PCA model, this may lead to the misinterpretation of the spectral loadings. A parsimonious model can be observed with Pareto method and UV might show potent systematic variations with minor change embedded in the height and multiplicity of NMR peaks. Furthermore, UV scaling method may show the importance of metabolites compared to Pareto scaling method. Since the high content of sugar in dragon fruit and possibility to hide important effect of certain compounds, the Pareto and UV scaling methods were compared on NMR data of CD_3OD extracts to get profound picture of their metabolic

differences. In addition, the possibility for the chemical markers to contribute to variation of these varieties is minor. Therefore, unit variance scaling method was applied on the methanol extracts for all compound signals to have similar importance. The targeted compound signals were included in the PCA model to avoid the effect of noise in the model. Therefore, the both PCA models CD_3OD and CD_3OD/D_2O extracts of the variation based on their metabolic characteristics of dragon fruit varieties were presented (Fig. 2). The dragon fruit samples were clustered according to cultivars, flesh color and location. The ^{1}H NMR metabolite profiling was applied to recognize the metabolites differences in the cultivars. Metabolite profiling of the samples was used to investigate the possible metabolites differences according to their species and location (Israel and Thailand). Both models showed similar trend in terms of grouping. The samples 1, 4, 5 and 6 were separated from samples 2, 3 and 7 by PC1. The methanol solvent showed clear clustering of samples compared to methanol/ water. Therefore, the methanol extracts were used to further differentiate the samples in terms of varieties, flesh color and location. Both PC1 and PC2 showed the samples variation of 73.0% and by position in the score plot, samples 4 and 5 were clustered together, suggesting their similarities. In addition, samples 1, 2, 6 and 7 were grouped similarly (Fig. 2B). However, sample 3 was totally separated from all samples. As can be seen from this finding, the fruits are differentiated based on their sizes, excluding flesh colors or geographical locations. Samples 2 and 3 have big fruit size, which is comparable to sample 7 (typical Thai dragon fruit). Thus, they are closely similar. The loading plot shows the compounds that contribute to the differentiation of samples (Fig. 2C). Samples 2, 3 and 7 were separated from the rest by the higher contents of GABA, aspartic acid, malic acid, glutamate, catechin and betalamic acid. These metabolites were significantly distinguished on the basis of different cultivars, where glucose and fructose was distinctively higher in samples 4 and 5. In dragon fruit, some soluble sugars affect its taste and quality, especially glucose and fructose, which is directly related to sweet taste of this fruit [14]. Samples 4 and 5 are very sweet as compared to others.

The metabolites in other region are affected and they do not show importance to the variation among dragon fruit varieties, based on Pareto scaling method, because of the high content of sugars and the intense signals of compounds in the carbohydrate region. In addition, the possibility for the chemical markers to contribute to variation of these varieties is minor. Therefore, unit variance scaling method was applied on the methanol extracts for all compound signals to have similar importance. The targeted compound signals were included in the PCA model to avoid the effect of noise in the model.

From the PCA score plot of methanol extracts using UV scaling method, the same trend of clustering was observed, where samples 2, 3 and 7 are clustered differently from others on the basis of fruit size (Fig. 3A). These results demonstrated that ^{1}H NMR-based metabolomics, as holistic approach, has high reliability. However, the ^{1}H NMR signals in the loading plot were scattered far from the zero point as significant contributor for classification. The levels of betalamic acid, betanin, arginine, glutamate, aspartic acid, 2′-*O*-Apiosyl-phyllocactin, and 2′-(5″-*O*-E-Sinapoylapiosyl) betanin were higher in sample 3. The samples 2 and 7 were clustered by caffeic acid, shikimate, while the levels of fructose and glucose were higher in samples 4 and 5. Other betacyanins compounds and inositol were higher in samples 1 and 6. Fig. 3 shows the hierarchical cluster analysis (HCA) of dragon fruit varieties extracted with methanol. The HCA dendrogram was generated based on the score value from six components of PCA model. There were seven clusters representing dragon fruit varieties. It is clearly observed that all samples are grouped in the relevant variety with 100% accuracy. It also shows that the chemotaxonomy of sample 2 is closely similar to sample 7, where samples 4 and 5 are largely comparable. In addition, the chemotaxonomy of sample 1 is closed to sample 6, where sample 3 was far from all samples. This result is mainly attributed to fruit size.

The relative quantification analysis was performed to further support the obtained PCA results and to show the variation in significant chemical markers on the basis of dragon fruit varieties (Fig. 4). The ^{1}H NMR signals of compounds of interest based on mean peak area of the respective metabolites were statistically compared. Pigments as anthocyanins and phenolics are mainly targeted as the active components in dragon fruit for their nutritional and health benefits [32].

3.6. Antioxidant profile of pytaya cultivars

The results of the antioxidant status of the investigated samples are presented in Table 2. The results of total polyphenols were as much as 1.2 times higher in water than in methanol extracts. The CUPRAC and DPPH values were in correlation with the polyphenol contents but not always significant. The obtained values are in line with recently published reports. The amount of polyphenols in two species of wild pitaya were 0.08–0.06% [8] lower than in cultivated samples. The application of microwave-assisted extraction and response surface methodology showed the yield of polyphenols of 463.8 ± 1.1 mg GAE per 100 g dry pitaya peel. Antioxidant assays showed that the ethanol extracts from red pitaya peel had stronger DPPH, hydroxyl and superoxide free-radical scavenging capabilities than vitamin C at 1.0 mg/mL. These results provide an alternative way to make good use of red pitaya peel to produce natural antioxidant [17]. The antioxidant activity of pitaya pulp (1266.3 μg mL ^{1}) was lower than in the pitaya peel (445.2 μg mL ^{1}). The antioxidant potential and the chemical properties of pitaya fruit can contribute to maintaining a healthy diet [39]. The obtained results were compared with cited literature [19], where total phenolic contents and antioxidant capacities of 33 fruits were evaluated. Phenolic content was 17.25 ± 1.08 mg GAE/100 g, according to Folin–Ciocalteu method. The results of polyphenols (Table 2) and DPPH values are in line with these cited results. Pitaya (white pulp) showed 10.39 ± 0.33 μmol VC/g wet weight (WW) in ascorbic acid as a standard by DPPH assay. Ferric reducing antioxidant power assay (FRAP, μmol TE/g) value was evaluated as 1.59 ± 0.04 and correlation between polyphenols and antioxidant values was 0.8447. $ABTS^{\bullet+}$radical cation scavenging activity (ABTS) value estimated 1.57 ± 0.01 μmol Trolox/g and coefficient of correlation is 0.8025. Four fruits, plum (sanhua) (*Prunus salicina* Lindl), red bayberry (*Myricarubra* Lour.), plum (green) (*P. salicina* Lindl), and mango (shuixian) (*Mangifera indica* Linn) showed the strongest antioxidant in comparison with pitaya [19].

4. Conclusions

The objective of the study was to assess the variation among of various cultivars of dragon fruit extracts obtained from different origins using ^{1}H NMR-based metabolomics approach. The different solvents were also optimized to maximize the number of compounds to get clear information about which solvent is suitable to extract those compounds contributing to the variation among various cultivars of dragon fruit extracts obtained from different origins. Therefore, the two solvents were compared and then only one solvent was used to conclude the differences among varieties. To the best of our knowledge, this is the first comprehensive examination on the phytochemical properties of Israeli dragon fruits was performed. The metabolic variations among dragon fruit cultivars were achieved using ^{1}H NMR-based metabolomics approach. Thirty six compounds were tentatively identified from dragon fruit extracts. Based on the results, the fruits of different genotypes are not differentiated by flesh color or geographical location and they are mainly differentiated in terms of compounds by fruit size. The big size of red fleshed fruit (samples 2 and 3) was closely comparable to typical pitaya sample (sample 7). The white and yellow fleshed fruits (samples 4 and 5) were higher in sugar content as compared to others. These values were in correspondence with the antioxidant status of the fruits and can be used for quality control of dragon fruit varieties and promoting the potential of the fruits in multiple purposes.

Table 2
Bioactive substances of methanol (M) and water (W) extracts of Pitaya samples per g dry weight (DW).

Indices	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Polyph M, mg GAE	8.75 ± 0.64^{ab}	10.40 ± 0.84^{a}	10.80 ± 0.92^{a}	4.61 ± 0.22^{c}	7.83 ± 0.46^{b}	6.84 ± 0.35^{bc}	2.43 ± 0.17^{d}
Polyph W, mg GAE	10.33 ± 0.87^{a}	11.86 ± 1.04^{a}	12.42 ± 1.11^{a}	5.41 ± 0.34^{bc}	9.16 ± 0.87^{ab}	7.93 ± 0.54^{b}	4.35 ± 0.32^{c}
CUPRAC M, μMTE	23.05 ± 2.14^{b}	33.21 ± 2.58^{a}	33.45 ± 2.22^{a}	12.17 ± 1.14^{c}	21.76 ± 1.23^{b}	21.10 ± 1.18^{b}	15.76 ± 1.38^{bc}
CUPRAC W, μMTE	27.77 ± 2.42^{ab}	39.25 ± 3.23^{a}	39.47 ± 3.39^{a}	14.48 ± 1.12^{c}	26.55 ± 2.43^{ab}	24.90 ± 2.35^{b}	19.25 ± 1.58^{bc}
DPPH M, μMTE	14.07 ± 1.08^{b}	19.93 ± 1.67^{a}	21.72 ± 1.75^{a}	7.43 ± 0.49^{bc}	13.41 ± 1.19^{b}	13.10 ± 1.28^{b}	4.80 ± 0.37^{c}
DPPH W, μMTE	15.20 ± 1.23^{b}	21.62 ± 2.03^{a}	23.46 ± 1.87^{a}	8.10 ± 0.67^{bc}	14.62 ± 1.21^{b}	14.54 ± 1.11^{b}	5.22 ± 0.45^{c}

Mean ± SD (standard deviation) of 5 measurements. Average in rows marked with different letters differ significantly ($P < 0.05$).
Abbreviations: Sample1, red-fleshed pitaya, average size with greenish scales; Sample 2, red-fleshed pitaya, big size with greenish scales; Sample 3, red-fleshed pitaya, big size with greenish scales, with very short leafy bracts; Sample 4, yellow-skinned pitaya with white flesh; Sample 5, pink-skinned pitaya with white flesh; Sample 6, red-fleshed pitaya, very small size with short leafy bracts; Sample 7, typical white Thai Pitaya. Polyph, polyphenols; GAE, gallic acid equivalent; CUPRAC, Cupric reducing antioxidant capacity; TE, trolox equivalent; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity.

CRediT authorship contribution statement

Nabil Ali Al-Mekhlafi: Methodology, Validation. **Ahmed Mediani:** Software, Validation. **Nor Hadiani Ismail:** Conceptualization, Resources. **Faridah Abas:** Conceptualization, Investigation, Writing - original draft. **Tomasz Dymerski:** Data curation, Validation. **Martyna Lubinska-Szczygeł:** Software, Validation. **Suchada Vearasilp:** Methodology, Formal analysis. **Shela Gorinstein:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105687.

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6.11 Recent Applications of 1D GC-MS and 2D GC-MS in Foodomics Studies

Recent Applications of 1D GC-MS and 2D GC-MS in Foodomics Studies

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Introduction

There is no doubt that without the gas chromatography coupled with the mass spectrometry (GC-MS, 1D GC), there would be no omics sciences as they are known today. This also applies to foodomics, one of the most rapidly developing branches of food analysis. It is not only focused on the identification, determination of food components but also on the assessment of the effects of food consumption, something that is of direct interest to us - consumers. In food science, the aim is to study the unknown, often using advanced data analysis methods. However, in addition to the matrix (in case of foodomics - food) and the mentioned data analysis, usually called bioinformatics, appropriate analytical instruments are also important factors. Nowadays foodomics, but also food analysis in general, cannot be achieved without a mass spectrometry, which can be used directly or in combination with separation techniques (Herrero et al., 2012). In the case of compounds considered volatile or semi-volatile, the method of choice for their separation is gas chromatography.

The beginnings of the coupling of mass spectrometry with gas chromatography can be traced back to 1959 (Bartle and Myers, 2002). This technique quickly became popular in various branches of science, including food analysis. The key advantage of GC-MS was the use of ionization energy of 70 eV, which is now used in almost every device (Scherer et al., 2013). As a result, the fragmentation of compounds takes place in an identical way, which enables comparison of the results, as well as the creation of databases containing information about fragmentation pattern of a wide range of compounds, for example, NIST libraries (Inoue and Toyo'oka, 2015a).

An optimal analytical method that could find its application in the omics sciences still wasn't found. The ideal solution would be a system with the widest possible versatility of use, that exhibits exceptional separation properties, high sensitivity and a wide linear range. It is obvious that there is no such solution at the time, so we strive for compromise, also in GC-MS. A particular challenge in foodomics is the fact that compounds are present in a wide range of concentrations, which makes it impossible to analyze all chemical compounds with a single analytical method (Scherer et al., 2013). On the other hand, with the use of currently available mass spectrometers, it is possible to achieve satisfactory sensitivity, and application of new approaches in gas chromatography have significantly improved the instrument resolution capabilities. In the 1970s, in order to increase the resolution of GC, multidimensional gas chromatography was developed (Bartle and Myers, 2002). This is a solution in which two chromatographic columns are connected in series. There are two basic approaches to the concept of multidimensional chromatography: the first, in which a selected fraction of eluent from the first column is introduced into the second column, called Heart-Cut two-dimensional Gas Chromatography (H/C GC-GC) and the second one in which eluate is continuously, in small portions, introduced into the second chromatographic column (García-Cañas et al., 2012). This second solution is known as comprehensive two-dimensional gas Chromatography (GC × GC, 2D GC) and is currently much more frequently used in food science than the H/C approach. The advantage of GC × GC is that with its application, it is possible to obtain the widest possible chemical profile, which is especially useful in non-targeted solutions (Dymerski, 2018). Use of its combination with high-resolution MS is a perfect solution in metabolite profiling or fingerprinting. A short history of GC × GC-MS development, main assumptions, the principle of operation and

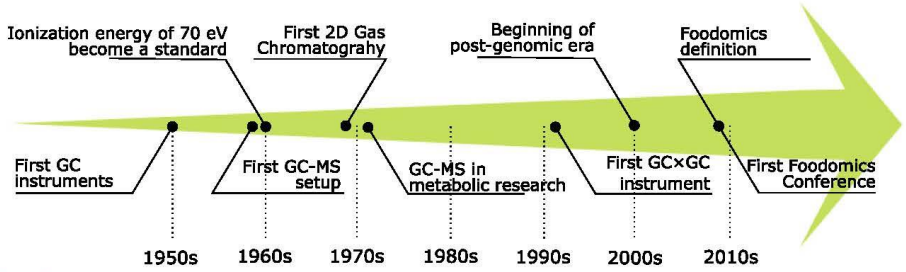

Figure 1 Milestones in the use of GC-MS in foodomics.

application in foodomics will be described in the following articles. A brief summary of GC-MS evolution and the history of its use in foodomics is illustrated as a timeline in Fig. 1.

The use of GC-MS in omics is limited by the range of compounds that can potentially be detected using this technique. It is assumed that the upper limit for the substances to be determined, including those subjected to derivatization, is 500 Da (Scherer et al., 2013). Considering that most of the metabolites, both primary and secondary, are chemical compounds with a mass below 1000 Da (Villaño et al., 2013), it is common for GC-MS to be used in metabolomics. The first attempt at coupling GC-MS and metabolic studies took place in 1971 (Horning and Horning, 1971), but in the post-genomic era this type of approach took on a new quality and it is possible to use the full potential of GC-MS.

In metabolomics, two dominant approaches can be distinguished: targeted and non-targeted (García-Cañas et al., 2012). Both of these solutions benefit from the advantages offered by GC-MS. In the case of targeted analysis, applied when it is known what kind of compound to expect, the possibility of obtaining good accuracy and precision is desirable. In the case of non-targeted, on the other hand, the main emphasis is put on exploration and thus, holistic handling of the sample. Then, the universal nature of compound detection and high resolution of measurement are used. In the non-targeted approach, one can either focus on the determination of a large group of compounds or the study of metabolic pathways, the so-called profiling, but also aimed at finding a characteristic fingerprint of the sample, which often leads to the discovery of new reaction mechanisms.

Due to the very broad framework that defines foodomics and a large amount of research involving GC-MS in this area, the authors decided to present a few out of the latest findings in this field. Moreover, the emphasis is put on the diversity of foodomics research. Thus, GC-MS can be used to determine trace contaminants in food as well as products of their metabolic conversion, determination of authenticity, quality and geographical origin of food, traceability and nutrients determinations. Additionally, application of GC-MS in foodomics also involves the determination of metabolic pathways, identification and determination of markers or profiling of substances exhibiting positive effects on human health. One of the most important fields of application of GC-MS is research on transgenic food, determination of its composition, the indication of differences in comparison to unmodified food, as well as the study of its effects on the human organism (Valdés et al., 2013). An interesting area of study is also the analysis of organic foods in terms of its pro-health potential (Vallverdú-Queralt and Lamuela-Raventós, 2016).

Specific new branches of foodomics, almost entirely reserved for GC-MS, are sensomics and flavoromics (Cordero et al., 2015). These approaches aim to analyze the compounds that may be responsible for the taste and smell of food, but also characterize the pathways of formation of these compounds, the possibility of manipulating their concentration (food engineering) and to determine their perception by the human senses of taste and smell. In those procedures, the key element is the coupling of advanced detection techniques is with sensory analysis and olfactometry. In this article, some examples of research in the field of sensory and flavoromics can be found.

General Workflow

Planning an analysis using GC-MS is extremely important for foodomics. In contrast to the classic targeted approach, where selected compounds or groups of compounds are measured, in foodomics, it is often important to use the high-throughput properties of GC-MS (Kanani et al., 2008). Therefore, each stage of the analysis should result in the smallest loss of information that can be useful for drawing logical conclusions. When developing the methodology, it is important to take into account such fundamental aspects as: an appropriate column (1D GC-MS) or pair of columns (2D GC-MS), a suitable temperature program, an injector suitable for a sample introduction and a type of MS (quadrupole, time-of-flight, ion trap, tandem). A general flowchart of handling food samples is presented in Fig. 2. As can be seen, output data maximization differs in the case of volatile and non-volatile metabolites by the fact that in the case of non-volatile metabolites we are dealing with derivatization. In the case of volatile metabolites, on the other hand, it seems essential to select an appropriate procedure to collect the volatile metabolites for GC-MS analysis, with simultaneous extraction and enrichment. Thereafter, the procedure is identical through data acquisition to data handling on the basis of

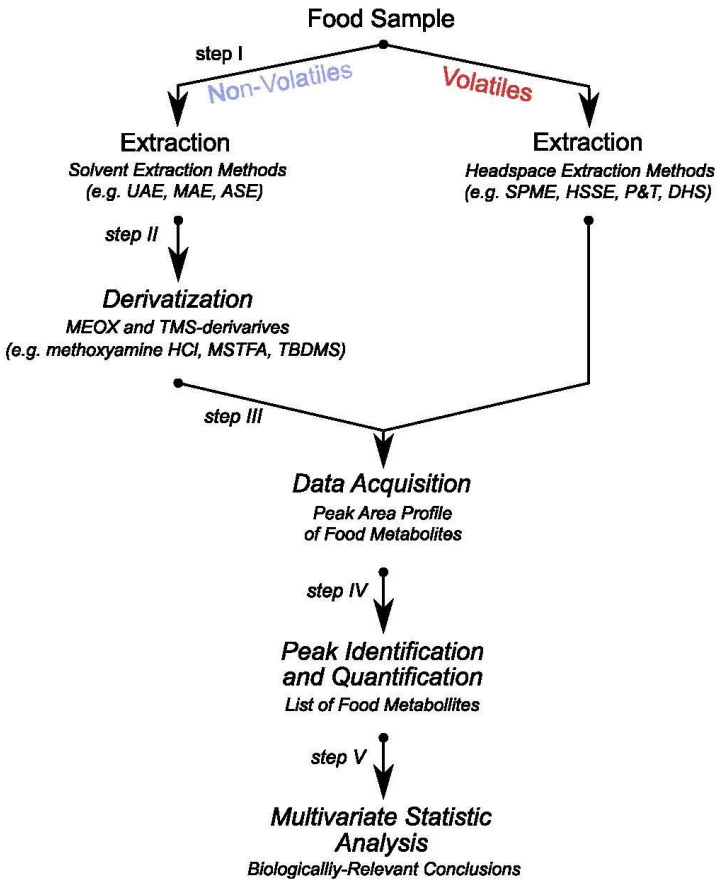

Figure 2 Scheme of foodomics workflows in GC-MS analysis both for primary (non-volatile) metabolites and secondary metabolites (volatile). Scheme modified from Kanani et al. (2008).

multivariate statistic to interpret the results as holistically as possible. For this purpose, advanced software and data contained in databases are often used.

The most commonly used extraction method is still liquid extraction, which uses solvents such as ethyl acetate, chloroform or methanol. In order to speed up the process, it is often assisted by ultrasound, microwaves or increased pressure. In the case of volatile compounds found in the headspace, sorption-based techniques are often applied. It is worth pointing out here the difficulties encountered while attempting to profile volatile metabolites. And it is not only about the difference in concentration levels of primary and secondary metabolites, but also about the development of a universal and holistic method for the collection and enrichment of analytes. In the past, the predominant method of choice of sampling was purge and trap (P&T), now almost completely replaced by solid phase microextraction (SPME). However, SPME has its limitations deriving mainly from the principle of measurement, namely sorption of analytes dependent on their affinity to sorbent (Bueno et al., 2019). Therefore, it is recommended to carry out an optimization stage in order to find appropriate extraction conditions, i.e., extraction time and temperature, type of sorbent or additives (e.g., NaCl in order to increase the volatility of compounds) (Aprea et al., 2011). In the absence of optimization possibilities, e.g. due to limited sample availability, it is recommended to use SPME fiber with universal sorbent divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS). The SPME may also be an alternative to derivatization, during which problems related to the possibility of overloading chromatographic columns with primary metabolites, limitations in

automation or time-consuming process may be encountered (Aprea et al., 2011). Using headspace SPME (HS-SPME), we focus only on compounds that are in the gaseous state over the surface of the sample and the use of sorbent guarantees their enrichment. In the literature, it is also possible to find other sorption methods in which the sorbent is placed directly in a liquid food sample, e.g. stir bar sorptive extraction (SBSE), which was successfully applied in foodomics (Cordero et al., 2013; Fan et al., 2011; Malowicki et al., 2008).

GC-MS, due to its advantages and good availability, is used for the analysis of non-volatile and thermolabile metabolites. However, the sample's components have to be derivatized, which generally involves conversion to methoxime (MEOX) and trimethylsilyl (TMS) derivatives (Fiehn et al., 2000). Thus, the obtained derivatives show reduced polarity and increased volatility (Halket et al., 2005). In short, the addition of methoxyamine hydrochloride in pyridine results in a more stable, non-polar compound with the MEOX group for each compound with the ketone group (Poole, 1978). Then these new MEOX derivatives and those metabolites that were not involved in the MEOX derivatization react with the silylating agent and thus more volatile TMS derivatives are formed (Poole, 1978). It should be noted that the ideal situation in which only one derivative for one chemical compound is created is often not the case for foodomics, especially if the holistic approach is applied (Gehrke et al., 2002). Kanani et al. have developed a strategy for the determination of non-volatile metabolites which considers three possible scenarios: (1) derivatization results in only one derivative; (2) metabolites react by forming two isomeric derivatives; (3) multiple derivatives are formed, which differ in the number of TMS-group or chemical formula (Kanani and Klapa, 2007). The authors of this article encourage readers to familiarize themselves with a comprehensive description of the reaction kinetics and workflow for these scenarios. In another paper, Osorio et al. provided a full step-by-step description of profiling primary metabolites using tomato fruit as an example, in which they focused mainly on the sample preparation and derivatization stage (Osorio et al., 2012). It should also be stressed that some of the metabolites that are present in the extract can be analyzed without derivatization, such as those present in essential oils (Halket et al., 2005).

Often, the ultimate goal of data extraction is to obtain a matrix containing detected chemical compounds, with assigned retention indices or retention times and quantitative information (for example, peak intensity). In order to achieve this, it is necessary to: correct the baseline, perform noise reduction, normalize the data (the most common procedure is to introduce internal standard(s)), carry out deconvolution, calculate retention indices, perform peak alignment and finally identify compounds based on MS databases (Lubes and Goodarzi, 2017; Tohge and Fernie, 2009). Due to the improvement of compounds picking in some databases, an additional retention index (RI) is implemented. Usually, in databases, it is possible to calculate RI based on the retention time of the n-alkanes mixture (e.g., from C8 to C30). Due to the fact that temperature-programming is commonly used, non-isothermal Kovats retention indices should be used (for further details see relevant web pages section). The most commonly used databases are the NIST library, Wiley database or for derivatized compounds Golm Metabolome Database (GMD) and the Fiehn/Binbase library (see relevant web pages section). Often in order to carry out all these activities, ready-made software such as MassHunter (Agilent Technologies, USA) or ChromaTOF (LECO Inc., USA) is used, but also independent all-in-one software such as M-IOLITE (Maga-Nteve and Klapa, 2016) with built-in KEGG pathways database or PARADISe (Johnsen et al., 2017) using the PARAFAC2 algorithm (Bro et al., 1999) are available. This algorithm can be an alternative to the calculations used in AMDIS (Automatic Mass Spectral Deconvolution and Identification System) (Stein, 1999).

However, the multivariate statistic is a very complex procedure, which according to omics research is often called bioinformatics. Workflow is often analogous in all high-through methods so the authors decided not to describe the use of multivariate statistics in GC-MS. Moreover, additional information regarding data preprocessing and processing can be found in high-quality reviews, e.g., the work published by Lubes et al. (Lubes and Goodarzi, 2017).

Basics of Two-Dimensional Gas Chromatography

Two-dimensional gas chromatography, as opposed to classical one-dimensional chromatography, uses two different separation mechanisms, due to the use of two different capillary columns. The two-dimensionality results from the fact that for each of the separated substances there are two independent parameters, namely the retention time of the first and the second column (or more precisely RI). Thus, in combination with the mass spectrometer, such a setup allows to provide three-dimensional data. Columns are connected through an interface called modulator. It enables trapping of analytes from sample matrix and provides a comprehensive introduction of these substances to a secondary column in narrow bands. This reinjection of effluent is continuous, thus all compounds reach the detector (Beens et al., 2001; Liu and Phillips, 1991; Ryan and Marriott, 2003).

Depending on the demand and complexity of the sample, two dimension analysis can be provided in various ways. Heart-cutting should be used when target regions (parts of first column effluent) are considered for analysis. However, in most cases a comprehensive approach is expected. Orthogonal separation conditions is key to provide a proper quality of the two-dimensional analysis. Typically, pairs of non-polar/semi-polar or non-polar/polar columns are used in most applications. The eluate originating from the first column is divided into a very large number of adjacent small fractions. To sustain the separation, these fractions should be no wider than one quarter or one-third of the peak width (Górecki et al., 2004). Moreover, activity coefficients in the first dimension (for highly non-polar stationary phase) are approximately equal to unity, because of the lack of specific interactions. Retention mechanism, in this case, is based only on vapor pressure and analytes are separated regarding their boiling points order. The separation in the second dimension has shape-selective nature and, thus actually affects the required

orthogonal separation conditions. A reverse-type of configuration (polar and non-polar set of columns) is also possible to use. In many papers it was proved that such configuration is also orthogonal (Górecki et al., 2004).

There are many systems, in which the second column is housed in a secondary oven to allow independent temperature programming. The limiting factor for the preparation of the temperature program is the thermal stability of the stationary phase in the second column (polar stationary phases are in general less thermolabile than non-polar). This is because the temperature in the second oven should be higher than the temperature in the first oven. It is worth to mention that in the case of 2D GC, peak capacity is the multiplication of peak capacities of both columns (Adahchour et al., 2008; Giddings, 1984). Another advantage of the use of two-dimensional systems is increased detectability. Abundance is enhanced in within two orders of magnitude, because of very effective peak compression obtained through focusing, trapping and desorption of analytes inside the modulator. As a result, a set of sharp and high peaks with a peak width ranging between 50 and 200 ms is obtained. The signal to noise (S/N) ratio is also improved (Gao X et al., 2010; Górecki et al., 2004).

So far, different types of modulators, have been developed to provide comprehensive transfer of the analytes from the first column to the second one. In general, two types of modulators were implemented, namely valve-based and thermal systems. For valve-based modulators, high-frequency sampling of the effluent is achieved via valve-switching. Focusing is obtained using a secondary circuitry of higher flow-rate carrier gas. Using these systems is rather challenging and complicated, even though drawbacks related to incomplete sampling and inherent temperature restrictions have recently been resolved with the introduction of a differential flow modulator (Liu and Phillips, 1991; Seeley et al., 2000). Thermal modulation, either heating or cryogenic, is based on trapping-desorption cycles (Liu and Phillips, 1991). For cryogenic modulators, trapping is achieved by the decrease in temperature (at least −100 °C) generated by the endothermic expansion of a cryogen such as carbon dioxide or liquid nitrogen (Marriott and Kinghorn, 2000). Desorption occurs when trapped fraction is exposed again to an oven temperature. The modulation period is a key parameter for chromatographic separations. The peak eluting from the first column is sampled by the modulator at a constant modulation period. Each fraction is focused and re-injected into the second column for further separation. The signal recorded by the detector is sliced according to the modulation period. The combination of chromatograms in the second dimension allows the reconstruction of two-dimensional chromatograms in a retention plane. Overlapping peaks are effectively deconvoluted into two series of modulated peaks. Avoiding wrapping around of analytes is the most important for creating proper chromatograms (Dallüge et al., 2002a,b; Phillips and Beens, 1999). This phenomenon occurs when highly retained peaks in the second dimension are eluted in the modulation cycle following that of their re-injection (Górecki et al., 2004).

In beginnings of two-dimensional gas chromatography, researchers turned toward a mass spectrometer as a potentially useful tool for identifying the multitude of peaks that can be seen in a two-dimensional gas chromatography chromatogram. Unfortunately, for most mass spectrometers the data acquisition rate in the scanning mode is too slow to handle the narrow peaks obtained from a two-dimensional gas chromatography system. For instance, when quadrupole MS is applied, it may happen that less than one scan per peak is acquired. Thus, to get a sufficient number of data points to accurately describe the shape of the peak (at least 10 points per peak), a detector that can collect data at a rate of at least 50 Hz is required. In order to collect full mass spectral information in a two-dimensional gas chromatography experiment, a high-speed time-of-flight mass spectrometer are commonly applied. Rapid spectral acquisition by TOFMS, with the presentation of at least 100 Hz and instantaneous measurement of all masses for each pulse, should ideally give unbiased spectra. Whilst faster detector data acquisition reduces signal-to-noize ratio by 1/2, so a 100 Hz detector data acquisition rate gives a signal-to-noize ratio about three times lower than a 10 Hz rate, the zone-compression effect leads to greater mass flux in the detector (Dalluge et al., 2002).

The quality of the modulator and detector performance is not the only challenge in gathering reliable information when two-dimensional mode is used. Proper treatment of raw data and the way they are visualized are equally important. A series of second-dimension chromatograms eluting continuously one after another from secondary column need to be shown in a relatively compact form. A good practice is when the greater number of peaks coming from the first column is sampled at least three or four times to the second dimension. Consequently, it is not clear which peaks in a series of second dimension chromatograms originate from the same compound and which ones are becoming from different compounds. Moreover, it is not reasonable to plot the entire raw chromatogram in one-dimensional mode to maintain sufficient details. These are only exemplary reasons why the data is usually converted into the two-dimensional or three-dimensional plot. Primary retention is plotted along the X-axis and secondary retention is plotted along the Y-axis. The peaks appear on performed chromatograms as spots of varying color or contour lines. The raw and original chromatographic signal is sliced into components to the second dimension chromatograms, which are aligned side-by-side. In this way, the two-dimensional retention plane is achieved (Górecki et al., 2004). The time at which each modulation is started provides the primary retention time for all of the peaks eluted in this certain modulation period. It is usual that single analyte occurs in a few modulation periods. Therefore, the quantification is based on summing up multiple peaks for each of them (Amador-Muñoz and Marriott, 2008). Currently, on the global market there are several software programs (e.g., ChromaTOF) providing proper visualization two-dimensional gas chromatography mode and further data treatment, including manual and automatic peak finding, quantification and automatic recognition of specific mass spectra (called scripts). Nevertheless, in some cases, scientists still develop their own software for data visualization and treatment. More than ten years ago, writing own software was the necessary and only way to present multi-dimensional data, due to the lack of commercial equivalents.

More recently, the chemometrics is much more popular to use and handle with two-dimensional gas chromatography data. Mainly, it can be used for pattern recognition or to lower detection limits by effective filtering of noise from the two-dimensional signal. In the first case, such tools as analysis of variance or principal component analysis techniques are used to categorize the groups of analytes according to the requirements for a given application. As the samples can be very similar in their

composition, the chemometrics can be a proper solution to find discreet differences, which gives later on qualitative information about the samples. Such a solution is invaluable in handling a large amount of data generated by a two-dimensional gas chromatography system. In the second case, the use of chemometrics, e.g., rank annihilation method, for two-dimensional gas chromatography treatment enables to obtain mathematically resolve chromatographic peaks that were only partially resolved on the two-dimensional retention plane. Therefore, it enables to speed up the separation by exchanging part of the chromatographic resolution for more rapid analysis, thus it improves separation (Pierce et al., 2008).

Recent Application of GC-MS in Foodomics

General Overview

Analysis of food is one of the most important fields of analytical chemistry, as quality and safety of consumed food and drinks have a direct impact on the consumers' well-being. With the application of foodomics methods, it is possible to obtain very accurate information on the food composition, which in effect can be used in a widely understood food quality assessment. The quality investigation concerns both the detection of exogenous substances and their metabolites in food as well as research in terms of food authenticity. Determining whether food is authentic is a significant challenge, due to the fact that there is often no single marker that could help to determine the geographical origin of a product, the raw material from which it was made or the way it was produced. Moreover, changes in its composition are often very subtle. Thus, it is possible to use metabolite profiling and chemometric based fingerprinting to sort out these challenges.

Each change of chemical composition in food results both from intrinsic and extrinsic factors, i.e., species variability, soil composition, climate and other meteorological parameters, hydrological conditions, application of appropriate agrotechnical treatments or maturity of the raw material. In addition, further elements that make up the production of food should be taken into account: the method of harvesting, treatment of the raw material, a method of conservation, etc. Finally, the conditions and method of storage should be mentioned. Collecting all these variables, the matrix of tens of dependent variables is obtained, which may potentially affect the composition of food and thus its quality. This only highlights the difficulties faced by researchers and even more rewards the use of foodomics, which seems to fit perfectly into this complex nature of food quality.

Gas chromatography-based methodologies are a method of choice when it comes to quality assessment. Both GC-MS and GC x GC-MS have found their use in the analysis of food and its composition due to their sensitivity, resolution and ease of application when the untargeted approach is needed. Since the origins of the use of GC-based methods for this purpose date back to the early days of the coupling of gas chromatography with mass spectrometry (Flath et al., 1969; Flath and Forrey, 1970; Mason and Johnson, 1967), the authors want to focus mainly on the latest reports, in which they are used for the non-targeted analysis of food composition. Examples of such uses are given in **Table 1**.

Wine Analysis

A flagship example of the use of GC-MS in foodomics is research concerning the evaluation of the quality and authenticity of wine. This is a particular challenge, as it is not easy to determine which parameters affect the quality of the wine, as many stages are carried out during the production process and thus, are potential variables (Majchrzak et al., 2018). These can, therefore, include climatic conditions, hydrogeological conditions, wine strain, agrotechnical activities, harvesting time, a method of production, storage, maturation or aging. What is more, wine may be adulterated, e.g., by admixing water to the wine, adding sugar (chaptalization) or actions related to mislabeling, both in terms of vintage year and origin (Setkova et al., 2007). Therefore, certificates of origin have been introduced in EU countries, such as a protected designation of origin (PDO). Moreover, this wine is one of the most frequently studied matrices in food analysis using 2D GC-MS (Dymerski, 2018).

It would seem logical, therefore, that foodomics researchers will try to use their tools to classify wines and detect adulteration. Thus, a separate branch of foodomics called wineomics (Moyano et al., 2019) was created. However, this is such a vast topic that it could be content for a separate book, so the authors will focus only on a few examples in which GC-MS is a crucial part. The first example is the use of headspace analysis to classify ice wines of Canadian and Czech origin. Thus, an attempt was made to differentiate wines on the basis of many parameters such as the origin, vintage year or the vessel in which the wine was fermented (oak barrels or stainless steel tanks) (Setkova et al., 2007). In other studies a new procedure of analysis of volatile aroma metabolites (VAMs) of wine was proposed, using dynamic headspace sorptive extraction (DHSE) and thermal desorption (TD). The proposed procedure was developed on the basis of experimental design, in which multivariate statistics were used and the validated method was applied in the quantitative analysis of white and red Spanish wines differing in Denomination of Origin (D.O.). The most significant groups of compounds were, for instance, esters, including ethyl acetate, butyl acetate, isoamyl acetate or ethyl hexanoate (Moyano et al., 2019). In other studies, using the P&T extraction method, 40 volatile compounds were determined, which were used to classify wines according to their origin (Aznar and Arroyo, 2007). Profiling of volatile secondary metabolites produced during wine manufacturing can be a valuable source of information on the fermentation process or further stages of production. Using the excellent separation properties of GC × GC and high resolving power of modern MS instruments it is possible to detect the vast majority of volatile compounds. Therefore, Welke et at. performed tentative identification of 179 compounds in Merlot wine (Welke et al., 2012), while Weldegergis et al. identified 206 volatile compounds in African Pinotage wine's headspace (Weldegergis et al., 2011). Moreover, it was found that the characteristic aroma of the Pinotage wine is mainly influenced by

Table 1 Selected applications of 1D and 2D GC-MS in foodomics studies

Type of sample	Sample preparation	Compounds no.	Type of food quality investigation	Technique	References
Apples	HS-SPME	399	Aroma profiling	2D-GC	Risticevic et al. (2012)
Baijiu (Chinese liquor)	SBSE	76	Variety classification	1D-GC	Fan et al. (2011)
Beef, pork and minced meat	HS-SPME	53	Authentication	1D-GC	Pavlidis et al. (2019)
Black tea	HS-SPME; extraction and derivatization (non-volatiles)	47	Traceability	1D-GC	Wu et al. (2019)
Brandy	HS-SPME	144	Variety classification	1D-GC	Zhao et al. (2009)
Cheese	Extraction and derivatization	44	Variety classification	1D-GC	Ochi et al. (2012)
Chinese dry-cured hams	MAE-SAFE	165	Aroma profiling	2D-GC	Wang et al. (2018)
Cocoa	HS-SPME	132	Botanical and geographical origin, influence of processing	2D-GC	Magagna et al. (2018)
Curcuma	Steam distillation	57	Variety classification	1D-GC	Xiang et al. (2011)
Fruit vinegar	SHS	9	Process conditions	1D-GC	Ubeda et al. (2011)
Germinating rice seed	Solvent extraction	174	Process monitoring	1D-GC	Shu et al. (2008)
Grapes and wines	HS-SPME	27	Traceability, maturation	1D-GC	Canuti et al. (2009)
Hazelnuts	HS-SPME	24	Influence of thermal treatment	2D-GC	Kiefl et al. (2012)
Honey	HS-SPME	110	Variety classification	1D-GC	Pontes et al. (2007)
Honeybush tea	HS-SPME	287	Flavor analysis	2D-GC	Ntlhokwe et al. (2018)
Ice wine	HS-SPME	201	Geographical origin	1D-GC	Setkova et al. (2007)
Jackfruit	SPME	37	Variety classification	1D-GC	Ong et al. (2008)
Olive oil	DHS-TD	230	Quality assessment	1D-GC	Danielsen et al. (2018)
Passion fruit	HS-SPME	51	Variety classification	1D-GC	Pontes et al. (2009)
Potato	Extraction and derivatization	115	Variety determination	1D-GC	Dobson et al. (2010)
Rum	HS-SPME	184	Variety classification	1D-GC	Pino (2007)
Seafood	HS-SPME	22	Storage influence, freshness determination	1D-GC	Zhang et al. (2010)
Tomatoes	SLE	267	Metabolic differentiation	2D-GC	Wojciechowska et al. (2014)
Vinegar	HS-SPME	56	Variety classification	1D-GC	Xiao et al. (2011)
White tea	HS-SPME	40	Process conditions	1D-GC	Qi et al. (2018)
Wine	HS-SPME	172	Vinification monitoring	2D-GC	Soares et al. (2015)

DHS, dynamic headspace; HS-SPME, headspace solid phase micro extraction; HSSE, headspace sorptive extraction; LLE, liquid-liquid extraction; MAE, microwave assisted extraction; SAFE, solvent assisted flavor evaporation; SBSE, stir bar sorptive extraction; SLE, solid-liquid extraction; TD, thermal desorption.

sulfides, terpenes, and methoxypyrazines. However, these compounds cannot be called key aroma compounds because in order to assess their contribution to the aroma it is necessary to perform a sensory or olfactometric evaluation. A more detailed explanation of workflow and the concept of flavoromics is described in Section "**Flavoromics and Sensomics**".

Honey Quality Determination

Honey is a unique matrix very interesting in terms of foodomics research, as it is one of the most complex mixtures of carbohydrates, consisting mainly of monosaccharides, such as fructose and glucose. It has health-promoting properties because, in addition to sugars, honey contains ingredients which have antioxidant activity, e.g., carotenes, phenolic compounds, flavonoids or ascorbic acid. Honey, therefore, has antibacterial, anti-cancer, anti-inflammatory and anti-viral functions (Viuda-Martos et al., 2008). The bioactive effect depends on the honey composition which is determined by several factors, such as the source of nectar, meteorological conditions, environmental and genetic factors as well as methods of their processing. For this reason, it is important to accurately establish its origin. It should also be noted that honey may also have an adverse effect on health due to allergenic or toxic properties of some ingredients, thus information about raw material is crucial (Bauer et al., 1996).

Aliferis et al. analyzed the headspace of Greek honeys, namely citrus and thyme using GC-MS technique (Aliferis et al., 2010). Using data analysis methods such as OPLS-DA or SIMCA, it was not only possible to distinguish between the two types of honey, but it was also able to indicate their geographical origin. Moreover, the compounds which had the greatest influence on the classification were selected, and so limonene and lilac aldehyde were responsible for the classification of citrus honey and phenylacetaldehyde or 1-phenyl-2,3-butanedione were characteristic for thyme honey. A similar challenge was also undertaken for headspace screening of Madeira Island honey, which was processed using PCA and LDA (Pontes et al., 2007).

Čajka et al. for the first time used the two-dimensional gas chromatography coupled to mass spectrometry for honey profiling (Čajka et al., 2007). The tested samples were obtained from various European countries and from Brazil. Then all the honey was

homogenized to get a universal material with a complex aroma. The number of 164 chemical compounds classified into different chemical groups as alcohols, aldehydes, ketones and esters were the most abundant groups. It should be remembered that using SPME with inadequately selected extraction parameters could result in negative analytical income. Too high extraction temperature and the presence of water can cause hydrolysis, thermal or Maillard reactions, leading to the formation of new compounds, so-called artifacts. Rivellino et al. identified hydroxymethylfurfural, methylfuran and furfural, which were considered as artifacts in honey samples (Rivellino et al., 2013). During monitoring of reactions and changes in food samples, it is extremely important to detect and identify artifacts, to avoid misclassification of them as unique chemical indicators of botanical or geographical origin. Considering the above aspects, attention should be paid to the sample preparation and extraction process. However, due to the use of appropriately selected and low aggressive extraction conditions, SPME may be an ideal coupling to complementary two-dimensional gas chromatography in food metabolic studies.

Another aspect of the honey analysis is quality control and authentication studies. The most common types of honey adulterations are: water or sugar addition, feeding bees with sugars or artificial honey as well as admixture of honey of different botanical or geographical origin. This last aspect is of particular importance for products with PDO. In order to distinguish between Corsican and non-Corsican honey, 374 samples, including 219 samples from Corsica and 155 samples from other European countries were analyzed. After profiling each sample using the GC × GC-TOFMS technique, it was found that it is possible to distinguish them in terms of botanical origin. In addition, due to a large number of detected chemical compounds, 26 markers with the highest discrimination efficiency were selected. Four different supervised methods were tested, namely linear discriminant analysis (LDA), soft independent modeling of class analogies (SIMCA), discriminant partial least squares (DPLS) and support vector machines (SVM) (Stanimirova et al., 2010). All models except SIMCA showed high classification efficiency. Both LDA and DPLS based models were characterized by high sensitivity and specificity, however, SVM achieved the best prediction capabilities, where the classification efficiency was as high as 91.5%. Čajka et al. used one supervised chemometric model - artificial neural networks with multilayer perceptrons (ANN-MLP) (Cajka et al., 2009). Therefore, it was possible to distinguish Corsican honey from other European honeys. The prediction and classification capability of this model was 94.6% and 96.5% respectively. Moreover, it was possible to distinguish Corsican honey markers, which is presented in Fig. 3.

The main components of honey are carbohydrates, such as monosaccharides, disaccharides and oligosaccharides. Due to the fact that the disaccharides form a very complex mixture of isomers, their complete separation using one-dimensional gas chromatography is difficult to achieve. For this reason, Brokl et al. used the GC × GC-TOFMS to determine content of disaccharides in honey (Brokl et al., 2010). For the analysis of disaccharides, derivatization was carried out, i.e., followed by trimethylsilylation. In this way, disaccharides have been transformed into derivatives of trimethylsilyl oximes (TMSO). During the tests, an atypical set of chromatographic columns was used, because the first column was characterized by moderately polar stationary phase and the second column was non-polar, which enabled the separation of most of the saccharide derivatives. Thanks to this approach, it was possible to separate most disaccharides except for maltose E and Z, nigerose E and turanose 1 and 2. Mayadunne et al. used the GC × GC-TOFMS technique to characterize amino acids (AAs) (Mayadunne et al., 2005). In this case, honey samples were derivatized with alkyl

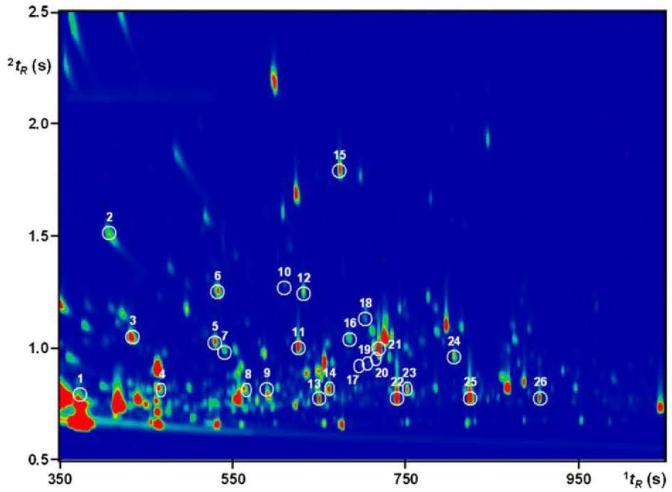

Figure 3 Volatile markers of corsician honey (marked as numbers) determined using HS-SPME–GC × GC–TOF-MS by Cajka et al. *For further details (table with markers) see (Cajka et al., 2009).*

chloroformate. Then a set of columns with a polar and non-polar stationary phases was selected. The main detected AAs in honey were proline, phenylalanine and aspartame acid. Using the proposed method it was possible to obtain very low LODs, even 0.01 mg/L for alanine, phenylalanine, isoleucine and serine.

Tea Foodomics Studies

Tea, actually *Camellia sinensis* infusion, is one of the most popular drinks and is valued for its exceptional taste, however, the authors may not be objective with this opinion. In addition, its numerous pro-health attributes, e.g., antioxidizing properties have been documented (Du et al., 2014). Depending on the processing procedure, tea can be classified into six main types: green, white, oolong, black, dark and yellow. Each of them has a different composition of both volatile and non-volatile metabolites. Taking care of the production procedure usually translates also into the quality and thus the price of tea, for example, the price of green tea ranges from $1 to even $100 per 100 g (Pongsuwan et al., 2007). Because of the above-mentioned reasons, there is a constant need for tea analysis. Foodomics seems to be the best option, as tracking metabolic changes at each stage of tea processing may be valuable information on the quality and authenticity of tea, and knowledge of the characteristic metabolites may enable a traceable production procedure.

Green tea is the least processed and therefore the least oxidized tea (Pongsuwan et al., 2007). Because of that, it exhibits the characteristic green and floral aroma. However, in tea evaluation, attention is paid not only to its taste but also to the smell, appearance of the leaves or the color of the infusion (Jumtee et al., 2011). Therefore, the literature contains studies in which one tries to link sensory impressions with the metabolic profile of green tea (Jumtee et al., 2011; Pongsuwan et al., 2007). There are two approaches to characterizing the metabolic profile of tea: the first is profiling of metabolites in tea leaves and the second focuses on infusion.

In order to determine hydrophilic primary metabolites, a series of single-phase extractions and subsequent derivatization by methoxyamine hydrochloride and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were performed (Pongsuwan et al., 2007). Therefore it was possible to identify such primary metabolites as acids (oxalic acid, malic acid, citric acid), several amino acids (including theanine responsible for the taste of tea), sugars as well as caffeine, inositol or silane. In addition, PCA fingerprinting was used to find the pattern, but it was not possible for different tea qualities. Using PLS together with Orthogonal Signal Correction (PLS-OSC) an approach was made to predict the quality of tea. Thus, on the basis of VIP values (variable importance in the projection), it was possible to identify the most significant metabolites, i.e., quinic acid, theanine and group of sugars.

For the determination of green tea VAMs, a similar approach was used in the data processing, however, sample preparation consisted of several tea infusions, followed by hydrodistillation and extraction with dichloromethane (Jumtee et al., 2011). After drying, the concentrate was introduced into the GC-MS injector. The 20 chemical compounds were identified and then the PLS-OSC algorithm was used to classify them according to their quality. Then key compounds responsible for the prediction of tea quality based on VIP were determined for good quality tea, namely indoles, *cis*-jasmone, linalool oxide (*trans*-pyranoid), methyl jasmonate, and 6-chloroindole, and *trans*-geranyl acetone. Summarizing the presented studies of green tea metabolites, it can be noted that GC-MS can guarantee the possibly comprehensive approach in the characterization of metabolites, both primary and secondary.

Unlike green tea, white tea undergoes a light fermentation process and the leaves are processed only in two stages: withering and drying (Qi et al., 2018). The tea is then aged to develop its characteristic organoleptic properties. Aging is a slow process and takes place in natural conditions. This, in turn, determines the processing capacity of the producers, so processes to accelerate the aging of the tea are demanded. However, it is doubtful whether rapidly aged tea has the same properties as natural aged one. An attempt to evaluate the aging procedure was made using foodomics based GC-MS approach (Qi et al., 2018). In these studies, rapid aged white tea (RAWT) was incubated for 180 days under proper temperature and relative humidity conditions (45–50 °C and 15%–20% RH) (Qi et al., 2018). Natural aged (NAWT), on the other hand, was aged for 12 years. The study focused only on HS analysis and SPME was used for this purpose. Using the Venn diagram it was possible to pre-select compounds that are specific for both teas as well as compounds that are markers for a given processing method. Such NAWT markers can be 2-ethyl-1-hexanol, phenylacetaldehyde, 1-phenylethanone or 1,2-dimethoxybenzene. On the other hand, 2-ethylpyrazine may be a marker of accelerated aging. Moreover, it has been suggested that rapid aging may contribute to the decomposition of β-cyclocitral, which is responsible for the green aroma. Based on fingerprinting it has been successfully demonstrated that there are clear differences between VAMs NAWT tea and RAWT.

And finally the last example, which is the metabolic profiling of black tea. Black tea is the most processed tea and at the same time one of the most consumed one. It is estimated that it accounts for 78% of all teas consumed (Li et al., 2013). The procedure for preparing black tea is multi-stage and can be divided into plucking, withering, rolling, fermentation and drying (Wu et al., 2019). During the whole process, oxidation reaction, degradation of amino acids, sugars, organic acids and many others occur, which in the final effect give appropriate sensory properties. Recent studies have attempted to trace changes in metabolic constituents during each stage of black tea production (Wu et al., 2019). The characterization of metabolites was carried out in two stages. Non-volatile compounds were isolated after ultrasonic-assisted extraction and derivatized with MSTFA. They were then determined using DI-GC-MS. The obtained normalized peak area was an input data for the analysis, in which the same multivariate statistic tools were used as in the case of previous green tea research. On the other hand, VAMs were extracted by SPME method and their identification was performed by authentic standards and MS matching to NIST MS spectra library. In total it was possible to detect 414 metabolite peaks of non-volatiles and 47 volatiles. In order to search for a pattern in non-volatile metabolites depending on processing stage, OPLS-DA and 2D projection were used. A clear change in the composition of non-volatile metabolites between the

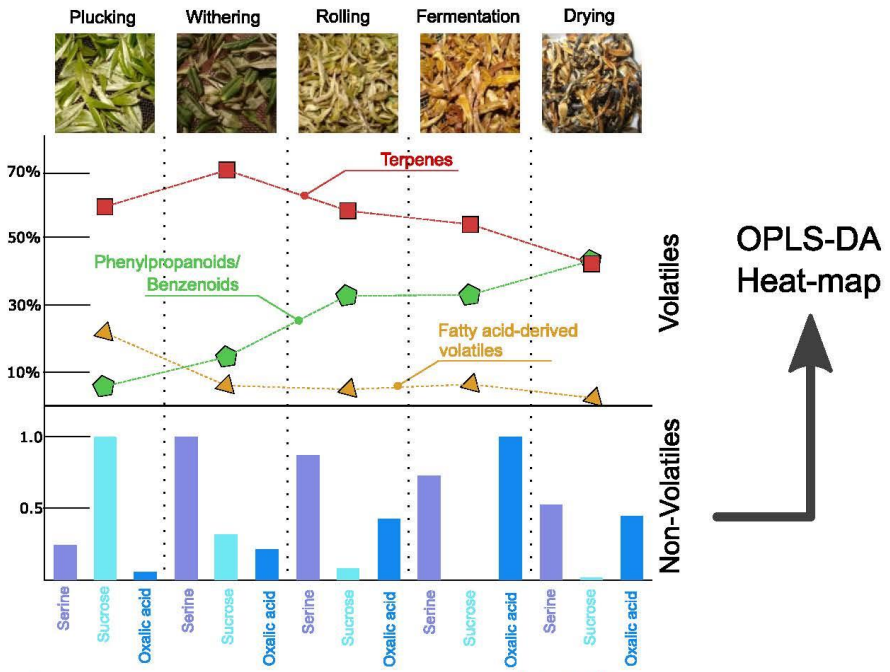

Figure 4 Graphical presentation of black tea metabolite determination performed by Wu et al. (2019). The different production stage is represented by different non-volatile primary and volatile secondary metabolites. Data matrix contained primary metabolites results was used in bioinformatics evaluation.

first four stages has been observed, while the differences between fermentation and drying are no longer so clear. Subsequently, for data reduction, the amount of non-volatile metabolites was limited to 62 using VIP values. The compounds which concentration decreased with the subsequent stage (catechins, sucrose) or increased (fructose, galactose) were identified by tracking individual stages. In turn, volatile metabolites were divided into biosynthetic pathways, namely fatty acid derivatives, terpene derivatives, phenylpropanoid/benzoid derivatives and carotenoid derivatives. The relationship between volatile and non-volatile metabolites at each stage of tea production was determined. A graphical summary of selected results from this study is presented in Fig. 4.

Organic Food Studies

The popularity of organic food and beverages is steadily increasing, as more and more consumers are interested in less processed food products with a simple ingredient list and with a decreased number of additives. Organic production is seen as healthier and less detrimental to the environment than conventional and thus, consumers often prefer it over more commercially available alternatives, even though it is usually more expensive and more difficult to obtain. However, there is still not enough information concerning the possible differences between food produced in conventional and organic conditions, therefore it is difficult to evaluate whether consumption of e.g., organic vegetables or dairy may be healthier than in the case of their nonorganic counterparts (Vallverdú-Queralt and Lamuela-Raventós, 2016).

There are numerous studies in which both targeted and untargeted analysis have been performed in order to evaluate how different agricultural practices may affect the metabolic profile of the food. However, it was established that quality of fruits and vegetables is influenced by a wide range of variables, such as climate, soil type, genetic differences of cultivars, post-harvest care or irrigation practices (Barros et al., 2010; Shepherd et al., 2014). Hence, the studies on organic and conventionally farmed food should be performed in controlled conditions, where these variables can be controlled, at least to the same extension.

Shephard et al. (Shepherd et al., 2014) performed a study in which they applied foodomics (namely, LC-MS and GC-MS coupled with chemometrics) in order to evaluate whether it is possible to differentiate between potatoes grown under the conventional and organic regime of crop protection and fertilization. Potatoes (*Solanum tuberosum* L.) were grown on a uniform land over a period of 2

years. They were subjected to two different crop protection regimes. Moreover, two different fertilizers were applied: composed cattle manure, which is a standard method for organic potato crops and ammonium nitrate, usually used for conventional, commercially-available crops.

With the application of gas chromatography-mass spectrometry approach, 93 polar metabolites were detected in the potato tubers extract during the experiments. Based on the results, it can be concluded that there were consistent differences in the concentration of the amino acids, with it being lower in organically grown potatoes as compared to those grown under conventional conditions. In addition, it was found that conventionally grown tubers exhibit approximately two-fold nitrogen content organic tuber exhibit of that measured in their organic counterparts. Shephard et al. discovered that the concentration of 83 identified polar metabolites was significantly different, with 63 out of them being present at lower levels in organic samples (Shepherd et al., 2014). With the use of GC-MS coupled with chemometrics, it was possible to distinguish between samples treated with different fertilizers, however, the differences between samples treated with different crop protection agents were less consistent and less prominent.

Foodomics in Transgenic Food Analysis

Genetic engineering is one of the trends which popularity is growing fast, as its application makes it possible to increase plants tolerance for herbicides, resistance to droughts or insects without decreasing food value. However, due to the risk of potential occurrence of unintended effects of genetic modification, as well as the existence of overall controversy related to recombinant DNA technology, there is a constant need for the analysis of both genetically modified organisms (GMOs) and food products that contain or derive from them (so-called transgenic food). Accepted strategy for the evaluation of transgenic food safety is to compare it with commercially available, non-modified food, as its consumption is generally assumed to be safe. However, since there is no one compound which presence may indicate that the consumption of food in question is unsafe, so no targeted analysis can be sufficient for establishing the GMOs' safety. Because of that, the European Food Safety Agency (EFSA) recommended the use of profiling technologies that could be applied in the broad, comparative analysis (EFSA, 2006). Foodomics can be a valuable tool for this analysis, as it implements various tools such as metabolomics, genomics, proteomics, lipidomics or transcriptomics and thus, offers complex information about modified organisms (Ibáñez and Cifuentes, 2014).

Roessner et al. (Roessner et al., 2000) applied GC-MS to perform quantitative analysis of a broad range of metabolites extracted from potato tubers. Based on the obtained data it was possible to recognize significant differences between modified and traditional, soil-grown potatoes, even though based on the data obtained previously with the different analytical systems they were deemed highly comparable. Moreover, 77 detected compounds were identified with the use of MS libraries, which provided valuable information concerning altered metabolic pathways in genetically modified potatoes. In addition data concerning unforeseen changes in some compounds concentration have been obtained, which further prove the need to conduct an untargeted analysis of GMOs. In another study, Linke et al. (Linke et al., 2007) demonstrated that GC-MS coupled with chemometrics, namely PCA and HCA, enables discrimination between modified and non-modified potato tubers extracts. Jiao et al. (Jiao et al., 2010) applied the combination of GC-MS coupled with chemometrics, near-infrared spectroscopy and high-performance liquid chromatography to identify unintended metabolic changes in transgenic rice and found that they exhibit different concentration of several amino acids and vitamins than their commercially available counterpart. Zhou et al. (Zhou et al., 2009) performed a similar study on genetically modified rice and have also found in it that the level of multiple species has prominently increased.

Another worth-mentioning approach to the application of foodomics is to identify the differences in metabolism caused by natural biological variation of metabolites in order to correctly differentiate between genetically modified and non-genetically modified species. Even though appropriately chosen data analysis techniques facilitate recognizing the changes caused by genetic engineering, this approach further reduces the risk of false discoveries. Tang et al. (Tang et al., 2017) analyzed 21 genetically diverse non-transgenic maize hybrids in order to assess the influence both the environment and genotype have on the metabolite in their forage and grain as well as to evaluate, which of them would be more susceptible to unintended metabolic changes caused by genetic modifications. In another study, metabolite profiles of transgenic maize kernels grown in Germany and South Africa were analyzed in order to identify possible causes of their variability (Frank et al., 2012). Based on the obtained results it was concluded that the metabolic changes are most likely related to natural variability and not caused by genetical modification, as the differences between maize grown in different countries were more pronounced than those between modified and non-modified samples. In another study, Barros et al. (Barros et al., 2010) analyzed transcriptome, proteome and metabolome of transgenic maize using cDNA microarray, two-dimensional gel electrophoresis, ^{1}H-NMR fingerprinting and GC-MS. Based on the obtained results, they also concluded that the differences caused by environmental factors (location, year etc.) are far more pronounced than those caused by genetic engineering. It is also worth mentioning that using several omics technologies is a relatively novel approach to foodomics that is steadily gaining popularity as with its application it is possible to obtain the data concerning a broad range of compounds with different properties and possible relationships between their presence or concentration in food.

Gas chromatography coupled with mass spectrometry was also applied in the profiling of aroma compounds found in transgenic fruits and vegetables. For example, Zawirska-Wojtasiak et al. (Zawirska-Wojtasiak et al., 2009) analyzed aroma compounds of transgenic and typical cucumbers using GC-TOFMS and GC-MS. Based on their results, it was possible to conclude that the levels of volatile compounds, particularly those seen as responsible for cucumber fruits aroma, are higher in genetically modified fruits.

Flavoromics and Sensomics

Investigations on flavor and aroma are great challenges for researchers, as the identification and determination of chemical compounds go together with an understanding of the ways in which these compounds affect the human senses of taste and smell. Flavor studies have been known for a long time in food analysis, but only the latest approaches, namely foodomics and sensomics guarantee a comprehensive approach to the identification of key flavor and aroma compounds. One of the assumptions for both concepts is that flavor and aroma sensations result not only from the presence of volatile compounds but also due to the presence of non-volatile substances as well as the type of matrix from which the volatile compounds are released. It should be noted that in both these approaches the core of the actions is the use of non-targeted profiling of aromatic compounds using GC-MS.

Flavoromics is based on the use of such analytical tools as metabolomics, bioinformatics and multivariate analysis together with advanced analytical equipment for the most comprehensive, non-targeted study of the chemical composition of food and for juxtaposition with sensory quality investigations (Charve, 2011). As the example, this approach was proposed in the study of the taste of three cultivars of Tasconia fruit, in which the data from GC-MS were compared with the results of gas chromatography-olfactometry (GC-O) (Martín et al., 2018). One of the most advanced coupling olfactometry and GC-MS was presented by Chin et al. where integrated GC × GC/MDGC-O/MS system was used (Chin et al., 2015). In a single analysis, it was possible not only to identify wine key aroma compounds but also to define their aroma, for instance, octen-3-ol was assigned as earthy odor, ethyl acetate as fruity or butanoic acid as sweaty odor. All sensitivity losses caused by splitting the flow into two systems were compensated by sample enrichment on SPME fiber. A diagram of the apparatus including an explanation of its main components is shown in Fig. 5.

Other examples are the differentiation of the characteristic flavor notes of Gouda, Cheddar and Parmigiano-Reggiano cheeses (Ochi et al., 2012), detection of off-flavor compounds in banana Terra spirit (Capobiango et al., 2015), profiling of aroma descriptors of different jackfruit (Ong et al., 2008) or melon cultivars (Verzera et al., 2011). However, this type of approach has one limitation - it is assumed that the taste of food is determined solely by volatile compounds. It is worth noting, however, that aroma compounds are only a component in the perception of food taste (Ronningen, 2016). Other important elements of taste are non-volatile compounds, for example, chemesthetic compounds (Charve, 2011). In short, they are irritants that change our perception of taste and can include capsaicin (burning), menthol (coolness) or carbon dioxide (tingling) (Green, 1996). An ideal example of flavoromics are studies on the taste of orange and mandarin juices, in which GC-MS and UHPLC-MS studies together with chemometrics have been combined (Charve et al., 2011; J. I. M. Charve, 2011).

Sensomics, in turn, differs from most of the procedures used in foodomics, as advanced data analysis is rarely used to interpret the results. However, the proposed solution guarantees a detailed and comprehensive analysis of the aroma of food. The concept of sensomics was developed by the Schieberle research group (Schieberle and Hofmann, 2012). It is possible to distinguish the following steps necessary to determine the compounds that are the main contributors to food aroma. The first step is to use aroma extract dilution analysis (AEDA) to pre-identify potential key aroma compounds. It should be emphasized that the use of AEDA itself only serves the purpose of selecting compounds, as their actual impact on the aroma is strictly dependent on the composition of the matrix, which is not taken into account in this method. After the determination of flavor dilution (FD) factors, the list of potential key aroma compounds is limited and the concentration of volatile compounds is determined using GC-MS together

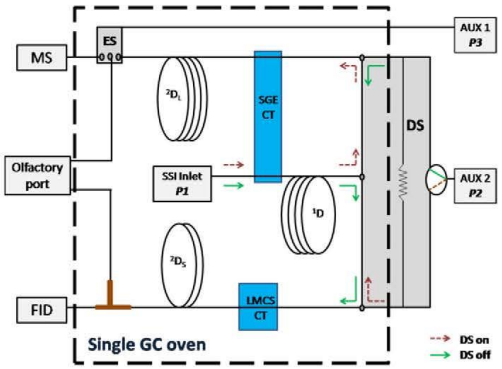

Figure 5 The system contains 1D-GC, heart-cut MDGC, and GC × GC coupled with olfaction, FID detector and MS. Split/splitless injection port was connected with the 1D column, while both 2D columns were connected to the deans switch (DS). When DS was on, effluent from the first column was transferred to the long 2D column, and then analytes were directed through the ES device the MS detector and the olfactory port. Whereas with DS off, the effluent from the first column was transferred to the short 2D column, then through the Y connection to the FID detector and the olfactory port; DS: Deans switch; ES: effluent splitter; AUX: auxiliary pressure port; 1D: first dimension column; 2DL: second dimension long column; 2DS: second dimension short column; CT: cryotrap; SSI: split/splitless injector; FID: flame ionization detector. *Scheme of integrated GC× GC/MDGC-O/MS system according to Chin et al. (2012).*

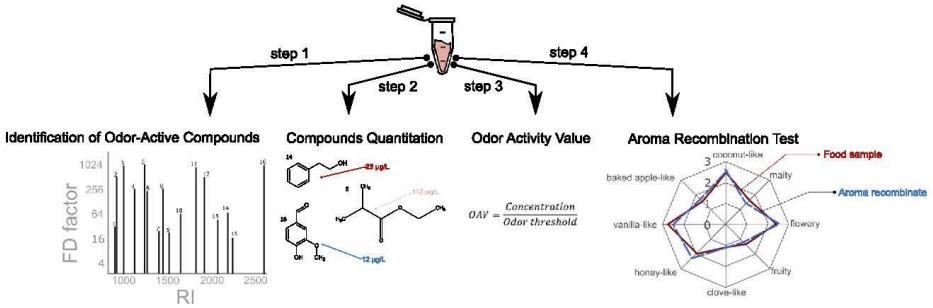

Figure 6 General workflow of sensomic approach.

with stable isotope dilution assays (SIDA; preferable). When the odor threshold of these compounds is determined and the odor activity value (OAV) calculated, the main compounds responsible for food odor can be selected. It is vital to determine the odor threshold of compounds dissolved in the medium similar to the matrix because the aroma release is very dependent on that (Uselmann and Schieberle, 2015). Any compound with an OAV above 1 denotes that it can be detected by the human sense of smell. The general procedure in senosmics is presented in Fig. 6. A particularly important stage is the aroma reconstruction stage, which plays the role of validation of the obtained results and also reveals the important role of the matrix in the release of aroma. Examples of the use of sensomics in the analysis of food aroma are presented in Table 2.

Pesticides, Endocrine Substances and Their Derivatives

Food quality and safety assessment are one of the most important fields of modern analytical chemistry, as it is important to assure both the health and well-being of the consumers. In particular, the application of mass spectrometry, often coupled with gas chromatography and chemometrics had a non-negligible impact on the field of food quality evaluation (Inoue and Toyo'oka, 2015b), e.g., determination of multiple residues of food contaminants, such as pesticides, endocrine disruptor compounds (EDCs) or microbial metabolites, which presence in food may cause adverse effects on the human body.

Zhiqiang et al. used GC-MS for multi-residue analysis of 102 pesticides in teas and were able to determine them even in low sub-ppb range (Zhiqiang et al., 2007). However, sample preparation step was rather time- and labor-intensive, as it consisted of extraction, filtration and purification by the means of gel permeation chromatography. Worth mentioning modification of this approach was used by Xu et al. (Xu et al., 2009) in order to determine 205 pesticides in fresh fruits, vegetables and beans. They applied large-volume injection (LVI) in order to further increase the efficiency of the GC-MS method and as a result, the method's limit of quantification was in the range of 0.5–600 μg/kg.

Cunha et al. 27 different pesticides in grapes, wines and must with good repeatability and with the time of the analysis not exceeding 20 min (Cunha et al., 2009a). In a different study, Spoznikova and Lehotay coupled low-pressure gas chromatography with tandem mass spectrometry in order to perform a multi-residue analysis of catfish samples (Sapozhnikova and Lehotay, 2013). They were able to simultaneously determine the concentration of 13 flame retardants, 18 pesticides, 16 polycyclic aromatic hydrocarbons, 7 polybrominated diphenyl ether and 14 polybrominated diphenyl ether congeners with a time of analysis being less than 10 minutes.

Walorczyk applied GC-MS/MS in order to establish whether it can be used in simultaneous analysis of 122 pesticides in dry food (Walorczyk, 2007). Based on the obtained results it was possible to conclude that with the use of this method, not only cereal grains, bran, maze and other dry food, but also by-products of oil manufacture can be analyzed with satisfying results and thus, it may find its application in the food industry. Przybylski et al. demonstrated that the modified version of this sample preparation method can be applied in the analysis of meat-based baby food by coupling gas chromatography to ion trap mass spectrometry (GC-IT-MS) (Przybylski and Segard, 2009). Using this approach, they were able to detect and quantify 236 pesticides and their metabolites in a relatively short time, which suggest that this method may find its application in routine analysis of food.

Mondello et al. stated that to separate and identify target contaminants from hundreds of interfering analytes in case of food samples, the best solution is certainly GC × GC-MS (Mondello et al., 2007). They analyzed a pesticide-contaminated red grapefruit extract to prove the effectiveness of a dual-filtered library search procedure (the first based on a linear retention indices (LRI) and the second on a minimum degree of spectral similarity) which enables a more reliable identification of experimental MS spectra.

Zrostlíková et al. carried out a study in which the twenty-four pesticides with a broad range of physico-chemical properties in fruit samples were compared. It was found that the LOD increased 1.5–50 times in the case of GC × GC comparing to GC-MS. Separation from matrix co-extracts was also achieved what allowed for reliable confirmation of pesticide residues at very low concentration level – LOD below 10 ng/mL (Zrostlíková et al., 2003).

Table 2 Selected key odorants determined using sensomics approach with their aroma descriptor and odor activity value

Sample	*Key odorants*	*Descriptor*	*OAV*	*References*
Bavarian wheat beer[a]	(E)-β-damascenone	Cooked apple-like	325	Langos et al. (2013)
	3-Methylbutyl acetate	Fruity, banana-like	231	
Cherry wine[a]	Ethyl hexanoate	Apple peel, fruity	86.7–162.7	Xiao et al. (2017)
	Ethyl octanoate	Fruity, fatty	46.2–164.0	
Chinese liquor[a]	Ethyl octanoate	Fruity	391–1500	Zheng et al. (2016)
	1,1-Diethoxyethane	Fruity	102–267	
	β-damascenone	Floral, honey	96–238	
	Ethyl acetate	Pineapple	65–99	
Cogniac[a]	(E)-β-damascenone	Cooked apple-like	2357	Uselmann and Schieberle (2015)
	Methylpropanal	Malty	850	
	Ethyl (S)-2-methylbutanoate	Fruity	195	
	Ethyl methylpropanoate	Fruity	120	
Icewine[a]	β-damascenone	Honey	5580.0	Ma et al. (2017)
	1-Octen-3-one	Mushroom	271.8	
	2,3-Butanedione	Cream	95.8	
	Ethyl hexanoate	Apple peel	94.9	
Pear brandy[a]	(E)-β-damascenone	Cooked apple-like	1800	Willner et al. (2013)
	Ethyl (S)-2-methylbutanoate	Fruity	1700	
	(S)-2-methylbutanoic acid	Sweaty, fruity	710	
Rapeseed oil	2-Isopropyl-3-methoxypyrazine	Earthy, pea-like	330[1]/290[2]/141[3]	Matheis and Granvogl (2016a,b), Pollner and Schieberle, (2016)
	Dimethyl trisulphide	Cabbage-like	37[1]/2900[2]	
	Ethyl 2-methylbutanoate[b]	Fruity	150[2]	
	2-Isobutyl-3-metoxypyrazine[b]	Bell pepper-like	92[2]	
Rum[a]	2,3-Butanedione	Butter-like	11–220	Franitza et al. (2016)
	3-Methylbutanal	Malty	12–110	
	Ethyl butanoate	Fruity	8–110	
Sheryy wine [a]	1,1-Diethoxyethane	Fruity	2475	Marcq and Schieberle (2015)
	2- And 3-methylbutanals	Malty	574	
	Methylpropanal	Malty	369	
Sour guava	Ethyl hexanoate	Fruity	7574	Cuadrado-Silva et al. (2017)
	(Z)-3-hexenal	Green	1132	
	Linalyl butyrate	Sweet	341	
Truffle	bis (methylthio)methane[WAT]	Sulfury, garlic-like	817000	Schmidberger and Schieberle (2017)
	2,3-butanedione[BT]	Buttery	1130	
	3-Methylbutanal	Malty	2140	
	3,4-Dihydro-2(H)-pyrrol	Amine-like, sperm-like	1530	

[a]Ethanol does not take into account; [b]Off-flavor compounds; [WAT]Key odorant of white Alba truffle; [BT]Key odorant of Burgundy truffle; [1]Matheis and Granvogl (2016a); [2]Matheis and Granvogl (2016b); [3]Pollner and Schieberle (2016).

Pesticides are not the only chemicals that occur in food at the trace level and can adversely affect the human body. The second group of chemical compounds with unfavorable influence on health, often detected in food are EDCs. This group includes polychlorinated dibenzodioxins (PCDD), polychlorinated biphenyls (PCB), phthalates and alkylphenols. These are chemical compounds which presence in food affects the proper functioning of the endocrine system, which in the result can have negative effects on the body. According to the US Environmental Protection Agency (EPA), endocrine disruptors are factors that interfere with the production, release of transport, metabolism, binding, action or elimination of natural hormones in the body (Diamanti-Kandarakis et al., 2009).

The use of two-dimensional gas chromatography for the determination of EDCs in food samples requires proper preparation of samples for analysis. Techniques commonly used for this purpose are ASE, GPC, DSPE and DLLME. Choosing the right method of sample preparation is a demanding issue in ECDs investigation, especially in the analysis of very fatty food, because using organic solvents when extracting lipophilic samples, several contaminants may also be co-extracted, as well as part of the matrix, which in turn causes that extraction is not selective. Moreover, the presence of salts and amino acids in the sample, as well as highly concentrated substances, can cause the suppression, which causes that trace level compounds may not be detected. Consequently, the determination of the analytes must be preceded by the purification of the obtained extract. The difficulty of analysis of fatty foods for residue compounds has been recently reviewed by Gilbert-Lopez et al. (Gilbert-López et al., 2009).

The literature contains information on the determination of EDCs in many food matrices. Examples of the use of the GC × GC technique for the determination of endocrine compounds in food samples have been compiled in **Table 3**. Planche et al. used a GC × GC technique for the determination of PCBs, PCDD/Fs in a sample of ground beef (Planche et al., 2015). To be able to determine these compounds present in trace level in a complex and high fat matrix such as ground beef, 2D GC-MS method

Table 3 Selected examples of the application of GC × GC-MS in pesticides and EDCs determination in food samples

Matrix	*Group of EDCs*	*Sample preparation*	*Compounds no*	*LOQ/LOD*	*References*
Ground beef	PCBs, PCDD/Fs	ASE, GPC	206	LOD: 50–100 pg/g for PCBs and 65–227 pg/g for PCDD/Fs	Planche et al. (2015)
Milk and cream	POPs	GPC, SPE	34	LOQ: 0.2–0.4 μg/kg ww	Hayward et al. (2010)
Grape and wine	Pesticides, PAHs, PCBs, bishpenol A	DSPE	160 pesticides and 12 PCBs, 12 PAHs, bisphenol A	LOQ: 12.5–25 μg/L	Dasgupta et al. (2010)
Cereal-based product	Pesticides	GPC, SPE	100	LOQ: 1–20 μg/kg	Van Der Lee et al. (2008)
Apple juice	Pesticides	DLLME	24	LOD: 0.06–2.20 μg/L.	Cunha et al. (2009b)
Tea	Pesticides	HS-SPME	36	LOQ: 1–28 μg/kg	Schurek et al. (2008)

was coupled to accelerated solvent extraction. As a result, researchers detected 206 ECDs, reaching the limits of detection at the level 50–100 pg/g for PCBs and 65–227 pg/g for PCDD/Fs. Hayward et al. carried out an analysis of milk and cream to determine Persistent Organic Pollutant (POPs) (Hayward et al., 2010). Due to the fat-rich matrix, in this case the GPC technique was also used to prepare samples for analysis.

The analysis of endocrine compounds is performed on a large scale in wines. These contaminants can get into the wine from the packages, primarily from the cork used for the production of wine stoppers, as well as from the as a result of using plant health products during viticulture. Dasgupta et al. used two-dimensional gas chromatography with time of flight mass spectrometry do for the simultaneous analysis of 160 pesticides, 12 dioxin-like polychlorinated biphenyls (PCBs), 12 polyaromatic hydrocarbons (PAHs) and bisphenol A in grapes and wine (Dasgupta et al., 2010). They presented the possibility of false negatives reduction with the lower detection limits offered by GC × GC and the well-resolved peaks allowing for improved identification capability.

Other Foodomics Examples

Both GC-MS and GC × GC-MS have found many other worth-mentioning application in the field of foodomics, in particular in the subject of various foods quality evaluation. An interesting example of GC-MS use for food quality assessment is the analysis of mono- and sesquiterpenes in ricotta cheese. Due to the fact that the composition of the flora and thus, the cattle's diet depends inter alia on the geographical location of pasture (Giuseppe et al., 2005), the presence of certain compounds (e.g., terpenes) in cheese is strongly affected by the type of the substances ingested by animals which milk is used for dairy production. Because of that, it was possible to determine at which season of the year the cheese was produced and to indicate each mountain pasture.

Another example of foodomics application is an analysis of rice. Kusano et al. performed the metabolic phenotyping of 70 varieties of brown rice (Kusano et al., 2007). For this purpose, rice grains were derivatized with methoxyamine hydrochloride, followed by trimethylsilylation of N-methyl-N-trimethylsilyl trifluoroacetamide with trimethylchlorosilane. The obtained solutions were analyzed using 1D GC-TOFMS and 2D GC-TOFMS, and the data were subjected to chemometric analysis using the principal component analysis and the partial least-squares discriminant analysis (PLS-DA) model. Based on chemometric analyzes, 10 metabolites (GABA, glycerol-3-phosphate, myristate, fructose, IAA, inositol-1-phosphate, trehalose, alpha-tocopherol, cholesterol, and raffinose) were selected that distinguish individual varieties of brown rice. In the literature there is plenty of worth-mentioning foodomics examples like metabolite profiling of fermented soybean paste during its fermentation process (Park et al., 2010), study of polar and non-polar metabolites of cultivated potatoes (Dobson et al., 2010), fingerprinting of meat microbial spoilage (Xu et al., 2010; Zhang et al., 2010) or foodomics studies on fruit analysis (Lubinska-Szczygieł et al., 2018; Ristic evic et al., 2012; Rocha et al., 2007).

Summary

In this article, the applications of mass spectrometer coupling to 1D and 2D gas chromatography in foodomics have been summarized. It should be emphasized, however, that these examples are only a small part of a rapidly developing trend, which in the coming years will only gain in popularity. The tendency to use solvent-free extraction methods and the related term 'green foodomics' (Gilbert-López et al., 2017), the use of automated systems and the development of foodomics platforms combining various analytical techniques can already be observed. The authors predict that in the nearest future the development of GC-MS application will be aimed at miniaturization and shortening the time of analysis (Fast-GC solutions), which could be beneficial for tracking changes in emissions of volatile primary metabolites in food. However, it is not foreseeable which direction food analysis will take, but we are confident that gas chromatography coupled with mass spectrometry will continue to be an integral part of it.

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8 List of scientific achievements

a) JCR-listed publications

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b) Chapters and articles in non-JCR listed journals,

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19. M. Rutkowska, J. Płotka-Wasylka, M. Lubinska-Szczygeł, A. Różańska, J. Możejko-Ciesielska, Justyna Namieśnik, Ptasie pióra jako biowskaźniki do uzyskiwania informacji o stanie środowiska, *Anal. Nauk. I Prak*t. 2 (2020) 26–33. MNISW (2020): 5.

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- A. Różańska, M. Lubinska-Szczygeł, T. Dymerski, J. Namieśnik, Classification of adulterated raspberry juice using ultra-fast gas chromatography, K. Nesměrák (Ed.), *Proc. 14th Int. Students Conf. 'Modern Anal. Chem*., Prague, Czech Republic, 2018: pp. 253–257.

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- M. Lubinska-Szczygeł, A. Różańska, T. Dymerski, J. Namieśnik, Study on the effect of steaming on the aromatic and pro-health properties of *Momordica charantia*, 2nd Food Chemistry Conference, Sevilla, Spain, September 2019.

- A. Różańska, M. Lubinska-Szczygeł, T. Dymerski, J. Namieśnik, The effect of chokeberry juice addition on raspberry juice aroma and pro-health properties, 2nd Food Chemistry Conference, Sewilla, Hiszpania, September 2019.

- A. Różańska, M. Lubinska-Szczygeł ,T. Dymerski, J. Namieśnik, The authenticity assessment of bilberry juice using GC×GC-TOFMS, XX EuroFoodChem Congress, Porto, Portugal, June 2019.

- M. Lubinska-Szczygeł**,** A. Kuczyńska-Łażewska, M. Rutkowska, Ż. Polkowska, E. Katrich, and S. Gorinstein, Kaffir Lime (*Citrus Hystrix*) Peel As A Source Of Functional Ingredients. XXXI International Conference On Polyphenols, Nantes, France, July 2023.

e) Academic internships and scientific missions

- April 2019 Israel, Hebrew University of Jerusalem, training trip as part of the "Erasmus + mobility of students and university staff with partner countries"

- July2019 Israel, Hebrew University of Jerusalem, training trip as part of the "Erasmus + mobility of students and university staff with partner countries"

f) Scholarships

- The Scholarship for Outstanding Scientific Achievements of the Minister of Science and Higher Education
- Gdańsk University of Technology Rector's Scholarship for best PhD Stu-dents
- European Union, "International scholarship exchange of doctoral students and academic staff", project number POWR.03.03.00-IP.08-00-P13 / 18, implemented as part of Measure: 3.3 Internationalization of Polish higher education, PO WER
- Scholarship from the Gdańsk University of Technology Development Program POWR.03.05.00-00Z044 / 17
- European Union, Project "Development of an interdisciplinary program of doctoral studies with an international dimension" co-financed by the European Union under the European Social Fund - Operational Program Knowledge

g) Resaerch projects

- Project „Determination of the metabolic pathway of selected terpenes in citrus fruits using the PTR-TOFMS technique„, (number of the application 201/31/N/NZ9/03255) financed by National Science Centre, Poland in a program „PRELUDIUM 16", project Manager
- Project „Antioxidant Power Series as a tool rational design and assessment of health-promoting properties of functional food based on antioxidant phytochemicals" (number of the application 2014/14/A/ST4/00640) financed by National Science Centre, Poland in a program „MAESTRO 6", project Researcher

h) Cooperation with foreign scientific units

- Israel, Hebrew University of Jerusalem, cooperation in realization of the "Erasmus + mobility of students and university staff with partner countries" and Antioxidant Power Series as a tool rational design and assessment of health-promoting properties of functional food based on antioxidant phytochemicals" (number of the application 2014/14/A/ST4/00640) financed by National Science Centre, Poland in a program „MAESTRO 6" programs.

Table 11 Individual contributions to co−authored publications

Publications	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	11	12	13	14	15	16	17	18	19
Author Contributions	**JCR publications**																	**Chapters non-JCR publications**	
collection of samples	**−**	**+**	**+**	**−**	**−**	**+**	**−**	**−**	**−**	**−**	±	−	na	na	±	−	−	**na**	na
sample analysis	**±**	**±**	**±**	**±**	**±**	**+**	**±**	**−**	**±**	**−**	−	−	na	na	±	±	±	**na**	na
conceptualization	**±**	**+**	**+**	**±**	**−**	**+**	**±**	**±**	**−**	**−**	±	−	−	−	−	±	±	**−**	−

statistical analysis	**±**	**±**	**±**	**±**	**±**	**+**	**±**	**±**	**±**	**−**	±	−	na	na	±	−	−	**na**	na
interpretation of results	**±**	**±**	**±**	**±**	**±**	**+**	**±**	**±**	**±**	**±**	±	±	±	±	±	±	±	**±**	
results description	**±**	**±**	**±**	**±**	**±**	**+**	**±**	**−**	**−**	**±**	±	±	±	±	±	±	±	**±**	±
preparation of tables and figures	**±**	**±**	**±**	**±**	**±**	**+**	**±**	**±**	**−**	**±**	±	−	±	±	−	−	−	**±**	±
manuscript writing	**±**	**±**	**±**	**±**	**±**	**+**	**±**	**±**	**±**	**±**	+	±	±	±	±	±	±	**±**	±

Na- not applicable

± partial contribution to the action

+ full participation in action

− no involvement in the action

publications used in the doctoral thesis are in bold