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Flavor and Aroma Analysis as a Tool for Quality Control of Foods

Edited by
Ángel Calín-Sánchez and Ángel A. Carbonell-Barrachina

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About the Editors

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Ángel Calín-Sánchez received his Ph.D. on Agrofood Resources and Technology in 2014. Currently, he is a teacher of vocational education at Centro Integrado de Formación y Experiencias Agrarias de Jumilla (CIFEA de Jumilla) and an honorary researcher at Miguel Hernandez University (UMH), where he belongs the Group on Food Quality and Safety. Dr. Calín-Sánchez has authored more than 30 research articles published in international journals within the areas of food sciences and technology and agriculture. At this time his main research topics are food quality and food safety; among his main interests are use of sensory evaluation in the food industry, industrialization of pomegranate and minor crops, drying effects on fruits and vegetables, and effects of deficit irrigation on the quality of foods. He has participated in national and international projects in Spain, Poland and Germany.

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Ángel Carbonell-Barrachina received his Ph.D. in chemistry in 1995 and currently is a full professor of food sciences and technology at the Miguel Hernandez University (UMH), where he leads the Group on Food Quality and Safety. Dr. Carbonell-Barrachina has authored more than 300 research articles published in international journals within the areas of food sciences and technology, environmental sciences and agriculture. At this time his main research topics are food quality and food safety; among his main interests are use of sensory evaluation in the food industry, industrialization of pomegranate and minor crops, drying effects on fruits and vegetables, effects of manufacturing on the quality of foods, such as ice creams, confections, chocolate, etc. In 2020, he was appointed as doctor honoris causa of the Wrocław University of Environmental and Life Sciences (Wrocław, Poland). Later, in August 2020, he was named General Director of Science and Research of the Valencian Government (Spain).

Preface to "Flavor and Aroma Analysis as a Tool for Quality Control of Foods"

The aroma composition of foods has been the subject of considerable research in recent years. It is well known that the presence of volatile compounds and their compositions determine the specific aroma of foods and the flavor of the resulting products. The main chemical families present in foods are monoterpenes, monoterpenoids, and phenylpropanoids. In lower amounts, alcohols, sesquiterpenes, sesquiterpenoids, aldehydes, and esters are also found. The composition and concentrations of volatile compounds depend on many factors, including climatic and soil conditions, seasonal variation, agronomical practices, processing, etc. On the other hand, sensory analysis is used to quantitatively determine the intensities of the main sensory properties and attributes of food as well as determine the preferences for foods. Such analysis requires the use of a trained panel and regular consumers.

The present Special Issue is aimed at gathering outstanding cross-disciplinary approaches applying the combination of instrumental analysis (volatile compounds) and sensory analysis as tools for quality control of foods as affected by both agronomical factors and processing conditions in order to provide very valuable information to farmers and manufacturers.

The editors appreciate the efforts of all the authors who participated in this Special Issue.

Ángel Calín-Sánchez, Ángel A. Carbonell-Barrachina
Editors

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Editorial

Flavor and Aroma Analysis as a Tool for Quality Control of Foods

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The aroma composition of foods has been the subject of considerable research in recent years. It is well known that the presence of volatile compounds and their composition determine the specific aroma of foods and the flavor of the resulting products. The composition and concentrations of volatile compounds depend on many factors including climatic and soil conditions, seasonal variation, agronomical practices and processing. On the other hand, sensory analysis is used to quantitatively determine the intensities of the main sensory properties and attributes of food as well as determine the preference. Such analysis requires the use of a trained panel and regular consumers.

Food quality constitutes the main concern of regular consumers with regard to sensory quality and economic factors. Farmers and manufacturers have made great efforts to achieve consumers' considerations; however, the requirements in terms of production optimization due to environmental deficiencies and energy saving still need the role of science and innovation. Accordingly, and within the idea and objective of bringing together original studies dealing with the aroma profile and sensory quality of foods, we edit this Special Issue on "Flavor and Aroma Analysis as a Tool for the Quality Control of Foods". From the twelve published papers, four main research topics were covered: (i) the effect of agricultural practices, (ii) the effect of different processing and conditions, (iii) the characterization of foods and (iv) consumer perception and acceptability.

In the first topic dealing with the effect of different agricultural practices on the quality of fruits, two papers can be categorized [1,2]. The first study by Noguera-Artiaga et al. [1] compares the different irrigation regimes (deficit, moderate and severe) of different pistachio cultivars and rootstocks. This study was conducted in Spain, a Mediterranean country with long periods of water scarcity. The results demonstrated that the application of a moderate deficit of irrigation during pistachio cultivation led to pistachios with the same morphological properties, total polyphenol content, antioxidant activity, volatile composition and sensory properties as pistachios obtained using full irrigation. Moreover, moderately irrigated pistachios led to the obtaining of a better profile of fatty acids and were the sample preferred by international consumers. On the contrary, when the deficit irrigation was severe, pistachio nuts had the lowest antioxidant activity, the lowest total polyphenols content and were the least preferred samples by consumers. In the case of pistachios obtained using different rootstocks, *P. integerrima* led to pistachio nuts with the highest weight, the lowest content of sucrose and better functional properties than *P. atlantica* and *P. terebinthus*. These results demonstrated that it is possible to save irrigation water in pistachio farming with a low environmental and economic cost and leading to pistachio nuts with same or even better quality attributes. The second study is by Aguilar-Hernández et al. [2], which was performed in order to determine whether the volatile profile of lemon peel oil was affected by the rootstock. The selected varieties of *Citrus limon* used in this study were "Bétera", "Verna", "Fino 49", "Fino 95" and "Eureka" grafted on to the rootstocks Forner-Alcaide Nº5 , Forner-Alcaide Nº13, Forner-Alcaide Nº517, *C. macrophylla* West and *C. aurantium* L. All of them reside in the European Union (BOE/04/12/2007)

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and were obtained by targeted hybridizations by Forner in IVIA (Valencia) [3]. The results demonstrated that the Forner-Alcaide rootstocks were the best rootstocks leading to a high content of volatile compounds followed by *C. aurantium* and *C. macrophylla*. The order of the total volatile contents was (in decreasing order): "Eureka" > "Bétera" > "Fino 95" > "Verna" > "Fino 49". These results confirmed that a strong relationship exists between the rootstock/scion combinations and the concentration of volatile compounds in the lemon peel oil. Aroma volatiles should be considered key parameters for the determination of rootstock-induced effects.

In the second topic of studies of different processing and conditions, six manuscripts have been published [4–9]. The authors provide an overview of recent advances made in the field of food processing establishing the best processing conditions in order to obtain food based products with a high quality in terms of aroma, flavor and, consequently, consumer satisfaction. The first study of this second topic by Zhang et al. [4] describes the main volatile compounds and marker substances during the concentration of coconut jam assessed by headspace solid phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS). The results showed that the concentration process of coconut jam occurs in three stages. In the early stage, alcohols and esters are responsible for the aroma, while ketones are the main compounds at the middle stage. Finally, in the final sterilization stage, a variety of aroma compounds are produced and form the unique flavor of coconut jam. The stepwise increase of the furfural content is consistent with the inflection point of the change in aroma during the whole process of coconut jam concentration. The logistic model has a higher degree of fit, which can be used as a marker of aromas to monitor the concentration of the product. However, the mechanisms of action of certain aroma compounds released from the coconut jam are still unclear. The second study by Cano-Lamadrid et al. [5] developed a confectionary product exclusively based on natural ingredients. This manuscript describes the quality parameters and consumer acceptance of jelly candies based on pomegranate juice. This study is a consequence of an upward trend towards reducing or suppressing food additives as well as reducing the use of E-numbers in labels, thus providing clean label foods. To reach this aim, the authors report that the formulation consisting of 20% gelatin with pure "Mollar de Elche" pomegranate juice, 1% citric acid and a sugar addition led to the best results in terms of color, texture, antioxidant capacity and sensory attributes. The most valued quality parameter of pomegranate products, the red color (a* coordinate), was not negatively affected by the process of preparing the jellies. The same authors claim that still this novel confectionary product must be improved and propose further investigations related to (i) hydrating gelatin in pomegranate juice instead of water and (ii) optimizing the drying step. The third study by Gasinski et al. [6] performed an assessment of volatile compounds among other determinators of beers enriched with dotted hawthorn (*Crataegus punctate*) by two ways of addition, fruit or juice. The study indicated that the addition of hawthorn led to a beer with an increased total content of polyphenolic compounds and antioxidant activity. Beer with a fruit addition was characterized by the greatest number of volatile compounds, eight times higher than in the control sample. The enrichment of the beer by hawthorn fruit mostly increased the concentration of the volatile compounds characterized by fruity and sweet aromas. The results of a sensory analysis indicated that the addition of hawthorn fruit resulted in an improvement of such characteristics as taste, aroma, clarity and overall impression to a higher degree than the addition of hawthorn juice. Hawthorn fruit and its juice can be used as a complementary raw material in the production of beer to increase its biological activity and improve its taste and aroma. It can also contribute to a greater consumer interest in the product. The fourth study by Issa-Issa et al. [7] had as main goal the investigation of the volatile composition by HS-SPME/GC-MS and a sensory profile by a descriptive sensory analysis of novel smoothies prepared by blending fig, jujube or quince purée with pomegranate juices (cv. "Mollar de Elche" or "Wonderful") at two ratios of purée to juice, 40:60 or 60:40. Twenty-three volatile compounds were present with the five predominant ones as follows: (i) 5-HMF; (ii) 3-hexen-1-ol; (iii) hexanal; (iv) 1-hexanol

and (v) 3-octanone. Fig smoothies were reported to be sweet and had a flavor and volatiles related to fig, pomegranate and grape. Meanwhile, jujube products were bitter and had jujube and pear notes. Finally, quince smoothies were sour and had quince, apple and floral notes. Thus, the type of fruit used clearly determined the flavor of the final product. The smoothies prepared with "Mollar de Elche" pomegranate juice were characterized by having a high intensity of pear odor/aroma and consistency while "Wonderful" smoothies were characterized by a lower consistency and a more intense pomegranate aroma and sour flavor. However, further research is still needed to fully optimize these novel products. Two ways of improvement can be researched: (i) increasing pomegranate notes and (ii) avoiding undesirable compounds after the Maillard Reaction. The fifth study by Kwasnica et al. [8] describes the volatile composition and the sensory properties as quality attributes of fresh and dried hemp flowers (*Cannabis sativa* L.). Flowers of hemp are widely used in cosmetics, food and in the pharmaceutical industry. The drying process plays a key role in the retention of the aroma and also in the quality of the products. Seven variants of hemp flower drying including convection drying, vacuum-microwave drying and combined drying consisting of convective pre-drying followed by vacuum-microwave finishing drying were checked in this study. During the drying process, losses found in 93 analyzed volatiles ranged from 48% for combined drying to 15% for vacuum-microwave drying at 240 W, which was finally chosen as optimal for the retention of aroma-active compounds. In that variant, a significant decrease of β-myrcene was observed. From a sensory point of view, the best drying treatment was vacuum-microwave drying at 240 W because it produced dried samples most resembling the fresh material with high intensities of key sensory descriptors such as hemp flower ID, fresh vegetables, citrus, balsamic and anise and therefore this drying treatment and conditions can be recommended as the best option for hemp flower drying. The sixth and last manuscript of this second topic by Calín-Sánchez et al. [9] is a review manuscript that compared traditional and novel drying techniques and describes their effect on the quality of fruits, vegetables and aromatic herbs. The quality of dehydrated fruits, vegetables and aromatic herbs is a key problem closely related to the development and optimization of novel drying techniques. This review reported the weaknesses of common drying methods applied for fruits, vegetables and aromatic herbs and the possible options to improve the quality of dried products using different drying techniques or their combination. In general, drying led to a reduction in all studied parameters. However, the behavior of each plant material was different. On the whole, the optimal drying technique was different for each of the materials studied and specific conditions were recommended after a proper evaluation of the drying protocols. However, a novel or combined technique must assure a high quality of dried products. Furthermore, the term 'quality' must englobe energy efficiency and the environmental impact leading to the production of sustainably dried products.

In the third topic that groups manuscripts dealing with the characterization of foods based on instrumental (GC-MS) and sensory analysis, three original papers have been published [10–12]. The first study of this group by Valli et al. [10] describes a screening support for panel tests in order to classify olive oils according to their volatile profile. A sensory evaluation, carried out by panel tests, is essential for a quality classification of virgin olive oils but is time consuming and costly when many samples need to be assessed; sensory evaluation could be assisted by the application of screening methods. Rapid instrumental methods based on the analysis of volatile molecules might be considered interesting to assist the panel test through fast pre-classification of samples with a known level of probability, thus increasing the efficiency of quality control. With this objective, a headspace gas chromatography-ion mobility spectrometer (HS-GC-IMS) was used to analyze 198 commercial extra virgin, virgin and lampante olive oils by a semi-targeted approach. The promising models were useful to predict the quality grade and presence of three sensory defects (musty, rancid, fusty/muddy sediment) providing percentages of correctly classified samples in external validation from 67% to 95% for the quality grade prediction model and from 48% to 80%, for the presence of each of the aforementioned defects.

However, additional investigations are needed before it can be implemented commercially; furthermore, to test the performance of this approach, inter-laboratory tests involving independent laboratories will be carried out in the future. The second study of this topic by Vichi et al. [11] led to the distinguishing of homozygous and heterozygous bitter genotypes in sweet almonds. Bitterness in almonds kernels is due to the presence of the cyanogenic glucoside amygdalin, which undergoes enzymatic hydrolysis by β-glucosidases upon the disruption of tissues to form glucose, hydrogen cyanide and benzaldehyde [13]. The results demonstrated the association between sweet almonds' genotype and a few volatile metabolites and provided for the first time chemical markers to discriminate between homo- and heterozygous sweet almonds. In particular, the amount of benzaldehyde, assessed by a simple, rapid, automatable and affordable technique such as SPME-GC-MS, allowed the differentiation between the homo- and heterozygous samples analyzed in the study and to tentatively classify almond kernels with an unknown genotype. The third and last research of this topic by Romero-Medina et al. [12] characterized a traditional Mexican corn beer by both sensory and instrumental analysis. The main objective of this study was to understand how the use of pigmented corn malt influences the chemical composition and sensory characteristics of beers. The authors demonstrated for the first time that among the groups of volatile compounds, ketones, terpenes and phenol volatiles as well as the presence of anthocyanins appeared as relevant criteria for the differentiation of corn beers. Moreover, the study of the relationship between the sensory attributes and the chemical parameters elucidated the effect of each type of malt (red corn, blue corn and barley malt) on the chemical parameters and their association with the sensory attributes. Finally, they declared that the sensory characteristics of these beers may carry the acceptance or rejection of consumers needs to be further investigated.

The last topic is composed by only one manuscript and is related to the sensory perception of novel fruit based products such as smoothies by a particular group of Spanish millennials. The work by Cano-Lamadrid et al. [14] applied Napping®, a descriptive sensory analysis and consumer studies in order to provide a new insight into the perception of smoothie products and comprehensive knowledge for the food industry to guide the design of new foods including functional and healthy fruit based products. The results showed that the descriptive sensory analysis and consumer studies preceded by the Napping® test seemed to be an appropriate combination to optimize the formulation of novel fruit and vegetable smoothies. The key attributes controlling the overall liking were the adequate intensity of a sour taste and notes of mango, banana and peach. Nevertheless, it should strive to improve recipes of smoothies to increase the consumption of fruits and vegetables in this form, which is considered a simple supplement in a balanced diet. The results of the penalty analysis gave a good direction to optimize these types of smoothies by avoiding vegetable ingredients with earthy or strong vegetal notes. The research provided a series of practical tips for the food industry to understand consumer preferences, select raw materials and improve marketing strategies.

In summary, the twelve papers published in this Special Issue highlight a great part of the research activities in the field of food quality as a tool for quality control, aiming to characterize and improve the sensory, chemical, nutritional, health and physical quality of fruits and fruit based products. This Special Issue, with the worldwide trend toward foods for nutrition and health, further states the importance of multidimensional and multidisciplinary approaches as exemplified in the papers described above. Finally, most authors who have contributed to this issue state that further research in their topic is required in every one of the presented papers and this assures an exciting time for future studies.

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Article

Volatile, Sensory and Functional Properties of HydroSOS Pistachios

Luis Noguera-Artiaga [1], Paola Sánchez-Bravo [1], David Pérez-López [2], Antoni Szumny [3], Ángel Calin-Sánchez [1], Armando Burgos-Hernández [4] and Ángel A. Carbonell-Barrachina [1,*]

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Abstract: Climate change, the increase in world population, and the intensification of urban and industrial activities, will cause a shortage of water for agriculture. This situation requires conscientious studies to manage water deficits without affecting the quality of the crops. In this study, regulated deficit irrigation (RDI) strategies and three rootstocks (*P. atlantica*, *P. integerrima*, and *P. terebinthus*) were applied to pistachio cultivation to study the quality of fruits obtained based on the morphological, functional, aroma, and their sensory properties. The results obtained demonstrated that RDI T1 (during phenological phase II of cultivation the stem water potential was maintained around −1.5 MPa) led to pistachios with same morphological properties, total polyphenol content, antioxidant activity, volatile composition, sensory properties, better profile of fatty acids, and being the favorite ones for international consumers, as compared to pistachios obtained under full irrigation treatments. On the other hand, when *P. integerrima* was used, pistachios obtained had the highest weight, the lowest content of sucrose and the best functional properties.

Keywords: antioxidant activity; fatty acid methyl esters; hydroSOS; *Pistacia vera*; pistachio flavor; quality; sensory analysis; total polyphenol content

1. Introduction

Mediterranean and South American countries, Southern California, Southern Australia and South Africa are characterized by partially wet springs and autumns, mostly rainy winters and hot dry summers. Water scarcity and water deficits in plants, mainly due to scarce rainfall, must be supplemented with irrigation treatments. In addition, different factors, such as climate change, the increase in world population, and the intensification of urban and industrial activities, will cause a shortage of water for agriculture, and it will become more and more severe in the near future [1]. This situation requires more conscientious studies to manage water deficits without affecting the quality of crops. These studies should focus on crops which are able to withstand deficit irrigation or have low water needs but without drastic impacts on production and fruit quality [2].

One of the techniques focused on the reduction of irrigation water during fruits and vegetables farming is regulated deficit irrigation (RDI). RDI consists of the imposition of water deficits in specific phenological stages, which are less sensitive to water stress without affect the crop yield or its economic benefits [3,4].

Pistachio (*Pistacia vera*) is considered the only commercially edible nut among the different species in the genus *Pistacia*, and it has been cultivated for centuries in Mediterranean areas and is considered resistant to both drought and salinity [5]. This tolerance is based mainly on crop yields, but the physicochemical, functional and sensory quality of nuts has not been fully characterized. For their vegetative propagation, pistachio trees requires the use of rootstocks, because they cannot be propagated by cutting and planting because this propagation material do not produce enough roots [6]. The main rootstocks used for pistachio cultivation are *P. atlantica* Desf., *P. integerrima* L., *P. terebinthus* L. and *P. vera* L. [7]. Cultivation of pistachio trees has become a very profitable business, because in recent years, their harvesting was fully mechanized, the inputs associated to their cultivation has decreased, and the prize paid to producers is constantly increased [2]. In the future, this trend is expected to keep increasing due to the many studies supporting the health benefits observed after pistachio consumption [8,9]. It has been proved that the pistachio antioxidant capacity, total phenolic content, monounsaturated and polyunsaturated acids, lutein, phytosterols, and another functional compounds (founded on the pistachio nuts) were responsible for the anti-inflammatory potential, helping to promote cardiovascular health, and foster protective effect against colorectal and breast cancer of this nuts [10–13].

For all the above reasons, it is necessary to establish or identify those parameters that allow characterizing the quality of pistachios. In this sense, the main objective of this study was to evaluate the quality of pistachio nuts obtained using three irrigation treatments and three rootstocks, based on their morphological properties, fatty acids content, antioxidant properties, total polyphenol content, volatile composition and their sensory properties.

2. Materials and Methods

2.1. Plant Material, Growing Conditions and Experimental Design

Pistachio nuts from trees (*P. vera*), cultivar "Kerman" were collected during 2016 from the experimental orchard "La Entresierra" located at Ciudad Real, Spain (3°56' W, 39° N; altitude 640 m above sea level). This area is characterized by a Mediterranean climate, with an average annual rainfall of 397 mm. The soil is a shallow clay-loam (Petrocalcic Palexeralfs) with a discontinuous petrocalcic horizon located at 0.5 m with a pH about 8.1, low electrical conductivity (0.2 dS/m), 1.05% organic matter, 0.12% N, 17×10^{-4} mol/kg K and a high cation exchange capacity (0.186 mol/kg).

Eighteen plots were used for this study with a completely randomized factorial design. Each of these plots had 12 trees (2 on the center for the analyses and 10 surrounding them) with same conditions of irrigation and rootstock. Pistachio trees were grafted over 3 rootstocks: (i) *P. atlantica*, (ii) *P. integerrima* and (iii) *P. terebinthus*, and, 3 irrigation treatments: (i) T0, in which trees were irrigated to ensure non-limiting water conditions in the soil (100% ETC of the previous week); (ii) T1, in which irrigation was suppressed (during phase II) until pistachio trees reached a stem water potential (SWP) below −1.5 MPa; and (iii) T2 with same irrigation protocol as T1 but with a SWP threshold of −2.0 MPa. Water relations were evaluated according to Memmi [14].

Pistachio nuts were collected from the field, and after being peeled and dried (convection oven with hot air at 60 °C until a moisture content of 5%), were immediately vacuum-packed and posted to the Universidad Miguel Hernández de Elche facilities in Orihuela (Alicante, Spain). Once there, samples were kept at 4–5 °C until analysis.

2.2. Volatile Compounds

The extraction of the volatile compounds of the samples of pistachios was carried out using the headspace solid-phase micro-extraction (HS-SPME) method. A sample of 1 g of ground pistachios was placed on a 50 mL vial, with a magnetic bar, and closed with an aluminum layer (foil). After equilibration time, 5 min at 45 °C, a 30/50 μm fiber (SUPELCO) covered by DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane) was exposed to the vial headspace at 45 °C, with continuous agitation (500 rpm) in a magnetic stirrer (IKA C-MAG HS 4, IKA-Werke GmbH & Co. KG, Staufen, Germany). After 25 min of exposure, fiber was put in a gas chromatograph to analyse.

The isolation and identification of the volatile compounds previously extracted by HS-SPME were performed using a Saturn 2000 Varian Chrompack gas chromatograph (Varian, Inc., Palo Alto, CA, USA) with an HP-5 column (5% Phenyl Methylpolysiloxane) 30 m × 0.53 mm ID × 1.0 μm (Agilent, Santa Clara, CA, USA). A mass spectrometer equipped with an ion-analyzer was set to 1508 V for all analyzes, and an electronic voltage factor to 1350 V. The analysis was carried out from 39 to 400 m/z, with an electronic impact (EI) of 70 eV, in 1 scan/s mode. Helium was used as carrier gas at a flow rate 1.0 mL/min and with a split ratio of 1:20. The injector and detector temperatures were 200 and 300 °C, respectively. The oven temperature started at 40 °C and, after 3 min, was increased by 5 °C/min up to 110 °C. Then, the temperature was increased by 20 °C/min up to 270 °C. The total analysis program lasted 25 min.

The volatile compounds were identified using three analytical methods: (i) Kovats Index (KI), (ii) GC-MS retention index (original chemical compound), and (iii) mass spectrum (original chemical compound and collection of the NIST05 and Adams 2012 spectrum library). The retention indexes were calculated using standard of aliphatic hydrocarbons in the range from C–5 to C–23. For the identification and determination of volatile compounds, the MS Workstation (Version 6.5, 2005 Varian, Palo Alto, CA, USA) and MestReNova (Version 9.0.1, 2014, Mestrelab Research, Santiago de Compostela, Spain) programs were used.

2.3. Sensory Analysis

The sensory analysis of samples was focused only on the analysis of the pistachios obtained under different irrigation treatments, in order to minimize the number of samples and, thus, maintain the panelists concentration to the maximum. Based on previous results [7], pistachio nuts obtained by *P. atlantica* rootstock were used on the sensory study.

To obtain information about the consumer opinion on the sensory properties of pistachios, a sensory evaluation with consumer panel was carry out in 3 countries: Mexico, Poland, and Spain. At least 60 consumers were recruited in each country. Consumers had to complete a screening questionnaire stating their age, gender, and allergies or diet restrictions. Consumers were asked about nut consumption frequency and willingness to taste pistachios. Consumers who stated that they were 18–70 years old, had no diet restrictions or allergies, ate any kind of nut at least once per week and were willing to taste pistachios were recruited for testing. In the specific case of Poland, the ballots, screeners and demographic questionnaires were translated from Spanish to Polish, and, then, back to Spanish.

Ten pistachio nuts were served, to each panelist, in odor-free disposable 60 mL covered plastic cups, coded using three-digit numbers, and at room temperature. Unsalted crackers and drinking water were used between samples to clean the panelists' palate. Natural illumination was used during the test, and testing room was at 20 ± 2 °C.

Consumers responded using a 9-point hedonic scale, where 9 = like extremely and 1 = dislike extremely. Consumers were, then, asked to indicate their order of preference for the samples, and mark the reasons of their preference regarding the attributes under study (size, peel, color, pistachio-ID, toasted, sweet, sour, aftertaste, oiliness, hardness, crunchiness, friability and adhesiveness). Then, consumers were asked about their "global" satisfaction degree for the samples under evaluation and for their intent to purchase.

2.4. Determination of Sugars and Organic Acids

Sugars and organic acids were identified and quantified according to Hernández [15], with some modifications. Approximately 1 g of sample was diluted in 5 mL of phosphate buffer (pH 7.8), homogenized by Ultra-TurraxTM (IKA L004640, Staufen, Germany) for 1 min, and centrifuged at 15,000× *g* for 10 min. Finally, samples were filtered through a 0.45 µm Millipore filter. For the determination of the content of sugars and organic acids on samples, an HPLC (high-performance liquid chromatograph) Hewlett-Packard series 1100 (Hewlett-Packard, Wilmington, DE, USA) was used. The elution buffer consisted of 0.1% phosphoric acid with a flow rate of 0.5 mL/min.

Sugars and organic acids were isolated using a Supelco column (Supelcogel TM C-610H column 30 cm × 7.8 mm, Supelco, Inc., Bellefonte, PA, USA) and a precolumn Supelguard (5 cm × 4.6 mm; Supelco), and the absorbance was measured at 210 nm using a diode-array detector (DAD). Standards of sugars (glucose, fructose, sucrose, raffinose, maltitol, and sorbitol) and organic acids (oxalic, citric, tartaric, malic, quinic, shikimic, succinic and fumaric) were obtained from Sigma (Poole, UK). Calibration curves were used for the quantification of sugars and organic acids, showing good linearity (R^2 = 0.999). Results for both organic acids and sugars were expressed as concentrations g/L of dry weight (dw).

2.5. Total Polyphenol Content and Antioxidant Activity

For the total polyphenol content (TPC) determination and the antioxidant activity of the pistachios affected by rootstock and irrigation treatments, a methanol extract was prepared. Half a gram of pistachios (crushed with a grounder) was introduced in a test tube with 10 mL of MeOH/water (80:20, *v*/*v*) in 1% HCl. Then, the mixture was sonicated at 20 °C for 15 min and left at 4 °C for 24 h. After that, the extract was sonicated again for 15 min and centrifuged at 10,000× *g* for 10 min [16].

TPC was quantified using the Folin-Ciocalteu colorimetric method described by Gao [17], with some modifications. To 0.1 mL of the methanolic extract was added 2 mL of distilled water, 0.2 mL of Folin-Ciocalteu reagent, and was incubated for 3 min at room temperature. After that, 1 mL of 20% sodium carbonate was added and the mixture was incubated again for 1 h [16]. The absorbance was determined by measurement at 765 nm using an UV-visible spectrophotometer (Thermo Fisher Scientific Helios Gamma model, UVG 1002E, Waltham, MA, USA). Quantification was carried out according to the standard curve of gallic acid. The results were expressed as gallic acid equivalents (GAE), mg/kg (dw).

For the analysis of the antioxidant activity, the methods $ABTS^+$ [18], FRAP [19], and $DPPH^\bullet$ [20] were used. Ten microliters of the supernatant of the methanolic extract was mixed with 990 µL of reagent $ABTS^+$ or FRAP. After a reaction time of 10 min, the absorbance was measured at 734 nm for $ABTS^+$ and 593 nm in case of FRAP method. For the DPPH method, 10 µL of the supernatant was mixed with 40 µL of MeOH and 950 µL of $DPPH^\bullet$ solution. Then, the mixture was shaken, placed under dark conditions (15 min), and its absorbance was determined at 515 nm. The results obtained on the analysis of the antioxidant activity of pistachio samples were expressed as mmol Trolox/kg dw.

2.6. Fatty Acids

The determination of fatty acid methyl esters (FAMEs) was carried out according to Noguera-Artiaga [16]. A gas chromatograph Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectrometer detector (GCMS QP-5050A) was used for the analysis of the organic layer of the pistachio extracts. The chromatograph was equipped with a column (polar) Suprawax 280, 100% polyethylene glycol (Teknokroma S. Co. Ltd., Barcelona, Spain; 30 m × 0.25 m × 0.25 µm film thickness). Helium (flow rate of 1.1 mL/min) was used as a carrier gas (split ratio 1:10). The temperature on the injector was 230 °C; on the detector, the temperature was 260 °C. The oven program and the identification of peaks were carry out following the method described by Carbonell-Barrachina [7] and Noguera-Artiaga [16].

2.7. Morphological Analysis

Twenty-five pistachio nuts from each treatment were randomly selected and used to determine the size, weight, and color. In addition, pistachios were classified as non-split open, split open, and others (uncommercial: unpeeled, broken shell, dark color, etc.). For the determination of the size, the height, width and length of the edible part from each pistachio were measured using a digital caliper (model 500-197-20 150 mm; Mitutoyo Corp., Aurora, IL, USA). In case of weight, the whole nut, shell and edible part were weighed (model AG204 scale; Mettler Toledo, Barcelona, Spain) with a precision of 0.1 mg. For the color, these 25 pistachios were ground (Taurus Aromatic Ver II; Taurus Group, Barcelona, Spain) and placed in Petri dishes (100 mm × 15 mm). A colorimeter Minolta model CR-300 (Minolta, Osaka, Japan) with an illuminant D65 and an observer of 10° was used for the measuring of color of samples. Color data were provided as CIE*L*a*b** coordinates.

2.8. Statistical Analysis

The data presented in this study are the mean values of 3 replicates and was subjected to two-way (irrigation treatment and rootstock) analysis of variance (ANOVA). Then, data were subjected to Tukey's multiple-range test to compare the means. Percentage data were transformed by Arcosen function before statistical analysis. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were done using XLSTAT software (Addinsoft, version 2014.1, Paris, France).

3. Results and Discussion

3.1. Volatile Compounds

Thirty-one compounds were identified in the volatile profile of pistachios under study (Table S1) and were characterized according their sensory descriptors. The three most abundant compounds were α-pinene (~35%), limonene (~14%), and β-myrcene (~11%), and it was demonstrated that both irrigation and rootstocks significantly affected the content these compounds (Table 1). These results agreed with those obtained by Hojjati [21] and Penci [22] in previous studies, who also found that the same main compounds predominated in the aromatic profile of pistachios and its essential oils, respectively.

The volatile composition of the T1 pistachios showed no significant differences with that of the control samples (T0). On the other hand, pistachios obtained under RDI T2 had lower amounts of α-pinene, dodecane and tridecane, but higher ones of β-myrcene and limonene than T0. Regarding the effect of the pistachio rootstock, *P. integerrima* had the highest content of α-pinene (the predominant compound); *P. atlantica* led to nuts with the highest content of β-myrcene, dodecane and tridecane; and, *P. terebinthus* had the highest content of limonene.

In previous studies, Carbonell-Barrachina [7] demonstrated that RDI treatments led to pistachio nuts with similar or even higher amounts of the main volatile compounds. These results are in concordance with those obtained in the current study, in the sense that both studies demonstrated that the application of RDI strategies had no negative effects on the volatile composition of pistachio nuts.

Based on the study of the interaction between the two studied factors (irrigation and rootstock), to obtain pistachios with the highest possible content of α-pinene, it is necessary to use the rootstock *P. integerrima* and the irrigation treatments T0 or T1. On the other hand, if the objective is to obtain pistachios with more citrus aroma (more limonene), the combination of *P. terebinthus* rootstock and irrigation treatment T1 will be the most successful one.

According to results obtained, the main volatile compounds found in the volatile profile of pistachios α-pinene, limonene, and β-myrcene (compounds sensory related with descriptors of woody, citrus and fruity, respectively) can be used as indicators to evaluate the pistachio aroma quality.

3.2. Sensory Analysis

Around 200 consumers from Mexico, Spain and Poland (at least 60 in each country) participated in the pistachio affective sensory analysis. In Mexico, a 68% of panelist were women, 37% in Poland, and 55% in case of Spain. Of the total number of consumers, 35% were between 18 and 25 years old, 32% were between 26 and 35, 15 % between 36 and 45 years old, 17% between 46 and 55 years old, and 2% were older than 55 years old.

The irrigation treatments significantly affected three out of the 13 sensory attributes under analysis (Table 2): pistachio-ID, oiliness, and overall. Pistachios obtained under RDI T1 obtained higher intensities of pistachio-ID (6.7) than control and T2 (6.4 and 6.4). This result was observed in each of the countries under study (Table 2).

The oiliness of T1 and T0 samples were slightly but statistically higher, 6.0 than T2 samples, 5.8. Mexican consumers liked the oiliness of the pistachio samples less than Polish and Spanish consumers; a similar trend was observed for hardness and crunchiness. The most consumed dried fruit in Mexico is peanut, so it is possible that consumers in this country are used to high intensities of these attributes and were expecting a little more oiliness, hardness and crunchiness in pistachio samples, hoping to find a texture similar to that of fried peanuts.

In addition, in case of overall liking (the attribute that define the final opinion of consumers about the overall quality of sample), the T1 treatment obtained the highest score (6.7), while T0 got 6.3; T2 was statistically related with both of them (score of 6.5). Regarding the factor country, this same trend was observed in Mexico and Spain. However, there were no statistically significant differences in the satisfaction degree of Polish consumers regarding the three irrigations treatments (Table 2).

When consumers were forced to choose (among the three samples studied) which was their favorite sample, T1 pistachios were the most liked ones in each of the countries (in case of Poland, no statistically significant differences were observed between T1 and T0). On the other hand, the least liked sample in all countries was T2 (Figure 1). Similar trends were observed when consumers were asked about their willingness to pay for the samples under study. Consumers mentioned that the main reasons for selecting the preferred sample (the most liked one) were: (i) pistachio flavor (~83%), (ii) crunchiness (~65%), (iii) aftertaste (~45%) and (iv) hardness (~30%) (Figure 1). Similar results were reported in previous studies; for instance, Carbonell-Barrachina [7] studied the purchase drivers of international pistachio consumers were pistachio flavor, saltiness, crunchiness and toasted flavor. Although, Noguera-Artiaga [23] concluded that international consumers preferred intense crunchy but low salty nuts.

Table 1. Relative content (%) of volatile compounds on pistachios affected by regulated deficit irrigation and rootstocks.

Compound	ANOVA Test †			Irrigation (%)			Rootstock (%)			Irrigation × Rootstock (%)								
	Irrigation	Rootstock	Irrigation × Rootstock	T0	T1	T2	AT	IN	TE	ATxT0	ATxT1	ATxT2	INxT0	INxT1	INxT2	TExT0	TExT1	TExT2
Acetic acid	NS	NS	NS	0.46	0.32	0.30	0.33	0.36	0.39	0.33	0.26	0.39	0.59	0.17	0.32	0.45	0.53	0.19
Ethyl acetate	NS	NS	NS	1.04	0.39	0.46	0.69	0.75	0.45	0.85	0.63	0.59	1.48	0.20	0.58	0.79	0.35	0.21
Pentanone	NS	NS	NS	0.25	0.20	0.17	0.19	0.21	0.22	0.20	0.18	0.19	0.33	0.13	0.16	0.21	0.28	0.18
1-Methyl-1H-pyrrole	NS	NS	NS	3.52	4.57	2.82	2.72	2.92	5.26	1.95	3.84	2.39	3.04	3.28	2.44	5.58	6.58	3.62
1-Pentanol	NS	NS	NS	0.92	0.55	0.43	0.67	0.47	0.76	0.90	0.72	0.39	0.67	0.27	0.45	1.18	0.65	0.46
(*Z*)-3-Octene	**	NS	**	0.26 b,‡	0.20 b	0.50 a	0.35	0.27	0.35	0.18 b	0.15 b	0.71 a	0.27 ab	0.13 b	0.39 ab	0.31 ab	0.33 ab	0.41 ab
Hexanal	NS	NS	NS	1.38	0.70	0.94	0.98	0.74	1.30	1.17	0.84	0.91	0.80	0.39	1.03	2.16	0.86	0.88
2-Octene	NS	NS	NS	0.18	0.20	0.27	0.22	0.18	0.24	0.17	0.17	0.31	0.15	0.15	0.24	0.22	0.27	0.24
1-Hexanol	NS	NS	NS	4.39	2.63	2.64	3.98	2.70	2.97	5.75	3.66	2.55	2.86	1.98	3.27	4.56	2.26	2.09
(*E*)-4-Nonene	NS	NS	**	0.21	0.21	0.30	0.27	0.20	0.24	0.18 b	0.20 ab	0.42 a	0.19 ab	0.14 b	0.26 ab	0.24 ab	0.27 ab	0.21 ab
(*Z*)-4-Nonene	NS	NS	NS	0.22	0.18	0.22	0.22	0.15	0.24	0.20	0.18	0.28	0.15	0.11	0.19	0.31	0.24	0.18
Nonane	NS	NS	NS	0.25	0.23	0.27	0.29	0.21	0.24	0.30	0.23	0.35	0.20	0.17	0.26	0.26	0.29	0.19
α-Pinene	**	**	**	36.90 a	35.08 a	33.54 b	30.19 b	42.41 a	32.92 b	31.29 c	33.13 c	26.15 d	49.27 a	46.25 a	31.71 c	30.14 c	25.86 d	42.76 b
2-Pentanol	NS	NS	NS	0.37	0.40	0.60	0.56	0.41	0.41	0.23	0.47	0.98	0.43	0.24	0.55	0.45	0.50	0.26
1-Decene	NS	NS	NS	0.83	0.40	0.47	0.75	0.36	0.60	1.10	0.53	0.62	0.47	0.30	0.31	0.94	0.38	0.47
Sabinene	NS	NS	NS	0.48	0.40	0.40	0.38	0.42	0.48	0.43	0.32	0.38	0.54	0.47	0.26	0.47	0.41	0.57
3-Decene	NS	NS	NS	0.62	0.35	0.49	0.51	0.34	0.61	0.44	0.47	0.61	0.41	0.16	0.43	1.00	0.41	0.42
β-Myrcene	**	**	NS	8.39 b	9.58 b	14.89 a	13.21 a	9.79 b	9.86 b	6.77	12.66	20.22	8.16	6.12	15.09	10.26	9.97	9.37
Decane	NS	NS	NS	2.07	2.53	2.74	2.92	2.26	2.16	2.13	3.27	3.34	2.09	1.96	2.73	1.99	2.35	2.14
3-Carene	NS	NS	NS	0.24	0.39	0.39	0.29	0.34	0.39	0.34	0.26	0.26	0.19	0.52	0.31	0.18	0.39	0.61
Limonene	***	**	**	12.19 b	13.72 b	15.01 a	11.19 b	12.24 b	17.49 a	14.87 c	8.11 de	10.60 d	7.91 e	12.73 cd	16.07 bc	13.80 c	20.32 a	18.36 b
(*E*)-3-Hexenol	NS	NS	NS	0.92	0.94	1.06	1.17	0.82	0.92	0.84	1.23	1.43	0.81	0.64	1.01	1.10	0.94	0.72
2-Octen-1-ol	NS	NS	NS	0.74	0.97	1.11	1.21	0.85	0.75	0.76	1.32	1.54	0.77	0.67	1.12	0.67	0.93	0.65
2-Methyl-decane	NS	NS	NS	0.68	0.45	0.49	0.68	0.46	0.48	0.83	0.60	0.61	0.48	0.31	0.58	0.72	0.45	0.26
Terpinolene	NS	NS	NS	0.66	0.95	0.76	0.81	0.84	0.72	1.06	0.79	0.57	0.40	1.43	0.70	0.52	0.62	1.02
Undecane	NS	NS	NS	8.59	9.40	8.77	9.53	8.77	8.46	9.87	7.73	11.00	7.67	9.17	9.47	8.22	11.32	5.84
2-Nonen-1-ol	NS	NS	NS	1.70	0.90	0.94	1.46	1.04	1.04	2.51	0.81	1.06	1.03	1.19	0.90	1.57	0.69	0.88
1-Nonanol	NS	NS	NS	1.54	1.20	1.01	1.72	0.90	1.13	2.34	1.68	1.13	0.75	1.02	0.94	1.55	0.90	0.95
Dodecane	**	**	**	6.25 ab	8.02 a	5.29 b	8.32 a	5.71 b	5.54 b	7.73 ab	10.51 a	6.71 ab	5.19 b	6.45 ab	5.46 ab	5.83 ab	7.09 ab	3.70 b
Decanal	NS	NS	NS	0.41	0.51	0.46	0.54	0.45	0.39	0.50	0.56	0.56	0.42	0.49	0.45	0.31	0.46	0.38
Tridecane	**	**	**	2.85 ab	3.45 a	2.26 b	3.66 a	2.44 b	2.47 b	3.75 ab	4.50 a	2.73 ab	2.26 ab	2.76 ab	2.28 ab	2.54 ab	3.09 ab	1.76 b

† NS: not significant at $p < 0.05$; **: significant at $p < 0.01$. ‡ Values (mean of 3 replications) followed by the same letter, within the same volatile compound and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test. *P. atlantica* (AT), *P. integerrima* (IN) and *P. terebinthus* (TE).

Table 2. Sensory affective test of hydroSOS pistachios conducted in three countries: Spain, Mexico, and Poland.

Factor	Liking													
	Size	**Peel**	**Color**	**Pistachio ID**	**Toasted**	**Sweet**	**Sour**	**Aftertaste**	**Oiliness**	**Hardness**	**Crunchiness**	**Friability**	**Adhesiveness**	**Overall**
ANOVA Test †														
Irrigation	NS	NS	NS	**	NS	NS	NS	NS	*	NS	NS	NS	NS	**
Country	NS	NS	NS	NS	NS	NS	NS	NS	*	*	**	NS	NS	NS
Country × Irrigation	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	NS	NS	*
Tukey's Multiple Range Test ‡														
Irrigation														
T0	6.6	6.8	6.4	6.4 b	6.4	5.8	5.8	6.3	6.0 a	6.5	6.4	6.0	5.7	6.3 b
T1	6.7	6.9	6.5	6.7 a	6.3	6.0	5.6	6.1	6.0 a	6.6	6.4	6.0	5.7	6.7 a
T2	6.5	6.7	6.3	6.4 b	6.5	5.8	5.9	5.9	5.8 b	6.5	6.5	6.2	5.4	6.5 ab
Country														
Mexico	6.5	6.8	6.3	6.3	6.5	5.8	5.8	5.8	5.6 b	6.2 b	6.0 b	5.9	5.5	6.4
Poland	6.9	6.9	6.5	6.7	6.4	5.9	5.8	6.3	6.0 a	6.8 a	6.7 a	6.2	5.9	6.7
Spain	6.5	6.6	6.3	6.5	6.2	5.9	5.6	6.3	6.2 a	6.6 ab	6.7 a	6.1	5.6	6.5
Country × Irrigation														
Mexico × T0	6.7	6.9	6.4	6.2 c	6.4	5.9	6.0	6.1	5.6 b	6.1	6.1	5.7	5.5	6.0 b
Mexico × T1	6.5	6.9	6.4	6.6 ab	6.5	5.8	5.5	5.9	5.7 b	6.4	5.9	6.0	5.7	6.6 a
Mexico × T2	6.2	6.6	6.2	6.2 c	6.7	5.7	5.8	5.4	5.6 b	6.2	5.9	6.1	5.1	6.4 ab
Poland × T0	6.7	6.9	6.5	6.6 ab	6.5	5.7	5.8	6.2	6.2 a	6.8	6.5	6.2	5.9	6.9 a
Poland × T1	6.9	6.9	6.4	6.8 a	6.3	6.3	5.8	6.1	6.1 a	6.8	6.7	6.1	6.0	6.7 a
Poland × T2	6.9	6.9	6.7	6.7 a	6.5	5.8	6.0	6.4	5.8 ab	6.8	6.8	6.2	5.7	6.6 a
Spain × T0	6.4	6.6	6.3	6.4 b	6.2	5.8	5.5	6.5	6.3 a	6.7	6.5	6.0	5.8	6.1 b
Spain × T1	6.8	6.8	6.5	6.8 a	6.0	6.0	5.6	6.3	6.2 a	6.5	6.7	5.9	5.5	6.8 a
Spain × T2	6.3	6.5	6.1	6.3 bc	6.4	5.8	5.8	6.0	6.0 ab	6.5	6.9	6.3	5.4	6.5 ab

† NS: not significant at $p < 0.05$; * and **: significant at $p < 0.05$ and 0.01, respectively. ‡ Values (mean of 200 replications) followed by the same letter, within the same sensory attribute and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test.

Figure 1. Preference of Mexican, Polish, and Spanish consumers about pistachios obtained using different irrigation treatments (T0, control; T1, moderate RDI regulated deficit irrigation); T2, severe RDI), together with their willingness to pay and the main reasons behind their election. Factors with the same letter were not significantly different ($p < 0.05$), Tukey's least significant difference test.

3.3. Sugars and Organic Acids

Three sugars (maltitol, raffinose and sucrose) and three organic acids (fumaric, oxalic, and shikimic) were identified and quantified in the pistachio samples under study (Table 3). In a previous study, Luh [24], identified four main sugars present in pistachios: sucrose, fructose, glucose and raffinose, being sucrose the main sugar, representing ~40% of the total content.

T1 samples had lower concentration of fumaric acid (0.287 g/L) than control pistachios (T0 = 0.315 g/L), while T2 nuts (0.287 g/L) were statistically related with both T0 and T1. In the rest of organic acids and sugars, no statistically differences were found among pistachios obtained under different irrigation treatments (Table 3).

Regarding rootstocks, *P. integerrima* led to pistachios with lower sucrose concentration (19.77 g/L) than *P. terebinthus* and *P. atlantica* (22.51 and 24.98 g/L, respectively). In the analysis of organic acids, *P. integerrima* was the rootstock having the highest concentrations of the three studied acids, while *P. terebinthus* had the lowest amount of oxalic and fumaric acids (Table 3).

Table 3. Sugars (g/L) and organic acids (g/L) on pistachios obtained under regulated deficit irrigation and different rootstocks.

Factor	Sugars (g/L dw)			Organic Acids (g/L dw)		
	Raffinose	**Sucrose**	**Maltitol**	**Oxalic**	**Shikimic**	**Fumaric**
ANOVA Test †						
Irrigation	NS	NS	NS	NS	NS	***
Rootstock	NS	***	NS	***	***	***
Irrigation × Rootstock	NS	***	NS	NS	NS	***
Tukey's Multiple Range Test ‡						
Irrigation						
T0	9.793	22.831	4.111	0.156	0.535	0.315 a
T1	9.077	20.449	4.848	0.127	0.581	0.287 b
T2	10.351	23.972	5.796	0.148	0.555	0.296 ab
Rootstock						
P. atlantica	10.345	24.977 a	5.772	0.165 a	0.467 b	0.260 b
P. integerrima	9.517	19.770 b	5.257	0.151 a	0.737 a	0.328 a
P. terebinthus	9.359	22.505 a	3.727	0.116 b	0.468 b	0.309 a
Irrigation × Rootstock						
T0 × *P. atlantica*	9.091	23.692 ab	4.441	0.189	0.456	0.273 bc
T1 × *P. atlantica*	8.963	21.574 ab	4.447	0.145	0.418	0.266 bc
T2 × *P. atlantica*	12.982	29.666 a	8.427	0.160	0.527	0.241 c
T0 × *P. integerrima*	10.418	20.981 ab	3.911	0.137	0.701	0.339 a
T1 × *P. integerrima*	9.237	18.240 b	6.503	0.140	0.832	0.299 ab
T2 × *P. integerrima*	8.895	20.090 ab	5.355	0.175	0.678	0.347 a
T0 × *P. terebinthus*	9.868	23.821 ab	3.982	0.141	0.448	0.332 a
T1 × *P. terebinthus*	9.032	21.534 ab	3.595	0.096	0.494	0.295 abc
T2 × *P. terebinthus*	9.176	22.160 ab	3.605	0.112	0.461	0.300 ab

† NS: not significant at $p < 0.05$; ***, significant at $p < 0.001$. ‡ Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test. dw: dry weight.

According to interaction of the two factors studied, pistachios obtained under *P. integerrima* and irrigation T1 had the lowest concentration of sucrose, while rootstock *P. atlantica* and T2 led to pistachios with the highest concentration of this sugar (Table 3).

Similar results were obtained by Lipan [25] in almonds affected by different treatments of regulated deficit irrigation (with sucrose, the predominant sugar showing no differences due to irrigation treatments).

According to our acknowledgement, these are the first results published regarding the effect of deficit irrigation on the acid and sugar composition of pistachios.

3.4. Antioxidant Activity (AA) and Total Polyphenol Content (TPC)

Results obtained on the study of AA and TPC are shown in Table 4. In general, pistachios have high functional potential based on their high total polyphenol content (~1350 mg GAE/kg, dw) and their antioxidant activity (~22 mmol Trolox/kg, dw, on the three methods studied). Similar results of TPC were obtained by Hojjati [21] in roasted pistachios, Lipan [25] in almonds, and Suárez [26] in chestnut.

Table 4. Total polyphenol content, TPC [mg gallic acid equivalents (GAE)/kg dry weigh, dw] and antioxidant activity, AA (mmol Trolox/kg dw) of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	TPC	DPPH	FRAP	ABTS
	(mg GAE/kg dw)	(mmol Trolox/kg dw)		
ANOVA Test †				
Irrigation	***	***	***	NS
Rootstock	***	***	***	***
Irrigation × Rootstock	***	***	***	***
Tukey's Multiple Range Test ‡				
Irrigation				
T0	1390 ab	21.70 a	23.89 a	23.5
T1	1409 a	20.50 ab	24.45 a	23.3
T2	1297 b	18.77 b	19.66 b	22.0
Rootstock				
P. atlantica	1310 b	19.02 b	20.08 b	21.53 b
P. integerrima	1522 a	22.09 a	25.47 a	28.08 a
P. terebinthus	1265 b	19.87 b	22.44 ab	19.07 c
Irrigation × Rootstock				
T0 × *P. atlantica*	1294 bcd	20.06 ab	22.07 abc	22.78 bc
T1 × *P. atlantica*	1450 abc	19.51 ab	21.80 abc	22.85 bc
T2 × *P. atlantica*	1184 d	17.48 b	16.37 c	18.95 c
T0 × *P. integerrima*	1615 a	24.28 a	26.57 ab	29.01 a
T1 × *P. integerrima*	1460 ab	22.53 ab	30.02 a	28.26 a
T2 × *P. integerrima*	1489 ab	19.46 ab	19.82 bc	26.96 ab
T0 × *P. terebinthus*	1260 bcd	20.76 ab	23.03 abc	18.56 c
T1 × *P. terebinthus*	1317 bcd	19.47 ab	21.51 abc	18.71 c
T2 × *P. terebinthus*	1216 cd	19.37 ab	22.77 abc	19.93 c

† NS: not significant at $p < 0.05$; ***: significant at $p < 0.001$. ‡ Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test.

The application of moderate regulated irrigation treatments (T1) on the cultivation of pistachios had no statistically incidence on the AA and TPC of nuts. On the contrary, when the water restriction was severe (T2), the AA of pistachios was reduced (according to DPPH and FRAP methods). Under situations of moderate water stress, plants redistribute the CO_2 to the formation of secondary metabolites as a physiological response for the removal the free radicals formed; while under high stress, this CO_2

is mainly used by primary metabolism [27,28]. Same results were found, in previous studies under similar conditions, by Noguera-Artiaga [16] who obtained that hydroSOS pistachios had same or even higher TPC than control samples. Similar results of AA and TPC were found in previous studies with pistachios affected by different irrigation treatments [4,7,29].

In case of the study of rootstocks, *P. integerrima* led to obtain pistachio nuts with the highest concentrations of TPC and AA than rootstocks *P. atlantica* and *P. terebinthus* (Table 4). In previous studies, no significant differences were found in the functional composition of pistachios obtained through these same rootstocks.

3.5. Fatty Acids

Nine fatty acids (FAMEs) were identified by GC-MS in pistachio samples (Table 5): two were polyunsaturated (PUFAs) [α-linolenic acid (C18:3) and linoleic acid (C18:2)]; three monounsaturated (MUFAs) [eicosenoic acid (C20:1), oleic acid (C18:1), and palmitoleic acid (C16:1)]; and, four saturated (SFAs) [arachidic acid (C20:0), stearic acid (C18:0), palmitic acid (C16:0), and myristic acid (C14:0)]. The three predominant compounds were C18:1 (~53% of the total), C18:2 (~31%), and C16:0 (~12%).

Table 5. Fatty acid composition of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	Fatty Acids (%)								
	C14:0	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**	**C20:1**
ANOVA Test †									
Irrigation	NS	NS	NS	NS	**	NS	**	NS	NS
Rootstock	NS	NS	NS	NS	NS	NS	*	NS	NS
Irrigation × Rootstock	NS	NS	NS	NS	*	*	NS	NS	NS
Tukey's Multiple Range Test ‡									
Irrigation									
T0	0.07	12.03	1.28	1.28	51.94 b	32.18	0.73 a	0.16	0.32
T1	0.07	11.60	1.21	1.52	54.95 a	29.54	0.62 b	0.16	0.33
T2	0.07	11.86	1.19	1.32	52.05 b	32.39	0.67 ab	0.15	0.30
Rootstock									
P. atlantica	0.07	11.90	1.20	1.38	53.34	31.00	0.63 b	0.18	0.31
P. integerrima	0.08	11.63	1.19	1.37	52.95	31.63	0.67 ab	0.15	0.33
P. terebinthus	0.07	11.96	1.29	1.36	52.66	31.48	0.73 a	0.15	0.31
Irrigation × Rootstock									
T0 × *P. atlantica*	0.07	12.13	1.26	1.28	52.09 b	32.01 ab	0.64	0.20	0.31
T1 × *P. atlantica*	0.07	11.59	1.17	1.29	52.95 b	31.87 ab	0.61	0.17	0.28
T2 × *P. atlantica*	0.06	11.97	1.17	1.58	54.98 ab	29.12 ab	0.63	0.16	0.33
T0 × *P. integerrima*	0.08	11.95	1.30	1.28	50.96 b	33.18 ab	0.78	0.14	0.33
T1 × *P. integerrima*	0.07	11.27	1.10	1.69	58.44 a	26.33 b	0.58	0.19	0.33
T2 × *P. integerrima*	0.08	11.66	1.18	1.16	49.45 b	35.38 a	0.66	0.11	0.33
T0 × *P. terebinthus*	0.07	12.01	1.28	1.28	52.76 b	31.36 ab	0.78	0.15	0.31
T1 × *P. terebinthus*	0.06	11.93	1.35	1.58	53.47 b	30.41 ab	0.68	0.14	0.37
T2 × *P. terebinthus*	0.08	11.93	1.23	1.23	51.73 b	32.65 ab	0.73	0.17	0.25

† NS: not significant at $p < 0.05$; * and **, significant at $p < 0.05$ and 0.01, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test.

Pistachios obtained under moderate RDI (T1) had the highest content of oleic acid and the lowest one of α-linolenic as compared to those of control (T0) and T2 treatment (Table 5). In case of rootstocks, no statistically differences were observed on the fatty acid composition except on the content of α-linolenic acid, where *P. atlantica* had the lowest values (Table 5). Regarding the interaction between the two factors studied, rootstock and irrigation, pistachios obtained by *P. integerrima* and T1 had the highest content of oleic acid (Table 5).

The application of treatment T1 affected the unsaturated fatty acid composition of pistachios, increasing the content of MUFAs, and decreasing that of PUFAs. The use of different rootstocks had no significant effect on the composition of the SFAs, MUFAs or PUFAs (Figure 2).

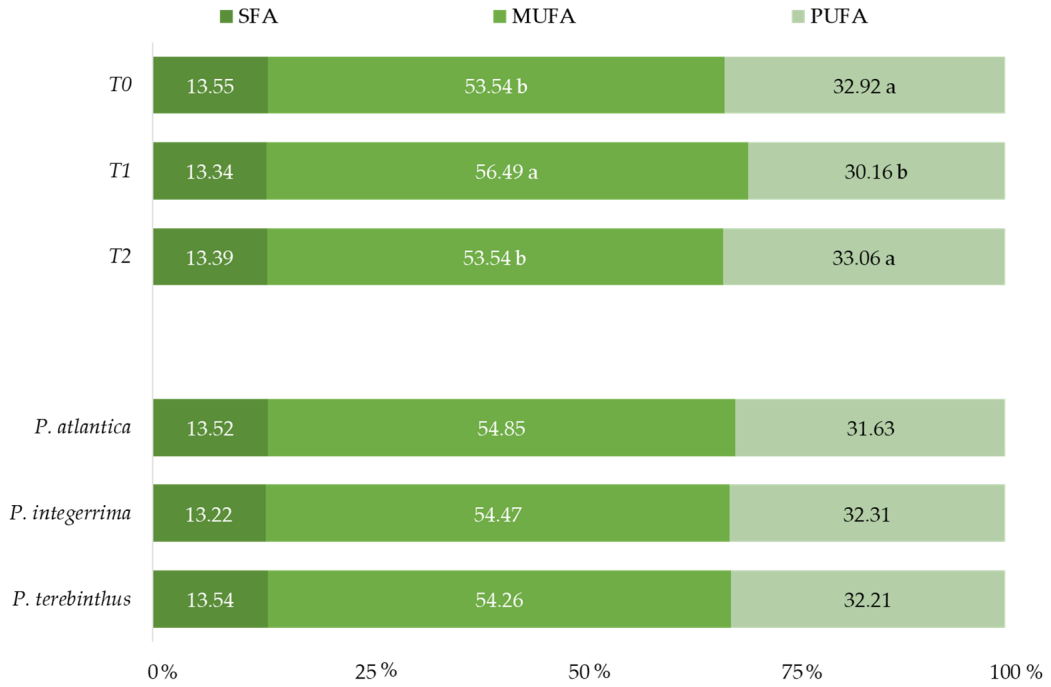

Figure 2. Fatty acid composition of pistachios as affected by deficit irrigation treatment and rootstock, grouped according to their saturation. Values (mean of 3 replications) followed by the same letter, within the same factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test. SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids.

In previous studies, carried out under similar conditions, Carbonell-Barrachina [7] obtained that moderate RDI increased the content of linoleic acid, while in this study the one that has been increased was that of the oleic acid. Acar [30] reported that main fatty acids found in pistachio were oleic, linoleic and palmitic acids as has been shown in the current study.

3.6. Morphological Analysis

On the analysis of split-open and non-split open pistachios (Table 6) no statistically significant differences were observed among samples obtained under different irrigation treatments, being the mean values 54% and 42%, respectively. In case of effect of rootstock, *P. terebinthus* had the highest number of split pistachios (60%) and, consequently, the lowest number of non-split open pistachios (35%); while data on *P. atlantica* and *P. integerrima* were statistically equivalent.

Table 6. Weight, size, and nature of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	Weight (g)			Size (mm)			Number per 100 Units			Color Coordinates				
	Whole	Edible Nut	Shell	Length	Height	Width	Split Open	Non-Split Open	Others §	*L*	*a**	*b**	*Chroma*	Hue
ANOVA Test †														
Irrigation	NS	***	NS	NS	***	***	NS	NS	NS	***	NS	NS	NS	***
Rootstock	NS	***	NS	NS	NS	***	***	***	NS	***	***	NS	NS	***
Irrigation × Rootstock	NS	***	NS	NS	***	***	*	*	NS	***	***	NS	NS	***
Tukey's Multiple Range Test ‡														
Irrigation														
T0	2.062	0.692 a	1.370	17.10	10.78 a	9.48 ab	55	42	4	67.74 a	−5.93	32.60	33.15	100.28 a
T1	2.051	0.695 a	1.356	17.23	10.74 a	9.56 a	53	43	4	66.37 b	−5.63	32.98	33.47	99.69 ab
T2	2.009	0.673 b	1.335	17.06	10.49 b	9.35 b	54	42	4	66.44 b	−5.54	32.67	33.16	99.61 b
Rootstock														
P. atlantica	2.036	0.686 ab	1.350	17.16	10.62	9.43 b	50 b	46 a	4	66.95 ab	−5.39 a	32.86	33.31	99.27 b
P. integerrima	2.047	0.695 a	1.352	17.18	10.68	9.61 a	52 b	46 a	2	67.32 a	−6.34 b	32.51	33.13	101.02 a
P. terebinthus	2.038	0.679 b	1.359	17.05	10.71	9.36 b	60 a	35 b	5	66.29 b	−5.38 a	32.88	33.33	99.29 b
Irrigation × Rootstock														
T0 × *P. atlantica*	2.061	0.691 ab	1.369	17.16	10.71 abc	9.37 bc	50 b	46 a	4	66.68 bc	−5.79 ab	33.16	33.68	99.87 ab
T1 × *P. atlantica*	2.029	0.688 ab	1.340	17.28	10.73 ab	9.41 abc	49 b	47 a	4	66.90 bc	−5.61 ab	32.79	33.27	99.69 ab
T2 × *P. atlantica*	2.018	0.678 ab	1.339	17.04	10.42 c	9.47 abc	51 b	44 a	5	67.26 b	−4.75 a	32.61	32.97	98.24 b
T0 × *P. integerrima*	2.062	0.702 a	1.360	17.15	10.91 a	9.67 ab	53 b	45 a	4	68.75 a	−6.59 b	32.56	33.23	101.43 a
T1 × *P. integerrima*	2.061	0.702 a	1.358	17.16	10.61 abc	9.74 a	51 b	46 a	4	66.49 c	−5.84 ab	32.64	33.16	100.15 a
T2 × *P. integerrima*	2.017	0.681 ab	1.336	17.24	10.54 bc	9.41 abc	51 b	47 a	3	66.74 bc	−6.58 b	32.34	33.01	101.48 a
T0 × *P. terebinthus*	2.062	0.682 ab	1.379	16.99	10.75 ab	9.37 bc	62 a	34 b	4	67.79 ab	−5.41 ab	32.07	32.53	99.52 ab
T1 × *P. terebinthus*	2.062	0.693 ab	1.368	17.25	10.88 a	9.54 ab	60 a	36 b	4	65.74 cd	−5.44 ab	33.51	33.96	99.23 ab
T2 × *P. terebinthus*	1.990	0.661 b	1.329	16.91	10.51 bc	9.17 c	59 a	36 b	5	65.34 d	−5.29 ab	33.07	33.51	99.11 ab

† NS: not significant at $p < 0.05$; * and ***: significant at $p < 0.05$ and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test. § Others' means unpeeled, broken shell, dark color, etc. L, *a**, *b**: CIE*L***a***b** color coordinates.

The moderated reduction of water during the phenological phase II of pistachios (T1) had no effect on the weight and size of the commercial nuts (Table 6). On the other hand, high reduction of water during this same phase (T2), led to pistachios with the lowest weight of their edible nut (T0 = 0.692 g and T2 = 0.673 g).

Regarding rootstocks, no statistically significant differences were found on the weight of whole nut and shell, and on the length and height of pistachio nuts. However, samples of *P. integerrima* and *P. atlantica* had the highest weight of the edible nut (Table 6).

The color of the samples had statistically significant differences in the parameters L^* and a^*, in case of irrigation and rootstock. These differences were minimum, and some authors have concluded that differences smaller than two units, as it is the current case (Table 6), are imperceptible for the human eye [31,32].

Similar results were previously reported by Carbonell-Barrachina [7] and Noguera-Artiaga [16], who showed that neither rootstocks nor RDI treatments significantly affected the morphological parameters of pistachios.

4. Conclusions

The results obtained in this study demonstrated that the application of a moderate deficit irrigation during pistachio cultivation (T1) led to pistachios with same morphological properties, total polyphenol content, antioxidant activity, volatile composition and sensory properties than pistachios obtained using full irrigation (T0). Moreover, T1 led to pistachios with better profile of fatty acids and being the sample preferred by international consumers. On the contrary, when the RDI was severe (T2), pistachio nuts had the lowest antioxidant activity, the lowest total polyphenols content, and the least preferred samples by consumers. In case of pistachios obtained using different rootstocks, *P. integerrima* led to pistachio nuts with the highest weight, the lowest content of sucrose and better functional properties than *P. atlantica* and *P. terebinthus*. These results demonstrated that it is possible to save irrigation water in pistachio farming, with low environmental and economic cost, and leading to pistachio nuts with same or even better quality attributes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/2/158/s1. Table S1: Identification and sensory descriptors of volatile compounds on pistachios affected by regulated deficit irrigation and rootstock.

Author Contributions: Contributed to experimental design, collected data, interpreted the results and drafted the manuscript, L.N.-A. and P.S.-B.; Contributed to experimental design of analysis of volatile compounds, A.S.; Contributed to experimental design of irrigation treatments, D.P.-L.; Interpreted the all results and revised the manuscript, A.A.C.-B, A.B.-H., and A.C.-S. All authors have read and agreed to the published version of the manuscript.

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Determination of the Volatile Profile of Lemon Peel Oils as Affected by Rootstock

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Abstract: *Citrus limon* (L.) Burm is an important crop that grows between latitudes 30° North and 30° South, the main producers being China, the USA, Mexico, India, Brazil, and Spain. In Spain, lemon grows mainly in Mediterranean areas such as Murcia, Valencia, and Andalucía. The most cultivated varieties are "Fino" and "Verna". In this study, five varieties of lemon, "Verna", "Bétera", "Eureka", "Fino 49", and "Fino 95" were evaluated on different rootstocks: three new Forner-Alcaide ("FA13", "FA5", "FA517"), *Citrus macrophylla*, Wester, and *Citrus aurantium* L. Hydrodistillation was used to obtain essential oil from fresh peels and then the volatile profile was studied by gas chromatography-mass spectrometry (GC-MS). A total of 26 volatile compounds were identified, limonene being the main one followed by β-pinene, γ-terpinene, sabinene, and α-pinene. The results revealed that Forner-Alcaide rootstocks ("FA5" > "FA517" > "FA13") proved to be the best rootstocks for the aroma quality as they led to high volatile contents, followed by *C. aurantium* and *C. macrophylla*. Among the other varieties, the most aromatic one was "Eureka". The whole trend was as follows (in decreasing order): "Eureka" > "Bétera" > "Fino 95" > "Verna" > "Fino 49".

Keywords: aroma composition; *Citrus limon* (L.); concentrations—monoterpene; GC-MS; limonene; sesquiterpenes; aldehydes

1. Introduction

Lemon is an important crop that grows in different parts of the world. The main lemon producers are in China, the United States, Mexico, India, Brazil, and Spain [1]. In Spain, it grows mainly in the Mediterranean areas of Murcia, Valencia, and Andalucía, which represent the highest productions [2]. These high productions are associated with selected, suitable, and compatible rootstocks [3]. Moreover, the use of rootstock influences quantitative and qualitative characteristics of agronomic variables which improve size, color, soluble solids, acidity, yield, and quality of the fruit [4–6].

Selecting the proper rootstock is decisive in order to succeed in a commercial citrus fruit plantation [4]. Fruit quality is currently valued, in addition to visual attributes (e.g., size, color), including chemical properties such as contents of vitamins, minerals, carotenoids, phenols, and volatile compounds [7]. The organic compounds are associated with the fruit aroma and are present in

peel, flowers, leaves, and juice [8]. In citrus species, the main quality characteristic is the aroma [9]. The quality of the lemon is highly influenced by the rootstock [10]. Several factors may modify the volatile profile of the lemon, including factors such as rootstock and variety [11–13]. Among these factors we can include environment, soil fertility, the content of beneficial microorganisms, the state of immaturity (green color), and unpeeled vs. peeled fruit juice [14–17]. Additionally, the volatile fraction may be altered by analytical method, sampling, and equipment used [18,19].

Throughout the world, citrus flavors are some of the most important flavors in the global market [20]. In this sense, fresh lemon peel can be used to obtain volatile compounds which give the characteristic citrus aroma and flavor [21,22]. For this, many citrus cultivars have been analyzed to identify their volatile profile [20]. Several authors have studied the volatile profile of the oil from the lemon peel [22–24], but information on how the rootstock influences the odorous compounds is very limited.

Thus, the purpose of this study was to identify and quantify the volatile profile of five varieties of lemon grafted on five rootstocks and analyze the influence that the rootstock–graft interaction can have on the volatile profile of lemons.

2. Materials and Methods

2.1. Plant Materials

Fruits were collected from 10-year-old, healthy trees, cultivated under the same pedoclimatic and cultural conditions. The climate was characterized by mild winters and slightly hot summers, temperatures ranging between 26 and 17 °C, and light rains concentrated in spring and autumn. Soil characteristics were as follows: sandy loam texture, 40% calcium carbonate, 8% active calcium, and pH = 8. The field was located at the IVIA (*Instituto Valenciano de Investigaciones Agrarias*) Experimental Station in Elche (latitude 38°14′56″ N, longitude 0°41′35.95″ E, altitude 149 m above sea level).

The selected varieties of *Citrus limon* used in this study were "Betera", "Verna", "Fino 49", "Fino 95", and "Eureka"; grafted on to the rootstocks Forner-Alcaide N°5 ("FA5"), Forner-Alcaide N°13 ("FA13"), Forner-Alcaide N°517 ("FA517"), *C. macrophylla* West, and *C. aurantium* L. The progenitors of hybrids Forner-Alcaide N°5 ("FA5") and Forner-Alcaide N°13 ("FA13") were "Cleopatra" mandarin × *Poncirus trifoliata* (L.) Raf., both characterized as being resistant to salinity and tolerant to waterlogging. Forner-Alcaide N°517 ("King" (mandarin) × *P. trifoliata*) is distinguished by its tolerance to limestone and its dwarfing character. All of them reside in the European Union (BOE/04/12/2007) and were obtained by targeted hybridizations by Forner in IVIA (Valencia) [25].

Twenty-five plots (a combination of variety and rootstock) were used for this study, with a completely randomized factorial design. Each plot was composed of six trees spaced at 6 m × 2.5 m.

Twenty lemons from each tree in each plot were collected. Next, the lemons were manually peeled with a peeler (no albedo was collected). Subsequently, the lemon peels were crushed with a grinder (Delhi model 180 W; Moulinex, Alençon, France) for 3 min and kept at −20 °C until analysis.

2.2. Determination of Volatile Compounds

For the determination of the volatile compounds, the essential oil was extracted using the protocol described by El-Zaeddi et al. [26] with slight modifications. Hydrodistillation (HD) using a Deryng system was used for isolating the essential oil in the lemons. Sixty grams of crushed lemon skin was placed in a 500 mL round-bottom flask with 200 mL of distilled water and 200 µL of isoamyl acetate, which was used as an internal standard. Once the mixture was boiling for 5 min, 2 mL of the essential oil was collected in a vial of 2.5 mL and maintained in refrigerated storage (4 °C) until the gas chromatography-mass spectrometry (GC-MS) analyses were conducted. All the samples were extracted in triplicate.

Volatile compounds were analyzed and identified using a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu QP-5050A mass-spectrometry detector (Shimadzu Corporation, Kyoto, Japan). The GC-MS system was equipped with a Supelco (Supelco, Inc., Bellefonte, PA, USA) SLB-5 MS column

(fused silica) 30 m × 0.25 mm, with a film thickness of 0.25 µm. The carrier gas used for this analysis was helium kept at a column flow rate of 0.6 mL min^{-1} and a total flow of 181.2 mL min^{-1} in a split ratio of 1:300. The program started with an increase of 3 °C min^{-1} from 80 to 170 °C. Afterwards, the temperature was increased at 25 °C·min^{-1} to 300 °C, maintaining this final temperature for 1 min. The temperature of the detector was 300 °C, and it was 230 °C for the injector.

Three methods were used to identify volatile compounds: (1) retention rates and their comparison with those in the literature; (2) retention times of pure chemical compounds; (3) mass spectra of authentic chemical compounds and the spectral library of the National Institute of Standards and Technology (NIST) database. In this study, only fully identified compounds have been described. The analysis of the volatile composition was run in triplicate for each extraction and the results were expressed as the concentration of each of the volatile compounds as well as the concentration of the main chemical families of compounds.

2.3. Statistical Analysis

Two-way analysis of variance (ANOVA) and Tukey's multiple range test were performed to compare experimental data and to determine significant differences among varieties and rootstock ($p < 0.05$). Principal component analysis (PCA) using Pearson correlation was also run. The software XLSTAT (Addinsoft 2016.02.270444 version, Paris, France) was used.

3. Results and Discussion

3.1. Identification of Volatile Compounds in Lemon Peels

Twenty-six volatile compounds in the lemon peel oils were identified by GC-MS (Table 1). These compounds can be grouped into four main chemical families: (i) monoterpenes (20 compounds); (ii) sesquiterpenes (3 compounds); (iii) aldehydes (2 compounds), and (iv) esters (1 compound). Moreover, Table 1 shows the main sensory descriptors of each of the volatiles identified in the lemon peel oils.

Table 1. Retention indexes of the volatile compounds by GC-MS in lemon peel oils.

	Compound	Chemical Family	Odor Properties	RT † (min)	KI (Exp.) ‡	KI (Lit.) *
1	α-Thujene	Monoterpene	Wood, green, herb ★	5.09	930	933
2	α-Pinene	Monoterpene	Pine, turpentine ★	5.28	939	944
3	Camphene	Monoterpene	Camphor ★	5.64	969	964
4	Sabinene	Monoterpene	Pepper, turpentine, wood ★	6.03	983	977
5	β-Pinene	Monoterpene	Pine, resin, turpentine ★	6.21	990	990
6	Octanal	Aldehyde	Strong and fruity smell ⊙	6.62	1004	1001
7	α-Phellandrene	Monoterpene	Turpentine, mint, spice ★	6.78	1010	1003
8	α-Terpinene	Monoterpene	Lemon ★	7.04	1019	1018
9	p-Cymene	Monoterpene	Woody and spicy ✠	7.25	1027	1026
10	Limonene	Monoterpene	Lemon, orange ★	7.44	1034	1031
11	γ-Terpinene	Monoterpene	Gasoline, turpentine ★	8.15	1059	1062
12	*cis*-Sabinene-hydrate	Monoterpene	Herbal ★	8.64	1076	1074
13	Terpinolene	Monoterpene	Herbal ★	8.99	1089	1089
14	Linalool	Monoterpene	Flower, lavender ★	9.39	1103	1098
15	Nonanal	Aldehyde	Rancid ✠	9.51	1106	1102
16	Citronellal	Monoterpene	Fat ★	11.17	1152	1165
17	Terpineol-4	Monoterpene	Peppery, woody, sweet, musty ★	12.46	1189	1184
18	α-Terpineol	Monoterpene	Oil, anise, mint ★	13.02	1204	1197
19	Nerol	Monoterpene	Sweet ★	14.11	1231	1228
20	Neral	Monoterpene	Lemon ★	14.63	1244	1239
21	Geraniol	Monoterpene	Rose geranium ★	15.16	1257	1255
22	Geranial	Monoterpene	Lemon, mint ★	15.82	1273	1277
23	Neryl acetate	Ester	Fruit ★	19.43	1360	1366
24	*trans*-Caryophyllene	Sesquiterpene	Wood and spicy ±	22.18	1425	1420
25	*trans*-α-Bergamotene	Sesquiterpene	Wood ★	22.59	1435	1437
26	β-Bisabolene	Sesquiterpene	Balsamic ★	25.68	1509	1509

† RT = retention time, ‡ KI (Exp.) = experimental Kovats indexes, * KI (Lit.) = literature Kovats indexes; ★ Tekgül and Baysal [23]; ⊙ Lewis and Wiley [27]; ✠ Bravo et al. [28]; ± Pino et al. [29].

3.2. Effects of Rootstock/Scion Combination in the Profile Volatile Compounds

Table 2 shows the concentration of the 26 compounds, expressed in mg·kg^{-1}, identified and quantified in lemon peel oils. The order from the highest to lowest concentration was: limonene, β-pinene, γ-terpinene, sabinene, α-pinene, geranial, neral, α-thujene, β-bisabolene, terpinolene, *trans*-α-bergamotene, α-terpineol, α-terpinene, neryl acetate, linalool, *p*-cymene, citronellal, *trans*-caryophyllene, terpineol-4, nerol, camphene, nonanal, geraniol, octylaldehyde, α-phellandrene, and *cis*-sabinene-hydrate. These results agreed with those previously obtained by Gonzalez-Mas et al. [30], Liu et al. [31], Cano-Lamadrid et al. [32], and Tekgül and Baysal [23].

The volatile profile of the five varieties of lemon studied was dominated by only five monoterpene hydrocarbon compounds (in decreasing order): limonene, β-pinene, γ-terpinene, sabinene, and α-pinene (Table 2). The most abundant volatile compound found in all varieties was limonene, and this volatile compound ranged from 19.76 g·kg^{-1} ("Verna") to 22.71 g·kg^{-1} ("Eureka"). Limonene was followed by β-pinene, the content of which ranged from 3.75 g·kg^{-1} ("Fino 95") to 5.01 g·kg^{-1} ("Verna"), γ-terpinene from 3.22 g·kg^{-1} ("Fino 49") to 3.84 g·kg^{-1} ("Verna"), sabinene from 0.61 g·kg^{-1} ("Fino 95") to 0.85 g·kg^{-1} ("Verna"), and α-pinene from 0.64 g·kg^{-1} ("Fino 95") to 0.79 g·kg^{-1} ("Verna"). Among the varieties, the highest concentration of total volatile compounds was found (in decreasing order) in "Eureka", followed by "Bétera" > "Fino 95" > "Verna" > "Fino 49". The essential oil composition of the current five varieties of lemon was similar to that reported by Di Vaio et al. [33], who analyzed the peel of 18 lemon cultivars, and by Lota et al. [34] who analyzed the peel and leaf essential oils of 15 species of mandarins. Another 15 monoterpene hydrocarbons which had not been previously identified in lemon peel were also identified and quantified, but at lower contents (<0.2 g·kg^{-1}). Di Vaio et al. [33] only identified 5 monoterpene in 18 lemon cultivars studied compared with the 20 monoterpenes identified in the present study. These differences may be due to the extraction methods, among other factors. Lu et al. [19] showed that differences in the presence or absence of volatile compounds depend on the oil distillation process; there is a greater presence of oxygenated compounds when hydrodistillated and a higher concentration of terpene compounds when pressed cold.

The results showed that rootstock strongly affected the total volatile contents (Table 2). The rootstocks of the Forner-Alcaide series ("FA517", "FA13", and "FA 5") showed the highest values of limonene and γ-terpinene (>22 g·kg^{-1} and >3.8 g·kg^{-1}, respectively), while the lowest values were in *C. macrophylla* and *C. aurantium*. In general, the series Forner-Alcaide rootstocks induced a greater content of all the volatile compounds identified compared to the traditional *C. aurantium* and *C. macrophylla* rootstocks. The reason for these differences among the rootstock of the Forner-Alcaide series and the *C. aurantium* and *C. macrophylla* rootstock might be to do with the specific rootstock/scion combinations which affect citrus fruit aroma volatiles levels, and these qualities may be governed by the level of rootstock/scion compatibility, which obviously affects the translocation of water, nutrients, plant growth regulators, and photosynthetic assimilates through the graft union.

The sesquiterpenes were the second most abundant chemical group in the lemon peel (Table 2). Only three compounds were identified (in decreasing order): β-bisabolene, *trans*-α-bergamotene, and *trans*-caryophyllene. Furthermore, the rootstocks of the Forner-Alcaide series showed the highest content for these three sesquiterpenes, while the *C. macrophylla* and *C. aurantium* had the lowest.

Two aldehyde compounds were identified: nonanal and octanal. The aldehyde concentrations were in the range of 14.7 to 28.9 mg·kg^{-1} in the varieties grafted on "FA 517" and *C. macrophylla* respectively for nonanal, and ranged between 10.2 mg·kg^{-1} to 19 mg·kg^{-1} in the varieties grafted on *C. aurantium* and "FA 5" respectively for octanal (Table 2).

Finally, regarding the esters, only one compound was identified: neryl acetate. No significant differences were observed in either the variety or the rootstock (Table 2).

Table 2. Concentrations (mg·kg^{-1}) of volatile compounds in lemon peel oils.

	ANOVA †			Variety					Rootstock				
Compound	**V**	**R**	**V*R**	**Verna**	**Betera**	**Eureka**	**Fino 49**	**Fino 95**	**FA 5**	**FA 13**	**FA 517**	***C. macrophylla***	***C. aurantium***
α-Thujene	***	***	***	195.1 a ‡	188.0 ab	170.0 bc	156.2 c	120.8 d	187.9 a	179.5 a	180.0 a	140.4 b	142.4 b
α-Pinene	***	***	***	797.3 a	765.8 ab	735.5 ab	704.8 b	648.5 b	798.1 a	743.8 ab	743.2 ab	685.5 b	681.2 b
Camphene	***	***	***	24.8 a	20.9 b	20.9 b	20.1 b	13.1 c	22.8 a	21.2 ab	18.9 b	21.4 ab	15.4 c
Sabinene	***	***	***	853.0 a	758.8 b	721.9 bc	730.3 bc	611.8 c	802.6 a	734.8 abc	691.9 bc	785.9 ab	660.5 c
β-Pinene	***	***	***	5011 a	4390 b	4417 b	4327 b	3757 b	4870 a	4451 abc	4193 bc	4501 ab	3888 c
Octanal	***	***	***	20.9 a	18.8 a	10.2 b	18.5 a	9.9 b	16.2 a	17.6 a	15.1 ab	19.0 a	10.2 b
α-Phellandrene	***	***	***	15.5 a	15.2 ab	14.6 ab	14.3 ab	11.7 b	16.0 a	15.4 a	15.9 a	12.3 b	11.7 b
α-Terpinene	***	***	***	101.0 a	98.1 ab	86.6 ab	87.1 ab	81.6 b	101.9 a	101.8 a	99.2 a	73.6 b	77.9 b
p-Cymene	NS	NS	***	88.1	80.0	88.5	86.6	55.8	85.6	92.0	72.9	79.6	68.9
Limonene	***	***	***	19,760 c	21,140 b	22,716 a	20,398 bc	22,107 ab	22,248 a	22,189 a	22,726 a	18,604 c	20,354 b
γ-Terpinene	***	***	***	3849 a	3786 a	3439 ab	3226 b	3299 ab	3967 a	3807 a	3882 a	2770 b	3172 b
cis-Sabinene-hydrate	***	NS	***	15.3 a	9.0 b	7.7 b	6.0 b	3.8 b	6.2	6.2	7.4	11.0	11.0
Terpinolene	***	***	***	167.8 a	166.2 a	151.5 ab	142.3 b	145.2 ab	175.5 a	167.6 a	171.2 a	119.9 b	138.8 b
Linalool	NS	NS	***	80.2	80.2	76.3	89.8	74.7	87.0	85.1	85.0	74.2	69.9
Nonanal	NS	**	**	21.1	21.4	17.8	23.9	14.8	19.0 ab	19.1 ab	14.7 b	28.9 a	17.4 b
Citronella	NS	***	***	74.1	76.7	68.3	68.5	60.0	85.3 a	77.2 a	85.4 a	49.7 b	50.0 b
Terpineol-4	*	NS	***	55.0 a	52.7 a	45.0 a	52.3 a	28.3 b	50.7	57.1	46.7	46.7	32.1
α-Terpineol	**	NS	***	141.5 a	141.7 a	116.8 ab	135.0 a	60.6 b	113.1	118.4	110.7	143.2	110.3
Nerol	NS	NS	**	38.4	38.0	33.4	51.1	22.3	42.6	44.5	37.5	40.3	18.3
Neral	NS	*	**	265.3	258.0	220.5	329.1	251.5	289.0 ab	317.0 a	287.3 ab	237.4 ab	193.5 b
Geraniol	NS	*	**	15.6	11.4	13.5	24.2	9.2	21.0 a	21.2 ab	14.5 ab	12.4 ab	3.8 b
Geranial	NS	NS	**	265.5	263.7	210.0	341.4	256.2	294.0	325.2	291.7	241.8	184.2
Neryl acetate	NS	NS	***	107.8	92.3	122.2	86.1	59.9	99.5	82.7	89.4	92.5	104.1
trans-Caryophyllene	***	***	***	64.8 ab	61.4 ab	75.8 a	55.2 b	46.9 b	61.5 ab	54.0 b	71.2 a	49.3 b	68.0 ab
trans-α-Bergamotene	***	***	***	139.0 b	142.5 ab	168.2 a	133.1 b	124.0 b	150.2 ab	138.3 bc	162.0 a	117.5 c	138.8 abc
β-Bisabolene	NS	***	***	170.8	174.6	203.9	167.1	156.1	185.1 a	171.9 ab	197.0 a	142.4 b	176.1 ab
Total	NS	***	***	32,338	32,851	33,950	31,473	32,029	34,796 a	34,037 a	34,310 a	29,100 b	30,399 b

† NS = non-significant F ratio ($p < 0.05$); *, ** and *** significant at $p < 0.05$, 0.01, and 0.001, respectively. ‡ Values followed by the same letter within the same row were not significantly different ($p < 0.05$) according to Tukey's least significant difference test ($n = 9$).

In this study, we examined the effects of five rootstocks, three new in the Forner-Alcaide series, and two commercially important rootstocks (i.e., *C. aurantium* and *C. macrophylla*) on volatile compounds in the lemon peel oils of five varieties. The results indicate that the effect of rootstock on the volatile compounds is a rather complex phenomenon that greatly depends on specific interactions between the rootstock and each particular scion variety. Our results agreed with those reported by Benjamin et al. [4] in varieties of mandarins, Seker et al. [35] in the fruits of peach, and Wang et al. [12] in grapevines and in pistachios [36]—they all noted that rootstocks influenced the concentration and availability of volatiles. This could be explained by the fact that grafted plants generally increase the uptake of water and minerals due to the roots of rootstock or the compatibility of graft and canopy [37].

3.3. Principal Component Analysis

To better understand the relationships among the volatile compounds found (26 volatile compounds) in the different samples (varieties and rootstocks), principal component analyses (PCAs) were applied to the experimental results (Figures 1 and 2). The PCA of the rootstocks (Figure 1) explained 92.05% of the variables in two axes, F1 (59.98%) and F2 (32.07%). Thanks to this statistical technique, it was very easy to observe that the *C. macrophylla* and *C. aurantium* rootstocks were isolated from the rest of the rootstocks, and were therefore characterized by volatile compounds such as nonanal and α-terpineol for *C. macrophylla* and neryl acetate in the case of *C. aurantium*. The rootstocks "FA517", "FA5", and "FA13" were linked to a higher number of volatile compounds, perhaps because genetically these rootstocks have a common parent and are characteristically smaller trees [25].

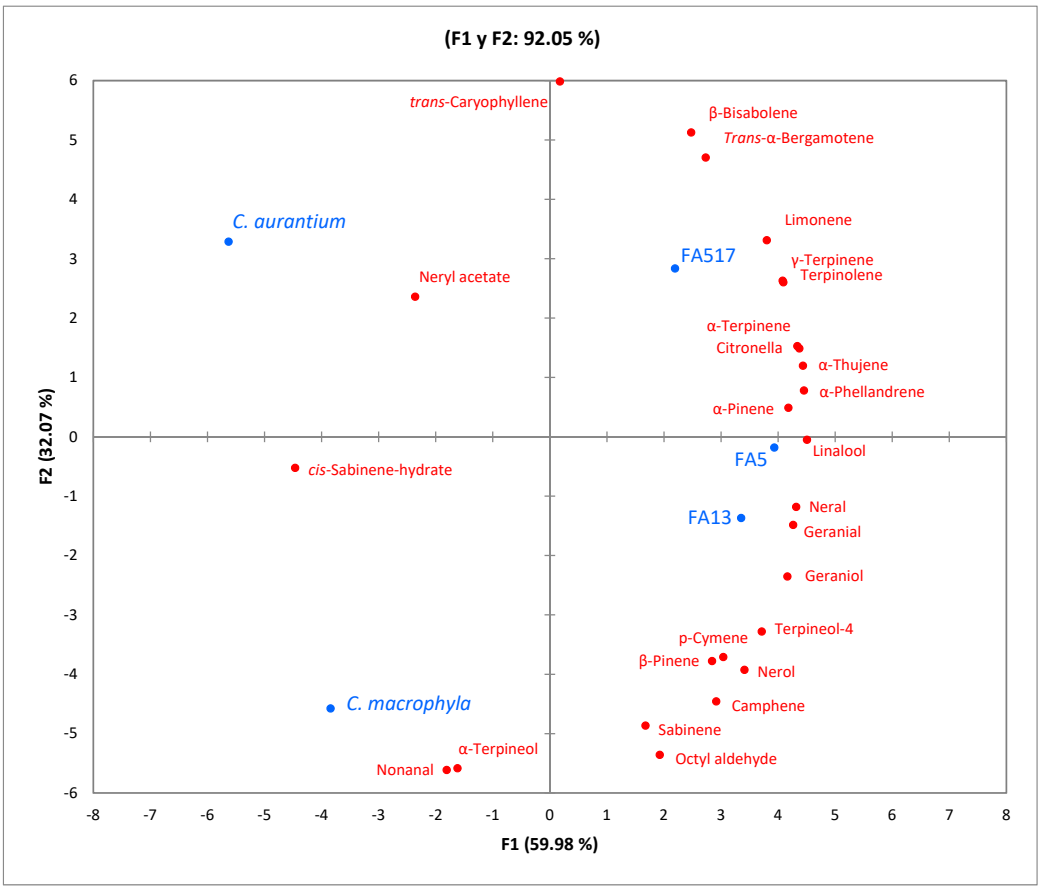

Figure 1. Principal component analysis (PCA) plot showing the relationships among volatile compounds and the factor "rootstock" (n = 9).

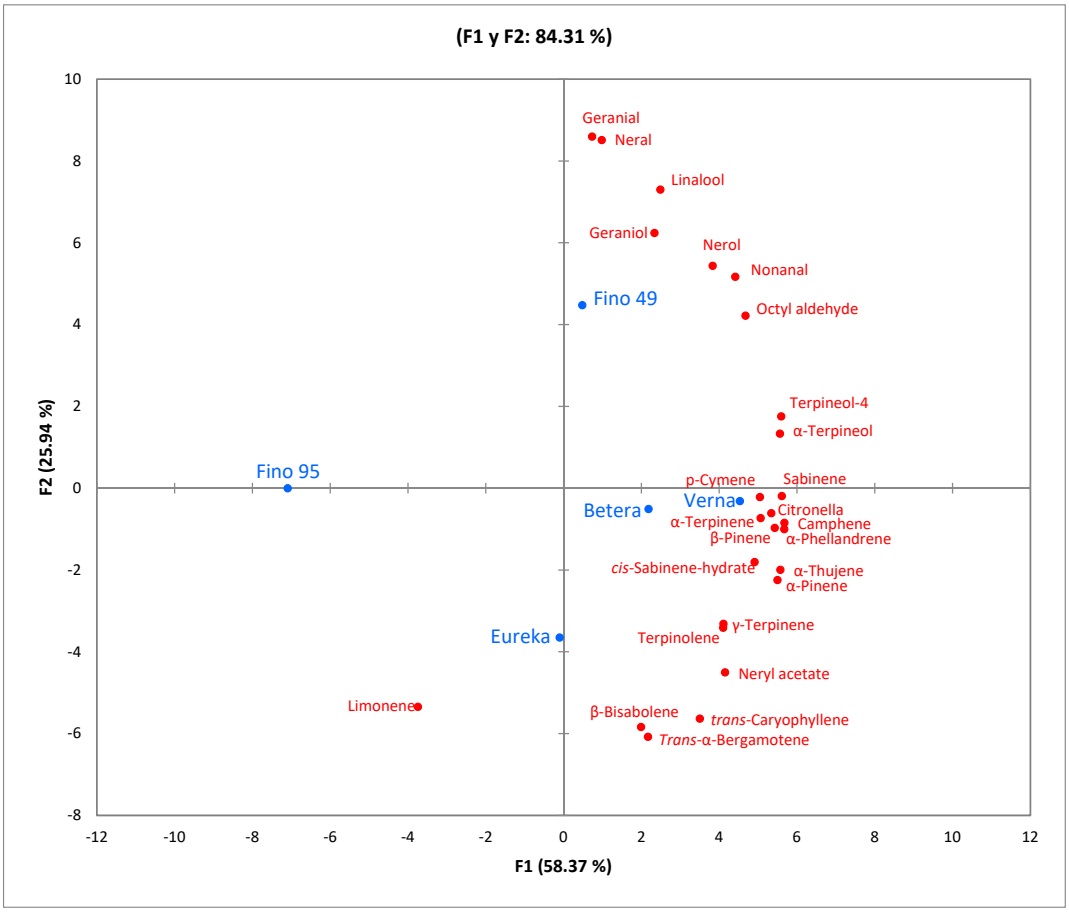

Figure 2. Principal component analysis (PCA) plot showing the relationship among volatile compounds and the factor "variety" (n = 9).

On the other hand, the PCA of the varieties (Figure 2) explained 84.31% of the variables in the F1 (58.37%) and F2 (25.94%) axes. This indicated that varieties such as "Betera", "Verna", and even "Eureka" had very similar aromatic profiles, while varieties such as "Fino 95" and "Fino 49" were isolated.

4. Conclusions

In this study, five rootstocks (three Forner-Alcaide rootstocks and two traditional *C. macrophylla* and *C. aurantium* rootstocks) were evaluated to study the effect on volatile composition of five commercial lemon varieties: "Bétera", "Verna", "Eureka", "Fino 49", and "Fino 95". A total of 26 aromatic compounds were identified and quantified by GC-MS in lemon peel oils. Of all the aroma compounds identified in lemon peel oils, five monoterpene hydrocarbons (limonene, β-pinene, γ-terpinene, sabinene, and α-pinene) were present at the highest levels, followed by sesquiterpenes, aldehydes, and esters. The present experimental results demonstrate that Forner-Alcaide rootstocks ("FA5" > "FA517" > "FA13") were the best rootstocks, leading to high content of volatile compounds, followed by *C. aurantium* and *C. macrophylla*. The order of total volatile contents was (in decreasing order): "Eureka" > "Bétera" > "Fino 95" > "Verna" > "Fino 49". These results confirm that a strong relationship exists between the rootstock/scion combinations and the concentration of volatile compounds in the lemon peel oil. Aroma volatiles should be considered key parameters for the determination of rootstock-induced effects.

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Characterization of Volatile Profiles and Marker Substances by HS-SPME/GC-MS during the Concentration of Coconut Jam

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Abstract: Characteristic aromas are usually key labels for food products. In this study, the volatile profiles and marker substances of coconut jam during concentration were characterized via sensory evaluation combined with headspace solid phase microextraction-gas chromatography-tandem mass spectrometry (HSPME/GC-MS). A total of 33 aroma compounds were detected by HSPME/GC-MS. Principal component analysis revealed the concentration process of coconut jam can be divided into three stages. In the first stage, esters and alcohols were the two main contributors to the aroma of the coconut jam. Next, a caramel smell was gradually formed during the second stage, which was mainly derived from aldehydes, ketones and alcohols. The concentration of aldehydes increased gradually at this stage, which may be the result of a combination of the Maillard reaction and the caramelization reaction. In the final sterilization stage, the 'odor intensity' of caramel reached the maximum level and a variety of aroma compounds were produced, thereby forming a unique flavor for the coconut jam. Finally, furfural fit a logistic model with a regression coefficient (r^2) of 0.97034. Therefore, furfural can be used as a marker substance for monitoring the concentration of coconut jam.

Keywords: coconut jam; volatile profiles; HS-SPME/GC-MS; marker substances; PCA

1. Introduction

The production of jam is one of the oldest food preservation techniques that allows people to enjoy all kinds of fruits during the off-season. According to European Union standards, jams, honey and dried fruits are classified as high-sugar, low-water products [1]. In China, coconut is mainly distributed in the Wenchang area along the coast of the Hainan province. Coconut water and pulp contain a variety of nutrients and have a unique flavor. Coconut water is considered a healthy beverage because it contains a variety of vitamins and minerals, enzymes with anti-inflammatory properties, and antioxidants [2]. In mature coconuts, coconut pulp is commonly used in coconut milk production [3,4]. The physical and chemical properties of coconut pulp make this food suitable for consumption in natural conditions. In addition, coconut pulp is an excellent raw material for the jam industry. However, little research has been conducted on coconut jam. Product development and formulation research are essential parts of the jam industry [5]. The quality of jam is normally defined according to its flavor, color and texture. Among these fundamental properties, aroma as the sensory indicator plays a key role in evaluating the quality of products. From an industrial point of view, an efficient procedure is

required to control the quality and stability of the products. Aroma may be a good option to monitor production as a marker [6]. Generally, heating to a high temperature can improve the variety of aroma compounds in coconut jam products and create a unique flavor. Aroma compounds have been identified in coconut water, including alcohols, aldehydes, esters, and acids [7]. Moreover, the flavor perceived by consumers is mainly due to many volatile compounds at different concentrations. Therefore, it is particularly important to explore the changes of the characteristic aroma compounds in the coconut jam during processing.

Due to the advantages of solvent free sample processing, high sensitivity and reliability, headspace solid phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) has been widely used for the analysis of volatile compounds [8–12]. The purpose of this study was to verify the volatile compounds of coconut jam produced during the concentration process. Through statistical analysis of a number of aroma compounds, the characteristic aroma compounds appearing in each concentration stage were determined. The marker aroma substances were combined with the concentration time to fit the kinetic equation, which may achieve the monitor production of coconut jam in the industry.

2. Materials and Methods

2.1. Materials

The fresh coconut pulp was procured from Hainan Taifengyuan Industrial Co., Ltd. (Haikou, China). The sugar was purchased from a local supermarket, while the pectin (degree of esterification<50%) and modified starch were purchased from Guangzhou Dilian Trading Co. Ltd. (Guangzhou, China). The other additives (sucrose ester, soy protein isolate, maltodextrin, fructose syrup and carboxymethyl cellulose) were procured from the Guangzhou Xinzhiwei Food Ingredients mall (Guangzhou, China).

The formula (Table 1) preparation method was provided by local food companies. Briefly, the desired amounts of sugar, maltodextrin and additives were added to the coconut pulp and then the mixture was transferred to an open stainless-steel pan. Batch heating was achieved in a convection oven, and the heating times were 0, 4, 8, 12, 16 and 20 min, respectively. The canning sterilization was conducted at 121 °C for 20 min. The prepared samples were placed in aluminum-foil pouches, sealed and stored at −20 °C.

Table 1. Detailed ingredient ratios of coconut jam (proportion of ingredients added per 100 g of coconut jam).

No.	Ingredient	Proportion (%)
1	Coconut pulp	40
2	Fructose syrup	18
3	Sugar	18
4	Maltodextrin	18
5	Modified starch	3.6
6	Soy protein isolate	1.8
7	Carboxymethyl cellulose	0.3
8	Sucrose esters	0.3
9	Monoglyceride	0.3
10	Pectin	0.2

2.2. Sensory Analysis of Aroma

The sensory evaluation panel consisted of ten members (aged 20–30) including male and female members of the college of Food Science and Engineering of Hainan University. They were trained in basic odor recognition tests before performing analysis, according to the references [13–15] (Table 2). All the evaluations were conducted at the Fruit and Vegetable Processing Laboratory of Hainan University. The coconut jam samples were stored at −20 °C and were taken out 2 h before serving.

Since the study focused on the characteristic aromas of coconut jams during the concentration process, the jams were evaluated for odor only. Before the formal odor assessment, the coconut jam packaging was checked to ensure that it was intact and free of air leaks. All the samples were placed in 50 mL cups and evaluated at room temperature. During the evaluation, the samples were sealed with polyethylene film to protect their aroma from volatilization. The samples were evaluated based on a 5-point intensity scale ranging from 1 'low' (barely detectable) to 5 'high' (moderately detectable). The mean values of these sensory properties were evaluated as the 'odor intensity'.

Table 2. Description and definition of the aroma of coconut jam.

Categories	Descriptors	Definitions
Odor/Flavor	Fruity	May resemble the odor of coconut, pineapple, apple, or other fruits
	Caramel	Cooked sugar, all which reminds sugar cooking, caramel
	Acid	Sour off-flavor due to acid-producing organisms such as *Lactococcus lactis ssp. cremoris*
	Fatty	Aromatics associated with stale fats
	Honey	Aromatics associated with the sweet fragrance of honey

2.3. Headspace Solid Phase Microextraction of Volatiles

The headspace solid phase microextraction (HS-SPME) method was appropriately modified based on the reference of Liu et al. [16]. Coconut jam (1 ± 0.01 g) was injected into a headspace vial (20 mL) with a syringe and incubated in a water bath at 40 °C for 40 min. The volatile components were extracted with SPME fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm Supelco, America) [17,18]. Before analysis, the fibers were conditioned and thermally cleaned by inserting them into the injector port of the GC system at 270 °C for 30 min in a stream of helium, and the aromatic compounds were absorbed by the SPME fiber in the headspace vial at 40 °C for 40 min. The desorption of volatile compounds from the SPME fiber in the GC injector was performed for 5 min, and then analyzed using the GC-MS.

2.4. Gas Chromatography-Tandem Mass Spectrometer

Gas chromatography-tandem mass spectrometry (GC-MS) method was appropriately modified based on the reference of Yang et al. [19]. The volatile compounds of the coconut jam were analyzed with a GC - MS instrument (GC-MS-QP2010, Shimadzu, Kyoto, Japan). The analysis was performed on a ZB-5MS silica capillary column (30 m × 0.25 mm, 0.25 μm) equipped with a mass detector. Helium (99.999% purity) was used as the carrier gas with a flow rate of 1.0 mL/min. The temperature of both the injector and detector was set at 270 °C. The programmed sequence for the column was set as follows: an initial temperature of 45 °C was held for 4 min and increased at 5 °C/min to 150 °C/min prior to being increased to 220 °C at 10 °C/min and held at 220 °C for 5 min. The mass detector was equipped and set in electron impact mode at an ionization voltage of 70 eV in the 50–500 amu (atomic mass unit) scan range for mass spectrum collection, and the ion source temperature was 250 °C.

Volatile compounds in coconut jam were identified according to the method of Choi et al. [20]. The volatile compounds were identified by searching the NIST spectrometry library and the retention index (RI) was calculated using a linear heating formula (Equation (1)). Finally, the chemical structure with the closest similarity to the mass spectrum and RI value was selected as the best identification result. Quantitative (relative content) analysis of aroma substances was achieved by peak area normalization [3]. The final volatile components and relative contents determined are shown in Table A1.

$$RI = 100_n + \frac{100(t_x - t_n)}{t_{n+1} - t_n} \quad (1)$$

where t_x, t_n, and t_{n+1} are the retention times of the outflow peaks of the component analyzed and the n-alkanes ($t_n < t_x < t_{n+1}$) with carbon numbers of n and n + 1, respectively.

2.5. Kinetics of Furfural Formation

Kinetic models can be used to predict the formation of compounds. Knol et al. used a logistic model to predict the formation and degradation of acrylamide in potato chips [21,22]. The formation kinetics of furfural in the coconut jams during the concentration process is consistent with the four-parameter logistic equation:

$$Y = \frac{A_1 - A_2}{1 + \left(\frac{t}{C}\right)^K} + A_2 \quad (2)$$

where A_1 is the minimum content of furfural, A_2 is the maximum content of furfural, t is the concentration time, C is the concentration at the inflection point during the formation of furfural and K is the slope at the inflection point during the formation of furfural.

2.6. Statistical Analysis

The data for different aroma compounds were presented as means ± standard errors. Each class of volatile compounds and sensory data were subjected to the analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range test ($p < 0.05$) using SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). The correlation matrix analysis was performed on the mean of aroma data by using principal component analysis (PCA). The cluster analysis was conducted by R (The University of Auckland, Auckland, New Zealand). The type of linkage method and distance measurement were "complete" and "Euclidean", respectively. Logarithmic transformation of raw data was required to be processed before constructing a heat map. Kinetic fit analysis used OriginPro8 (Origin Lab Inc., Northampton, Massachusetts, USA) software.

3. Results and Discussion

3.1. Sensory Analysis

The aroma sensory evaluation of the coconut jam samples from 0 min to post-sterilization are shown in Figure 1. According to the sensory evaluation radar fingerprint chart, all the samples displayed a combination of fruity, honey, caramel and fatty, but presented almost no acid flavor. As the time of concentration increased, the 'odor intensity' of the aroma increased, especially for post-sterilization, which presented higher values significantly than those of other samples ($p < 0.05$). These results showed that the comprehensive aroma of the coconut jams enhanced greatly after the high temperature sterilization. The coconut jam samples from 0 to 8 min seemed to be characterized by more fruity and honey flavors (induced by the higher contents of ester and alcohol in coconut jam) [23]. According to the results of GC-MS as shown in Table A1, the relative contents of ethyl decanoate and 2-octanol were higher than those of other esters and alcohols in the time range of 0–8 min. It is worth noting that ethyl decanoate had a fruity odor and 2-octanol had aromatic characteristics [24,25]. Therefore, the odor intensity of fruit and honey in coconut jam was more obvious during this period. As the heating continued from 8 min to the end of the sterilization, the caramel aroma gradually became the dominant aroma. This may be due to the Maillard and caramelization reaction during high temperature because hexanal, furfural and benzenecarbonal were detected during this period [26,27]. It is worth mentioning that the relative contents of hexanal and furfural began to rise significantly after 8 min, and benzenecarbonal itself has the characteristics of caramel aroma [28,29]. Therefore, it is possible that the combined effect of these three compounds ultimately leads to a significant increase in the caramel odor intensity.

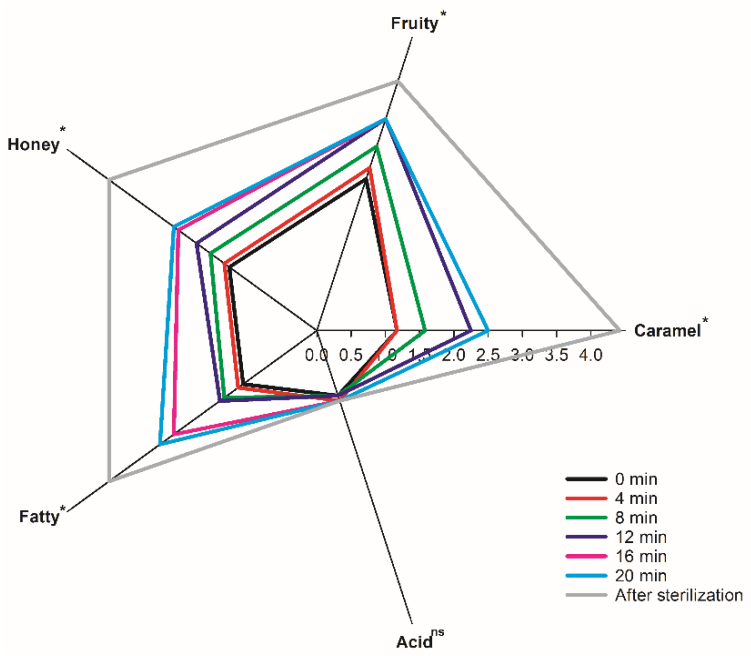

Figure 1. Flavor profiles for the coconut jams (0, 4, 8, 12, 16, 20 min, and after sterilization). The treatments were evaluated in triplicate by 10 panelists (n = 30). Asterisks and "ns" indicate significant (* $p \leq 0.05$) differences and no significant differences of means, respectively.

3.2. HS-SPME/GC-MS Analysis Results

A total of 33 different aroma compounds were detected. According to the relative content of each group of aroma components determined, the components were arranged in order from highest to lowest: aldehydes, ketones, esters, lactones, alcohols, acids, alkenes, furfurans and pyrazines (Figures 2 and 3). The relative content of aldehydes was the highest among the nine types of aroma compounds, accounting for approximately 29.7% of the total flavor components of each coconut jam sample.

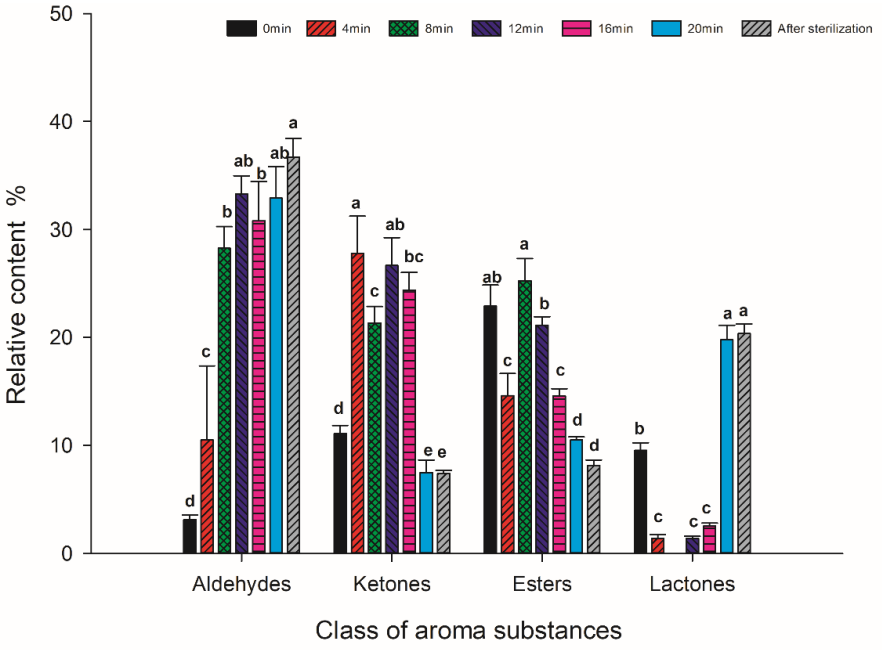

Figure 2. Relative contents of ester, lactone, alcohol and ketone aroma compounds for the coconut jam obtained by GC-MS. Values identified with different letters represent significant differences for each class of compound.

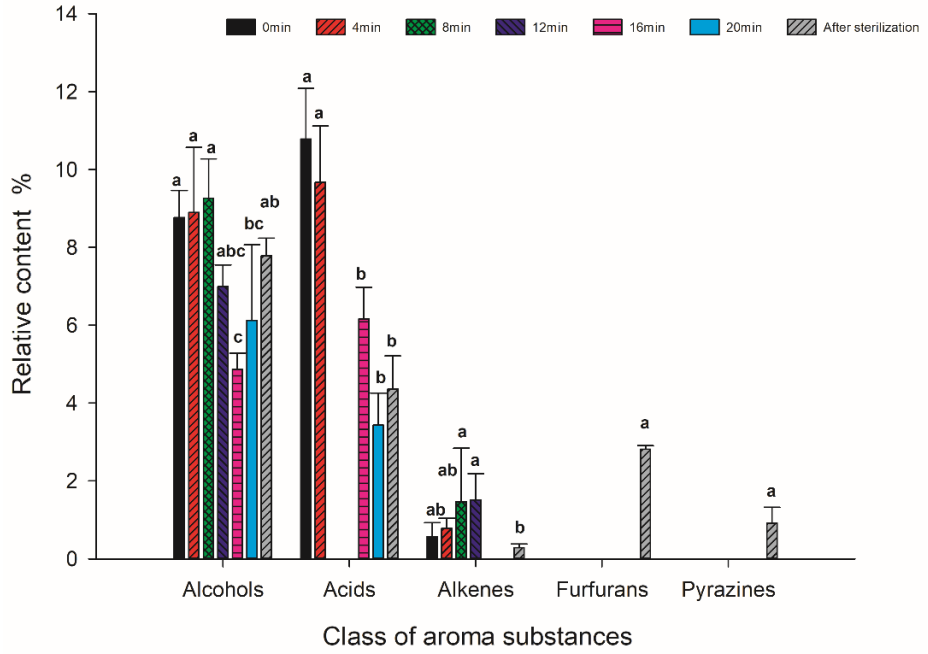

Figure 3. Relative contents of aldehydes, acids, alkenes, furfurans and pyrazines for the coconut jam obtained by GC-MS. Values identified with different letters represent significant differences for each class of compound.

3.2.1. Esters and Lactones

Ester compounds play an important role in the aroma of coconut products. Esters are formed by the enzymatic condensation of organic acids and alcohols [30]. In fresh coconut water, the main esters are ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate. In particular, ethyl decanoate and ethyl dodecanoate are present at the highest concentrations and represent approximately half of all the esters present [23,31]. Ethyl decanoate, ethyl dodecanoate and hexyl formate represented about 82%–92% of the total ester content of the coconut jam samples. As shown in Table A1, coconut jam has the same ester aroma compounds as fresh coconut water, with only a few differences, indicating that the coconut jam basically retains the original flavor of the coconut.

Lactones are cyclic esters with a fruity aroma. In this study, two lactones (delta-nonalactone and delta-dodecalactone) were detected. Delta-nonalactone has fruit and dairy odor characteristics [32]. Delta-dodecalactone has a sweet and fruity scent [33]. It is worth noting that the content of delta-nonalactone kept rising during the heating process and reached the highest value after sterilization, which enhanced the fruit aroma of coconut jam and made its flavor more unique.

3.2.2. Alcohols

Alcohols are formed either by anabolism or catabolism (Ehrlich pathway) of amino acids [30]. These aroma compounds have both positive and negative effects. There are more alcohols in fresh coconut water than in coconut jam. Cappelletti et al. reported that fresh coconut water contained 13 types of alcohols, of which 3-methyl-1-butanol, hexanol, 2-ethyl-1-hexanol, 1-octanol and 1-decanol were present in high amounts, accounting for 72% of all the alcohols [23]. The GC-MS results are shown in Table A1. Five alcohols present in the coconut jam samples were identified as isopentyl alcohol, 2-methyl-1-butanol, pentyl alcohol, 2-octanol and furfuryl alcohol. The content of 2-octanol was the highest among the alcohols from 0 to 20 min. However, furfuryl alcohol became the dominant aroma compound after sterilization and was present at a significantly higher content than the other alcohols ($p < 0.05$). The compound 2-octanol has aromatic odor characteristics, and furfuryl alcohol has caramel aroma characteristics [34]. The formation of furfuryl alcohol and the interaction of this compound with other the aroma compounds imparted a special flavor to the sterilized coconut jam.

3.2.3. Ketones

In total, seven types of ketones were detected in the coconut jam samples. Table A1 shows that 3-hydroxy-2-butanone, 2-octanone, 2-nonanone and 5-hexyl-4-methyldihydro-2(3H)-furanone were detected from 0 to 20 min. Ethylidene acetone, dihydro-2-methyl-3(2H)-furanone and 3-6-dimethyl-tetrahydropyran-2-one were detected after sterilization. The contents of 2-octanone and 5-hexyl-4-methyldihydro-2(3H)-furanone in the ketones were significantly higher than the contents of the other ketones from 0 to 20 min before sterilization ($p < 0.05$), and 2-octanone has soapy and fruity odor characteristics [28]. After sterilization, dihydro-2-methyl-3(2H)-furanone, with sweet and creamy characteristics, interacted with the other ketones to contribute to the aroma of the coconut jams.

3.2.4. Aldehydes

The aldehydes generated during the concentration process of the coconut jams were important precursors in the formation of aromatic compounds such as higher alcohols and esters [35]. From 0 min to post-sterilization, hexanal and furfural always dominated the aldehyde aroma. Hexanal and furfural are common aroma substances in baked and caramel goods flavors. The appearance of these compounds brought a caramel and baking aroma to the coconut jams, which had a positive effect, but the contents of these compounds should not be too high because this condition would mask the fruit flavor of the coconut jams.

3.2.5. Acids and Alkenes

The analysis results of the GC-MS show that only two types of acids and one alkene were detected in the coconut jam samples. The acids were mainly composed of decanoic acid, and the alkenes were mainly composed of (3E)-6-methyl-3-undecene. In general, the contributions of acids and alkenes to the aroma of the coconut jams were comparatively low, due to the presence of compounds that were not particularly odor active. Decanoic acid was present at a relatively high content in the acids, and has rancid and fatty odor characteristics [28] that may negatively affect the overall aroma of coconut jam. As seen from Table A1, the content of decanoic acid gradually decreased from 0 min to post-sterilization. The acids were formed during the early stages of the concentration process. Therefore, to reduce the adverse effects of volatile acids on the overall aroma, the concentration temperature and the agitation speed should be closely controlled in the early processing of coconut jam.

3.2.6. Furans and Pyrazines

The furan and pyrazine aromatic compounds were detected after sterilization. These compounds may have been the result of a combination of the Maillard reaction and caramelization reaction during high temperature sterilization [36,37]. As shown in Table A1, 2-pentylfuran comprised the largest fraction of the furans, and this compound has a fruity fragrance [38]. Therefore, this compound was an aroma compound that had a positive effect on the flavor of coconut jam post-sterilization.

3.3. Changes in the Aroma Components of Coconut Jams during Concentration

The statistical analysis software, R, was used to form cluster heat maps to visually show the differences in the aroma compound contents at different concentration times. As shown in Figure 4, the main aroma compounds displayed various trends. All the aroma compounds in the seven groups of processed coconut jam can be divided into two clusters. R2–R27 (from 3-hydroxy-2-butanone to ethyl decanoate) were grouped into one class, and R12–R32 (from furfuryl alcohol to delta-dodecalactone) were grouped into another class. The amount of furfural and hexanal constantly increased from 0 min to post-sterilization, whereas the amounts of 2-octanone and ethyl decanoate first increased from 0 to 12 min but then gradually decreased. Conversely, the contents of pentyl alcohol, isopentyl alcohol, hexyl formate, ethyl caproate 2-octanol, 2-nonanone and ethyl dodecanoate decreased gradually. These phenomena may be due to the use of sugar as a precursor substance to generate important flavor

substances; sugar generates a variety of aroma compounds when heated to a high temperature and produces aroma substances such as furan derivatives and ketone aldehydes [39]. In addition, during the heating process, the original heat unstable aroma substances in the coconut water were degraded, such as alcohols, ketones, and esters [40]. The Maillard reaction also causes changes in flavor, carbonyl compounds forming by oxidation of ketones and aldehydes, and reduction of sugars. Further, carbonyl compounds and amino acids undergo oxidation, decarboxylation, condensation, and cyclization to form a series of reactive intermediates [41]. However, these reactive intermediates continue to react with amino acids, ultimately causing changes in the flavor of the product.

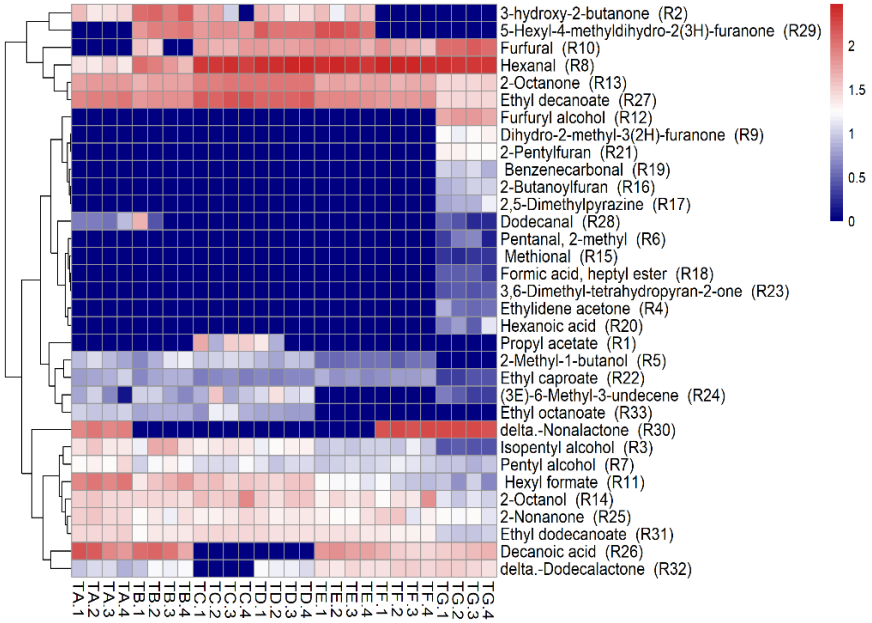

Figure 4. Heat map of the contents of the main volatile compounds during the concentration times and after sterilization. The content value increases with the color varying from blue to red. (TA1 … 4, TB1 … 4 to TG1 … 4) represent samples in quadruplicate at different time points.

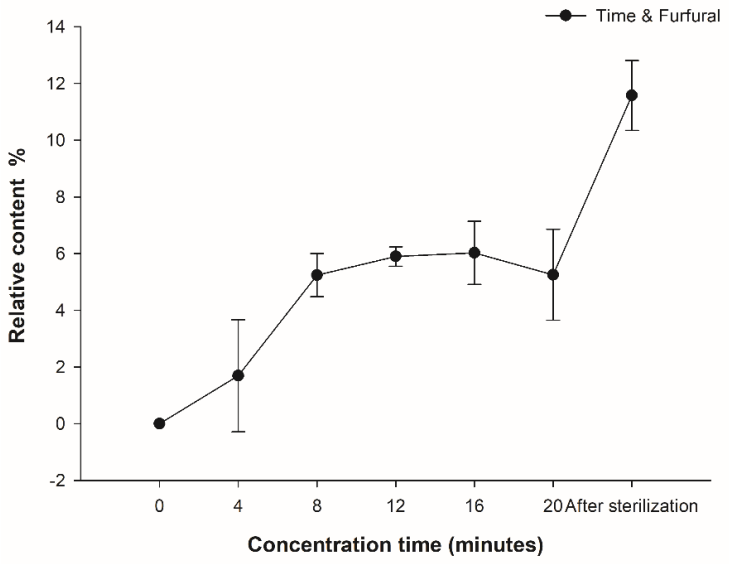

Figure 5. The variation of furfural content during different concentration times.

It was a reasonable and novel choice to use aromatic compounds as production indicators [6,42]. As shown in Figure 5, the relative content of furfural increased with increasing concentration time,

and the two turning points in the growth process occurred between 4 and 8 min and between 20 min and post-sterilization, which was consistent with the PCA analysis results. Moreover, the formation kinetics of the furfural in the coconut jam samples during concentration were fitted to a logistic model. We inserted the parameter values in the model equation to ultimately obtain the kinetic equation of the furfural formation (Equation (3)). The logistic model has a high degree of fit (r^2 = 0.97034).

$$Y = \frac{-5.72899}{1 + (\frac{t}{4.78625})^{4.90798}} + 5.73277 \quad (3)$$

Therefore, furfural can be used as a marker aroma in the production of coconut jam to monitor the degree of concentration of the product.

3.4. Principal Component Analysis (PCA) of the Characteristic Aroma of Coconut Jams

Although the quantitative and qualitative analysis could measure the aroma compounds present in the coconut jams at different concentration times, these methods were not able to determine the characteristic aroma components in the samples. PCA is a multivariate statistical analysis method that employs multiple variables to linearly transform the data to select fewer important variables [43]. Thus, this method can be used to determine the characteristic aroma components of the overall aroma of the dominant coconut jam. The first principal component (PC1) and the second principal component (PC2) explain changes in the data variance of 59.35% and 17.84%, respectively. The cumulative contribution rates of PC1 and PC2 reached a high level, which was sufficient to explain the maximum variation of the aroma substances in the coconut jams during the concentration process.

As shown in the scatterplot of PC1 and PC2 in Figure 6a, seven coconut jam samples were clearly distributed in the three spatial regions of the PCA plot. The after-sterilization samples were located in the region covering the positive axis of PC1 and the positive axis of PC2. The associated aroma components could be ethylidene acetone (4), pentanal-2-methyl (6), dihydro-2-methyl-3(2H)-furanone (9), furfuryl alcohol (12), methional (15), 2-butanoylfuran (16), 2,5-dimethylpyrazine (17), formic acid, heptyl ester (18), benzenecarbonal (19), hexanoic acid (20), 2-pentylfuran (21), 3,6-dimethyl-tetrahydropyran-2-one (23), delta-nonalactone (30) and delta-dodecalactone (32) (Figure 6b); these 14 compounds may be the characteristic aroma components of the post-sterilization samples.

The 0 min and 4 min samples were located in the top left corner of the region covering the negative axis of PC1 and the positive axis of PC2. The aroma associated with the 0 min and 4 min samples could be 3-hydroxy-2-butanone (2), decanoic acid (26), dodecanal (28), ethyl octanoate (33), isopentyl alcohol (3), pentyl alcohol (7), hexyl formate (11), ethyl caproate (22) and 2-methyl-1-butanol (5), implying that these compounds may be the main aroma components of the 0 min and 4 min samples (Figure 6b).

The 8 min, 12 min, 16 min and 20 min samples were mainly located in the bottom left corner of the region covering the negative axis of PC1 and the negative axis of PC2 (20 min was situated along the positive axis of PC1), implying that the aroma was steady from 8 min to 20 min. The aroma associated with these times could be propyl acetate (1), 2-octanone (13), 2-octanol (14), 2-nonanone (25), (3E)-6-methyl-3-undecene (24), ethyl decanoate (27) and 5-hexyl-4-methyldihydro-2(3H)-furanone (29), (Figure 6b), suggesting that these compounds could be the characteristic aroma components at these times. It is worth noting that at this stage it can be divided into two subgroups of 8–12 min and 16–20 min. Among them, the related compounds 2-octanol (14), ethyl decanoate (27), 2-octanone (13) and (3E)-6-methyl-3-undecene (24) decreased significantly from 12–16 min, which may be the continuous rise in temperature leading to the compounds undergoing the process of first synthesis and degradation. Eventually, the flavor of coconut jam changes at this stage.

According to PCA analysis results, the change in the aroma of coconut jam during the concentration process can be divided into three stages: the initial stage, the middle heating stage, and the sterilization stage (Figure 6a). The aroma of coconut jam after high temperature sterilization significantly differed from the aroma of the first two stages, and new flavor compounds such as furans and pyrazines were

formed. Therefore, after sterilization, the coconut jam had a relatively obvious caramel aroma. Overall, the results of the PCA show that the odor of coconut jam at each stage was formed by the combined action of multiple individual aroma compounds.

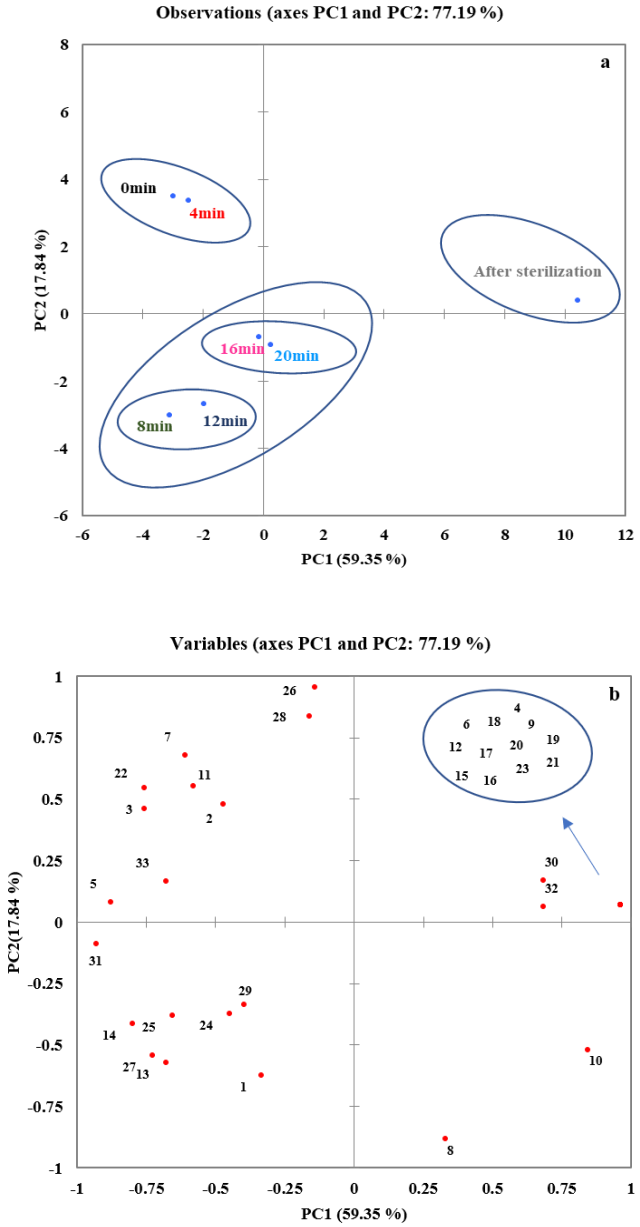

Figure 6. Analysis score (**a**) and correlation loading (**b**) plot of principal components 1 and 2 for the volatile compounds in coconut jam. Coconut jam sample distribution at different concentration times (**a**). Distribution of 33 volatile compounds in coconut samples (**b**).

4. Conclusions

In conclusion, the volatile profiles and marker substances of coconut jam processing were characterized by HSPME/GC-MS. According to PCA analysis, the concentration process of coconut jam can be regarded as occurring in three stages. The results suggested that esters and alcohols, such as isopentyl alcohol, pentyl alcohol, hexyl formate and ethyl caproate, were the main contributors to the aroma of the coconut jam in the early stage, while 2-octanone, 2-octanol, 2-nonanone and 5-hexyl-4-methyldihydro-2(3H)-furanone were the main aroma components of the middle stage. In the final sterilization stage, a variety of aroma compounds were produced, such as benzenecarbonal,

dihydro-2-methyl-3(2H)-furanone and furfuryl alcohol, forming the unique flavor of the coconut jam. The stepwise increase of the furfural content is consistent with the inflection point of the change in aroma during the whole process of coconut jam concentration, and the logistic model has a higher degree of fit, which can be used as a marker of aroma to monitor the concentration of the product. However, the mechanisms of action of certain aroma compounds released from the coconut jam are still unclear. Therefore, it is necessary to find new methods to further study the formation mechanism of aroma compounds in coconut jam.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

Appendix A

Table A1. Aroma compounds of coconut jam treated by different concentration time.

No.	Category	RI [f]	Component Name [g]	Identification	Area (%)						
					0 min	4 min	8 min	12 min	16 min	20 min	After Sterilization
	Aldehydes										
1		817	Hexanal	MS, RI	2.61 ± 0.49d	7.63 ± 3.10c	23.04 ± 2.55b	27.39 ± 1.47a	24.79 ± 2.67ab	27.67 ± 1.68a	23.48 ± 0.68b
2		1405	Dodecanal	MS, RI	0.52 ± 0.22b	1.6 ± 2.55a	-	-	-	-	0.23 ± 0.07b
3		914	Furfural	MS, RI	-	1.69 ± 1.98c	5.24 ± 0.76b	5.9 ± 0.34b	6.03 ± 1.11b	5.25 ± 1.61b	11.58 ± 1.23a
4		-	Pentanal,2methyl	MS	-	-	-	-	-	-	0.32 ± 0.16
5		1014	Methional	MS, RI	-	-	-	-	-	-	0.18 ± 0.01
6		1190	Benzenecarbonal	MS, RI	-	-	-	-	-	-	0.93 ± 0.18
	Ketones										
1		-	3-hydroxy-2-butanone	MS	2.9 ± 0.75b	11.81 ± 2.18a	2.87 ± 2.74b	3.41 ± 0.94b	3.31 ± 1.22b	-	-
2		998	2-Octanone	MS, RI	6.08 ± 0.32b	5.43 ± 0.54c	9.62 ± 0.81a	9.66 ± 0.13a	5.42 ± 0.23c	4.91 ± 0.34c	3.1 ± 0.17d
3		1281	2-Nonanone	MS, RI	2.12 ± 0.71ab	2.00 ± 0.42ab	2.28 ± 0.34ab	2.25 ± 0.23ab	2.04 ± 0.36ab	2.59 ± 1.16a	1.60 ± 0.26b
4		1480	5-Hexyl-4-methyldihydro-2(3H)-furanone	MS, RI	-	8.57 ± 1.55c	6.6 ± 0.57c	11.35 ± 2.12b	13.64 ± 2.47a	-	-
5		-	Ethylidene acetone	MS	-	-	-	-	-	-	0.62 ± 0.16
6		844	Dihydro-2-methyl-3(2H)-furanone	MS, RI	-	-	-	-	-	-	1.82 ± 0.21
7		1105	3,6-Dimethyl-tetrahydropyran-2-one	MS, RI	-	-	-	-	-	-	0.28 ± 0.02
	Esters										
1		1396	Ethyl decanoate	MS, RI	9.15 ± 0.96c	6.76 ± 0.44d	14.39 ± 1.37a	13.26 ± 0.32b	7.45 ± 0.27d	6.72 ± 0.42d	2.99 ± 0.05e
2		1099	Ethyl caproate	MS, RI	0.75 ± 0.17a	0.66 ± 0.13ab	0.45 ± 0.05c	0.46 ± 0.04c	0.58 ± 0.11bc	0.59 ± 0.07bc	0.24 ± 0.03d
3		1594	Ethyl dodecanoate	MS, RI	3.05 ± 0.25a	2.20 ± 0.2c	2.90 ± 0.12a	2.65 ± 0.11b	2.47 ± 0.11b	2.10 ± 0.08c	0.97 ± 0.09d
4		1183	Ethyl octanoate	MS, RI	0.98 ± 0.11a	0.7 ± 0.04b	0.98 ± 0.42a	0.62 ± 0.03b	-	-	-
5		969	Hexyl formate	MS, RI	9.01 ± 0.01a	4.29 ± 1.79b	3.47 ± 0.45b	3.4 ± 0.39b	1.54 ± 0.22c	1.1 ± 0.31c	0.76 ± 0.29c
6		-	Propyl acetate	MS	-	-	3.08 ± 1.82a	0.78 ± 1.12b	-	-	-
7		1060	Formic acid, heptyl ester	MS, RI	-	-	-	-	-	-	0.27 ± 0.05
	Lactones										
1		1480	delta-Nonalactone	MS, RI	8.58 ± 0.81b	-	-	-	-	16.84 ± 1.38a	17.42 ± 0.80a
2		1692	delta-Dodecalactone	MS, RI	0.97 ± 0.18d	1.41 ± 0.34c	-	1.41 ± 0.2c	2.54 ± 0.27b	2.96 ± 0.23a	2.94 ± 0.34a
	Alcohols										
1		-	Isopentyl alcohol	MS	2.69 ± 0.6ab	3.57 ± 1.80a	2.28 ± 0.22b	1.81 ± 0.24bc	0.99 ± 0.07cd	1.06 ± 0.22cd	0.27 ± 0.02d
2		-	2-Methyl-1-butanol	MS	0.88 ± 0.22a	0.95 ± 0.45a	1.02 ± 0.08a	0.80 ± 0.11a	0.36 ± 0.02b	0.37 ± 0.04b	-
3		-	Pentyl alcohol	MS	2.09 ± 0.46a	1.59 ± 0.39b	1.27 ± 0.21bc	1.11 ± 0.12c	1.04 ± 0.12c	1.24 ± 0.19bc	0.86 ± 0.10c
4		1010	2-Octanol	MS, RI	3.10 ± 0.26abc	2.79 ± 0.16abc	4.7 ± 2.21a	3.28 ± 0.41ab	2.47 ± 0.41bc	3.47 ± 2.44ab	1.17 ± 0.21c
5		947	Furfuryl alcohol	MS, RI	-	-	-	-	-	-	5.49 ± 0.59

Table A1. *Cont.*

No.	Category	RI [f]	Component Name [g]	Identification	Area (%)						
					0 min	4 min	8 min	12 min	16 min	20 min	After Sterilization
	Acids										
1		1373	Decanoic acid	MS, RI	10.78 ± 4.3a	9.67 ± 3.45a	-	-	6.16 ± 0.82b	3.43 ± 0.82b	3.72 ± 0.5b
2		1081	Hexanoic acid	MS, RI	-	-	-	-	-	-	0.64 ± 0.43
	Alkenes										
1		1160	(3E)-6-Methyl-3-undecene	MS, RI	0.57 ± 0.36ab	0.78 ± 0.26ab	1.47 ± 1.37a	1.51 ± 0.67a	-	-	0.29 ± 0.09b
	Furfurans										
1		1015	2-Butanoylfuran	MS, RI	-	-	-	-	-	-	0.91 ± 0.18
2		1090	2-Pentylfuran	MS, RI	-	-	-	-	-	-	1.9 ± 0.11
	Pyrazines										
1		1020	2,5-Dimethylpyrazine	MS, RI	-	-	-	-	-	-	0.92 ± 0.41

a–e Different letters in the same row are significantly different. [g] The compounds were identified by comparing retention indices of authentic n-alkanes (C8–C40) and mass spectra with those from NIST08 and NIST08s. [f] Retention indices (RIs) were based on a series of alkanes (C8–C40). Labels 0, 4, 8, 12, 16, 20 min and after sterilization represent the different concentration processing times.

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Quality Parameters and Consumer Acceptance of Jelly Candies Based on Pomegranate Juice "*Mollar de Elche*"

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Abstract: There is an upward trend towards reducing or suppressing additives in foods, as well as reducing the use of E-numbers in labels providing clean label foods. Therefore, the development of confectionary products based exclusively on natural ingredients with antioxidant properties may offer valuable solutions to the confectionery industry. Fruit juices and purées may provide functional and organoleptic properties in jelly candies in a natural way. The consumption of pomegranate fruit and derivative products has increased due to their association with health benefits. The aim of this study was to determine consumer insights about pomegranate-based jellies, cultivar "*Mollar de Elche*", as affected by formulation (100% pomegranate juice or 50%–50% pomegranate juice–apple purée) and type of sweetener (sugar or honey), and to link affective and descriptive data from sensory studies. The most valued quality parameter of pomegranate products, red color (measured by the green–red coordinate, *a**), was not negatively affected by jelly preparation. It was determined that the main liking drivers for pomegranate jellies were intense red color and high brightness. The results might be used by pomegranate processing companies to improve their manufacturing protocols and to develop successful products meeting consumer demands and needs. The formulation containing 20% gelatin, pure "*Mollar de Elche*" pomegranate juice, 1% citric acid, and sucrose as sweetener provided the best quality of jellies in terms of color, texture, antioxidant capacity, and sensory attributes.

Keywords: *Punica granatum*; *Malus domestica*; honey; sensory analysis; descriptive; PDO

1. Introduction

Gummies consist of food products whose main ingredient is sugar, normally incorporated in the form of sucrose syrup and/or glucose, combined with gelling agents, acids, aromas, and food colorants [1]. Within this category, two confections can be differenced according to the type of gelling agent used: (i) jellies and (ii) gummies, using gelatin and other hydrocolloids such as pectin, respectively [2]. Due to the high contents of sugar and food additives, as well as the nondesirable compounds generated by the heat treatment, it has been reported that a high consumption of such

confections may have negative effects on human health [3–5]. In spite of this knowledge, both jellies and gummies are consumed by a large and heterogeneous group of people from children to elderly persons [3,6].

According to recent studies, it has been reported that (i) most of the Spanish population consume more sugar than recommended [7], (ii) adult American males and females ingest up to 150 and 100 kcal of added sugars per day, respectively [8], and (iii) 56% of United Arab Emirates students were considered heavy sugar consumers [9]. The high consumption of sugar in children has been linked with obesity, impulsiveness, addictive behavior, and stress-driven anxiety [10,11]. Recently, it has been found that honey-fed rats showed significantly less anxiety throughout the study as compared with those fed with sucrose [11]. Taking this into account, both a reduction of sugar content and the replacement of sugar with other alternatives [12,13] such as honey could be healthier alternatives for gummies and jellies [14]. The use of healthier alternatives such as orange, strawberry, and black mulberry juices as well as honey has been considered for the manufacturing of jellies [5].

Additives (color agents, flavorings, etc.) have been considered as potential carcinogens and/or neurotoxic agents by themselves or by the contaminants they contain [15]. Although these compounds can be used due to their safety, there is an upward trend towards their suppression and a high demand for clean label foods, without food additives or E-additives. Therefore, the development of confectionary products based on natural ingredients with antioxidant properties is a current trend to obtain new and healthier products [3]. Because gummies and jellies are widely consumed, they can be considered a good vehicle to increase the intake of functional substances such as fiber and phenolic compounds [3,6]. Fruit juices and purées are the current alternative by the food industry to improve the organoleptic properties of gummies and jellies in a natural way (color, flavor, and texture), and even fruit by-products have been used [2,3,6].

Pomegranates (*Punica granatum* L.) are not only consumed fresh but are also used in the preparation of industrialized foods, such as jams, fermented milks, juices, smoothies, jellies, wines, and dried snacks [16–19]. During the last decades, pomegranate consumption has increased due to its benefits on human health [20,21]. It has been demonstrated that pomegranate presents a low caloric index and its composition highlights the presence of citric acid, polyphenols, and vitamin C. Apart from these functional compounds, pomegranate juice presents techno-functional properties, being useful, for example, as a natural colorant. Additionally, fruit purée or fruit pomace with high pectin content, such as that from apples, has been reported as suitable to obtain jellies and gummies with a smooth texture [22,23]. A previous study described the production of a reduced-sugar pomegranate juice jelly supplemented with an aqueous extract of pomegranate by-product (peel) [24].

Considering all the above, the aim of this study was to determine consumer insights about pomegranate-based jellies (cultivar "*Mollar de Elche*") as affected by formulation (pure pomegranate juice or 50%–50% pomegranate juice–apple purée) and type of sweetener (sugar or honey), and to link consumer data to descriptive sensory analysis. The results might be used by companies producing or selling pomegranate coproducts to improve their procedures and make their products more successful by meeting consumer demands and needs. In addition, the following parameters were studied to make the correct decision: (i) physical parameters (color), (ii) mechanical properties (texture), (iii) antioxidant capacity (DPPH, FRAP, and $ABTS^{+}$), and (iv) affective and descriptive sensory analyses.

2. Materials and Methods

2.1. Experimental Design

The research consisted of two parts:

In the first part of the study, the effects of three gelatin doses (15%, 17%, and 20%), two sweeteners (sucrose or honey), and two juice–purée ratios (100–0 and 50–50) on properties of pomegranate jelly candy were investigated. The parameters controlled were antioxidant capacity, color, and texture. Additionally, the effect of the addition of citric acid (1%) was evaluated on color and antioxidant capacity.

In the second part of the study, a sensory evaluation was carried out using only confections prepared using the selected gelatin dose (20%) and with citric acid addition (1%) but still evaluating the effect of two sweeteners (sucrose or honey) and two juice–purée ratios (100–0; 50–50).

With this design, the number of formulations in the first stage was 12 (n = 3 × 2 × 2) while in the second part of the study, the number was 4 (n = 2 × 2). Three jelly candies for each formulation were obtained as replicates for mechanical analysis, color coordinates, and antioxidant capacity.

2.2. Chemicals

The acetic acid, hydrochloric acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH), potassium persulfate, TPTZ (2,4,6-tripyridyl-1,3,5-triazine), and $FeCl_3$ were purchased from Sigma-Aldrich (Steinheim, Germany).

2.3. Plant Material and Jelly Candy Preparation

Pomegranate fruits, cultivar "*Mollar de Elche*", were collected from a farm located in Elche, Alicante (Spain) with Protected Designation of Origin (PDO) and apple fruits, cultivar "*Smith*", were obtained from a local supermarket. Pomegranate fruits were hand-harvested at a commercial maturity stage (14° Brix and 0.20% citric acid for "*Mollar de Elche*" pomegranates), whereas apples were characterized by having 8 °Brix and 0.75 % malic acid. Both types of fruits were stored at optimal conditions (8 °C and HR 90%–95%, and 1 °C and HR 90%–95%, respectively, for pomegranates and apples).

The different stages of the fruit-based jelly candy preparation process were:

1. Purée preparation: apples were cut, ground, and heated at 80 °C in Thermomix device (Vorwerk, Wuppertal, Germany); 10 mL of citric acid per 1 kg of fruit was added to prevent enzymatic browning of the fruit [25]. Finally, the particle size of the mixture was reduced in a blender until a thin purée was obtained (apple purée, AP).
2. Pomegranate juice preparation: pomegranate fruits were cut in halves, arils were manually separated from the husk, and juices were prepared using only arils (pomegranate juice, PJ).
3. Gelatin hydration: gelatin was hydrated with water at 25 °C for 10 min (using ratios of 13%, 17%, and 20 %).
4. Heat treatment and homogenization: the final blend included 25% of sweetener (sucrose or honey) and 31%, 29%, and 27.5% of pomegranate juice for the product with 13%, 17%, and 20% of gelatin, respectively. These blends were heated at 60 °C for 4 min in a Thermomix device (Vorwerk, Wuppertal, Germany) and then the same percentage (31%, 29%, and 27.5%) of pomegranate juice or apple purée was added and mixed at 60 °C for 2 min. Finally, the different formulations were obtained (Table 1).

The molding and maturation step was carried out at 4 °C for 24 h.

Table 1. Formulation of jelly candies consisting of pomegranate juice and apple purées.

Code †	Gelatin	Honey	Sucrose	Juice	Purée	Citric Acid
	(%) ‡					
PJH-13	13	25	-	62		0
PJH-17	17	25	-	58		0
PJH-20	20	25	-	55		0
PJS-13	13	-	25	62		0
PJS-17	17	-	25	58		0
PJS-20	20	-	25	55		0
PJAPH-13	13	25	-	31	31	0
PJAPH-17	17	25	-	29	29	0
PJAPH-20	20	25	-	27.5	27.5	0
PJAPS-13	13	-	25	31	31	0
PJAPS-17	17	-	25	29	29	0
PJAPS-20	20	-	25	27.5	27.5	0
PJH-20c	20	25	-	55		1
PJS-20c	20	-	25	55		1
PJAPH-20c	20	25	-	27.5	27.5	1
PJAPS-20c	20	-	25	27.5	27.5	1

Note: † PJ, pomegranate juice; S, sucrose; H, honey; AP, apple purée. ‡ The percentage of each component was expressed in weight: weight, w: w.

2.4. Antioxidant Capacity (DPPH, FRAP, ABTS+) and Total Polyphenol Content

Methanol extract was prepared as follows: jelly candies (1 g) were mixed with 10 mL of MeOH/water (80:20, v/v) + 1% HCl, sonicated at 20 °C for 15 min, and left for 24 h at 4 °C. Then, the extract was again sonicated for 15 min (TSD-J 0.7 L, 50 W, 40 kHz) and centrifuged at 15,000× *g* for 10 min (Sigma 3–18 K, Osterode and Harz, Germany). The antioxidant capacity (AOC) and total polyphenol content (TPC) were determined using DPPH, $ABTS^+$, and FRAP assays, and quantified using Folin–Ciocalteu reagent [26,27]. As to DPPH, 10 µL of the supernatant was mixed with 40 µL of MeOH and added to 950 µL of DPPH solution. The mixture was shaken and placed under dark conditions for 15 min. The absorbance was measured at 515 nm. Additionally, 10 µL of the supernatant was mixed with 990 µL of ABTS or FRAP solutions. After 10 min of reaction, the absorbance was measured at 734 nm for ABTS and 593 nm for FRAP. Determinations were performed using a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). Calibration curves, in the range 0.5–5.0 mmol Trolox L^{-1}, were used for the quantification of the three methods of antioxidant activity showing good linearity ($R^2 \geq 0.998$). Analyses were run in triplicate per each jelly candy replicate (n = 9) and results were expressed as mg/100 g fw (fresh weight).

2.5. Color Parameters

Color coordinates (*L**, *a**, and *b**) were determined by reflectance measurement with a Color Quest XE Hunter Lab colorimeter (Illuminant D65 and 10° observer angle). Measurements were run in triplicate per each jelly candy replicate (n = 9).

2.6. Texture

A texture profile analysis (TPA) was performed using a texturometer (TA-XT2i, Stable Micro Systems, Surrey, England), following a previous method for jelly candies [5] with slight modifications. Instead of using a sphere probe, a P0.5R cylinder (AOAC reference for gelatin analysis) was used, and the test conditions were as follows: pretest speed 2 mm/s, test speed 1 mm/s, post-test speed 1 mm/s, waiting time between cycles 2 s, trigger force 5 g, and 75% compression. The parameters measured were the following: hardness (H), adhesiveness (A), springiness (S), cohesiveness (Co), gumminess (G), and chewiness (Ch). The texture profile analysis was performed within the same gelation container, executing four measurements per each jelly formulation replicate (n = 12).

2.7. Descriptive Sensory Analysis

In order to run the sensory test, jellies were prepared in a teddy bear candy shape. Eight trained panelists from the *Escuela Politécnica Superior de Orihuela* (EPSO), University Miguel Hernández de Elche, UMH (aged 26 to 55 years old, four females and four males), and with more than 500 h of experience in sensory testing, participated in this study. The panel was selected and trained following the ISO standard 8586-1 (1993), and it is specialized in descriptive sensory evaluation of pomegranate products [28,29]. For the present study, the panel worked during two orientation sessions (90 min for each one) discussing the main organoleptic characteristics of pomegranate jelly. The lexicon used for describing the flavor and texture attributes was previously developed by other authors [30,31]. Both lexicons were adapted for jelly based on pomegranate during the orientation sessions. The methodology for serving samples and the scale used for quantifying the intensity of each evaluated attribute were those indicated in a previous study [28]. The pomegranate jellies used for the descriptive sensory analysis were prepared using nonstick molds which were flexible for easy removal with 50 teddy bear cavities (size: 19 × 14 cm).

2.8. Consumer Study

A consumer panel of 100 volunteers recruited from the SensoFood Solutions database evaluated the samples. At the beginning of the test, consumers were presented with a consent form with general information about the test and their willingness to participate in the study. Two experimental conditions were investigated: blind and informed. Participants were randomly divided into two groups of 50 people: one of the groups performed a blind evaluation of the samples, whereas the other performed an informed evaluation. Information on the exact formulation used in the preparation of each samples was provided to the consumers participating in this section of the study; this was (i) sample PJAPH-20c contains honey, pomegranate juice PDO "*Mollar de Elche*", and apple; (ii) sample PJH-20c consists of honey and pomegranate juice PDO "*Mollar de Elche*"; (iii) PJAPS-20c: sugar, pomegranate juice PDO "*Mollar de Elche*", and apple; and (iv) PJS-20c: sugar, pomegranate juice PDO "*Mollar de Elche*". The consumer study was carried out in the official testing room of UMH and each consumer evaluated all four samples in a single session in a randomized way. Consumers were asked about their overall liking on a nine-point hedonic scale followed by questions about the appearance, flavor, and texture attributes. Additionally, a nine-point Likert scale was used for the Just About Right (JAR) questions to determine possible improvements of the attributes: overall liking, color, appearance, fruity-ID, pomegranate-ID, sweetness, sourness, hardness, solubility, and adhesiveness.

2.9. Statistical Analysis

Multifactor analysis of variance (ANOVA) [factor I: formulation (pomegranate juice, or pomegranate juice and apple purée); factor II: gelatin percentage; factor III: sweetener (sugar or honey); and/or factor IV: information] was performed using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD), followed by Tukey's multiple range test. Partial least-square regression (PLS) analysis was carried out to study the relationship of the descriptive analysis (x: independent variables) with the overall liking data (y: dependent variable) using XLSTAT Premium 2016 (Addinsoft, Barcelona, Spain). Finally, penalty analysis was conducted to provide extra information about the possible improvements of some of the samples.

3. Results and Discussion

3.1. Percentage of Gelatin Selection

The antioxidant capacity of jellies, assayed by 3 methods ($ABTS^+$, DPPH, and FRAP), TPC, color coordinates, and several texture parameters were the parameters which determined the proper percentage of gelatin addition (Table 2). $ABTS^+$ values ranged from 2.46 (PJAPS-20) up to 3.69 mg/100 g fw (PJAPS-13) with no statistically significant differences being found for formulation and sweetener; however, the percentage of gelatin caused significant differences. The highest values of $ABTS^+$ were found for samples with the addition of 13% or 17% of gelatin. DPPH values varied from 10.6 to 14.9 mg/100 g fw for PJAPS-20 and PJS-20, respectively. In this case, the factor sweetener did not cause significant differences but the factors formulation and concentration of gelatin produced significant differences. FRAP analysis reported values ranging from 1.41 mg/100 g fw for the case of PJAPH-20 up to 4.59 mg/100 g fw in the case of PJS-13. Statistical analysis of FRAP antioxidant capacity values showed a similar trend to that of DPPH. TPC ranged from 72.0 mg/100 g fw up to 159 mg/100 g fw. It is well known that the antioxidant capacity is positively correlated with the polyphenol content, especially in the case of pomegranate fruit [32]. In general, it can be stated that the higher the gelatin content, the lower the functional properties of jelly candies, considering both the antioxidant capacity and the TPC [1]. On the other hand, there was a clear effect on the addition of honey instead of sucrose regarding the antioxidant capacity because honey has significantly higher antioxidant power [14].

Table 2. Effect on antioxidant capacity, color coordinates, and texture properties of pomegranate jellies as affected by formulation, gelatin percentage, and type of sweetener.

Samples	Antioxidant Capacity				Color			Texture Properties $^{\gamma}$					
	ABTS^{+}	DPPH	FRAP	TPC				H	A	S	Co	G	Ch
	(mg/100 g fw)				*L**	*a**	*b**	(g)	(g s^{-1})	(g)		(g)	(g mm^{-1})
PJH-13	2.61cd	14.8a	4.51a	159a	25.1d	0.74c	3.12d	885.0d	−4790abc	0.74	0.31	269.6	209.5
PJH-17	2.51d	13.8b	3.30b	133b	23.9de	0.65cde	3.15d	1224cd	−7191.8bcde	0.55	0.31	384.8	226.9
PJH-20	2.48d	11.9de	3.57b	77.8f	22.6ef	0.49ef	3.19d	3664a	−8629cde	0.76	0.26	959.0	791.6
PJS-13	2.70bcd	11.1ef	4.59a	116cd	24.8d	1.24a	2.25ef	1542c	−2138a	0.94	0.30	467.2	444.6
PJS-17	3.64a	12.1d	4.27a	115de	25.7d	0.70cd	1.90f	820.9d	−4248abc	1.06	0.45	203.4	213.8
PJS-20	2.90bcd	14.9a	1.47f	108d	21.4f	0.68cd	2.43e	2795b	−3304ab	1.05	0.30	847.8	900.8
PJAPH-13	3.13b	12.6cd	1.86df	127bc	30.7abc	0.55de	4.19bc	791.5d	−4535abc	1.08	0.56	442.8	478.9
PJAPH-17	2.89bcd	10.8f	2.44cd	106d	29.07bc	0.13g	3.70c	1080cd	−8026cde	0.90	0.66	716.2	643.8
PJAPH-20	2.79bcd	12.1cd	1.41f	72.0f	29.6bc	0.38f	4.20b	1298cd	−9898e	1.02	0.53	692.7	704.7
PJAPS-13	3.69a	12.2cd	2.67c	92.2e	32.7a	0.47ef	5.25a	747.3d	−4309abc	1.02	0.52	386.8	393.8
PJAPS-17	3.09bc	12.9c	3.21b	159a	31.1ab	0.93b	3.76bc	836.8d	−7018bcde	0.93	0.71	595.2	551.3
PJAPS-20	2.46d	10.6f	2.04de	109d	28.9c	−0.15h	1.66f	950.5d	5662.7bcd	0.99	0.58	551.5	550.4
Multifactor ANOVA †													
Formulation (F)	NS	**	***	NS	***	***	***	***	***	**	***	NS	NS
% Gelatin (G)	**	*	***	**	***	***	***	**	***	NS	***	NS	NS
Sweetener (Sw)	NS	NS	NS	NS	NS	***	***	NS	***	**	NS	NS	NS
F × G	**	NS	***	***	NS	NS	NS	***	NS	NS	NS	***	***
F × Sw	NS	NS	***	***	NS	NS	NS	NS	*	**	NS	NS	NS
G × Sw	NS	*	***	***	*	*	NS	***	***	NS	*	NS	
F × G × S	***	***	***	***	***	***	***	***	***	NS	NS	NS	NS
Tukey Test ‡													
F													
PJ $^{\gamma}$	2.80	13.1a	3.62a	118	23.9b	0.75a	2.68b	16345a	−5180a	0.84 b	0.32 b	478.4	418.9
PJAP	3.01	11.9b	2.27b	111	30.3a	0.38b	3.82a	642.6b	−6643b	1.00a	0.57a	372.7	372.4
G													
13%	4.01a	12.7a	4.13a	145a	28.3a	0.75a	3.70a	991.6b	−3943a	0.94	0.42 b	391.6	381.7
17%	3.60ab	12.2ab	3.26b	129ab	27.4a	0.60b	3.13b	787.0b	−6462b	0.86	0.50 a	323.0	275.8
20%	2.81b	11.5b	2.43b	107b	25.6b	0.35c	2.92b	1638a	−7320b	0.94	0.42 b	561.9	526.4
S													
Honey	3.42	12.2	3.55	122	26.8	0.49b	3.59a	1223	−7249b	0.84b	0.42	403.5	384.7
Sucrose	3.53	12.1	2.99	133	27.4	0.65a	2.91b	1054	−4568a	1.00a	0.47	447.6	406.6

Note:† NS = not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same column, were not significantly different ($p < 0.05$), according to Tukey's least significant difference test. $^{\gamma}$ Texture Properties: H, Hardness; A, Adhesiveness; S, Springiness; Co, Cohesiveness; G, gumminess; Ch, Chewiness; PJ, pomegranate juice; S, sucrose; H, honey; AP, apple purée.

The color analysis results determined that L^*, a^*, and b^* parameters of jelly candies were significantly affected by the formulation, the gelatin dose, and the addition of sweetener (only a^* and b^*) (Table 2). The color of the jelly candies enriched with apple purée got darker during the hot mixing step; this may be as a result of nonenzymatic browning reactions, such as the Maillard reaction and caramelization at high temperatures, among other reasons. The Maillard reaction occurs among amino groups and reducing sugars, with high temperatures accelerating such reactions and resulting in changes in the aroma, taste, and especially color of foods [33]. Although a pretreatment was made to avoid enzymatic reaction by heating at 80 °C [25] and adding citric acid to the apple purée, it can be possible that it was not enough due to the high browning rate of Granny Smith as compared to other cultivars [34]. In addition, a previous study about pomegranate jelly obtained higher reddish color (a^* values between 4.4 and7.6), due to type and percentage of gelling agent (guar, xanthan, and tragacanth gums at 1%), and probably a different cultivar of pomegranate juice which was not indicated [24].

The TPA test consists of compressing a food sample in a reciprocating motion that imitates the action of the jaw. Table 2 shows the results of several texture parameters (hardness: H; adhesiveness: A; springiness: S; cohesiveness: Co; gumminess: G; and chewiness: Ch). Regarding hardness, both formulation and gelatin percentage had significant effects, with the highest and lowest values being those of the samples PJH-20 and PJAPS-13, respectively. At this point, honey-sweetened jellies would be preferred because honey is healthier than sucrose and no significant effects on the type of sweetener were observed on their hardness [14]. The rest of texture parameters showed a particular behavior depending on the different variables. All three factors evaluated significantly affected the jellies' adhesiveness, with the PJH-20 sample having an intermediate value which can be accepted as optimal; similar intermediate values were obtained for jellies prepared using 20% gelatin. Therefore, samples with this percentage of gelatin (20%) were selected for the next part of the experiments. It is worth mentioning that in future research, it may be of interest to test pectin as a gelling agent as it has been suggested in recent studies [35].

3.2. Addition of 1% Citric Acid

The addition of 1% citric acid to pomegranate jellies significantly affected the color coordinates (L^*, a^*, and b^*) and the AOC (Table 3). Jellies prepared with 1% citric acid had higher values of L^* (darker color) and also a^* and b^* (more intense red color) than those without acid addition. One of the most important quality characteristics of the pomegranate is the intense red (garnet) pigmentation of its arils, juices, and related products [17]. This red color depends on total anthocyanin content but also on the chemical structure of the individual anthocyanin [36]. It is known that the lower the pH of the juice, the more stable the anthocyanins [37]. This red/garnet color is highly attractive for consumers and its preservation during the jelly preparation is essential. The color behavior in this study is similar to those reported in other studies [28].

On the other hand, the response of the AOC values, evaluated using three methodologies ($ABTS^+$, FRAP, and DPPH), to the citric acid addition showed different trends for each assay. In this way, the addition of 1% citric acid caused an increase in the DPPH values but a decrease in the FRAP values, as compared with control jellies, while no significant effect of citric acid addition was observed on $ABTS^+$ values (Table 3). This disagreement is due to the fact that each method is specific for only one antioxidant mechanism, so they do not necessarily have to behave in the same way. In this way and according to previous studies [38], one of the major problems with the evaluation of the antioxidant activity of biological materials is the choice of the proper assay.

The addition of citric acid increased the TPC by 16% as compared to the control jellies. These results were similar to those previously reported by Kim and Padilla-Zakour [39], who reported values of 132.9 mg GAE 100 g^{-1} for cherry jelly and 144.3 mg GAE 100 g^{-1} for plum jam. In addition, the current findings agreed with a recent study [40], which observed that the processing of sapota pulp jelly showed an increase of 4.8 times from the fresh fruit to the processed fruit. Heat treatment can modify

the content of phenolic compounds due to disruption of the plant cell wall, with the consequent release of these compounds [41].

Table 3. Effect on color coordinates, antioxidant capacity, and total polyphenol content of pomegranate jellies as affected by formulation, gelatin percentage, and type of sweetener.

Samples	*L**	*a**	*b**	ABTS^{+}	DPPH	FRAP	TPC
				(mg/100 g fw)			
PJH-20	23.9bc	0.65c	3.15b	2.51c	13.8ab	3.30b	74.1d
PJS-20	25.7b	0.70c	1.90c	3.34b	12.1c	4.27a	124bc
PJAPH-20	29.1a	0.13d	3.70b	2.99bc	10.8d	2.44bc	107c
PJAPS-20	31.1a	0.93bc	3.76b	3.09bc	12.9bc	3.21b	139ab
PJH-20c	19.7d	0.85bc	1.72cd	2.45c	13.9ab	1.18d	133abc
PJS-20c	23.2c	1.94a	0.98d	3.68b	13.6ab	3.31b	115bc
PJAPH-20c	29.6a	1.06b	6.24a	4.83a	14.4a	2.81bc	107c
PJAPS-20c	28.7a	0.17d	5.82a	2.46c	12.5c	1.17d	160a
Multifactor ANOVA †							
Formulation (F)	***	***	***	NS	NS	NS	NS
Citric acid (C)	***	***	***	NS	***	***	*
Sweetener (Sw)	***	***	**	NS	NS	NS	*
F × C	*	NS	***	NS	NS	NS	NS
F × Sw	*	NS	***	***	NS	***	NS
C × Sw	NS	NS	NS	***	NS	NS	NS
F × C × Sw	***	***	*	*	***	**	***
Tukey Test ‡							
F							
PJA $^{\gamma}$	23.1b	1.05a	1.95b	3.07	13.3	3.02	112
PJAP	29.6a	0.58b	4.89a	3.32	12.6	2.41	128
C							
0%	27.4a	0.61b	3.14b	3.03	12.4b	3.31a	111b
1%	25.3b	1.02a	3.70a	3.35	13.6a	2.11b	129a
Sw							
H	25.6b	0.69b	3.13b	3.17	13.2	2.43	105b
S	27.2a	0.94a	3.72a	3.22	12.8	2.99	134a

Note: † NS = not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same column, were not significantly different ($p < 0.05$), according to Tukey's least significant difference test. $^{\gamma}$ PJ, pomegranate juice; S, sucrose; H, honey; AP, apple purée.

It was found that the higher the DPPH values, the higher the TPC values. This was in accordance with recent studies [42,43], which reported this positive and significant relationship (R^2 = 0.733 for different plant extracts and R^2 = 0.899 for jellies with *Musa acuminata* Colla peels). The strong relationship between TPC and free radical scavenging activity might be due to the combined effect of various phenolic compounds and their high hydrogen atom donating abilities [44]. In summary, it can be concluded that citric acid addition at 1% improves the color, antioxidant activity, and TPC of the pomegranate jellies.

3.3. Descriptive Sensory Analysis, Consumer Acceptability, and Driving Sensory Attributes

The conditions rendering the best functional results (20% gelatin and 1% citric acid) were used to prepare the pomegranate jellies used for the descriptive and affective sensory studies. This study will provide us with information on the drivers of liking for pomegranate jelly candies and offer industry-relevant information regarding their formulation.

Descriptive sensory analysis was performed to assess the sensory profile of the pomegranate jelly candies and to check significant differences among formulations. The effect of formulation (the addition of pomegranate juice and/or apple purée) and sweetener (honey and sucrose) was studied, and 10 flavor and texture attributes were evaluated (Table 4).

Table 4. Variation of formulation and sweetener on descriptive sensory attributes of pomegranate jelly candies (PJH, PJS, PJAPH, PJAPS).

Samples	Color	Brightness	Sweetness	Sourness	Fruity	Pomegranate ID	Honey ID	Apple ID	Solubility	Hardness
PJH-20c	0.6b	8.8a	1.8b	0.9	2.3ab	0.8	0.2b	1.8a	5.7	3.4
PJS-20c	4.0a	8.4a	1.5b	0.4	1.9b	0.3	0.3b	0.3b	6.3	2.9
PJAPH-20c	0.1b	6.3b	2.1ab	0.4	2.2ab	0.5	0.4b	1.5a	5.9	3.1
PJAPS-20c	0.1b	6.3b	2.7a	0.8	2.7a	0.8	1.4a	1.6a	5.0	3.3
ANOVA Multifactor †										
Formulation (F)	***	***	*	NS	NS	NS	**	*	*	NS
Sweetener (Sw)	***	NS	NS	NS	NS	NS	**	**	NS	NS
F × Sw	***	***	***	NS	*	NS	**	**	**	NS
Tukey Test ‡										
F										
PJc $^{\gamma}$	2.3a	8.6a	1.6b	0.7	2.1	0.5	0.2b	1.0b	6.0a	3.2
PJAPc	0.1b	6.3b	2.4a	0.6	2.4	0.6	0.9a	1.5a	5.5 b	3.2
Sw										
Honey	0.3b	7.5	1.9	0.7	2.2	0.6	0.3b	1.6a	5.8	3.3
Sucrose	2.0a	7.4	2.1	0.6	2.3	0.5	0.8a	0.9b	5.7	3.1

Note: † NS = not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same column, were not significantly different ($p < 0.05$), according to Tukey's least significant difference test. $^{\gamma}$ PJ, pomegranate juice; S, sucrose; H, honey; AP, apple purée.

The highest color intensity (the higher the value, the more intense reddish the color) was observed when pomegranate juice and sucrose were used (PJS-20c), with lower values being found when honey was used. Previous studies reported that the addition of honey prevented browning reactions in preparation of several fruit juices and fruit-based products, but current results showed the opposite behavior [45]. The presence of amino acids in honey that can easily react with the sugars when heating begins breaking the molecular bonds in the honey (nonenzymatic reaction), thus changing the color of the jellies. Additionally, although citric acid was added to the apple purée to avoid enzymatic browning and Maillard browning, the addition of apple purée reduced color values and brightness. It has been demonstrated that the addition of apple purée increases brownish notes due to enzymatic browning, although it depends on the cultivar used [34]. The cultivar used (Granny Smith) could be substituted by other cultivars with less polyphenol oxidase activity such as Fuji or Golden [34]. The sweetness of these jellies was adjusted to low values because consumers are used to the high-sugar commercial confections (with at least 7% of sugar content). One of the purposes of this work was to reduce the sugar content. Apart from that, it is important to highlight that no differences in sweetness were found between sweeteners, but a nonsignificant increase was noticeable when apple purée was used. It is worth mentioning no off-flavor was detected and higher values of apple ID were observed as compared to those of pomegranate ID. Previous studies indicated that "*Mollar de Elche*" pomegranate juices presented apple notes [46]; this could be the reason for the observed trend. In addition, previous studies indicated that the use of apple juice in a pomegranate-based product led to samples with low color intensity and low pomegranate flavor but high intensity of apple; the apple flavor masks that of pomegranate [17].

Mean scores for liking of color, appearance, some flavor notes (fruity, pomegranate), basic taste (sweetness, sourness), texture attributes (hardness, solubility, and adhesiveness), and overall liking of samples are shown in Table 5. In general, all the tested jellies obtained scores above 5.0, on a nine-point hedonic scale, and consequently can be considered as acceptable, although their acceptance can be clearly improved. Besides, it is important that the effect of providing information about the nature of the jellies to consumers was also evaluated. Regarding the formulation factor, there were no statistical differences for both the overall liking and that of the rest of attributes. Regarding the sweetener factor, the use of sugar was the most liked one by consumers, presenting the highest scores for overall, color, appearance, and fruity ID liking. Making consumers aware of the nature of the jellies they were consuming made a difference, leading to higher scores for overall liking and the satisfaction degree for most of the evaluated attributes (appearance, pomegranate ID, sourness, hardness, solubility, and adhesiveness). A recent study found similar results, with the overall liking of pomegranate-based drinks increasing after information which was described previously in the Materials and Methods section was provided to the consumers [47].

Table 5. Mean scores and ANOVA for color, appearance, flavor notes, basic taste, texture, and overall liking for consumers.

Samples	Overall	Color	Appearance	Fruity ID	Pom ID	Sweetness	Sourness	Hardness	Solubility	Adhesiveness
PJH-20c	4.8b	4.4b	5.0c	4.4b	4.3	5.2	5.0	5.1	5.7	5.5
PJS-20c	5.9a	6.1a	6.2ab	5.1ab	4.7	5.2	4.8	5.5	5.2	5.7
PJAPH-20c	4.8b	4.4b	5.0c	4.4B	4.3	5.2	5.0	5.1	5.7	5.5
PJAPS-20c	5.0ab	4.6b	5.3bc	4.6ab	4.3	5.1	5.0	5.2	5.5	5.5
PJH-20c+inf	5.2ab	4.7b	5.5abc	4.5b	4.4	5.1	5.1	5.5	5.7	5.9
PJS-20c+inf	6.0a	6.0a	6.5a	5.5a	5.0	5.7	5.5	6.0	6.0	6.0
PJAPH-20c+inf	5.2ab	4.7b	5.5abc	4.5b	4.4	5.1	5.1	5.5	5.7	5.9
PJAPS-20c+inf	5.5ab	5.1ab	6.0ab	4.9ab	4.9	5.2	5.4	5.4	5.7	5.8
ANOVA Multifactor †										
Formulation (F)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Sweetener (Sw)	*	***	**	**	NS	NS	NS	NS	NS	NS
Information (Inf)	*	NS	**	NS	*	NS	***	*	**	*
F × Sw	***	***	***	**	NS	NS	NS	NS	NS	NS
F × Inf	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Sw × Inf	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
F × Sw × Inf	***	***	***	**	NS	NS	NS	NS	NS	NS
Tukey Test ‡										
F										
PJc $^{\gamma}$	5.5	5.3	5.8	4.9	4.6	5.3	5.1	5.5	5.6	5.8
PJAPc	5.4	5.1	5.7	4.8	4.6	5.0	5.2	5.3	5.4	5.6
Sw										
H	5.2b	4.9b	5.6b	4.6b	4.5	5.1	5.1	5.3	5.5	5.7
S	5.6a	5.6a	6.0a	5.1a	4.7	5.3	5.2	5.5	5.5	5.7
Inf										
Yes	5.6a	5.3	6.0a	4.9	4.8a	5.3	5.4a	5.6a	5.8a	5.9a
No	5.2b	5.2	5.5b	4.7	4.4b	5.1	4.8b	5.2b	5.3b	5.5b

Note: † NS = not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same column, were not significantly different ($p < 0.05$), according to Tukey's least significant difference test. $^{\gamma}$ PJ, pomegranate juice; S, sucrose; H, honey; AP, apple purée.

Figure 1 represents a correlation mapping of consumer overall liking with the descriptive attributes (only statistically significant ones were used) to determine the drivers of liking. Two well-separated groups of consumers were observed. On the right side, the majority of consumers were grouped close to the attributes color and brightness. Current results agreed with previous studies, indicating that color of pomegranate-based products is one of the most important parameters [28]. On the opposite side, fewer consumers were grouped close to fruity, apple ID, honey, and sweetness attributes, indicating that a smaller possible cluster of consumers could prefer these sensory attributes. These results indicated that most consumers would choose samples which are characterized by reddish color (PJcS). Industry could use these liking drivers as quality indicators for improving their commercial products. It is worth mentioning that some expected sensory attributes as consumer drivers were not selected since the values generally were not sufficiently high in the present developed pomegranate jellies.

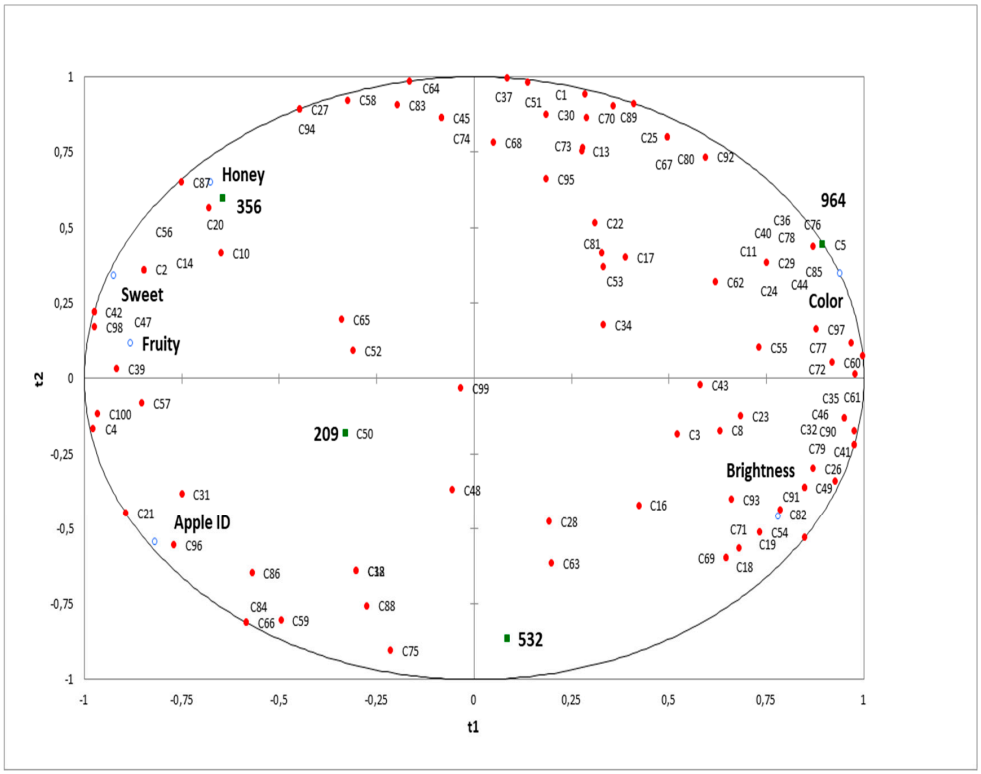

Figure 1. Partial least squares regression (PLS) of the descriptive sensory profile (X) and consumer overall liking (Y) of the samples (squares = samples; filled circle = consumers; unfilled circle = descriptive attributes).

Apart from the evaluation of the liking of specific attributes, some JAR questions were also included in the affective study. For a better understanding of the relationships among JAR scores and consumer liking (with information and without information), a penalty analysis was conducted (Figure 2). The improvable attributes were those in which at least 20% of consumers caused a drop of at least 1 point for liking (critical corner). Significant differences were found for the attributes located in the critical corner among samples, with perhaps the most interesting findings being the significant effect of the information on the opinion of consumers. Among samples evaluated after providing information to consumers about formulation of jellies, all samples needed to have higher intensity of color, fruity ID, and pomegranate ID, except PJH-20c (no improvements were required). Additionally, low sweetness was detected in samples elaborated with apple purée and pomegranate juice. On the other hand, when no information was provided to consumers, the attributes to be improved in the honey- and pomegranate-based jellies (PJAPH-20c and PJH-20c, respectively) were pomegranate ID, sweetness, hardness, and solubility (all needing higher intensities).

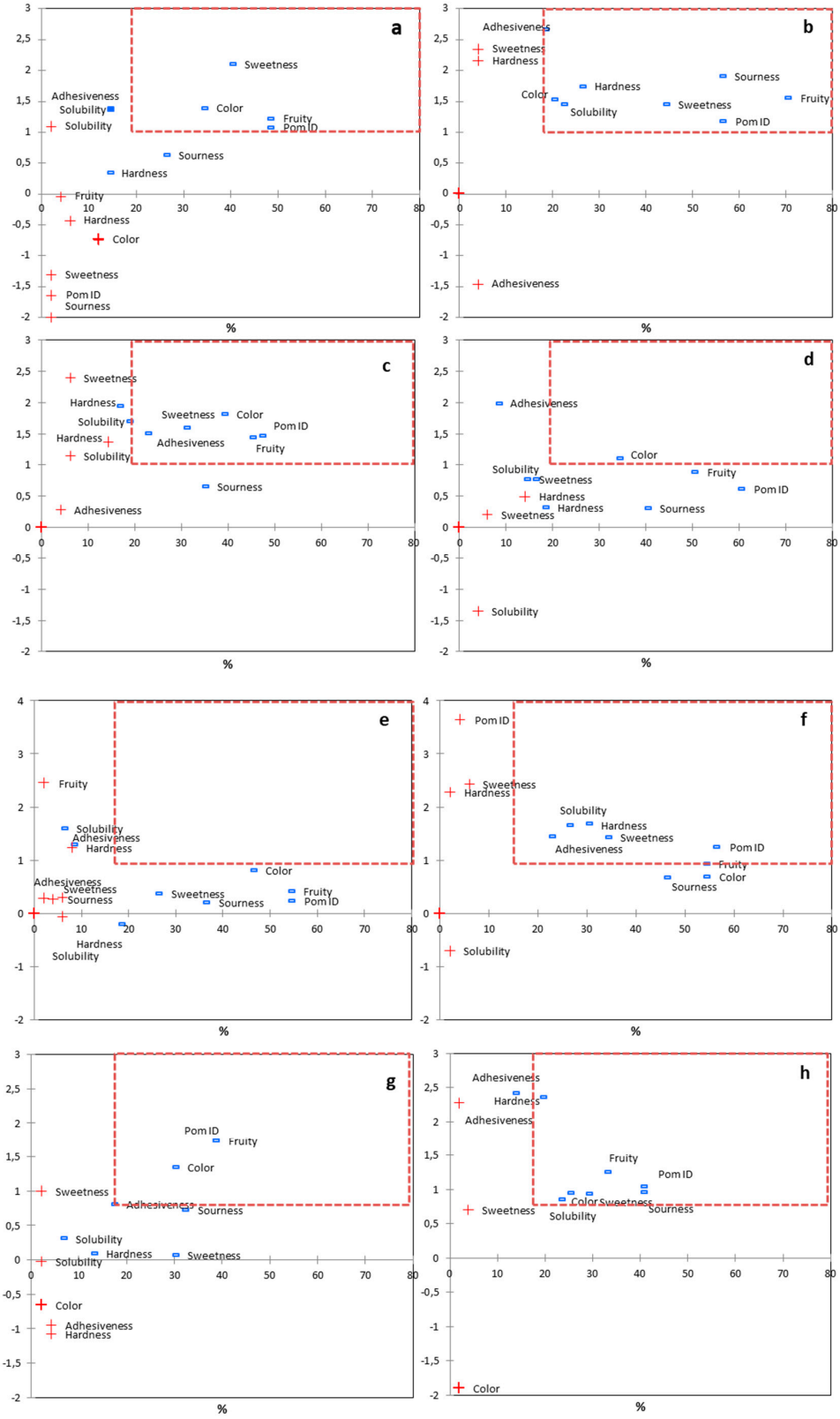

Figure 2. Penalty analysis of samples (sample code indicated on the top left of each figure; "too low intensity" indicated with "—", and "too high intensity" indicated with "+"); (**a**) indicated PJAPH-20c+inf; (**b**) indicated PJAPH-20c; (c) indicated PJAPHS-20c+inf; (**d**) indicated PJAPHS-20c; (**e**) indicated PJH-20c+inf; (**f**)indicated PJH-20c; (**g**) indicated PJS-20c+inf; (**h**) indicated PJS-20c.

Future studies focused on improving the fruity and pomegranate flavors and texture attributes should be optimized starting with the information reported in this study and the attributes indicated by consumers as those that needed to be improved (those located in the critical corner of Figure 2).

4. Conclusions

The formulation consisting of 20% gelatin with pure "*Mollar de Elche*" pomegranate juice, 1% citric acid, and sugar addition led to the best results in terms of color, texture, antioxidant capacity, and sensory attributes. The most valued quality parameter of pomegranate products, red color (a^* coordinate), was not negatively affected by the process of preparing jellies. Additionally, the affective data and its relationship with descriptive sensory data showed that the main liking drivers for this specific product were: (i) high reddish color and (ii) high brightness. Application of penalty analysis showed the hardness of the jellies can be improved in at least 50% of samples assayed. It is necessary to mention the high water activity values in all samples, which will restrict the shelf life of these confections. However, further research is still needed to fully optimize this novel product; for instance, two ways of improvement can be: (i) hydrating gelatin in pomegranate juice instead of water, and (ii) optimizing the drying step. Additionally, it was clearly demonstrated how important it is to provide the consumers with the proper information. Therefore, after studying the physico-chemical, functional, and sensory parameters of the developed products, it is worth continuing this research topic and studying the effects of a blend of pectin:gelatin in the formulation of the confections, their behavior during the maturation stage and during storage, and the impact of labeling information on consumer acceptance. The development of pomegranate jelly is a good strategy to promote the consumption of pomegranate products and the present study provides useful information to understand consumer preferences and what they expect to find in these types of products.

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Assessment of Volatiles and Polyphenol Content, Physicochemical Parameters and Antioxidant Activity in Beers with Dotted Hawthorn (*Crataegus punctata*)

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Abstract: Beer with the addition of dotted hawthorn (*Crataegus punctata)* fruit and juice was prepared and analysed. The content of carbohydrates, glycerol and ethanol in beers was determined by high-performance liquid chromatography (HPLC). Analysis of the total content of polyphenols was also performed using the Folin-Ciocalteu method, as well as determining antioxidant capacity by DPPH$^{\bullet}$ and ABTS$^{+\bullet}$ assay, and the ability to reduce iron ions by FRAP assay. Content of volatile compounds was analysed by means of solid-phase microextraction and gas chromatography coupled with mass spectroscopy. Beers with addition of hawthorn, both juice and fruit, had higher antioxidative potential and higher polyphenols concentration compared to control beer. The content of polyphenols in beers was in the range 200.5–410.0 mg GAE/L, and the antioxidant activity was in the range of 0.936–2.04 mmol TE/L (ABTS$^{+\bullet}$ assay), 0.352–2.175 mmol TE/L (DPPH$^{\bullet}$ assay) and 0.512–1.35 mmol TE/L (FRAP assay). A sensory evaluation of beers was also carried out. Beer with hawthorn fruit addition obtained the best scores in sensory analysis for criteria such as aroma, taste and overall quality. This beer had the highest content of volatile compounds (287.9 µg/100 mL of beer), while the control beer had lowest concentrations (35.9 µg/100 mL of beer).

Keywords: antioxidant activity; *Crataegus punctata*; beer; total polyphenol content; gas-chromatography; headspace solid-phase microextraction; volatile compounds; aroma; sensory analysis

1. Introduction

Beer polyphenols come from malt (70–80%) and hops (20-30%) [1]. Polyphenols and melanoidins affect the antioxidant activity of beer, as well as its sensory properties [2]. Antioxidant compounds play a role in reducing the amount of free radicals present in human body. The imbalance between antioxidants and pro-oxidants is called oxidative stress, which is a cause of many diseases. Exogenous antioxidants, introduced into the body with food, can scavenge free radicals, chelate metal ions, or inhibit pro-oxidative enzymes, protecting our body from the negative effects of oxidative stress [3].

Moderate consumption of beer has a positive effect on some biomarkers associated with human health [4,5]. Food rich in polyphenols can reduce the possibility of developing many civilization-related diseases such as arteriosclerosis, cancer and neurodegenerative diseases. This means that there is a public interest in products with high content of polyphenols [6,7]. Quifer-Rada et al. identified

47 phenolic compounds in beer, including simple phenolic acids, hydroxycinnamoylquinics, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha-acids and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavonoids [8]. Phenols and polyphenols can directly contribute to the characteristics of beer, mainly to body, haze, flavour, fullness and astringency [9]. However, volatile compounds play a major role in creating the taste and aroma [10]. Sensory active chemicals in beer belong to various classes, such as esters, carbonyl compounds, alcohols, fatty acids, volatile phenols, furanic compounds or terpenoids [11]. In traditional beer styles volatile compound content depends on yeast metabolism and type of malt and hops used [12,13]. The aroma profile of beer is one of the main factors evaluated by consumers, so the analysis of volatile compound content is an important part of testing product quality.

Research on the impact of brewing raw materials and the production process on the content of polyphenolic compounds, antioxidant activity and beer physiochemical properties have already been described [14,15]. Beer characteristics can be modulated by using unusual ingredients in brewing technology such as fruits. Consumer interest in fruit beers is increasing [16]. The fruits or fruit juices used as raw materials in beer production, apart from changes in taste and aroma, add another source of bioactive compounds to the product [17,18]. Studies have been conducted on beers with the addition of cherry, raspberry, peach, apricot, grape, plum, orange and apple, but also less popular fruit like persimone or Cornelian cherry [18–20]. Researchers have also evaluated beers with the addition of lemon, grapefruit, raspberry and cranberry juice available on the market [21]. The possibilities of using industrial waste products, e.g., orange peels and eggplant peels, in the production of beer have also been explored [18,22].

The dotted hawthorn (*Crataegus punctata*) is a shrub that grows naturally in North America [23]. Analytical studies have shown that hawthorn fruit contain flavonoids such as kaempferol, apigenin, quercitrin, rutin, hesperidin and arbutin; phenolic acids such as ursolic and isovanillic acid; anthocyanins such as cyanidine-3-rutinoside, cyanidine-3-galactoside and cyanidine-3-arabinoside [24, 25]. No work has been found in which it was analysed how the addition of hawthorn fruit or juice to beer modifies the physicochemical parameters, the content of polyphenols, the antioxidant activity and the content of volatile compounds in beers. This article describes comprehensive research on the use of hawthorn in beer production. A distinctive aspect of this research is the comparison of whole fruit or juice application. The influence of the additive form (juice or fruit) on beer properties shaping the quality of the product was discussed. Therefore, analysis of impact of fruit addition on content of volatile compounds and sensory analysis is an interesting aspect of conducted study.

This study aimed to determine whether the addition of dotted hawthorn fruit or juice could influence the physicochemical properties, polyphenol content, antioxidant activity, volatile compounds concentration and results of sensory analysis.

2. Materials and Research Methods

2.1. Reagents and Standards

Reagents used in this study were 1,1-diphenyl-2-picrylhydrazil (DPPH$^{\bullet}$) radical, diammonium salt of 2,2′-azobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS$^{+\bullet}$), 2,4,6-tripyridyl-S-thiazine was used (TPTZ), 20% aqueous sodium carbonate solution, Folin-Ciocalteu reagent, acetic acid, sulfuric acid, $FeCl_3$, sodium acetate and diatomaceous earth. Internal standard was 2-undecanone (Sigma-Aldrich, Saint Louis, MI, USA) with a content of 100 mg of compound per 100 mL of distilled water.

2.2. Biological Material

Saccharomyces cerevisiae var. diastaticus with the trade name "3724 Belgian Saison" from Wyeast company (Hood River, OR, USA) were used for wort fermentation. Yeasts were bought in liquid starter form which contains 1.2×10^{12} cells/mL. The wort was inoculated in dose of 4.3 mL/L according to the producers' recommendations. The yeast strain used was low flocculating and possess high degree of fermentation (76–80%), with an optimal fermentation temperature in the range of 21–35 °C.

2.3. Research Material

The research material was American Saison-style beer in three variants: without any additions (BC), enriched with the addition of hawthorn fruit (BF) and enriched with hawthorn fruit juice (BJ). The raw materials were Pilsen malt, wheat malt, Vienna malt, Caramel Pale malt, Light Caramel malt, acidified malt. All malts were acquired from the Viking Malt company (Strzegom, Poland). Amarillo and Calypso hops (USA) were purchased from Twój Browar company (Wrocław, Poland). The hawthorn fruit (*Crataegus punctata)* of the variety "Aurea" and juice pressed from this fruit were added in the production process. The juice was obtained from fruits by the use of a manual winepress.

2.4. Brewing Technology

Mashing was carried out under laboratory conditions with an infusion system in the following conditions: 64 °C for 60 min and 72 °C for 15 min. Next, the whole mash was heated to 78 °C to inactivate the malt enzymes, filtered and 25 L wort was obtained. The wort was boiled for 75 min with addition of hops in three doses: first, Calypso (15 g), 75 min of boiling; second, Amarillo (15 g), 30 min of boiling; third, Amarillo (15 g) and Calypso (10 g), 15 min of boiling. The hopped wort was cooled to 25 °C, filtered and aerated. The initial extract content was set at 12.5% *(w/w)* measured using Densito 30PX densimeter (Mettler Toledo, DC, USA). Fermentation was carried out in fermentation vessels at 25 °C. Hawthorn juice or fruits were added to beers at a dose of 10% (*w*/*v*) in the last 3 days of fermentation. The resulting beer was aged in bottles for 3 weeks.

2.5. Physicochemical Parameters

The pH value of beer was measured using Mettler-Toledo MP 220 (Greifensee, Switzerland). The extract content was measured at 20 °C with a Densito 30P density meter (Mettler-Toledo, Columbus, OH, USA). The measurements were performed in triplicate.

2.6. Determination of Carbohydrate Profile, Ethanol and Glycerol Content

The sugar profile and the content of ethanol and glycerol were examined by means of High-Performance Liquid Chromatography (HPLC) [26]. Degassed and centrifuged (5500 rpm, 10 min) beer samples were diluted two-fold (1:1) with ultrapure water and filtered through nylon syringe filters with 0.45 µm pore size for chromatographic vials. The samples were then analysed using a Prominence liquid chromatography system (Shimadzu Corp., Kyoto, Japan) equipped with a Rezed ROA-Organic Acid H + column (300 × 4.6 mm) from Phenomex (Torrance, CA, USA). The following measurement parameters were used: sample volume: 20 µL, separation temperature: 60 °C, mobile phase flow rate: 0.6 mL/min, mobile phase: 0.005 M H_2SO_4, detection temperature: 50 °C. The concentration of ethanol, glycerol, dextrins, maltose, glucose and maltotriose was based on five-point calibration curves using Chromax 10.0 software (Pol-Lab, Wilkowice, Poland). All measurements were performed in triplicate.

2.7. Analysis of Total Polyphenol Content and Antioxidative Activity

2.7.1. Analysis of Total Polyphenol Content

The total polyphenol content of the beer was determined using the Folin-Ciocalteu (F-C) spectrophotometric method [27]. Degassed and centrifuged beer samples or fruit juice samples (0.1 mL) and 0.2 mL of F-C reagent were pipetted into cuvettes. After 3 min, 1 mL of a 20% aqueous solution of sodium carbonate (Na_2CO_3) and 2 mL of distilled water were added. The absorbance at 765 nm was measured after 1 h using Beckmann DU650 spectrophotometer (Brea, CA, USA) and the results were expressed as mg of gallic acid equivalents (GAE) per L of beer. Data were expressed as the mean value for three measurements.

2.7.2. Free-Radical-Scavenging Ability by the Use of a DPPH• Radical Assay

The antiradical activity was determined using a DPPH• radical assay [28]. Samples of beer or fruit juice (0.1 mL) were mixed with 2 mL of 0.04 mmol/L DPPH• in ethanol and 0.4 mL of distilled water. After 10 min of incubation at room temperature, the absorbance was measured with a spectrophotometer at 517 nm using disposable polystyrene cuvettes. A calibration curve was prepared with Trolox solution (0.005 mmol/L). The data were expressed as Trolox equivalent (TE) of antioxidative capacity per 1 L of the beer (mmol TE/L). All measurements were performed in triplicate.

2.7.3. Ferric Reducing/Antioxidant Power (FRAP) Assay

The FRAP assay is based on the reduction of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe (III)-TPTZ] to the ferrous complex at low pH, followed by spectrophotometric analysis [29]. Briefly, the reagent was prepared by mixing 10 mmol 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ)/L reagent with 20 mmol/L ferric chloride in acetate buffer (pH 3.6). Quantitative analyses were performed by the external standard method using ferrous sulphate (0.2 mmol/L) as the reference standard and correlating the absorbance (λ = 593 nm) with the concentration. 0.1 mL of beer/fruit juice sample was mixed in polystyrene cuvettes with 0.9 mL of distilled water and 3 mL of ferric complex. The data were expressed as Trolox equivalent (TE) of antioxidative capacity per 1 L of the beer (mmol TE/L). All measurements were performed in triplicate.

2.7.4. Free-Radical-Scavenging Ability by the Use of an $ABTS^{+\bullet}$ Radical Cation

Another method used to measure the antioxidant activity of beers was the $ABTS^{+\bullet}$ radical cation assay [30]. Sample of beer or fruit juice (0.03 mL) were mixed with 3 mL of $ABTS^{+\bullet}$ solution with measured absorption of 0.700 at a wavelength of 734 nm. After 6 min the absorbance of samples was measured. Each sample was tested in triplicate. The data were expressed as mmol Trolox equivalent (TE) of antioxidative capacity per 1 L of the beer (mmol TE/L).

2.8. Adsorption of Volatile Compounds Using Solid Phase Microextraction (SPME)

Centrifuged and degassed beer (4 mL) was added to a 20 mL glass vial. 5 µL of 100 mg/100 mL aqueous emulsion internal standard solution (2-undecanone) was added. A magnetic stir bar was placed in the vial which was then closed with an aluminium membrane. The vial was placed on a IKA RCT basic magnetic stirrer (Guangzhou, China). The SPME holder needle with three-component universal fibre for SPME (DVB/CAR/PDMS 50/30 µm) was used to puncture the membrane. Heating to 40 °C and stirring was started and fibre was extended and held over the beer for 20 min. An analogical approach was used for each type of beer. The adsorption of volatile compounds contained in peeled hawthorn fruits added to beer was performed in order to find out which chemical compounds are characteristic for hawthorn fruit. Adsorption of compounds isolated from the hawthorn fruit was carried out in an analogous manner to the adsorption of volatile compounds from beer, but 5 g of hawthorn fruit instead of 4 mL of beer was added to the vial. The analysis of juice squeezed out of hawthorn fruit was carried out in an analogous manner to beer (4 mL juice volume). Measurements for each type of beer were performed in duplicate.

2.9. Gas Chromatography and Mass Spectrometry of Compounds Adsorbed on Fibre

Separation, identification and quantification of volatile compounds adsorbed on the fibre was carried out using a gas chromatograph connected to a Saturn 2000 MS Varian Chrompack mass detector (Palo Alto, CA, USA) with a ZB-5 column (Phenomenex, Torrance, CA, USA) (30 m length × 0.25 µm film thickness × 0.25 mm diameter).

Chromatographic conditions were carried out in accordance with the methodology of Calin-Sanchez et al. [31]. Scanning (1 scan/s) was carried out in the 35–400 m/z range using 70 mV electron ionization. The analyses were carried out using helium as a carrier gas with a flow rate of

1 mL/min using the following program for oven temperature: 40 °C at the beginning of the process, 5 °C/min up to 110 °C, 20 °C/min up to 270 °C. The initial temperature was maintained for 3 min. The injection port temperature of the chromatograph was 220 °C.

2.10. Sensory Analysis

The beers prepared in this study were subjected to an organoleptic assessment on a five-point scale using features such as clarity, aroma, colour, taste and overall impression. Beers were evaluated by a group of 15 people (21 to 32 years old), which consisted of 9 women and 6 men. Samples were given in coded plastic cups with a capacity of 200 mL. The temperature of the served beer was 8 °C.

2.11. Data Analysis

Volatile compounds separated from the beer were identified by comparing retention indexes (RI) with Kovats standards (KI exp. and KI lit.) and with NIST11 chemical standard libraries and by mass spectral analysis. Two standard matrices were also created (for the control sample chromatogram, i.e., beer without addition and hawthorn fruit). Chromatograms of the remaining samples (i.e., beer with addition of fruit and beer with addition of fruit juice) were integrated using retention time of compounds in those two standard matrices, using Mnova MS 12.0.1 software (Mestrelab Research, Santiago de Compostela, Spain). Data concerning content of volatile compounds in hawthorn juice and hawthorn fruit is available in Supplementary Materials (Table S1).

The results in this work were statistically analysed in the Statistica 12.5 program from Statsoft (Tulsa, OK, USA) using one-way ANOVA ($\alpha = 0.05$). Differences between means were calculated using Duncan's test ($\alpha < 0.05$).

3. Results and Discussion

3.1. Concentration of Carbohydrates, Glycerol, Ethanol and pH Value of Tested Beers

The ethanol content in the tested beers ranged from 35.43–41.63 g/L (Table 1). The control beer without fruit addition (BC) had the highest content of ethanol, glycerol, maltose, maltotriose, as well as the highest pH value in relation to the other samples: beer with hawthorn fruit (BF) and beer with hawthorn juice (BJ). The addition of juice diluted and acidified the sample. This is indicated by a lower sugar content and lower pH characterising the samples with additives. BF compared to BJ, had a higher content of ethanol, glycerol, as well as maltose, maltotriose and dextrins.

Table 1. Concentration of carbohydrates, glycerol, ethanol and pH value in tested beers.

Beer Type [1]	Ethanol [g/L]	Glycerol [g/L]	Maltose [g/L]	Maltotriose [g/L]	Dextrin [g/L]	Glucose [g/L]	pH
BC	41.63 ± 0.02 [a]	2.23 ± 0.01 [a]	1.05 ± 0.002 [a]	1.13 ± 0.003 [a]	17.58 ± 0.01 [a]	n.d.	3.87 ± 0.02 [a]
BJ	35.42 ± 0.2 [c]	1.95 ± 0.01 [c]	0.8 ± 0.015 [c]	0.92 ± 0.007 [c]	14.21 ± 0.05 [c]	n.d.	3.5 ± 0.02 [c]
BF	37.23 ± 0.03 [b]	2.07 ± 0.04 [b]	0.9 ± 0.001 [b]	0.98 ± 0.015 [b]	15.77 ± 0.3 [b]	n.d.	3.7 ± 0.01 [b]

[1] BC—control beer; BJ—beer with hawthorn juice; BF—beer with hawthorn fruit, Values are expressed as mean ($n = 3$) ± standard deviation. Mean values with different letters (a, b, c) within the same column are statistically different (p-value < 0.05).

The use of hawthorn fruit did not dilute the beer as much as juice addition. The fruit, after the primary fermentation process, was separated from the beer, so it did not significantly increase its final volume, in contrast to the samples with the addition of juice. This is reflected in the content of sugar, ethanol and glycerol per litre of beer, as well as in the pH value. Liu et al. analysed the sugar content in hawthorn fruits. The sugars identified in the fruits were: fructose, glucose, sucrose, sorbitol and myo-inositol [32]. The addition of hawthorn fruit or juice does not affect the content of maltose and maltotriose. The whole amount of these sugars was introduced into the beer with malt. The differences in sugar content between the samples are caused by the dilution of the samples by the addition of fruit or juice. Glucose content was not detected in any of the tested beers. Glucose is the sugar preferentially

used by the yeast *Saccharomyces cerevisiae*. The final product usually does not contain this sugar or contains a small amount of it [33]. The addition of fruit or fruit juices reduces the pH of the finished product. Nardini and Garaguso showed that the pH value for the classic ale beer style ranges from 4.39 to 4.73, while for fruit beers the pH reached even around 3.5 [18]. This was also confirmed in the studies by Kawa-Rygielska et al., where the effect of adding Cornelian cherry varieties on beer parameters was analysed. Control beer, without the addition, had a pH value of 4.59, while beers with the addition of juice had a pH in the range of 3.43–3.71 [20]. The content and composition of sugars and acids in the product is an important factor shaping the quality of fruit, and thus also fruit products, by affecting the taste and reception of the product by consumers [34].

3.2. Concentration of Total Polyphenols and Antioxidative Activity

The use of hawthorn fruit or juice resulted in a significant increase in the polyphenol content as well as the antioxidant activity tested by FRAP, $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ methods. The addition of fruit caused an increase in the content of polyphenolic compounds in beer by 1.4 times (279.6 mg GAE/L), while the use of fruit juice more than twice (410.0 mg GAE/L) (Table 2). A smaller increase in the polyphenol content was observed in BF compared to BJ. This can be explained by the poorer extraction of polyphenolic compounds from fruit into solution. Similar trends were observed for the beer's antioxidant activity. The addition of juice increases the total antioxidant capacity ($ABTS^{+\bullet}$) 2.2 times, while the addition of fruit only 1.4 times. The ability to reduce the $DPPH^{\bullet}$ radical solution increased the in BJ up to 6 times while in BF the increase was much less noticeable (only about 1.2 times). The FRAP assay showed a 2.6 times increase in antioxidant activity for BJ, and 1.7 times BF compared to BC.

Table 2. Antioxidant activity and concentration of polyphenols in tested beers and juice pressed from the hawthorn fruit.

Analysed Sample [1]	Polyphenol Concentration mg GAE/L	$ABTS^{+\bullet}$ mmol TE/L	$DPPH^{\bullet}$ mmol TE/L	FRAP mmol TE/L
BC	200.5 ± 1.9 ^{d}	0.936 ± 0.09 ^{d}	0.352 ± 0.03 ^{c}	0.512 ± 0.01 ^{d}
BJ	410.1 ± 11.8 ^{b}	2.041 ± 0.12 ^{b}	2.175 ± 0.01 ^{b}	1.35 ± 0.02 ^{b}
BF	279.6 ± 2 ^{c}	1.356 ± 0.11 ^{c}	0.443 ± 0.04 ^{c}	0.869 ± 0.01 ^{c}
J	2633.9 ± 27.9 ^{a}	11.03 ± 0.32 ^{a}	17.22 ± 0.29 ^{a}	8.52 ± 0.13 ^{a}

[1] BC—control beer; BJ—beer with hawthorn juice; BF—beer with hawthorn fruit, J—hawthorn juice. Values are expressed as mean (n = 3) ± standard deviation. Mean values with different letters (a, b, c, d) within the same column are statistically different (p-value < 0.05).

The addition of fruit or fruit juices in beer production is a method for significantly increasing the content of bioactive polyphenolic compounds in product. In the studies of Nardini and Garaguso, classic ale beers showed total polyphenol content in the range of 383–482 mg GAE/L, while beers with fruits (cherry, orange peel, grape, plum, raspberry, peach, apricot or apple) from 399 to up to 767 mg GAE/L [18]. The addition of fruits or fruit juices to beer can almost double the content of these biologically active compounds. BC contained 200.5 mg GAE/L polyphenolic compounds.

Fruit and vegetable peels can also be an interesting addition to the production of beer. Beers with eggplant skin extract were tested in a study by Horincar [22]. Polyphenol content in beers assessed in this study ranged from 0.443 to 0.610 mg GAE/mL, antioxidant capacity measured by $DPPH^{\bullet}$ assay was 1.287–1.306 mmol TE/mL, while $ABTS^{+\bullet}$ 0.095–0.107 mmol TE/mL. The content of polyphenols was of similar order as polyphenol content in BJ, but the antioxidant capacity of beer with addition of eggplant peel extract was significantly higher than antioxidant capacity of BJ. The main reason for such differences in tested beers was the amount of the addition used. The use of peels may be an interesting object for further research.

In addition to increasing antioxidant activity, polyphenolic compounds can affect the sensory characteristics of beer. They can introduce unpleasant bitter taste to beer and participate in the creation of turbidity [35]. Beer styles vary in polyphenol content. The type and dose of malts, hops, and the

additions and technological processes used are factors which determine polyphenol content of the finished product. Habschied et al. [2] tested 12 commercially available beers including pilsner, lager, dark and black beers. The content of polyphenolic compounds ranged from 464.34 to 855.45 mg/L. Antioxidant activity of these beers was tested by DPPH• assay and ranged from 0.4 to 0.61 mmol TE/100 mL. These values were higher than those obtained by fruit beers in our experiment. In a study by Kawa-Rygielska et al. [20] on beers with the addition of Cornelian cherry juice, it was shown that Cornelian cherry juice increased the content of polyphenols in a greater extent than the addition of hawthorn juice. However, another study on non-alcoholic beers with the addition of Cornelian cherry juice, carried out by Adamenko et al. [36] showed a smaller increase in the antioxidant activity and the content of polyphenols in beers than the increase in the antioxidant activity and content of polyphenols in beer with hawthorn juice, but greater than determined in beer with fruits.

3.3. Concentration of Volatile Compounds in Tested Beers

The main volatile chemicals in alcoholic beverages are ethanol and carbon dioxide. Other compounds, such as esters, play a crucial role in creating beer aroma, despite being present at very low concentrations [37]. We found almost no research about volatile compounds in beers with fruit addition [38] and no works in which authors compared differences in volatile composition of beer made with addition of fruit or juice. In BC, 45 volatile compounds were identified (Table 3). The largest groups among them were esters (20 compounds), sesquiterpenes (8 compounds), and alcohols (6 compounds). The greatest number (51) of volatile compounds was identified in BF. Like in the BC, the largest groups among the constituents were esters (25 compounds), sesquiterpenes (8 compounds) and alcohols (8 compounds). The analysis, in the case of BF, also revealed two peaks recognized as volatile constituents, which could not be identified (the mass spectra of unidentified constituents are available in the Supplementary Materials Figures S1 and S2). Other volatile compounds identified in BF, BJ and BC belonged to such groups as aldehydes, monoterpenes, ketones, organic acids and hydrocarbons. In a study by Gao et al. [39] about volatile compounds in hawthorn fruits, 61 volatile compounds were identified. Although more volatile constituents could be identified in fruit, our study managed to characterise more of the esters. The main reason for this phenomenon is probably yeast metabolism. Yeast cells can form esters by enzymatic chemical condensation of alcohols and organic acids [40]. The content of compounds identified in hawthorn fruit, such as ethyl butyrate, 1-hexanol, ethyl hexanoate, α-terpineol, ethyl octanoate, ethyl (4E)-4-decenoate and ethyl trans-2-decenoate was determined in all tested beers. The largest amount of compounds characteristic for hawthorn was recorded in BF and the smaller (except ethyl octanoate) in BJ. It is also worth noting that these compounds occur in a smaller amount, in the control sample. Therefore, they may also come from malt, hops or be products of yeast metabolism [41]. The addition of hawthorn fruits or hawthorn juice to beer introduced only six new compounds (acetic acid hexyl ester; propanoic acid 2-methyl, 2-methylbutyl ester; limonene, ethyl 5-methylhexanoate; octanoic acid methyl ester; isopentyl hexanoate). However, there were increases in the amount of almost all volatile compounds characteristic for the beer. The compound derived from hawthorn, which was found in the largest amount in BF, was α-terpineol, characterised by resinous and citrus aromas [42,43]. This constituent was found in the research about craft beers produced by mixed fermentation by *Torulaspora delbrueckii* non-conventional yeast and typical ale yeast [44]. In the control sample fermented solely with brewer's yeast, α-terpineol was not detected. In the samples fermented with *Torulospora* culture, the amount of α-terpineol was 3 times higher than in BJ, but 4.5 times lower than in BF. As in the study by Canonico [44], the amount of α-terpineol in BC was minuscule (0.1019 µg/100 mL). Another compound, one of the esters characteristic to the hawthorn fruit, was ethyl hexanoate. Among the esters characteristic of hawthorn fruit aroma, the concentration of ethyl hexanoate in BF was the highest. It is a compound with a sweet, fruity aroma [45,46]. Its presence was demonstrated in all tested samples: BF (11.2222 µg/100 mL), BJ (1.3959 µg/100 mL) and BC (0.9681 µg/100 mL). It is a compound which was characterised in a study about dotted hawthorn by Kucharska et al. [46]. In dotted hawthorn, ethyl hexanoate constituted 26.5% of all

volatile components, but in beer, ethyl hexanoate constituted only 3.9% of all volatiles. In a study about wines made from hawthorn fruit juice, conducted by Zhang et al. [47], it was shown that concentration of ethyl hexanoate in wines was 7 times higher than in BJ, 10 times higher than in BC, and similar to the concentration in BF. Another ester identified in hawthorn fruit and all tested beers was ethyl (4E)-4-decenoate, which is characterised by fruit (pineapple), cognac and wax flavours [48,49]. It was detected in the tested samples in the amount of 0.1267 μg/100 mL for BJ, 0.4505 μg/100 mL for BF and 0.0985 μg/100 mL for BC. Concentration of ethyl octanoate was highest in BJ (8.3716 μg/100 mL) and lowest in BC (0.0656 μg/100 mL). This compound is characterised by a fruity aroma and can originate from hawthorn fruit as well as yeast metabolism. Ethyl octanoate has been found in various beers analysed by other authors, such as in beer with sorghum addition made by Chenge et al., where its determined concentration was two times higher than in BJ [41,46]. A compound not belonging to the group of esters, which was identified in the fruits of hawthorn, was 1-hexanol, which is a compound from chemical family of alcohols. It has a sweet, alcoholic aroma [50,51]. Its highest concentration was found in BF (0.0819 μg/100 mL). BJ contained 0.0327 μg/100 mL of this compound, while BC contained 0.0164 μg/100 mL. This compound was also found in greater concentration in fruit beers with addition of quince than in control beer in study of Zapata et al. [38]. The most abundant sesquiterpenes in beers were humulene and humulene epoxide I. Concentration of humulene was the highest in BF (1.9659 μg/100 mL), and the lowest in BJ (0.4772 μg/100 mL). Meanwhile, humulene epoxide I was found in BF in the amount of 3.1127 μg/100 mL and BJ in the amount of 0.2812 μg/100 mL. In BC, the concentration of these compounds was greater than in BJ (respectively 0.5118 μg/100 mL and 0.2969 μg/100 mL). In classic beers, these compounds come from hops [52], while this study may indicate that these sesquiterpenes can be found in hawthorn fruit, but only in the flesh (because in BJ their quantity is smaller than in BC). Another compound, β-farnesene, with the lowest concentration in BJ, which is naturally found in classic beers, also comes from the sesquiterpene group [53]. It is a compound characterised by woody and citrus aromas [54,55]. Its concentration in BJ was 0.0136 μg/100 mL, while in BC it was 0.0492 μg/100 mL, and in BF 1.6383 μg/100 mL. The total concentration of all volatile compounds in the tested beers was the highest for BF which was 287.8529 μg/100 mL, which was 6.5 times higher than in BJ, which contained 43.8884 μg of volatile compounds per 100 mL of beer, and 8 times higher than BC, which contained 35.9348 μg of volatile compounds per 100 mL of beer.

3.4. Organoleptic Analysis

The aroma, colour, clarity, foaminess and overall impression of beer were assessed. The beer that achieved the highest rating in terms of aroma, taste, clarity and overall impression was BF (Table 4, Figure 1). This is in line with the results of research carried out by Adamenko et al. [36] on beers with the addition of Cornelian cherry, where beers with the addition of fruit also received better marks in terms of taste than control beers. Statistical analysis showed no significant differences between BC and BF clarity, although the addition of juice negatively affected this attribute. The use of whole fruit caused a significant deterioration in beer foaminess. Control beer got the highest grade for foaminess; however, statistical analysis did not show a significant difference between the BC and BJ results. The addition of juice does not worsen the beer foam quality. It is possible that a high concentration of polyphenols in beer can have a detrimental effect on beer foaming [2]. Although some authors show a positive effect of high polyphenols on beer foam stability [56]. The results obtained are contradictory. It has not been clearly defined how polyphenolic compounds affect beer foam. BF achieved the best note for taste criterion. In this aspect of sensory analysis, it obtained significantly higher results than other analysed beers. BJ and BF beers received better aroma ratings than BC. This may be due to a higher concentration of esters characterised by floral and fruity notes (ethyl hexanoate or ethyl octanoate). Zapata et al. [38] presented similar results. They analysed the composition of the quince beer aroma. The study showed that the higher concentration of ethyl esters resulted in better scores in sensory analysis.

Table 3. Content of volatile compounds in tested beers.

				Kovats Indices			Concentration in Beer μg/100 ML [1]		
	Compound Name	**Retention Time**	**Chemical Family**	**KI Exp.**	**KI NIST**	**CAS Number**	**BJ**	**BF**	**BC**
1	1-Butanol, 3-methyl-	4.208	Alcohols	724	735	123-51-3	5.7104 ± 2.8229 c	11.9594 ± 4.9811 b	18.4103 ± 9.6497 a
2	Isobutyl acetate	5.145	Esters	763	771	110-19-0	0.0257 ± 0.0133 b	0.2048 ± 0.0954 a	0.0328 ± 0.0147 b
3	Butanoic acid. ethyl ester	5.852	Esters	799	802	105-54-4	0.0812 ± 0.0622 b	0.5324 ± 0.3472 a	0.0492 ± 0.0251 b
4	1-Hexanol	7.899	Alcohols	872	868	111-27-3	0.0327 ± 0.0142 b	0.0819 ± 0.0533 a	0.0164 ± 0.0086 c
5	1-Butanol, 3-methyl-, acetate	8.095	Esters	872	876	123-92-2	3.0275 ± 1.3892 b	18.4305 ± 6.4922 a	1.6409 ± 0.7294 b
6	5-Hepten-2-one, 6-methyl-	11.656	Ketones	987	986	110-93-0	0.0139 ± 0.0068 b	0.0819 ± 0.0412 a	0.0153 ± 0.0089 b
7	β-Myrcene	11.782	Monoterpenes	994	991	123-35-3	0.0137 ± 0.0101 b	0.1229 ± 0.0844 a	0.0164 ± 0.0114 b
8	Hexanoic acid, ethyl ester	12.034	Esters	1001	1000	123-66-0	1.3959 ± 0.9982 b	11.2221 ± 6.4921 a	0.9681 ± 0.06742 b
9	Acetic acid, hexyl ester	12.508	Esters	1017	1011	123-35-3	0.0059 ± 0.0036 b	0.1229 ± 0.0712 a	Trace
10	Propanoic acid, 2-methyl-, 2-methylbutyl ester	12.577	Esters	1019	1016	2445-69-4	0.0042 ± 0.0021 b	0.6963 ± 0.3152 a	Trace
11	p-Cymene	12.83	Aromatic hydrocarbons	1028	1025	99-87-6	0.0079 ± 0.0023 c	0.6144 ± 0.2197 a	0.0328 ± 0.0094 b
12	Limonene	12.955	Monoterpenes	1032	1030	5989-54-8	0.0218 ± 0.0074 b	0.1638 ± 0.0852 a	Trace
13	Butanoic acid, 3-methylbutyl ester	13.937	Esters	1062	1056	106-27-4	0.0041 ± 0.0019 c	0.1229 ± 0.0622 a	0.0164 ± 0.0072 b
14	Ethyl 5-methylhexanoate	14.035	Esters	1066	1072	10236-10-9	0.0119 ± 0.0041 a	0.0819 ± 0.0332 a	Trace
15	1-Octanol	14.316	Alcohols	1075	1071	111-87-5	0.0972 ± 0.0386 c	7.4541 ± 2.8264 a	0.5743 ± 0.2848 b
16	2-Nonanone	14.96	Ketones	1096	1092	821-55-6	0.0119 ± 0.0049 c	41.5231 ± 11.9227 a	2.7894 ± 1.0048 b
17	Linalool	15.2	Alcohols	1099	1099	78-70-6	0.8554 ± 0.2652 b	1.1468 ± 0.4392 a	0.0824 ± 0.0312 c
18	2-Nonen-1-ol	15.311	Alcohols	1099	1105	22104-79-6	0.1584 ± 0.0466 c	43.4859 ± 13.5984 a	2.7894 ± 0.9982 b
19	Phenylethyl Alcohol	15.607	Esters	1114	1116	1960-12-08	3.0156 ± 0.9954 a	1.1877 ± 0.05572 b	0.0492 ± 0.0235 c
20	Octanoic acid, methyl ester	15.945	Esters	1127	1126	106-32-1	0.0396 ± 0.0178 b	0.2431 ± 0.0861 a	trace
21	Endo-Borneol *	17.199	Monoterpenes	1177	1167	464-43-7	0.0158 ± 0.0084 c	0.2457 ± 0.0973 a	0.0656 ± 0.0221 b
22	Benzoic acid, ethyl ester	17.311	Esters	1180	1171	93-89-0	0.0976 ± 0.0394 c	10.1163 ± 3.8572 a	0.7548 ± 0.2362 b
23	Octanoic acid	17.674	Organic acids	1192	1180	124-07-2	1.3425 ± 0.5283 a	0.5734 ± 0.2271 b	0.0492 ± 0.0198 c
24	α-Terpineol	17.772	Monoterpene	1194	1190	98-55-5	5.7758 ± 2.2853 b	73.1488 ± 23.0739 a	0.1019 ± 0.0476 c
25	Octanoic acid, ethyl ester	17.858	Esters	1196	1196	106-32-1	8.3716 ± 2.3497 a	0.2457 ± 0.0612 b	0.0656 ± 0.0227 c
26	Decanal	18.028	Aldehydes	1207	1206	112-31-2	0.1465 ± 0.0381 b	1.2287 ± 0.4776 a	0.0164 ± 0.0048 c
27	Acetic acid, octyl ester	18.126	Esters	1217	1211	112-14-1	0.0178 ± 0.0061 c	4.0547 ± 1.1286 a	0.3774 ± 0.0982 b
28	Citronellol	18.46	Alcohols	1238	1229	106-22-9	0.5307 ± 0.2163 a	Tr ace	Trace
29	Benzeneacetic acid, ethyl ester	18.709	Esters	1254	1246	101-97-3	0.0139 ± 0.0047 c	20.3555 ± 4.3992 a	2.0182 ± 0.8642 b
30	Isopentyl hexanoate	18.752	Esters	1256	1252	2198-61-0	0.0079 ± 0.0032 b	0.0287 ± 0.0092 a	trace
31	Acetic acid, 2-phenylethyl ester	18.879	Esters	1263	1258	103-45-7	2.9661 ± 1.0873 a	0.3277 ± 0.0932 b	0.0164 ± 0.0052 c
32	1-Decanol	19.077	Alcohols	1277	1273	112-31-2	0.0812 ± 0.0272 a	0.0416 ± 0.0138 b	0.0328 ± 0.0098 b
33	2,4-Heptadienoic acid, 6-methyl-, ethyl ester	19.146	Esters	1282	1293	10236-06-03	0.1327 ± 0.4921 b	0.2457 ± 0.0733 a	0.0164 ± 0.0043 b
34	Methyl geranate	19.728	Esters	1339	1326	214-712-6	0.4396 ± 0.0963 b	1.4744 ± 0.3896 a	0.3282 ± 0.1158 b
35	Octanoic acid, 2-methylpropyl ester	19.938	Esters	1352	1348	5461-06-03	0.2198 ± 0.0624 b	0.3686 ± 0.1374 a	0.1641 ± 0.0556 b
36	Citronellol acetate	20.023	Esters	1357	1354	150-84-5	0.0973 ± 0.0372 b	0.3277 ± 0.0992 a	0.0985 ± 0.0313 b
37	Unknown sesquiterpene	20.149	Sesquiterpenes	1369			1.8197 ± 0.6294 a	0.6553 ± 0.3278c	1.2799 ± 0.3775 b
38	Ethyl (4E)-4-decenoate	20.291	Esters	1384	1377	76649-16-6	0.1267 ± 0.0352 b	0.4505 ± 0.0966 a	0.0985 ± 0.0217 b
39	Ethyl trans-2-decenoate	20.347	Esters	1389	1389	7367-88-6	0.7702 ± 0.2432 b	7.5366 ± 2.8836 a	0.5087 ± 0.1764 b
40	Decanoic acid, ethyl ester	20.418	Esters	1396	1396	110-38-3	4.1086 ± 1.2883 a	1.6792 ± 0.4732 b	0.5127 ± 0.2296 c
41	Dodecanal	20.561	Aldehydes	1412	1409	112-54-9	0.0396 ± 0.0084 b	0.3277 ± 0.0912 a	0.0492 ± 0.0068 b
42	β-Caryophyllene	20.786	Sesquiterpenes	1440	1419	87-44-5	0.0772 ± 0.0252 b	0.3686 ± 0.0992 a	0.0476 ± 0.0118 b
43	Octanoic acid, 3-methylbutyl ester	20.87	Esters	1453	1446	2035-99-6	0.1148 ± 0.0326 b	1.4335 ± 0.3774 a	0.0985 ± 0.0264 b

Table 3. *Cont.*

				Kovats Indices			Concentration in Beer μg/100 ML [1]		
	Compound Name	**Retention Time**	**Chemical Family**	**KI Exp.**	**KI NIST**	**CAS Number**	**BJ**	**BF**	**BC**
44	(E)-β-Famesene	20.984	Sesquiterpenes	1464	1457	18794-84-8	0.0436 ± 0.0092 b	1.6383 ± 0.4694 a	0.0492 ± 0.0111 b
45	Humulene	21.069	Sesquiterpenes	1474	1454	6753-98-6	0.4772 ± 0.0958 b	1.9659 ± 0.5312 a	0.5118 ± 0.1322 b
46	α-Muurolene	21.224	Sesquiterpenes	1493	1485	10208-80-7	0.0178 ± 0.0054 b	0.2457 ± 0.0618 a	0.0164 ± 0.0051 b
47	Pentadecane	21.296	Hydrocarbons	1500	1500	629-62-9	0.0139 ± 0.0058 c	0.3686 ± 0.1372 a	0.0492 ± 0.0126 b
48	δ-Cadinene	21.561	Sesquiterpenes	1538	1524	483-76-1	0.1267 ± 0.0344 b	0.9838 ± 0.2258 a	0.1149 ± 0.0376 b
49	Unknown compound	21.687		1557			0.0317 ± 0.0076 a	0.0023 ± 0.0017 b	Trace
50	β-Calacorene	21.744	Sesquiterpenes	1565	1563	50277-34-4	0.0475 ± 0.0122 b	0.0819 ± 0.0315 a	0.0328 ± 0.0087 b
51	Nerolidol	21.786	Sesquiterpenes	1574	1565	7212-44-4	0.0297± 0.0102 b	0.4505 ± 0.0936 a	0.0245 ± 0.0097 b
52	Dodecanoic acid, ethyl ester	21.955	Esters	1590	1595	106-33-2	0.9722 ± 0.2318 b	14.2939 ± 3.5972 a	0.6563 ± 0.2532 b
53	Humulene epoxide I	22.167	Sesquiterpenes	1607	1604	19888-33-6	0.2812 ± 0.0676 b	3.1127 ± 0.9136 a	0.2969 ± 0.0754 b
	Total						43.8884	287.8529	35.9348

[1] BC—control beer; BJ—beer with hawthorn juice; BF—beer with hawthorn fruit. Values are expressed as mean (n = 2) ± standard deviation. Mean values with different letters (a, b, c) within the same line are statistically different (p-value < 0.05).

Table 4. Organoleptic analysis of tested beers.

Beer Type [1]	Aroma	Clarity	Foaminess	Taste	Overall Impression
BC	3.02 ± 0.2 [c]	4.07 ± 0.21 [a]	4.13 ± 0.19 [a]	2.81 ± 0.17 [b]	2.87 ± 0.19 [b]
BJ	3.73 ± 0.15 [b]	2.93 ± 0.18 [b]	3.87 ± 0.19 [ab]	2.67 ± 0.21 [b]	2.67 ± 0.19 [b]
BF	4.13 ± 0.19 [a]	3.87 ± 0.24 [a]	3.53 ± 0.13 [b]	4.13 ± 0.19 [a]	4.47 ± 0.17 [a]

[1] BC—control beer; BJ—beer with hawthorn juice; BF—beer with hawthorn fruit. Values are expressed as mean (n = 15) ± standard deviation. Mean values with different letters (a, b, c) within the same column are statistically different (p-value < 0.05).

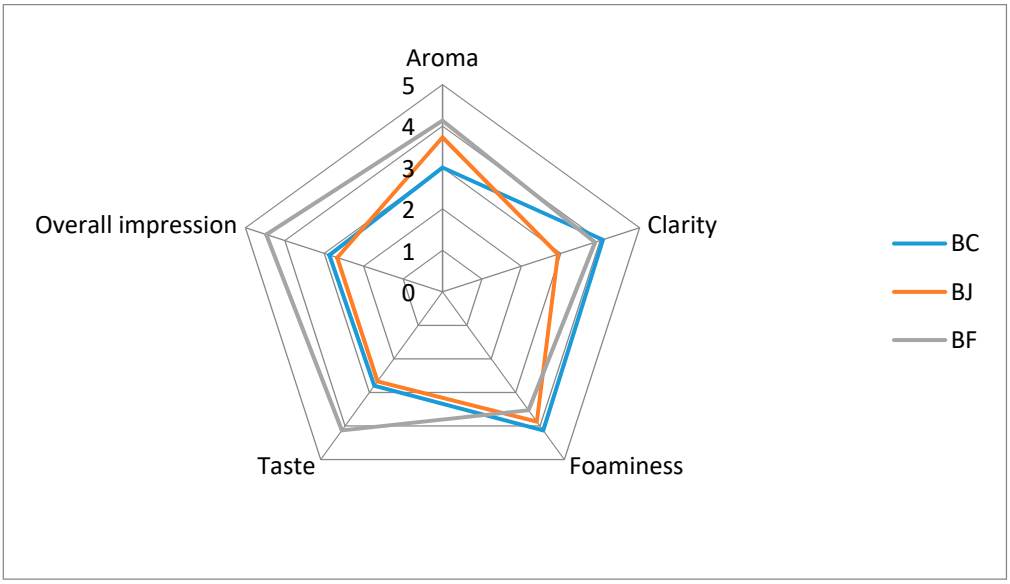

Figure 1. Organoleptic analysis of tested beers.

4. Conclusions

The study indicates that the addition of hawthorn (*Crataegus punctata*) makes it possible to obtain beer with an increased total content of polyphenolic compounds and antioxidant activity. Beer without hawthorn addition had the highest content of ethanol carbohydrates, glycerol and highest pH value. The total content of polyphenolic compounds was affected by both the addition of fruit and the form of the fruit added. Hawthorn in the form of juice made it possible to obtain a much higher content of bioactive polyphenolic compounds in beer than addition of fruit. Nevertheless, beer with fruit addition was characterised by the greatest amount of volatile compounds, eight times higher than in the control sample. Enrichment of the beer by hawthorn fruit mostly increased the concentration of the volatile compounds characterised by fruity and sweet aromas. The results of sensory analysis indicate that addition of hawthorn fruit results in an improvement of such characteristics as taste, aroma, clarity and overall impression in a higher degree than hawthorn juice addition. Hawthorn fruit and its juice can be used as complementary raw material in the production of beer to increase its biological activity and improve its taste and aroma. It can also contribute to greater consumer interest in the product.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/6/775/s1, Table S1: Content of volatile compounds in hawthorn fruit and juice, Figure S1: Mass spectrum of unknown sesquiterpene, Figure S2: Mass spectrum of unknown compound.

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Volatile Composition and Sensory Attributes of Smoothies Based on Pomegranate Juice and Mediterranean Fruit Purées (Fig, Jujube and Quince)

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Abstract: To increase the intake of fruits and vegetables—especially among young people—the food industry is trying to develop new, easy-to-eat and long-shelf-life products, such as smoothies. Nowadays, consumers are choosing their foods based not only on nutritional/functional properties (content of polyphenols, vitamins, minerals, among others), but also on sensory attributes. The aim of this study was to investigate the volatile composition by HS-SPME and the sensory profile by descriptive sensory analysis of novel smoothies prepared by blending fig, jujube or quince purée with pomegranate juices (cv. *Mollar de Elche* or *Wonderful*) at two ratios purée:juice (40:60 or 60:40). Twenty-three volatile compounds were identified by GC-MS and classified as alcohols, aldehydes, esters, furans, ketones, terpenes and terpenoids. Among volatile compounds, the five predominant ones in the studied smoothies were: (i) 5-HMF (30.6%); (ii) 3-hexen-1-ol (9.87%); (iii) hexanal (9.43%); (iv) 1-hexanol (8.54%); and (v) 3-octanone (7.67%). Fig smoothies were sweet and had flavor and volatiles related to fig, pomegranate, and grape. While jujube products were bitter and had jujube and pear notes. Finally, quince smoothies were consistent, sour and had quince, apple and floral notes. Thus, the type of fruit purée used clearly determined the flavor of the final product. The smoothies prepared with *Mollar de Elche* pomegranate juice were characterized by having high intensity of pear odor/aroma and consistency, and the *Wonderful* smoothies were characterized by lower consistency and more intense pomegranate aroma and sourness.

Keywords: *Punica granatum*; *Ficus carica*; *Ziziphus jujuba*; *Cydonia oblonga*; descriptive sensory analysis; volatile profile

1. Introduction

Nutrition is the most important external factor influencing children's development. Its influence is essential from birth through childhood. Consumption of fruit and vegetables is important as it plays an essential role in preventing childhood obesity, and preventing many diseases, including certain cancers, osteoporosis, diabetes, coronary heart disease, stroke, neuronal degeneration, and type II diabetes [1–3]. Therefore, the World Health Organization (WHO) recommends eating a minimum of 400 g of fruits and vegetables per day to improve health and prevent the above-mentioned chronic diseases. Because current consumption is lower than the recommended intake, the development of

easy-to-eat fruit-based products such as smoothies could be a good option. Despite of the natural sugar content, smoothies could reach the promotion into the children's diet [4,5]. Smoothies are beverages containing a blend of fruit pulp, fruit juice, ice, yoghurt, and/or milk. They are becoming a so popular way to consume fruits, especially among young people. These products are typically purchased freshly prepared from juice bars or as a processed product (mildly pasteurized) from the refrigerated section of retail outlets. Even after the economic crisis of 2007–2008, smoothies remained a popular and convenient way of consuming fruit [6]. Fruit components of smoothies could be considered as natural foods because of their nutrient profile or health-protecting qualities [7].

The southeastern part of Spain is one of the most intensively Mediterranean agricultural areas dominated by fruit orchards and vegetables fields. Some of these crops grown in this area, include fruits with appropriate characteristics for developing new products (organoleptic and functional properties), but underutilized, such as figs (*Ficus carica*), jujubes (*Ziziphus jujuba*) and quinces (*Cydonia oblonga*). It was recently found that the blend of pomegranate juice with the above mentioned fruits purée seemed a great opportunity to promote their use in an easy, sustainable and healthy way [5]. A positive effect of the addition of fig, jujube, and quince purée was observed in the nutritional and functionality of the novel pomegranate smoothies. For example, the addition of jujube contributed to an enrichment of the final smoothies in terms of vitamin C and organic acid content, while an increase of pectin content was found in fig and quince pomegranate-based smoothies [5].

On the other hand, the high interest in consuming fruit and vegetable products clearly shows that consumers are choosing products based not only on nutritional/functional properties (content of polyphenols, vitamins, minerals, among others), but also on sensory attributes (taste, smell, appearance, or even satisfaction) [8]. For this reason, fruit smoothies have become popular among health-conscious consumers and are among the major sources of bioactive compounds in daily diet [9,10]. Therefore, after knowing the nutritional and functional quality of the smoothies previously developed, the next steps should be to evaluate their volatile compositions and sensory profiles.

Taking all above mentioned into account, the aim of this study was to study the volatile composition and sensory profile of 12 different smoothies prepared using pomegranate juice (from 2 cultivars, cv., *Mollar de Elche* and *Wonderful*) and purée of Mediterranean crops (figs, jujubes and quinces) at different ratios purée:juice.

2. Materials and Methods

2.1. Plant Material

Pomegranates (*Punica granatum*) cv. *Mollar de Elche* and *Wonderful*, figs (*Ficus carica*) cv. *Colar*, jujubes (*Ziziphus jujuba*), cv. *Grandes de Albatera* and quinces (*Cydonia oblonga*) cv. *Gigante de Vranja* were hand-harvested in between mid-August and mid-October 2016 at a commercial maturity stage. The different stages of the smoothie preparation process as well as the ratio of purée:juice were previously described by Cano-Lamadrid et al. [5]. Briefly, the stages of the smoothie preparation process were:

i. Purée preparation: figs (F), jujubes (J), or quinces (Q) were peeled, ground, and heated at 80 °C in a Thermomix device (Vorwerk, Wuppertal, Germany); 10 mL of rhubarb juice per 1 kg of fruit were added to prevent enzymatic browning of the fruit due to the high oxalic acid concentration which chelates copper from the active site of polyphenol oxidase (PPO) [11,12]. After, the particle size of the mixture was reduced in a blender (Symbio, Zelmer, Rzeszów, Poland) until getting a thin purée. Then, the samples were cooled to room temperature.
ii. Pomegranate juice preparation: pomegranate fruits (*Mollar de Elche* and *Wonderful*, PM and PW, respectively) were cut in halves, and arils were manually separated from the husk and ground in a Thermomix to obtain the pomegranate juices.
iii. Partial products in appropriate proportions preparation: purée and juices samples, immediately after their preparation, were mixed in the proportions 40:60 (40F:60 PM; 40F:60PW; 40J:60 PM;

40J:60PW; 40Q:60 PM; 40Q:60PW) and 60:40 (60F:40 PM; 60F:40PW; 60J:40 PM; 60J:40PW; 60Q:40 PM; 60Q:40PW), obtaining 12 samples. Then, the products were heated to 100 °C, put into glass jars (130 mL) and pasteurized (10 min at 90 °C).

2.2. Volatile Compounds

Volatile composition of the samples under analysis was obtained by headspace solid phase microextraction (HS-SPME). Five g of each smoothie + 10 mL ultrapure water were placed into 50-mL vials with polypropylene caps and PTFE/silicone septa. A magnetic stirring bar was added, together with NaCl (15%) and the vial was placed in a water bath with controlled temperature and automatic stirring. A 50/30 µm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA) (high capacity of trapping fruit volatile compounds) was exposed to the sample headspace for 50 min at 40 °C to simulate the mouth temperature during the chewing process. Desorption of the volatile compounds from the fiber coating was carried out in the injection port of the GC-MS for 3 min.

The identification and semiquantification of the volatile compounds was performed on a gas chromatograph (GC-MS), Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectrometer detector GC-MS QP-5050A. The chromatographic set up and conditions were identical to those recently reported, with the only exception that the column used was a Restek Rxi-1301 Sil MS (Restek Corporation, Palo Alto, USA) of 30 m × 0.25 mm internal diameter, 0.25-µm film thickness. Analyses were carried out using helium as carrier gas at a flow rate of 6 mL min^{-1} in a split ratio of 6 and a program: (a) initial temperature 80 °C; (b) rate of 3.0 °C min^{-1} to 210 °C and hold for 1 min; (c) rate of 25 °C min^{-1} from 210 to 300 °C and hold for 3 min. Injector and detector temperatures were held at 230 and 300 °C, respectively.

Most of the compounds were simultaneously identified by using 3 different analytical methods: (1) retention indices, (2) GC-MS retention times (authentic chemicals), and (3) mass spectra (authentic chemicals and Wiley spectral library collection). Identification was considered tentative when it was based only on mass spectral data. The volatile composition analysis was run in triplicate and results were expressed as percentage of the total area represented by each one of the volatile compounds.

2.3. Sensory Evaluation with Trained Panel

Eight trained panelists (aged 30 to 55 years; 4 females and 4 males) from the *Escuela Politécnica Superior de Orihuela* (EPSO), *Universidad Miguel Hernández de Elche* (UMH) with more than 500 h of training in sensory testing participated in this study. The panel was selected and trained following the ISO standard 8586-1 (1993), and it is specialized in descriptive sensory evaluation of pomegranate products [13–16]. For the present study, the panel worked during 2 orientation sessions (90 min for each one) discussing the main organoleptic characteristics of commercial smoothies and fruit-based: pomegranate, figs, jujube, and quinces. The lexicon used for describing the flavor and texture attributes was based on the previously developed by other authors [17,18]. Both lexicons were adapted for smoothies based on pomegranate during the orientation sessions (Table 1). Samples were served into odor-free, disposable 90 mL covered plastic cups, at room temperature and were coded using 3 digit numbers as previous studies indicated [15]. Unsalted crackers and distilled water were provided to panelists to clean their palates between samples. The panel used a continuous numeric scale (0–10) for quantifying the intensity of smoothie attributes, where 0 represents none and 10 extremely strong, with 0.5-unit increments.

Table 1. Sensory descriptors for odor, flavor, basic tastes and defects.

Descriptor	Definition	References
	Odor/Flavor attributes	
Pomegranate	Sweet and fruity flavor associated with pomegranate	Freshly harvested pomegranate at optimum maturity index, cv. *Wonderful* = 8.0 Freshly harvested pomegranate at optimum maturity index, cv. *Mollar de Elche* = 5.0
Fig	Sweet and fruity flavor associated with figs	Freshly harvested fig, cv. *Colar* = 9.0
Jujube	Sweet and fruity flavor associated with jujube	Freshly picked jujubes at best picking time, cv. *Grande de Albatera* = 9.0
Quince	A floral, fresh, and fruity aromatics associated with quince	Freshly harvested quinces, cv. *Vranja* = 6.5
Apple	Aromatic compounds associated with processed apple juice and cooked apples	Hacendado mango–apple nectar = 5.5
Pear	Sweet, slightly musty, floral, honey/caramel-like, fruity aromatic associated with ripe pears	Hacendado pear nectar = 6.5
Grape must	Sweet and fruity aromatics from fresh grapes	Grape juice (Welch's Concord) = 10
Cranberry	Aromatic associated with cranberries	Fresh cranberries = 10
Floral	Sweet, heavy aromatics blend of a combination of flowers	Geraniol (1000 mg L^{-1}) = 4.0
Green	Green, fresh aromatics associated with green vegetables and newly cut vines and stems; related to cucumber	Trans-2-hexen-1-ol 5000 ppm = 4.0 Heinz tomato ketchup (vinegar) = 4.5 Freshly sliced tomatoes = 10.0
	Basic tastes	
Sweet	The fundamental factor associated with a sucrose solution	3% sucrose solution = 2.0 6% sucrose solution = 4.0 12% sucrose solution = 8.0
Sour	The taste factor associated with some organic acid, specifically citric acid	0.043% citric acid solution = 2.0 0.064% citric acid solution = 3.0 0.120% citric acid solution = 5.0 0.168% citric acid solution = 7.0
Bitter	The taste factor associated with a caffeine or quinine solution	0.008% caffeine solution = 1.0 0.15% citric acid solution = 2.0
Astringent	Dry sensation on the surface of the tongue or mouth associated with alum solution	0.03% alum solution = 1.5 0.05% alum solution = 2.5 0.1% alum solution = 5.0
	Texture	
Fiberness	Geometric property of the texture linked with the perception of the shape and orientation of the particles in the product	Fresh jujube purée (100%) = 9 Diluted jujube purée (1:1) = 4.5
Consistency	The force required to move the product across the tongue	Distilled water = 1 Condensed milk = 10
	Defect	
Cooked	Reminiscent aromatic compounds of fruit and/or vegetables after heating	Frozen orange concentrate (Minute Maid)-reconstituted = 4

2.4. Statistical Analysis

Data were subjected to analysis of variance (ANOVA), after checking the normality and homogeneity of the variance, and later to Tukey's multiple-range test to compare the means. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed using Statgraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD, USA). Instrumental parameters correlated with sensory descriptors were used to perform a principal component analysis (PCA regression map) and a dendrogram analysis using XLSTAT Premium 2016 (Microsoft Corporation, Redmond, WA, USA). Euclidean distance by Ward method was performed for the dendrograms of clusters.

3. Results and Discussion

3.1. Volatile Profile and Composition

HS-SPME is a standard method used for the isolation of volatile compounds; and it is considered as an environmentally friendly technique (due to no solvents are used), selective and very sensitive [19]. This technique has been successfully used to establish the volatile profiles of different matrices such as herbs, wines, vegetables, and fruits [20]. Volatile composition has been investigated in different pomegranate products, such as pomegranate juice [21,22] and dehydrated pomegranate arils [16]. It was also studied in different fruits such as jujube [23], quinces [24] and figs [25]. However, the present study is the first one evaluating the combined effects of two factors (pomegranate cultivar and ratio purée:juice) on the volatile profile of smoothies blended with different Mediterranean fruits. Table 1 shows the retention indices used for the identification of the compounds, together with the main sensory descriptors of each of the volatiles. Twenty-three volatile compounds were isolated, identified, and their relative abundance determined in the pomegranate smoothies blended with fig, jujube and quince purée samples using this method. Previously, 12 and 14 different compounds were identified in the PW and PM juices [22], indicating that the addition of Mediterranean fruits increased the volatile profile on pomegranate products. Among identified volatile compounds (Table 2), several common compounds were previously detected in *Mollar de Elche* and *Wonderful* pomegranate juices (V1, V3, V4, V7, V11, V13, V15, V17, V18, V19 and V20) [22], and in heat treated pomegranate-based products (dried arils) (V1, V3, V4, V13, V17, V18 and V21) [16]. On the other hand, V4, V6, V11, V12, V13, V14, V15, V18 and V19 were previously identified in quinces fruits [24], while V1, V3, V4, V8, V11, V12,V14, V14, V17 and V19 were identified in previous studies of jujube fruits [23,26,27]. As to figs fruits and dried figs, V1, V2, V4, V5, V6, V8, V10, V11, V13, V14, V15, V16 and V18 were already detected [25,28]. The combinations of different fruit matrix and heat treatments for pasteurization could generate other volatile compounds not previously described and identified (V9, V22 and V23).

Table 2. Retention indices and sensory descriptors of the volatile compounds in smoothies prepared by blending pomegranate juice with fig, jujube, or quince purée.

Code	Compounds ‡	Material ᵞ	RT (min)	Retention Indexes †		Descriptors ‡
				Exp	Lit	
V1	hexanal	F, J, Q	6.76	830	835	Fatty, green
V2	furfural	F, J, Q	8.58	894	899	Almond, woody
V3	3-hexen-1-ol	F, J, Q	8.85	902	905	Banana
V4	1-hexanol	F, J, Q	9.11	909	912	Green, herbaceous
V5	3-heptanone	F, J, Q	9.81	927	923	Green, fruity, fatty
V6	α-pinene	F	10.13	935	937	Woody
V7	β-pinene	F, Q	12.47	995	998	Woody
V8	2-heptenal	F, J, Q	12.99	1007	904	Apple, lemon, green, spicy
V9	3-octanone	F, J, Q	13.33	1015	1024	Banana, berry, spicy, green
V10	α-terpinene	J, Q	14.12	1032	1034	Berry, lemon, vegetable
V11	octanal	F, J, Q	14.52	1042	1029	Honey, fruity, fatty, citrus
V12	hexyl acetate	F, J, Q	14.57	1042	1042	Apple, cherry, floral, pear
V13	limonene	F, J	14.75	1046	1046	Lemon, orange, citrus
V14	1-octanol	F	15.75	1068	1123	Citrus, fatty, woody
V15	2-ethyl-1-hexanol	F, Q	15.83	1069	1070	Oily, rose, sweet
V16	linalool oxide	J	17.82	1112	1114	Floral
V17	linalool	F, J, Q	19.08	1138	1142	Lemon, orange, floral
V18	nonanal	F, J	19.29	1142	1154	Apple, coconut, grape
V19	ethyl octanoate	F	22.95	1218	1231	Apricot, floral, pear
V20	terpinen-4-ol	F	23.18	1222	1226	Citrus, woody, herbaceous
V21	5-HM	F, J, Q	28.84	1340	1362	Butter, caramel, musty
V22	β-damascenone	F, J	33.46	1438	1459	Apple, herbaceous, woody
V23	α-gurjunene	F	33.64	1442	1436	Woody

ᵞ F = fig; J = jujube; Q = quince; † RT = retention time; Exp = experimental; Lit = literature. ‡ National Institute of Standards and Technology, NIST (2020); SAFC (2012).

Eleven compounds were common in all 3 types of smoothies (fig, jujube, and quince), including for instance hexanal, furfural, 3-heptanone, hexyl acetate, linalool and HMF (Table 2).

At the beginning, statistics were preformed individually for each type of smoothie because of the completely different nature of the products under study, and later, the effect of purée fruit was also analyzed. Table 3 shows the relative abundance of the volatile compounds, grouped by chemical family, in the smoothies prepared with the 2 pomegranate cultivars (*Mollar de Elche* and *Wonderful*) and 3 fruits purée (figs, jujubes and quinces) for 2 ratios purée:juice (40:60 and 60:40). To make the discussion of this section easier, the pomegranate volatile compounds have been grouped into 7 chemical families:

i. Alcohols (ALCs): 3-hexen-1-ol (V3), 1-hexanol (V4), 1-octanol (V14), and 2-ethyl-1-hexanol (V15);
ii. Aldehydes (ALDs, total aldehydes): hexanal (V1), 2-heptenal (V8), octanal (V11), and nonanal (V18);
iii. Esters (ESTs): hexyl acetate (V12), and ethyl octanoate (V19);
iv. Furans (FURs): furfural (V2), and 5-HMF (V21);
v. Ketones (KETs): 3-heptanone (V5), 3-octanone (V9), and β-damascenone (V22);
vi. Terpenes (TEs): α-pinene (V6), β-pinene (V7), α-terpinene (V10), limonene (V13) and α-gurjunene (V23);
vii. Terpenoids (TOs): linalool oxide (V16), linalool (V17), and terpinen-4-ol (V20).

The main chemical groups of the pomegranate smoothies were: (i) furans, representing (32.8% ± 6.8%) of the total concentration of aroma compounds, followed by (ii) aldehydes (20.2% ± 4.0%), (iii) alcohols (19.3% ± 3.8%), (iv) ketones (10.8% ± 4.4%), (v) terpenoids (6.6% ± 2.7%), (vi) terpenes (5.9% ± 1.2%), and (vii) esters (4.6% ± 1.4%). It can be observed that volatile profile differed between the fresh pomegranate juices in which ALCs were the predominant family (67%, mainly 1-hexanol and 3-hexen-1-ol) in PW juices, while ALDs (30%) played an important role and were the most abundant chemical family in the PM juices [22].

The 5 predominant compounds in the studied smoothies were: (i) 5-HMF (mean for all samples 30.6%); (ii) 3-hexen-1-ol (9.87%); (iii) hexanal (9.43%); (iv) 1-hexanol (8.54%); and (v) 3-octanone (7.67%). The fact that the predominant compound was 5-HMF was unexpected. The furanic compound 5-HMF forms as an intermediate in the Maillard reaction between hexoses and amino components, and from direct dehydration of sugars under acidic conditions (caramelization) during thermal treatments applied to foods [29]. For instance, this compound is used as an indicator of the intensity of thermal treatment in honey [30]. In a previous study, 5-HMF and furfural were even found in the optimized dehydrated pomegranate arils [16]. However, the novel pomegranate smoothies highlighted by having less content than the above mentioned product. Without any doubt this compound is generated during the two heating steps of the smoothie preparation. The other 4 compounds are typical of fruits and fruit-based products; for instance, 3-hexen-1-ol, hexanal and 1-hexanol are key compounds of the peach flavor [31]. As 3-octanone was a predominant compound in the volatile composition of fresh wild mushrooms [32].

Hexanal, 3-hexen-1-ol and 3-octanone were more abundant in *Mollar de Elche* samples, while linalool and 5-HMF predominated in *Wonderful* smoothies (Table 3). It is worth mentioning that certain compounds can be used as an indicator of the fruit purée used in the smoothies. For instance, the volatile compounds exclusively identified in figs smoothies were: α-pinene, 1-octanol, ethyl octanoate, terpinen-4-ol and α-gurjunene. On the other hand, linalool oxide was exclusively present in the jujube smoothies. Quinces did not provide any exclusive compound to the smoothies. Formulation with a higher percentage of pomegranate juice (60%) led to higher abundance of 3-hexen-1-ol and linalool, while 60% of fruit purée increased the content of 3-octanone (Table 3).

Table 3. Relative abundance (%) of volatile compounds in smoothies prepared by blending pomegranate juice with fig, jujube, or quince purée.

ANOVA (AN) [†]		Fig Smoothies				AN	Jujube Smoothies				AN	Quinces Smoothies							
		40F:60 PM	60F:40 PM	40F:60PW	60F:40PW		40J:60 PM	60J:40 PM	40J:60PW	60J:40PW		40Q:60 PM	60Q:40 PM	40Q:60PW	60Q:40PW	AN	F	J	Q
ALCs																			
V3	***	25.1 ± 5.02a [‡]	0.46 ± 0.09c	6.80 ± 1.36b	3.19 ± 0.64bc	***	13.0 ± 3.73a	11.6 ± 0.94a	4.71 ± 2.33b	12.7 ± 6.05a	***	23.6 ± 9.75a	2.28 ± 0.80c	4.02 ± 0.32c	10.9 ± 2.17b	NS	8.89 ± 11.1	10.5 ± 3.91	10.2 ± 9.66
V4	***	13.7 ± 2.74a	nd	8.38 ± 1.68b	4.48 ± 0.90bc	**	9.34 ± 2.32b	5.73 ± 0.79bc	3.94 ± 1.15c	10.8 ± 2.17a	***	18.2 ± 6.19a	7.56 ± 1.02b	5.12 ± 1.07b	15.2 ± 3.05a	NS	8.85 ± 4.62	7.46 ± 3.18	11.5 ± 6.20
V14	NS	1.02 ± 0.20	0.84 ± 0.17	0.66 ± 0.13	nd [υ]		nd	nd	nd	nd		nd	nd	nd	nd	–	–	–	–
V15	*	4.14 ± 0.83a	0.87 ± 0.17b	0.56 ± 0.11b	0.72 ± 0.14b		nd	nd	nd	nd	NS	0.44 ± 0.09	nd	0.38 ± 0.08	0.56 ± 0.11	NS	1.57 ± 1.72	–	0.34 ± 0.24
∑ALCs		44.0	2.17	16.4	8.39		22.3	17.3	8.65	23.5		42.24	9.84	9.52	26.7				
ALDs																			
V1	***	19.0 ± 3.80a	2.23 ± 0.45c	3.81 ± 0.76c	8.41 ± 1.68b	***	3.89 ± 1.52c	19.7 ± 3.73a	5.32 ± 0.86c	10.9 ± 1.98b	***	29.8 ± 13.7a	1.06 ± 0.15c	3.09 ± 0.62bc	5.91 ± 1.18b	NS	8.36 ± 7.55	8.94 ± 7.15	9.96 ± 13.4
V8	*	3.88 ± 0.78a	1.05 ± 0.21b	1.24 ± 0.25b	1.49 ± 0.30b	***	4.55 ± 2.74c	19.5 ± 3.90a	7.31 ± 1.46b	19.1 ± 3.83a	NS	0.37 ± 0.04	nd	0.27 ± 0.05	0.32 ± 0.06	*	1.91 ± 1.32b	12.6 ± 7.82a	0.24 ± 0.16c
V11	**	2.66 ± 0.53ab	5.63 ± 1.13a	1.13 ± 0.23b	1.66 ± 0.33b	*	1.06 ± 1.42b	4.69 ± 0.94a	2.84 ± 0.57b	3.60 ± 0.72ab	**	0.55 ± 0.11b	0.22 ± 0.03b	18.3 ± 3.66a	0.61 ± 0.12b	NS	2.77 ± 2.01	3.05 ± 1.53	4.92 ± 8.93
V18	**	5.97 ± 1.19a	1.80 ± 0.36b	3.45 ± 0.69ab	4.75 ± 0.95a	NS	1.45 ± 1.60	3.48 ± 0.67	3.45 ± 0.67	2.60 ± 0.50		nd	nd	nd	nd	NS	3.99 ± 1.79	2.65 ± 0.93	–
∑ALDs		31.5	10.7	9.63	16.3		11.0	47.4	18.9	36.2		30.7	1.28	21.7	6.84				
ESTs																			
V12	***	4.22 ± 0.84b	3.26 ± 0.65b	2.21 ± 0.44b	16.2 ± 3.23a	**	1.82 ± 4.74b	3.37 ± 0.67b	8.20 ± 1.64a	2.87 ± 0.57b	**	3.30 ± 0.66b	nd	4.68 ± 0.94a	0.53 ± 0.11c	NS	6.46 ± 6.52	4.06 ± 2.83	2.13 ± 2.23
V19	NS	0.96 ± 0.19	1.53 ± 0.31	0.05 ± 0.01	1.75 ± 035		nd	nd	nd	nd		nd	nd	nd	nd	–	–	–	–
∑ESTs		5.18	4.79	2.26	18.0		1.82	3.37	8.20	2.87		3.30	nd	4.68	0.53				
FURs																			
V2	**	0.50 ± 0.10b	1.10 ± 0.22b	3.65 ± 0.73a	0.72 ± 0.14b	NS	2.95 ± 0.39	1.55 ± 0.52	2.59 ± 0.31	2.09 ± 0.42	**	1.91 ± 0.32b	3.17 ± 0.36a	1.80 ± 0.45b	3.65 ± 0.73a	NS	1.49 ± 1.46	2.29 ± 0.61	2.63 ± 0.92
V21	***	0.55 ± 0.11c	19.2 ± 3.84b	47.7 ± 9.55a	22.3 ± 4.46b	***	37.7 ± 12.3a	7.19 ± 1.44c	45.1 ± 9.01a	13.7 ± 2.74b	NS	14.2 ± 2.83d	79.0 ± 15.8a	25.7 ± 5.13c	55.3 ± 11.1b	NS	22.5 ± 19.4	25.9 ± 18.3	43.5 ± 29.3
∑FURs		1.05	20.3	51.4	23.0		40.7	8.74	47.7	15.8		16.1	82.2	27.5	59.0				
KETs																			
V5	***	0.46 ± 0.09b	0.35 ± 0.07b	Nd	15.5 ± 3.10a	*	nd	2.78 ± 0.56a	nd	nd	NS	nd	1.00	nd	nd	–	–	–	–
V9	***	0.99 ± 0.20b	52.8 ± 10.6a	0.86 ± 0.17b	0.65 ± 0.13b	***	2.00 ± 1.77c	14.5 ± 2.50a	4.00 ± 0.80b	13.8 ± 2.37a	*	0.41 ± 0.08b	nd	0.41 ± 0.08b	1.61 ± 0.32a	NS	13.8 ± 26.0	7.57 ± 5.38	0.61 ± 0.69
V22	*	2.46 ± 0.49b	nd	Nd	5.67 ± 1.13a	**	5.47 ± 0.94a	nd	3.00 ± 0.60b	0.94 ± 0.19bc		nd	nd	nd	nd		2.03 ± 2.69	2.35 ± 2.43	–
∑KETs		3.91	53.2	0.86	21.8		7.47	17.3	7.00	14.7		0.41	1	0.41	1.61				
TEs																			
V6	*	3.86 ± 0.77a	0.60 ± 0.12b	1.27 ± 0.25b	0.89 ± 0.18b		nd	nd	nd	nd		nd	nd	nd	nd	–	–	–	–
V7	*	1.21 ± 0.24b	0.23 ± 0.05b	3.46 ± 0.69a	1.04 ± 0.21b		nd	nd	nd	nd	***	3.58 ± 1.56a	2.38 ± 0.10ab	0.50 ± 0.34b	1.61 ± 0.32bc	NS	1.48 ± 1.38	–	2.02 ± 1.30
V10		nd	nd	Nd	nd	**	8.60 ± 2.61a	0.47 ± 0.09b	2.86 ± 0.57ab	1.00 ± 0.20b	*	2.89 ± 1.06a	1.90 ± 0.27ab	0.70 ± 0.14b	1.64 ± 0.33ab	NS	–	3.22 ± 3.70	1.78 ± 0.90
V13	NS	1.91 ± 0.38	1.35 ± 0.27	0.71 ± 0.14	2.26 ± 0.45	*	7.10 ± 1.68a	2.04 ± 0.41b	3.13 ± 0.63b	3.08 ± 0.62b		nd	nd	nd	nd	NS	1.56 ± 0.68	3.84 ± 2.23	–
V23	**	2.14 ± 0.43b	0.60 ± 0.12c	4.48 ± 0.90a	0.66 ± 0.13c		nd	nd	nd	nd		nd	nd	nd	nd	–	–	–	–
∑TEs		9.12	2.78	9.92	4.85		15.7	2.51	5.99	4.08		6.47	4.28	1.20	3.25				
TOs																			
V16		nd	nd	Nd	nd	NS	0.73 ± 0.89	2.67 ± 0.36	2.58 ± 0.34	2.26 ± 0.31		nd	nd	nd	nd	–	–	–	–
V17	**	4.00 ± 0.80a	1.07 ± 0.21b	1.05 ± 0.21b	2.03 ± 0.41b	NS	0.40 ± 0.49	0.73 ± 0.15	1.00 ± 0.20	0.49 ± 0.10	***	0.83 ± 0.17bc	1.45 ± 0.20b	35.1 ± 7.01a	2.14 ± 0.43b	NS	2.04 ± 1.39	0.66 ± 0.27	9.87 ± 16.8
V20	**	1.28 ± 0.26c	4.99 ± 1.00b	8.50 ± 1.70a	5.65 ± 1.13ab		nd	nd	nd	nd		nd	nd	nd	nd	–	–	–	–
∑TOs		5.28	6.06	9.55	7.68		1.13	3.4	3.58	2.75		0.83	1.45	35.1	2.14				

Note: Alcohols (ALCs): 3-hexen-1-ol (V3), 1-hexanol (V4), 1-octanol (V14), and 2-ethyl-1-hexanol (V15); Aldehydes (ALDs, total aldehydes): hexanal (V1), 2-heptenal (V8), octanal (V11), and nonanal (V18); Esters (ESTs): hexyl acetate (V12), and ethyl octanoate (V19); Furans (FURs): furfural (V2), and 5-HMF (V21); Ketones (KETs): 3-heptanone (V5), 3-octanone (V9), and β-damascenone (V22); Terpenes (TEs): α-pinene (V6), β-pinene (V7), α-terpinene (V10), limonene (V13) and α-gurjunene (V23); Terpenoids (TOs): linalool oxide (V16), linalool (V17), and terpinen-4-ol (V20); F: fig; J: jujube; Q: quinces; [†] NS = not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same row for the same fruit purée, were not significantly different ($p < 0.05$), according to Tukey's least significant difference test; [υ]nd: not detected, meaning below the quantification limit.

As a brief summary of this section, it can be stated that *Mollar de Elche* pomegranate juice and smoothies prepared using fig and jujube purées were less sensitive to heat treatment than *Wonderful* and quince smoothies, as reflected by lower 5-HMF contents.

Recently, consumers' overall liking ("drivers of liking") of pomegranate-based products (dehydrated arils) was positively linked with the presence of aldehydes, esters, aliphatic alcohols and terpenes [16] which were presented in the novel developed products. Industry could use these liking drivers as quality indicators for improving their commercial and future novel smoothies.

3.2. Descriptive Sensory Analysis

An appropriate performance of the panel was observed with a good reproducibility by the end of the orientation sessions. Sixteen attributes (odor, basic tastes, flavor, texture, and defects) were used to fully describe the smoothies and are presented in Table 4. The smoothies prepared with *Mollar de Elche* pomegranate juice presented higher intensity of the fruit purée (F, J or Q) and pear odor, while the *Wonderful* smoothies had more intense pomegranate odor. The use of figs intensified the grape and pomegranate odor notes, while jujubes increased the pear notes. Regarding the basic tastes, *Mollar de Elche* pomegranate smoothies led to sweeter notes compared to the scores of *Wonderful* which were defined as sourer samples. With respect to the fruit purée, the use of figs intensified the sweetness (Table 4).

Regarding to flavor attributes, similar trends as odor attributes was observed. *Wonderful* samples led to higher scores of pomegranate compared to *Mollar de Elche* smoothies that presented higher intensities of fruit purée (F, J or Q). In accordance with our results, previous study indicated that the use of *Wonderful* juices in pomegranate products enhanced pomegranate notes [16].

The fiberness and consistency were the texture attributes evaluated and highly significant differences were observed, especially in the product fiberness. The use of jujubes and quinces led to the highest intensities of fiberness and consistency, respectively. Therefore, the type of fruit purée used had an important effect on fiberness, with jujubes leading to the highest intensity and quinces to intermediate values. Finally, it is important to highlight that the factor having the lowest influence on the sensory profile was the ratio purée:juice, which only influenced the consistency (with the 60:40 products being the most consistent ones). Recently, it was observed that the pectin content increased when using the highest content of fruit purée in smoothies [5]. Pectin has techno–functional characteristics which enhance texture of the smoothies by the reaction of certain water-soluble pectic substances with Ca ions to form some Ca pectates [5]. The purée:juice ratio can, therefore, be adjusted accordingly to the consistency preferred by the consumers. The price of the fruits used for the purée should be also be taken into consideration in order to produce a smoothie with high qualitative characteristics, but at affordable price.

It is worth mentioning that a previous study indicated that consumer's overall liking in a jujube fruit consumer study was highly correlated with jujube flavor (high intensity), sweetness (high intensity), and bitterness (low intensity) [23]. In our study, the highest jujube notes and sweetness was found for the ratio 40J:60 PM. It represents a good starting point for further development/exploitation of novel smoothies. Similar trend was found in dried pomegranate arils, where the consumer´s overall liking was linked with fresh pomegranate flavor [16].

Table 4. Descriptive sensory analysis of smoothies prepared by blending pomegranate juice with fig, jujube, or quince purée.

ANOVA (AN) †		Fig Smoothies				AN	Jujube Smoothies				AN	Quinces Smoothies				AN Purée Type			
		40F:60 PM	60F:40 PM	40F:60PW	60F:40PW		40J:60 PM	60J:40 PM	40J:60PW	60J:40PW		40Q:60 PM	60Q:40 PM	40Q:60PW	60Q:40PW		F	J	Q
Odor																			
Pomegranate	***	3.5 ± 0.5b ‡	2.8 ± 0.7b	5.4 ± 0.7a	4.9 ± 1.1a	NS	2.3 ± 1.0	1.0 ± 0.4	1.6 ± 0.5	2.0 ± 0.7	NS	1.9 ± 1.5	1.1 ± 0.6	2.3 ± 1.3	2.1 ± 1.1	**	4.2 ± 1.2a	1.7 ± 0.5b	1.9 ± 0.5b
Fig	***	7.0 ± 0.1a	7.2 ± 0.5a	5.6 ± 0.5c	6.3 ± 0.8b											***	6.5 ± 0.7a	0.0 ± 0.0b	0.0 ± 0.0b
Jujube						NS	4.6 ± 0.9	3.8 ± 0.5	2.8 ± 1.0	3.5 ± 1.9						***	0.0 ± 0.0b	3.7 ± 0.8a	0.0 ± 0.0b
Quince											***	7.0 ± 0.0ab	8.6 ± 0.5a	5.8 ± 0.5b	8.0 ± 1.1ab	***	0.0 ± 0.0b	0.0 ± 0.0b	7.4 ± 1.3a
Apple	NS	1.5 ± 0.5	0.9 ± 0.7	1.3 ± 0.6	1.5 ± 0.4	NS	2.3 ± 1.2	2.3 ± 1.2	1.9 ± 0.9	3.0 ± 0.7	*	2.5 ± 0.6ab	3.1 ± 1.0a	1.5 ± 0.6b	3.0 ± 0.7ab	**	1.3 ± 0.3b	2.4 ± 0.5a	2.5 ± 0.7a
Pear	*	4.7 ± 0.5a	3.4 ± 1.3ab	2.9 ± 0.8b	2.8 ± 1.5b	*	7.4 ± 0.5a	6.1 ± 0.6ab	5.1 ± 1.0b	5.6 ± 1.5ab	NS	2.6 ± 0.8	3.6 ± 1.1	2.4 ± 1.1	2.6 ± 1.3	***	3.5 ± 0.8b	6.1 ± 1.0a	2.8 ± 0.6b
Grape must	*	5.8 ± 0.8a	3.3 ± 1.9b	4.4 ± 1.4ab	2.9 ± 1.7b	*	4.6 ± 1.5ab	3.0 ± 1.4b	2.9 ± 1.3b	2.6 ± 1.1a	NS	1.0 ± 0.4	1.5 ± 1.5	1.0 ± 0.9	0.8 ± 0.6	**	4.1 ± 1.3a	3.3 ± 0.9a	1.1 ± 0.3b
Cranberry	NS	0.7 ± 0.6	0.4 ± 0.4	1.7 ± 0.3	1.2 ± 0.8	NS	0.5 ± 0.4	0.3 ± 0.3	0.3 ± 0.3	1.3 ± 0.5	NS	0.4 ± 0.3	0.6 ± 0.6	1.3 ± 1.2	1.4 ± 0.9	NS	1.0 ± 0.6	0.6 ± 0.5	0.9 ± 0.5
Floral	NS	0.4 ± 0.5	0.3 ± 0.4	0.6 ± 0.5	0.5 ± 0.4	*	2.1 ± 0.3a	1.0 ± 0.7ab	1.0 ± 0.8ab	0.6 ± 0.5b	NS	2.0 ± 0.4	2.5 ± 1.2	1.8 ± 1.2	1.9 ± 1.4	**	0.5 ± 0.1b	1.2 ± 0.6b	2.1 ± 0.3a
Green	NS	1.4 ± 0.6	0.8 ± 0.5	1.8 ± 0.9	1.4 ± 1.0	***	0.6 ± 0.3b	0.4 ± 0.3b	0.6 ± 0.3b	1.6 ± 0.3a	NS	0.8 ± 0.3	0.6 ± 0.5	0.8 ± 0.9	1.0 ± 1.1	NS	1.4 ± 0.4	0.8 ± 0.6	0.8 ± 0.2
Basic tastes																			
Sweet	***	7.5 ± 0.8a	4.3 ± 0.8c	6.8 ± 0.4ab	6.3 ± 1.4b	***	6.4 ± 0.3a	4.1 ± 0.9b	5.3 ± 0.3ab	4.5 ± 0.6b	NS	4.0 ± 0.7	4.4 ± 1.4	3.6 ± 1.1	4.0 ± 0.7	**	6.2 ± 1.4a	5.1 ± 1.0ab	4.0 ± 0.3b
Sour	***	1.1 ± 0.7c	0.9 ± 0.6c	4.8 ± 0.4a	3.8 ± 1.3b	***	1.9 ± 0.3b	2.1 ± 1.0b	5.6 ± 0.5a	5.0 ± 0.0a	***	1.8 ± 0.9b	2.1 ± 1.4b	5.9 ± 1.3a	5.3ab	NS	2.7 ± 1.9	3.7 ± 1.9	3.8 ± 2.1
Bitter	NS	0.5 ± 0.5	0.5 ± 0.4	0.5 ± 0.5	0.9 ± 0.7	NS	1.0 ± 0.4	1.1 ± 0.3	0.9 ± 0.9	0.9 ± 0.5	NS	0.8 ± 0.6	1.0 ± 0.7	1.1 ± 0.9	0.6 ± 1.3	*	0.6 ± 0.2b	0.8 ± 0.1ab	1.0 ± 0.2a
Astringent	NS	0.3 ± 0.4	0.3 ± 0.4	0.7 ± 0.6	0.7 ± 0.5	NS	1.0 ± 0.6	2.1 ± 0.8	1.1 ± 0.5	1.8 ± 1.0	NS	1.3 ± 1.2	1.5 ± 1.2	1.5 ± 0.4	1.1 ± 0.8	**	0.5 ± 0.2b	1.5 ± 0.5a	1.4 ± 0.2a
Flavor																			
Pomegranate	***	2.4 ± 0.9c	1.6 ± 0.6c	5.8 ± 0.9a	4.7 ± 1.2b	***	4.4 ± 0.5b	2.3 ± 0.5c	6.1 ± 0.6a	2.9 ± 0.9c	**	2.6 ± 0.9ab	1.5 ± 0.7b	3.9 ± 0.6a	3.0 ± 0.9ab	NS	3.6 ± 1.9	3.9 ± 1.7	2.8 ± 1.0
Fig	*	5.7 ± 0.6a	3.9 ± 0.8b	4.2 ± 1.0b	4.9 ± 1.2ab											***	4.7 ± 0.8a	0.0 ± 0.0b	0.0 ± 0.0b
Jujube						*	4.1 ± 0.3b	4.5 ± 0.6ab	5.5 ± 0.4a	4.8 ± 1.0ab						***	0.0 ± 0.0b	4.7 ± 0.6a	0.0 ± 0.0b
Quince											*	4.9 ± 1.7b	6.3 ± 1.3a	4.4 ± 1.1b	6.6 ± 1.4a	***	0.0 ± 0.0b	0.0 ± 0.0b	5.6 ± 1.1a
Apple	*	1.1 ± 0.8b	0.7 ± 0.4b	2.6 ± 1.2a	1.8 ± 1.8ab	NS	3.3 ± 1.3	2.9 ± 1.5	2.9 ± 1.9	2.8 ± 1.0	NS	2.8 ± 1.7	4.0 ± 1.1	2.4 ± 1.1	3.4 ± 1.0	**	1.6 ± 0.8b	3.0 ± 0.2a	3.2 ± 0.7a
Pear	NS	2.9 ± 1.4	1.7 ± 0.8	1.8 ± 1.0	1.9 ± 1.4	NS	6.6 ± 0.5	5.0 ± 1.4	4.8 ± 0.5	5.0 ± 1.1	NS	3.1 ± 1.8	3.4 ± 1.1	2.6 ± 1.5	3.1 ± 1.0	***	2.1 ± 0.6b	5.4 ± 0.9a	3.1 ± 0.3b
Grape must	**	4.8 ± 1.7a	1.5 ± 0.8c	3.6 ± 1.7ab	2.3 ± 1.2bc	NS	2.8 ± 1.5	1.6 ± 1.1	2.5 ± 1.3	2.4 ± 1.5	NS	1.4 ± 0.8	1.6 ± 1.6	1.6 ± 1.1	1.6 ± 2.0	NS	3.1 ± 1.4	2.3 ± 0.5	1.6 ± 0.1
Cranberry	***	0.3 ± 0.4c	0.3 ± 0.4c	3.8 ± 1.9a	2.2 ± 1.6b	***	0.6 ± 0.5b	0.3 ± 0.3b	3.5 ± 0.4a	1.5 ± 1.5b	NS	0.5 ± 0.4	0.4 ± 0.5	1.8 ± 1.2	1.5 ± 1.1	NS	1.7 ± 1.5	1.5 ± 1.4	1.1 ± 0.7
Floral	NS	0.3 ± 0.3	0.2 ± 0.3	0.3 ± 0.9	0.3 ± 0.3	NS	1.3 ± 0.6	0.6 ± 0.6	1.0 ± 0.7	0.9 ± 0.5	NS	1.1 ± 0.6	1.1 ± 0.9	1.1 ± 0.8	0.9 ± 0.8	***	0.3 ± 0.1b	1.0 ± 0.3a	1.1 ± 0.1a
Green	***	0.9 ± 0.4b	0.6 ± 0.4b	2.8 ± 1.3a	0.9 ± 0.7b	NS	0.9 ± 0.5	0.8 ± 0.9	2.0 ± 0.7	2.0 ± 1.1	NS	1.8 ± 1.0	1.4 ± 0.9	2.3 ± 0.9	1.3 ± 1.8	NS	1.3 ± 1.0	1.4 ± 0.7	1.7 ± 0.4
Texture																			
Fiberness	NS	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.1 ± 0.2	***	9.0 ± 0.0ab	9.9 ± 0.3a	8.6 ± 0.5b	9.1 ± 1.0ab	NS	3.9 ± 1.9	6.4 ± 3.1	4.9 ± 3.2	5.4 ± 3.1	***	0.1 ± 0.0c	9.2 ± 0.5a	5.2 ± 1.0b
Consistency	***	1.2 ± 0.4c	8.9 ± 0.2a	1.3 ± 0.4c	3.8 ± 1.2b	***	1.0 ± 0.0d	6.3 ± 0.3a	2.0 ± 0.7c	5.0 ± 0.0b	NS	2.8 ± 0.3c	6.8 ± 0.3b	2.5 ± 0.4c	8.1 ± 0.3a	NS	3.8 ± 3.5	3.6 ± 2.5	5.1 ± 2.8
Defect																			
Cooked (odor)	*	1.1 ± 1.5ab	0.0 ± 0.0b	1.8 ± 1.3a	0.0 ± 0.0b	NS	1.4 ± 0.6	1.5 ± 0.9	0.4 ± 0.5	0.8 ± 0.6	***	0.3 ± 0.3	0.6 ± 0.8	0.3 ± 0.3	0.1 ± 0.3	NS	0.7 ± 0.6	1.0 ± 0.5	0.3 ± 0.2
Cooked (flavor)	NS	0.8 ± 0.9	0.0 ± 0.0	0.8 ± 1.0	0.0 ± 0.0	NS	0.6 ± 0.9	0.6 ± 0.3	0.4 ± 0.5	0.4 ± 0.5	NS	1.4 ± 0.9	0.6 ± 0.8	0.1 ± 0.3	0.3 ± 0.3	NS	0.4 ± 0.3	0.5 ± 0.1	0.6 ± 0.5

Note: † NS = not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same row for the same fruit purée, were not significantly different ($p < 0.05$), according to Tukey's least significant difference test.

3.3. Principal Component Analysis and Pearson's Correlations

A principal component analysis (PCA) was conducted to clearly see the relationships among the 12 smoothies, their volatile composition and sensory profile. Figure 1 shows that the first principal component (F1) explained 31.40% of the total data variance and the second one (F2) explained 18.56% of the total variance.

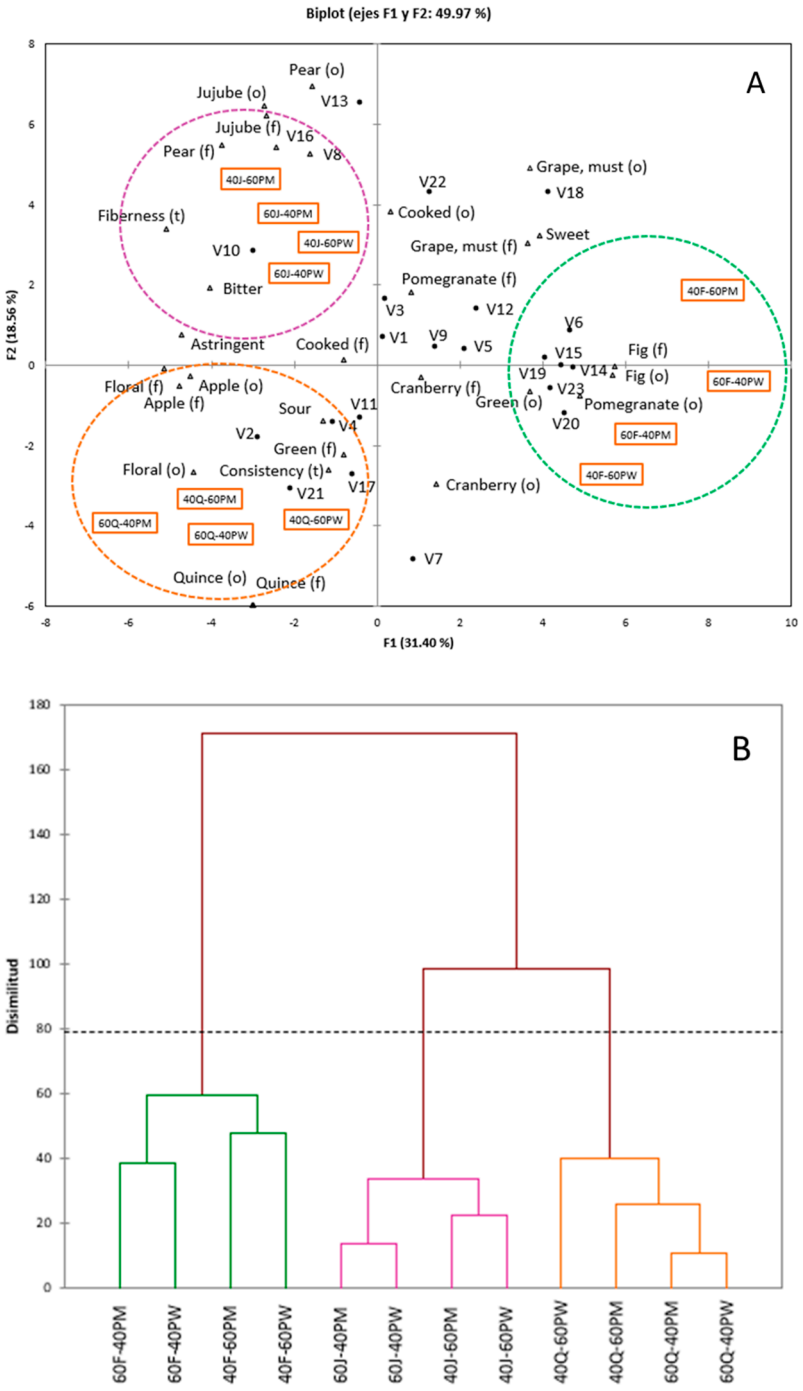

Figure 1. (**A**) PCA and (**B**) cluster maps prepared using (•) volatile compounds and (Δ) sensory profile of smoothies prepared by blending pomegranate juice (PM and PW for "*Mollar de Elche*" and "*Wonderful*", respectively) with fig (F), jujube (J), or quince (Q) purée.

As can be seen, samples were grouped mainly according to the type of fruit purée in the smoothies, regardless of the purée:juice ratio and the pomegranate cultivar. Figure 1B shows how samples are grouped into 3 main clusters. Fig smoothies were characterized by high intensity of fig, pomegranate, cranberry, green, grape notes and sweetness, and with the following volatile compounds: V6, V14, V15, V19 V20, and V23, which present the following odor descriptors: woody, floral, citrus, and green. The relationship between volatile compounds and fig odor and aroma were backed up by significant values of the Pearson's correlations (Table 4). Jujube smoothies were characterized by high intensity of jujube and pear notes, fiberness and bitterness, and were associated with V8, V10, V13, and V16, being citrus and floral the main descriptors. Values above 0.7 for Pearson's correlations were found among jujube and these volatile compounds (Table 4). Finally, quince smoothies were characterized by high intensity of consistency and quince, green, apple and floral notes, and sourness, and were linked with V2, V4, V17 and V21. The descriptors of these volatiles are caramel, woody and floral.

Pearson's correlation coefficient (Table 5) showed that fig odor and fig flavor was positively correlated ($R > 0.6$; p-value < 0.05) with V6, V14, V15, V18, V19, V20, V23. No significant (p-value >0.05) correlation was observed among pomegranate flavor with volatile compounds, while pomegranate odor was well-correlated with V20 and V23 ($R > 0.7$; p-value < 0.05). In addition, no significant correlation between volatile compounds and cranberry aroma, cranberry odor, green aroma, and sour was found. As to jujube odor and flavor, Pearson's coefficient showed that was positively correlated ($R > 0.6$; p-value < 0.05) with V8, V13 and V16. Quince odor and flavor was not positively correlated with any identified volatile compound. Pear odor and flavor, and grape odor and flavor were positively correlated with V8, V13 and V16, and V6, V14, V18 and V23, respectively ($R > 0.6$; p-value < 0.05). Sweet was the highest correlated basic taste with V6, V18, V22 and V23 ($R > 0.6$; p-value < 0.05).

Table 5. Pearson correlations between volatile compounds and sensory descriptive attributes in smoothies prepared by blending pomegranate juice (Pom) with fig (F), jujube (J), or quince (Q) purée.

R	Odor Attributes								Basic Taste				Flavor Attributes						
	Pom	F	J	Q	Apple	Pear	Grape	Floral	Green	Sweet	Bitter	Astringency	F	J	Q	Apple	Pear	Grape	Floral
V1																			
V2																			
V3																			
V4																			
V5																			
V6																			
V7																			
V8																			
V9																			
V10																			
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V19																			
V20																			
V21																			
V22																			
V23																			

Green: $R > 0.6$, p-value < 0.05; Pink: $R < -0.6$, p-value < 0.05; Orange: $0.0 < R < 0.6$, NS; and Blue: $-0.6 < R < 0.0$, NS. Cranberry aroma, cranberry odor, green aroma, sour and pomegranate do not appear in this table since no significant correlation was found.

4. Conclusions

The volatile composition and the sensory profile of novel smoothies prepared blending fig, jujube or quince purée with pomegranates juices (cv. *Mollar de Elche* or *Wonderful*) at two ratios of purée:juice (40:60 or 60:40) were studied. Twenty-three volatile compounds were identified, the five

predominant ones: (i) 5-HMF (mean for all samples 30.6%); (ii) 3-hexen-1-ol (9.87%); (iii) hexanal (9.43%); (iv) 1-hexanol (8.54%); and (v) 3-octanone (7.67%). Fig smoothies were sweet and had flavor and volatiles related to fig, pomegranate, and grape. Meanwhile, jujube products were bitter and had jujube and pear notes. Finally, quince smoothies were sour and had quince, apple and floral notes. Thus, the type of fruit used clearly determined the flavor of the final product. The smoothies prepared with *Mollar de Elche* pomegranate juice were characterized by having high intensity of pear odor/aroma and consistency. While *Wonderful* smoothies were characterized by lower consistency and more intense pomegranate aroma and sour. However, further research is still needed to fully optimize these novel products. Two ways of improvement can be researched: (i) increasing pomegranate notes; and (ii) avoiding undesirable compounds after the Maillard Reaction. After knowing the volatile compounds and sensory profiles of these developed smoothies, it is worth to continue researching in this area studying the volatile composition of other types of smoothies and other types of pasteurization treatments to avoid undesirable compounds and aromas. To know the active odor compounds of these products also can be necessary in the future studies. Moreover, consumer studies should be carried out to know the drivers of smoothie's consumer acceptance.

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Volatile Composition and Sensory Properties as Quality Attributes of Fresh and Dried Hemp Flowers (*Cannabis sativa* L.)

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Abstract: Flowers of hemp (*Cannabis sativa* L.) are widely used in cosmetics, food, and in the pharmaceutical industry. The drying process plays a key role in retention of aroma and also in the quality of products. Seven variants of hemp flower drying, including convection drying (CD), vacuum–microwave drying (VMD), and combined drying consisting of convective pre-drying followed by vacuum–microwave finishing drying (CPD-VMFD) were checked in this study. For each process, we applied the two-term model. Dried material was submitted to color and chromatographical assessments. Analyses of obtained essential oil showed the presence of 93 volatile compounds, predominantly β-myrcene, limonene, and β-(*E*)-caryophyllene, as well as α-humulene. Application of 240 W during VMD and 50 °C during CD gave the highest retention of aroma compounds, amounting to 85 and 76%, respectively, but with huge color changes. Additionally, sensory analysis proved that drying with a microwave power of 240 W provides a product most similar to fresh material.

Keywords: *Cannabis sativa* L.; drying methods; essential oils; sensory evaluation

1. Introduction

Cannabis sativa L. is an annual herbal plant of the cannabis species (*Cannabaceae*) that has been known and cultivated especially in Asia since ancient times [1]. Nowadays, it is already cultivated all over the world and has a wide range of applications, including food, dietary supplements, medicines, body care products, fuel, paper, and as a building material, as well as a role in textiles [2]. Hemp contains around 750 natural chemical compounds, which can be classified into different classes [3]. The abundance of chemicals in cannabis flowers stems from the biosynthesis, particularly of terpenes and cannabinoids in the extracellular secretory cavity, known as the trichome. The active substances are secreted into

the trichomes to prevent damage to plant cells and are the first line of defense against the external environment [4].

The most common and basic technique for conserving herbs and retaining bioactive compounds is drying. The research shows that, depending on the choice of drying method and parameters, different chemical and biological activity of herbs is obtained, due to different content of chemical compounds in their composition [5]. The selection of the drying method has a major influence on the content of volatile essential oils present in herbs, as shown in earlier studies [6–10]. During the drying process, the following changes in the composition of the essential oil may occur—an increase or decrease in the concentration of volatile substances, or the formation of new chemical compounds [5]. The most commonly used drying method is convection drying (CD), a process using a continuous flow of hot air to remove moisture from the biological material [11]. With the development of technology, alternatives for better drying performance have emerged, such as vacuum–microwave drying (VMD) and combined methods consisting of convectional pre-drying followed by vacuum–microwave finishing drying (CPD-VMFD). The VMD method allows faster drying of the material while avoiding high temperatures [12]. Due to the high cost of production, an alternative drying method, CPD-VMFD, has been proposed due to the requirement of high vacuum during drying. This multi-stage drying process coupling the two CD and VMD methods together allows a satisfactory degree of drying of the material as it offers the advantages of the VMD method with the high performance that the CD method provides [11].

Recently, there has been a focus on the use of secondary metabolites from hemp flowers, which are characterized by low tetrahydrocannabinol (THC) content, which are known as nonpsychotropic cannabinoids, terpenoids, and flavonoids [13–15]. Essential oils extracted from hemp flowers are widely used in cosmetology as ingredients used in the production of creams, soaps, and shampoos, as well as in the food industry as aromas for alcoholic and non-alcoholic beverages and additives in baking [16]. Hemp essential oils have shown an interesting antimicrobial effect and can constitute an economic, effective antiseptic. They are therefore used to treat wounds and infections such as food poisoning and nosocomial infections, and can be used against antibiotic-resistant bacterial strains [17]. In addition, they are commonly used as insecticides [18,19], fungicides [20], and growth inhibitors for unwanted plants [21], and can be used in plant protection as a means of stopping plant diseases and pest attacks [19]. Cannabinoids contained in hemp also play a special role. They are responsible for the modulation of hunger/satiety and participate in peripheral metabolic reactions of the liver, fat, muscles, and anti-inflammatory reactions in blood cells [22]. Despite this, they can also cause side effects such as imbalance, hallucinations, nausea, and drowsiness [23].

Hemp can also be used as a source of food because of its health-promoting properties. Hemp seed oil is characterized by high levels of exogenous fatty acids (EFA) and polyunsaturated fatty acids (PUFA) [24]. The oil contains linoleic acid and L-linolenic acid, as omega-6 and omega-3 acids, which are optimal for nutrition because of their proportions (3:1, LA:LNA). Hemp seed oil is additionally enriched with gamma-linolenic acid (GLA), which makes the nutritional value ultimately higher than most seed oils. A properly balanced oil prevents excessive accumulation of certain metabolic products and also provides the necessary intermediaries for the body to work efficiently. The benefits of hemp seed oil as a food product and food supplement are that it can enrich the diet of the potential consumer [25].

The leading theme of the research was to define the composition of the volatile profile of hemp flowers and to verify how selected drying methods influence the profile of volatile compounds and the contribution of cannabinoids. For this purpose, we distilled the essential oil from the material under study and then analyzed it using gas chromatography in combination with the mass spectrometry technique (GC–MS).

2. Materials and Methods

2.1. Plant Material

Approximately 20 kg of hemp flowers var. Henola were harvested on 15 October 2019 from commercial field in Oborniki Śląskie (16°55′ E, 51°18′ N) Poland. The whole flowers, after being manually detached from the stem, were mixed and immediately subjected to drying, the distillation processes, as well as subsequent chemical analyses. The initial moisture content of the material was 68%$_{wb}$ (wet basis), assessed by a vacuum dryer SPT-200 ZEAMIL (Horyzont, Krakow, Poland). Plant material was subjected to various drying processes, which were suspended when no changes in weight were observed. The voucher specimen of investigated flowers were deposited in local herbarium at the Department of Chemistry.

2.2. Drying Methods

In the study, three different drying methods were applied: convective drying (CD), vacuum–microwave drying (VMD), and combined drying consisting of convective pre-drying and vacuum–microwave finishing drying (CPD-VMFD). Approximately 60 g of hemp flowers were used in each case, and the process was carried out until the final moisture content of the sample was below 10%$_{wb}$. This initial loading mass of sample allowed for thin layer drying without of the raw material compacting, which was necessary to meet the requirements for modeling the drying kinetics and to provide a sufficient amount of dry material for quality tests.

2.2.1. Convective Drying (CD)

The CD was conducted on an apparatus located at the Institute of Agricultural Engineering (Wrocław University of Environmental and Life Sciences, Wrocław, Poland). The hemp flowers were placed in a special container (d = 100 mm) and dried in 50 °C, 60 °C, and 70 °C with an airflow of 0.5 ms^{-1}. The preliminary tests revealed that that drying at temperatures below 50 °C takes too long to be applicable in industrial conditions in terms of energy consumption and thus operating costs, while temperatures above 70 °C lead to a drastic degradation of the chemical composition of the dried product. Due to difficulties in low temperature drying of hemp flowers, the temperatures used in this study were slightly elevated compared to the temperatures used for drying of some other herbal products such as thyme [6], sweet basil [8], or marjoram [7].

2.2.2. Vacuum–Microwave Drying (VMD)

The VMD was performed using the SM 200 dryer (Plazmatronika, Wrocław, Poland). The samples were placed in a glass cylindrical drum that rotated at 6 rpm. The dryer was equipped with a BL 30P vacuum pump (Tepro, Koszalin, Poland), MP 211 vacuum manometer (Elvac, Bobolice, Poland), and a 0.15 m^3 compensation tank. During the drying process, we used three power levels (240, 360, and 480 W) and pressure in the range of 4–6 kPa. Microwave powers were selected on the basis of the results from previous studies where similar materials were successfully dried in these conditions [6]. The maximum temperature of the dried hemp flowers was measured after removal from the dryer using an i50 infrared camera (Flir Systems AB, Stockholm, Sweden).

2.2.3. Combined Drying Consisting of Convective Pre-Drying Followed by Vacuum–Microwave Finishing Drying (CPD-VMFD)

During combined drying, the samples were initially placed in drying baskets at the convective dryer for 60 min at 60 °C; then, the samples were moved to a vacuum–microwave dryer where finishing of drying at 360 W occurred until its final moisture content was below 10%$_{wb}$. The time of convective pre-drying was proposed on the basis of preliminary studies, which showed that by that time water was effectively removed from the raw material at a satisfactory drying rate.

2.2.4. Modelling of Drying Kinetics

Drying kinetics of hemp flowers were presented using a moisture ratio MR defined by relationship (1):

$$MR = \frac{M_{(t)} - M_e}{M_0 - M_e} \quad (1)$$

where $M_{(t)}$ is the moisture content of the sample at given time, M_0 is the initial moisture content, and M_e is an equilibrium moisture content that is usually omitted as the values of M_e are relatively small (compared to $M_{(t)}$ and M_0), and therefore the simplified relationship (2) was used in the study without any significant influence on the drying kinetics modeling [26]:

$$MR = \frac{M_{(t)}}{M_0} \quad (2)$$

On the basis of obtained experimental data, we fitted several empirical drying models, including Newton, Midelli et al., logarithmic, two-term, and Page's models, performed using TableCurve 2D software. The results of preliminary tests revealed that only the two-term equation (Equation (3)) can be considered, as it takes into account the best fit determined according to the highest values of R^2 and the lowest values of root mean square error (RMSE).

$$MR = a \cdot e^{-k_1 \cdot t} + b \cdot e^{-k_2 \cdot t} \quad (3)$$

where k_1 and k_2, and a and b denote drying constants and model coefficients, respectively.

2.3. Color Analysis

Color of the samples was measured in five repetitions using a Minolta Chroma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan). The results were obtained in reference to International Commision on Illumination (CIE) $L^*a^*b^*$ color space, where L^* stands for lightness, a^* values vary between negative (green) and positive (red), and b^* values vary between negative values indicated as blue and positive values indicated as yellow hues. The total change in color of dried material was expressed as DE* according to the following formula:

$$\Delta E^* = \sqrt{\left(L_0^* - L^*\right)^2 + \left(a_0^* - a^*\right)^2 + \left(b_0^* - b^*\right)^2}$$

where L_0*, a_0*, and b_0* denote the values of fresh material.

2.4. Distillation of Essential Oil (EO)

In the process of extraction of essential oils (EOs), we used the Deryng apparatus. A suitable quantity of weighed fresh or dried material was transferred to a 250 mL round-bottomed flask. The hemp flowers were poured with 100 mL of distilled water. The flask was placed in a heating mantel and the mixture was brought to boiling point and kept at this temperature for 45 min. When the boiling point was reached, we added 1 mL of cyclohexane to collect the essential oil, which contained 1 mg of 2-undecanone as internal standard (Sigma-Aldrich, Saint Louis, MO, USA). After the extraction process, organic phase with the essential oil was collected and stored at −18 °C until chromatographical analysis.

2.5. GC–MS Analyses

The profile of volatile compounds was analyzed using a gas chromatograph coupled with a mass spectrometer (Shimadzu GCMS QP 2020, Shimadzu, Kyoto, Japan). Separation was obtained by a capillary column Zebron ZB-5 (30 m, 0.25 mm, 0.25 µm; Phenomenex, Torrance, CA, USA). The GC–MS analysis was carried out according to the following parameters: scanning in the range from 35 to 320 m/z in electron ionization mode at 70 eV, in the option of 3 scans s^{-1}. Analyses were performed

using helium as a carrier gas at a flow rate of 1.11 mL min^{-1} in a split ratio of 1:20. The GC oven temperature was programmed from 45 °C as initial temperature to 150 °C at a rate of 2 °C/min, then to 270 °C at a rate of 15 °C and kept for 5 min.

Identification of compounds were based on 3 independend methods: (a) comparison of obtained spectra with databases NIST 17 (National Institute of Standards and Technology) [27] and FFNSC (Mass Spectra of Flavors and Fragrances of Natural and Synthetic Compounds) [28], (b) comparison of calculated retention indices (RI) using a retention indices calculator [29] with values presented in NIST 17 and FFNSC, and (c) comparison of retention times of unknown compounds with authentic standards. For comparison of mass spectra, we used the AMDIS (v. 2.73) and GCMS solution (v. 4.20) programs. Additionally, all experimental RI were compared with those published in Adams [30].

2.6. Sensory Evaluation

The intensity of the main sensory attributes of dried hemp flowers were evaluated by a trained sensory panel. The panel consisted of 7 panelists (4 males and 3 females), aged between 34 and 53 years old. Panelists belonged to the research group "Food Quality and Safety" of the Universidad Miguel Hernández de Elche (UMH) and had over 1000 h of evaluation experience. The panel was selected and trained following the International Organization for Standardization ISO standard 8586-1 (1993), and it is specialized in descriptive sensory evaluation of fruits and vegetables and has a wide expertise in studying the effects of drying on different matrixes, such herbs, fruits, vegetables, and mushrooms [31].

Descriptive sensory analysis (DSA) was used to describe the dried hemp flowers. During 1 orientation session of 90 min, panelists discussed about the main odor (perception of volatile compounds with the product outside the mouth) and agreed on their use of key attributes/descriptors. Panelists agreed that the sensory profile of these dried samples could be described using 11 attributes: (i) fresh material: hemp flower ID, fresh vegetables, citrus, balsamic, spicy, and anise odor; and (ii) dried material: cooked, hay-woody, camomile, earthy, and burnt. Reference products of these attributes, with intensity similar to those of the samples under evaluation were prepared and provided to the panel.

The evaluation was performed in normalized individual booths with controlled illumination and temperature, 23 ± 2 °C. Samples coded with 3-digit random numbers were randomly presented to each panelist in odor-free plastic beakers of 100 mL with lids; samples were left for 15 min at room temperature prior to analysis. The 9 samples under analyses were analyzed in 3 sessions in which 3 samples per session were randomly presented to the panel; this design was selected to avoid sensory fatigue. The intensity of the sensory attributes was scored using a scale from 0 to 10, where 0 = none or not perceptible intensity and 10 = extremely high intensity.

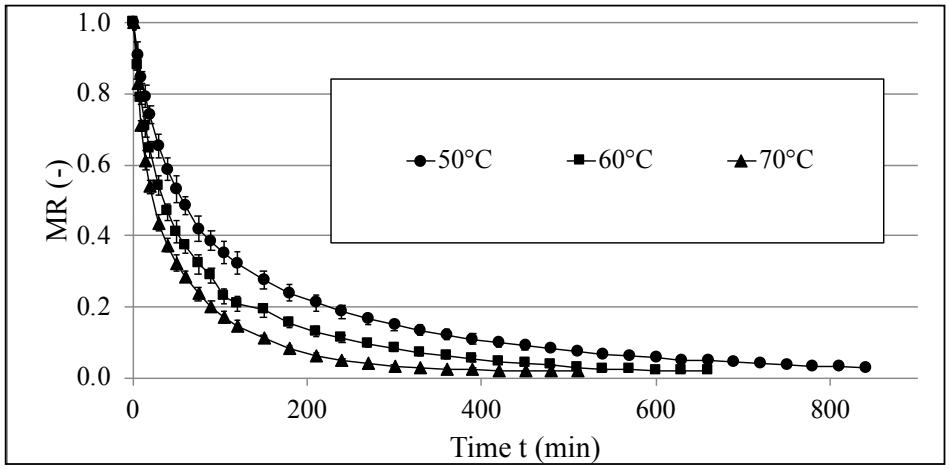
2.7. Statistical Analysis

All analyses were performed using the STATISTICA 13.3 software (StatSoft, Krakow, Poland). Data are expressed as means values ± standard deviation. Before analyses, all data were screened for normality using the Shapiro–Wilk test. The results from drying kinetics were subjected to the analysis of variance using Tukey's test ($p < 0.05$) and the results from analyses of essential oils were subjected to the analysis of variance using Duncan's test ($p < 0.05$). The data from the sensory analysis were also subjected to the Honest Significance Difference (HSD) Tukey's test ($p < 0.05$).

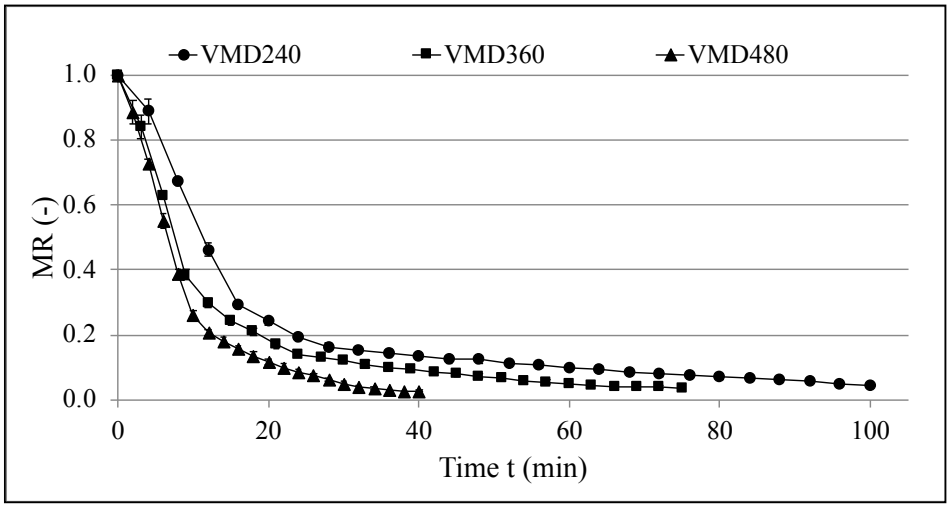
3. Results and Discussion

3.1. Drying Methods

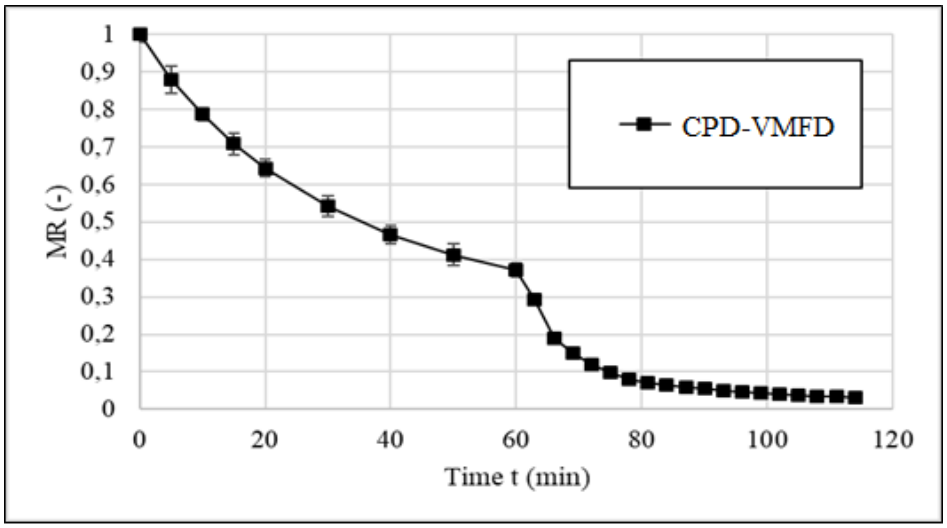
Figure 1 shows the drying kinetics of hemp flowers treated by convective drying (CD) (a), vacuum–microwave drying (VMD) (b), and combined drying (CPD-VMFD) (c), whereas Table 1 presents model constants with drying times and maximum temperature during drying using different methods.



(**a**)



(**b**)



(**c**)

Figure 1. Drying kinetics of hemp flowers treated by convective drying (CD) at 50, 60, and 70 °C (**a**); vacuum–microwave drying (VMD) at 240, 360, and 480 W (**b**); and combined drying consisting of convective pre-drying (CPD) at 60 °C for 1 h and following vacuum–microwave finishing drying (VMFD) at 360 W (**c**).

Table 1. Model parameters (a, k_1, b, k_2); maximum temperature of the hemp flowers during drying (TMax); and drying times t_{CD} and t_{VMD} of CD and VMD, respectively.

Drying Methods	Parameters				Statistics		Drying Time (min)		T_{Max} (°C)
	a	k_1, (min^{-1})	b	k_2, (min^{-1})	RMSE	R^2	t_{CD}	t_{VMD}	
CD50	0.54412	0.02469	0.44193	0.00350	0.00537	0.9997	840	-	50
CD60	0.58617	0.03623	0.40373	0.00521	0.00692	0.9995	660	-	60
CD70	0.53544	0.06093	0.46011	0.00912	0.00810	0.9993	510	-	70
VMD240	0.95054	0.08296	0.11951	0.00742	0.03445	0.9827	-	112	59
VMD360	0.91787	0.11572	0.12779	0.01484	0.02596	0.9903	-	78	54
VMD480	1.04449	0.12333	0.01818	0.00014	0.03465	0.9883	-	40	61
CPD-VMFD	**0.29965**	**0.12680**	**0.06519**	**0.01216**	**0.00785**	**0.9938**	**60**	**54**	**55**

The two-term model was used to describe the drying kinetics of hemp flowers. The good fit of this model was confirmed by high values of coefficient of determination ($R^2 > 0.98$ for all the drying methods) and low values of RMSE (Table 1). The model was previously successfully used in studies on pomegranate rind and arils [32] and ginger slices [33]. This was due to the model structure that specifically describes two stages of drying, with coefficient a referring to the breadth of the first stage of drying where intensive evaporation occurs, illustrated by high values of drying constant k_1, and the second stage (coefficient b), with decreasing drying rate characterized by low values of drying constant k_2. On the other hand, $1/k_1$ and $1/k_2$ are time constants that express the time required for 37% decrease of a and b values, respectively [34]. Therefore, it can be stated that the higher the value of the drying constant k_1 or k_2, the lower the value of the time constant $1/k_1$ or $1/k_2$ and thus the shorter the time of the relevant stage of drying.

It is worth mentioning that during the first stage of drying, the mass transfer mainly consists of water evaporation from the surface of the material at a high drying rate assured by sufficient water diffusion at a relatively high external moisture content, whereas during the second stage of drying, the mass transfer occurs at a lower drying rate limited by internal water diffusion hindered by decreasing moisture content [5]. The drying kinetics of CD might be affected to some extend by shrinkage of the dried material manifested by curling of the whole flower at the end of the first stage of drying, leading to a decrease of surface evaporation and thus hindering the mass transfer, which results in the reduction of drying rate. It can be seen that the change of temperature of hot air during convective drying (from 50 to 70 °C) results in an increase of drying rate followed by higher values of k_1 and k_2 constants (increase from $k_1 = 0.02469$ min^{-1} in case of CD50 to $k_1 = 0.06093$ min^{-1} in the case of CD70 during first stage of drying). Similar behavior was previously reported in studies on orange slices [35] and red pepper [36].

As for VMD, it should be noted that values of coefficient a were very high (over 0.9), which means that most of the drying occurred in the first stage, while coefficient b was over nine times lower than coefficient a, which marks shorter period of the second stage of drying. Moreover, with higher microwave power, k_1 constant increased, which shows that the drying rate was greatly influenced by the power of magnetrons in the first stage of drying. However, in the second stage of drying, the drying rate decreased with an increase of microwave power. It was due to the extended first phase of the process (defined by coefficient a) with high k_1 values that mostly contributed to the water evaporation and reduction of moisture content and thus moisture ratio (MR). Still, the second stage greatly affected the drying kinetics and the time of the process, even though it had little impact on MR values, which decreased at low rate. The course of drying curves showed that application of 480 W instead of 360 W did not contribute to an increase of the drying rate at the initial phase of drying, which was confirmed by similar values of k_1 amounting to 0.1157 and 0.1233, respectively. This means that from a practical point of view, starting with a microwave power higher than 360 W is not reasonable during VMD of hemp flowers. However, the elevation of microwave power from 360 to 480 W is reasonable after the initial phase of VMD lasting around 8 min (Figure 1b). It can be presumed that during the initial phase of VMD at high microwave power, the water removal rate was

restricted by the increased resistance of mass transfer caused by the thickening of the water molecules in the outer layer of the material as a result of a transport mechanism of the Darcy type [37] that had too much intense pressure at the limited possibility of water evaporation from the surface of the dried material. From this perspective, shrinkage of the dried material described above additionally hindered water evaporation from the decreased surface area and promoted the thickening of the water molecules in the outer layer of the material.

In case of CPD-VMFD, convective pre-drying can be easily described by the model parameters fitted and adjusted for CD at 60 °C; thus, only vacuum–microwave finishing drying is provided in Table 1. During combined drying, the most intensive evaporation occurred throughout the pre-drying period, and therefore the joint contribution of finishing drying (defined as the sum of a and b coefficients) was quite low. However, the drying constant k_1 reached the highest value (0.12680 min^{-1}) among all applied drying methods, which was almost 10% more than in the case of the sole use of VMD360, while k_2 was also very high and was only lower than in the case of VMD360. The relatively high values of k_1 and k_2 may be somewhat surprising, taking into account the fact that VMFD started when the surface of plant material had been already reduced due to the shrinkage formed during CPD. This can be explained by the recovery of the water molecule distribution within the entire volume of the pre-dried material during the time necessary to reload the sample.

Obtained data show that an increase of drying temperature resulted in shorter drying times during CD. An increase by 20 °C (from 50 to 70 °C) resulted in 39% time reduction (from 840 to 510 min), which is consistent with previous studies on true lavender leaves [38], carrot slices [39], and cornelian cherry fruit [40]. Moreover, VMD was much shorter than CD. This was due to the volumetric heating occurring during VMD that sped up the process by increasing the temperature inside of the sample [41,42]. As a result, samples treated by VMD at 480 W were dried 21 times faster when compared to CD at 50 °C. Furthermore, the power of magnetrons during VMD influenced water removal and resulted in shorter processing times when higher powers were applied [43]; namely, an increase from 240 W to 480 W decreased the time of drying by 72 min (reduction from 112 to 40 min for VMD at 240 W and VMD at 480 W, respectively).

On the other hand, combined methods (CPD-VMFD) resulted in a much shorter time of drying compared to CD, yet were still longer than VMD, which is consistent with previous studies on pomegranate arils [32] and quinces [44]. This method proved to be beneficial in other studies, i.e., on sweet basil [8] and thyme [6], where high quality products were obtained in a significantly shorter time period (compared to CD).

It is worth noting that the duration of VMD did not depend on the maximal temperature (T_{Max}) of the samples treated by microwaves. Usually the maximal temperature is achieved by VMD samples at the end of drying when the heat energy generated by water dipoles in the microwave field are in excess the energy necessary for water evaporation [45]. In the case of VMD of hemp flowers, the highest values of T_{Max}, amounting to 61 and 59 °C, were obtained at 480 and 240 W, respectively. The relatively high value of T_{Max} for samples dried at 240 W can be explained by the longer exposition to microwave radiation and thus the accumulation of the heat energy delivered at low microwave power [9]. The lowest values of T_{Max}, amounting to 61 and 59 °C, were found for VMD360 and CPD-VMFD, respectively. During both the drying protocols, the same microwave power of 360 W was applied, which suggests that the mean value of microwave wattage maintained the thermal energy balance at the lowest temperature of VMD material. Generally, the temperature of VMD herbal products is relatively low due to their specific morphological structure, preventing the excessive increase of inner pressure caused by microwave heating [6].

It can be seen that all the applied drying protocols differed in terms of the form of heat energy delivery, duration, and dried material temperature. Therefore, it is crucial to evaluate these drying protocols also in terms of their impact on the color, chemical composition, and sensory attributes of hemp flowers.

3.2. Color Analysis

Color of the samples is presented in the Table 2. The drying process increased lightness, yellowness, and greenness of the samples, which was confirmed by higher values of parameters L^* and b^* and lower values of a^* parameter. The lightest samples (highest L^* values) with yellowest hues (one of the lowest values of a^* parameter) were obtained after being subjected to VMD at 480 W. On the other hand, samples of VMD at 240 W were significantly darker (lowest L^*) than the samples obtained by other drying methods. Moreover, VMD at 360 W resulted in the greenest samples (highest values of a^*) and lowest values of b^* parameter, which had yellow hues. Generally, an increase of temperature during CD resulted in the obtaining of darker samples with a higher share of green and yellow hues, despite a slight decrease in a^* and b^* values. Although a similar effect on greenness and yellowness was observed when increasing the microwave power during VMD, these color alterations were associated with lightening of the samples. It is worth noting that the values of the color parameters determined for CPD-VMFD samples were between the relevant values obtained for samples dried by CD at 60 °C and VMD at 360 W. The significance of the color changes can be estimated by the values of total color change ΔE^*. According to Sumic at al. [46], if ΔE^* is less than 1.0, it is assumed that the difference in color would not be perceptible by the human eye. The total color change of the dried samples was much higher than 1.0 for all cases. However, the lowest change of 19.2 was stated for the VMD240 sample, whereas the highest change of 24.20 was found for the VMD480 sample. This indicates that microwave power during VMD had a higher impact on the color change of the dried material than hot air temperature during CD.

Table 2. Color parameters of hemp flowers subjected to drying using different methods.

Drying Method	Color			
	L^*	a^*	b^*	ΔE^*
Fresh	20.61 ± 5.37	−1.98 ± 0.61	8.41 ± 2.87	-
CD50	42.48 ± 1.59 ^{a,b,1}	−4.49 ± 0.83 ^{a,b}	12.06 ± 1.01 ^{a,b}	22.31
CD60	41.88 ± 0.7 ^{a,c}	−4.99 ± 0.6 ^{a}	10.77 ± 0.4 ^{a,c}	21.61
CD70	41.72 ± 1.26 ^{a,c}	−4.82 ± 0.25 ^{a}	11.25 ± 0.72 ^{a,b}	21.49
VMD240	39.78 ± 0.43 ^{c}	−3.57 ± 0.66 ^{b}	9.6 ± 0.34 ^{c}	19.27
VMD360	43.56 ± 1.28 ^{a,b}	−3.5 ± 0.62 ^{b}	11.34 ± 0.61 ^{a,b}	23.19
VMD480	44.27 ± 0.93 ^{b}	−4.83 ± 0.37 ^{a}	12.62 ± 0.57 ^{b}	24.20
CPD-VMFD	42.09 ± 1.09 ^{a,b}	−4.59 ± 0.52 ^{a,b}	10.99 ± 1.14 ^{a,c}	21.79

[1] Mean values followed by the same letter were not significantly different ($p < 0.05$) according to the HSD Tukey's least significance difference test.

3.3. Volatile Constituents of Fresh Cannabis sativa Flowers

Analysis of GC–MS of the distilled essential oils from the hemp flower revealed 93 peaks, which were considered to be volatile substances (chromatograms from the GC–MS analysis of volatile compounds content of hemp flowers are available in supplementary data; Figures S1 and S2). These compounds are collected in Table 3. Of all the volatile components identified in the tested samples of hemp flowers, we could distinguish the following main substances: β-myrcene (26.66%), β-(*E*)-caryophyllene (17.50%), limonene (10.45%), α-humulene (7.26%), caryophyllene oxide (3.79%), β-pinene (2.51%), terpinolene (2.50%), and α-pinene (2.16%). In smaller quantities, (E,E)-α-farnesene (1.80%), α-selinene (1.65%), β-chamigrene (1.41%), humulene epoxide II (1.25%), pseudowiddrene (1.24%), and β-trans-ocimene (1.12%) were identified. The substances above have a considerable influence on the fragrance quality of the hemp flowers.

In previous studies, similar results were found, where the identified compounds were as follows: β-(*E*)-caryophyllene (23.8%), α-pinene (16.4%), and β-myrcene (14.2%). The remaining compounds worth showing had the following percentages: terpinolene (9.6%), α-humulene (8.3%), β-pinene (5.2%), β-(*E*)-ocimene (5.1%), and β-(*E*)-farnesene (3.0%) [47]. Benelli in other studies also showed a

similar profile of volatile components to those mentioned above, where β-(E)-caryophyllene (21.4%), β-myrcene (11.3%), α-pinene (7.8%), terpinolene (7.6%), α-humulene (7.1%), β-(*E*)-ocimene (3.9%), and β-pinene (2.9%) were identified as the most representative compounds [48].

Considering the analysis of the obtained fractions, we observed a similarity with the paper published by Nissen. Depending on the cannabis cultivar, the following substances can be distinguished, β-myrcene (12.46–29.22%), α-pinene (10.9–16.99%), β-(*E*)-caryophyllene (10.56–13.90%), β-pinene (6.38–9.33%), α-humulene (4.84–6.71%), terpinolene (3.42–10.73%), limonene (3.11–4.99%), and β-(*E*)-ocimene (2.03–9.34%) as those occurring in the highest amount [17]. Mediavilla and Steinemann also studied the profile of volatile compounds on different cannabis strains. The main compounds isolated were β-myrcene (29.4–65.8%), β-(*E*)-caryophyllene (3.8–37.5%), α-pinene (2.3–31.0%), terpinolene (0.4–23.8%), β-(*E*)-ocimene (0.3–10.2%), α-humulene (0.7–7.9%), β-pinene (0.6–7.8%), and limonene (0.2–6.9%) [49]. Iseppi et al. reported β-myrcene (4.5–39.2%), β-(*E*)-caryophyllene (8.5–29.8%), β-pinene (4.8–25.4%), terpinolene (1.9–9.6%), α-pinene (3.4–8.2%), β-(*E*)-ocimene (2.2–7.1%), α-humulene (2.2–6.6%), and limonene (0.1–5.7%) as the main volatile components of hemp flower essential oils. These discrepancies may result from the use of different cultivars for research and from the condition of raw material [50].

Table 3. Complete volatile constituents of fresh *Cannabis sativa* flowers.

Compound	RT (min)	Retention Indices (RI)				Content (%) [5]
		RI_lit [1]	RI_lit [2]	RI_lit [3]	RI_exp [4]	
Octane	4.585	800	800	800	800	0.08 ± 0.01
(2*E*)-2-Hexenal	6.130	855	851	850	855	tr [6]
(3*Z*)-3-Hexen-1-ol	6.240	859	857	853	857	0.06 ± 0.02
1-Hexanol	6.705	870	868	867	869	tr
2-Heptanone	7.485	892	891	892	890	0.05 ± 0.01
Heptanal	7.900	902	901	906	900	0.51 ± 0.12
(2*E*,4*E*)-2,4-Hexadienal	8.395	909	911	914	909	tr
Artemisia triene	8.620	929	929	922	926	0.50 ± 0.21
α-Thujene	9.035	930	929	927	928	0.06 ± 0.08
α-Pinene	9.310	939	937	933	932	2.16 ± 0.82
Fenchene	9.985	952	952	948	953	0.25 ± 0.05
Benzaldehyde	10.585	960	962	960	960	tr
(2*E*)-2-Hepten-1-ol	11.025	965	978	964	970	tr
Sabinene	11.290	975	974	972	976	tr
β-Pinene	11.400	979	979	978	976	2.51 ± 0.32
***trans*-Isolimonene**	11.815	984	983	984	983	tr
6-Methyl-5-hepten-2-one	12.115	985	985	986	987	tr
β-Myrcene	12.455	990	991	991	994	26.66 ± 1.79
α-Phellandrene	12.970	1002	1005	1007	1004	0.13 ± 0.06
3-Carene	13.280	1011	1011	1009	1007	0.12 ± 0.09
α-Terpinene	13.665	1017	1017	1018	1017	0.12 ± 0.03
o-Cymene	13.910	1026	1022	1024	1021	tr
p-Cymene	14.115	1024	1030	1025	1025	tr
Limonene	14.415	1029	1030	1030	1030	10.45 ± 1.21
Sylvestrene	14.745	1030	1027	1031	1035	tr
β-*cis*-Ocimene	15.055	1037	1038	1035	1041	0.09 ± 0.03
β-*trans*-Ocimene	15.660	1050	1049	1046	1051	1.12 ± 0.14
Prenyl isobutyrate	15.930	1052	1052	1050	1052	tr
Oct-(3*Z*)-enol	16.025	1054	1059	1059	1054	tr
γ-Terpinene	16.205	1059	1060	1058	1062	0.14 ± 0.07
***cis*-Sabinene hydrate**	16.695	1070	1070	1069	1071	0.14 ± 0.09
2-*trans*-Octenol	17.285	1074	1072	1073	1075	0.05 ± 0.01
Terpinolene	18.030	1088	1088	1086	1087	2.50 ± 0.43
6,7-Epoxymyrcene	18.445	1092	1090	1096	1091	0.06 ± 0.02
***trans*-Sabinene hydrate**	18.650	1094	1093	1099	1096	tr
Linalool	18.895	1096	1099	1101	1099	0.09 ± 0.11
Nonanal	19.150	1100	1104	1107	1105	0.32 ± 0.14
Fenchol	19.565	1116	1113	1119	1116	0.82 ± 0.03
***trans*-Pinene hydrate**	20.025	1122	1120	1121	1118	0.82 ± 0.21
***cis*-Pinene hydrate**	21.340	1143	1143	1144	1139	0.11 ± 0.07
β-Terpineol	21.720	1144	1144	1149	1145	0.06 ± 0.01
Ipsdienol	21.875	1145	1147	1146	1147	0.1 ± 0.02
Myrcenone	22.000	1149	1145	1149	1150	0.09 ± 0.10

Table 3. *Cont.*

Compound	RT (min)	Retention Indices (RI)				Content (%) [5]
		RI_lit [1]	RI_lit [2]	RI_lit [3]	RI_exp [4]	
α-Pinene oxide	22.635	1159	1157	1156	1158	0.09 ± 0.05
3-Thujanol	23.005	1168	1167	1169	1165	0.09 ± 0.03
Terpinen-4-ol	23.740	1177	1177	1177	1177	0.12 ± 0.09
Isogeranial	23.880	1180	1182	1179	1177	tr
α-Terpineol	24.670	1188	1189	1195	1189	0.43 ± 0.12
Hexyl butanoate	25.035	1192	1192	1195	1194	0.14 ± 0.07
***trans*-4-Caranone**	25.530	1196	1197	1200	1196	tr
Bornyl acetate	30.975	1285	1285	1285	1282	tr
α-Cubebene	35.095	1351	1351	1349	1345	tr
α-Ylangene	36.410	1375	1372	1371	1368	0.12 ± 0.01
α-Copaene	36.700	1376	1376	1375	1373	tr
Hexyl hexanoate	37.405	1383	1384	1387	1383	tr
7-*epi*-Sesquithujene	37.705	1391	1402	1389	1387	0.07 ± 0.03
Isocaryophyllene	38.610	1408	1406	1405	1402	0.54 ± 0.12
α-Gurjunene	38.800	1409	1409	1406	1404	tr
β-(*E*)-Caryophyllene	39.480	1419	1419	1424	1415	17.50 ± 1.75
β-Duprezianene	39.735	1422	1422	1427	1420	0.09 ± 0.03
α-*trans*-Bergamotene	40.560	1434	1435	1432	1435	0.17 ± 0.09
β-Humulene	40.660	1438	1440	1440	1435	0.11 ± 0.05
Guaia-6,9-diene	40.925	1444	1443	1444	1440	0.17 ± 0.11
α-Humulene	41.485	1454	1454	1454	1453	7.26 ± 1.48
Khusimene	41.650	1455	1451	1451	1455	0.21 ± 0.14
β-(*E*)-Farnesene	41.870	1456	1457	1452	1457	0.33 ± 0.21
9-*epi*-(*E*)-Caryophyllene	41.985	1464	1466	1464	1459	0.39 ± 0.18
Dodec-(2*E*)-enal	42.380	1466	1468	1469	1464	0.15 ± 0.08
γ-Gurjunene	42.865	1474	1475	1476	1473	0.10 ± 0.01
β-Chamigrene	43.290	1477	1476	1479	1479	1.41 ± 0.56
γ-Selinene	43.425	1479	1479	1480	1481	0.62 ± 0.19
α-Selinene	43.990	1496	1494	1495	1490	1.65 ± 0.77
α-Zingiberene	44.330	1498	1495	1496	1495	0.31 ± 0.16
δ-Amorphene	44.785	1504	1505	1506	1499	0.38 ± 0.09
(*E*,*E*)-α-Farnesene	45.100	1505	1508	1504	1509	1.80 ± 0.64
Pseudowiddrene	45.385	1509	1510	1510	1512	1.24 ± 0.57
δ-Cadinene	45.755	1523	1524	1518	1514	0.21 ± 0.03
γ-Cuprenene	46.320	1533	1532	1530	1532	0.56 ± 0.12
Selina-4(15),7(11)-diene	46.575	1546	1542	1540	1533	2.13 ± 0.86
α-Cadinene	46.705	1538	1538	1538	1538	0.82 ± 0.31
(*E*)-α-Bisabolene	47.025	-	1512	1540	1538	0.28 ± 0.16
***cis*-Muurol-5-en-4-β-ol**	47.300	1551	1549	1548	1548	0.07 ± 0.03
Germacrene B	47.555	1561	1557	1557	1551	0.16 ± 0.05
Lippifoli-1(6)-en-5-one	47.995	1552	1553	1551	1557	0.69 ± 0.32
***epi*-Longipinanol**	48.310	1563	1566	1558	1564	0.44 ± 0.11
Caryophyllene oxide	49.055	1583	1581	1581	1576	3.79 ± 0.44
Humulene epoxide I	49.965	-	1604	1604	1590	0.18 ± 0.05
Humulene epoxide II	50.540	1608	1606	1613	1607	1.25 ± 0.14
1,10-di-*epi*-Cubenol	51.115	1619	1615	1614	1612	0.62 ± 0.23
***epi*-γ-Eudesmol**	51.455	1623	1622	1624	1623	0.64 ± 0.45
α-Acorenol	51.935	1633	1631	1632	1633	0.14 ± 0.04
Caryophylla-4(12),8(13)-dien-5α-ol	52.350	1640	1637	1642	1646	0.80 ± 0.23
α-Bisabolol	54.140	1685	1684	1688	1686	0.44 ± 0.13

Retention indices according to [1] Adams [30], [2] NIST 17 database [27], [3] FFNSC [28]; [4] % calculated from TIC data; 5 experimental retention indices calculated against n-alkanes; 6 tr < 0.05%.

3.4. Effects of Different Drying Methods on the Volatile Compound Content in Cannabis sativa

In the conducted studies of the biological material consisting of fresh hemp flowers, we found that the content of essential oil was 0.21 g/100 g^{-1} of dry weight (DW). Considering the yield of essential oils in cannabis flowers, we can conclude that it was high, as 0.1–0.25% [17] and 0.25% [51] of essential oils were reported in previous studies. Furthermore, Liang et al. [52] and Chalchat and Özcan [53] reported the yield of essential oils in the inflorescences of sage and basil as 0.2% and 0.5%, respectively. A comparison of changes in the content of 11 main volatile components in hemp with reference to different drying methods is shown in Table 4. As far as the total content of essential oils is concerned, it is not analogous to the 11 main compounds, as they represent 77.88% of the total.

Table 4. Comparison of changes in volatile composition of fresh and dried hemp flowers.

Compound	Drying Method							
	Fresh [1]	CD 50 °C	CD 60 °C	CD 70 °C	VMD 240 W	VMD 360 W	VMD 480 W	CPD-VMFD
	Content (%)							
α-Pinene	2.16 [a,3]	10.79 [g]	7.16 [e]	8.27 [e]	3.67 [b]	5.86 [d]	9.58 [f]	11.13 [g]
β-Pinene	2.51 [a]	4.47 [a]	3.49 [c]	4.43 [d]	2.17 [a]	2.89 [b]	4.96 [e]	5.12 [e]
β-Myrcene	26.66 [a]	9.95 [f]	10.34 [e]	19.27 [b]	10.78 [e]	7.54 [g]	13.03 [d]	16.88 [c]
Limonene	10.45 [a]	2.17 [e]	2.16 [e]	4.13 [b]	3.55 [c]	1.58 [g]	2.02 [f]	2.77 [d]
β-*trans*-Ocimene	1.12 [a]	0.80 [d]	0.62 [e]	0.99 [b]	0.50 [f]	0.78 [d]	0.68 [e]	0.92 [c]
Terpinolene	2.50 [a]	0.97 [e]	1.60 [b]	1.66 [b]	0.69 [f]	0.29 [g]	1.25 [c]	1.29 [c]
β-(*E*)-Caryophyllene	17.50 [a]	25.35 [e]	23.41 [c]	17.91 [a]	24.26 [d]	22.62 [b]	16.92 [a]	24.24 [d]
α-Humulene	7.26 [a]	11.51 [e]	9.62 [c]	7.22 [a]	9.23 [c]	8.90 [b]	8.41 [b]	10.32 [d]
(*E*,*E*)-α-Farnese	1.80 [a]	1.82 [a]	0.58 [e]	1.05 [d]	1.14 [c]	1.44 [b]	1.48 [b]	1.03 [d]
Selina-4(15),7(11)-diene	2.13 [a]	1.21 [f]	1.20 [f]	1.35 [e]	1.96 [b]	1.43 [d]	1.52 [c]	1.47 [d]
Caryophyllene oxide	3.79 [a]	4.96 [b]	7.82 [c]	10.71 [e]	11.04 [e]	8.65 [d]	12.16 [f]	5.84 [b]
EO yield [2]	**0.21 [a]**	**0.16 [c]**	**0.14 [d]**	**0.12 [e]**	**0.18 [b]**	**0.15 [d]**	**0.15 [d]**	**0.11 [e]**
% recovery of EO	**100**	**76.19**	**66.67**	**57.14**	**85.71**	**71.42**	**71.42**	**52.38**

[1] Dry mass calculated; [2] mL/100 g^{-1} according to distillation on Deryng apparatus; [3] values followed by the same letter within a row are not significantly different ($p > 0.05$, Duncan's test).

By analyzing the content of essential oils in relation to different drying processes, we observed that each of them had an impact on its final yield. VMD at 240 W (0.18 g/100 g^{-1}) turned out to be the most effective drying method, followed by CD at 50 °C (0.16 g/100 g^{-1}), while VMD at 360 W and VMD at 480 were the next methods with the same oil yield (0.15 g/100 g^{-1}). The least effective method was CPD-VMFD, with an essential oil yield of only 0.11 g/100 g. The above data are also presented as percentage oil recovery, which is included in Table 3. Other works describing the drying of marjoram [7] and thyme [6] also prove that the VMD at 240 W is the best solution to stop volatile compounds. Vacuum–microwave drying has also proven to be the most effective method for oregano drying [9]. However, this is in contradiction with the paper by Łyczko's et al. [54] that described the drying of lavender flowers, where this method was proven to be the least effective. However, the separate systematics of hemp and lavender flowers must be taken into consideration.

In addition, the study also showed the relationship between the intensity of the treatment and the losses that occur. It was observed that with increasing intensity of drying conditions, the level of losses of volatile compounds in CD increased in comparison to VMD. In CD, the loss of volatile compounds was recorded from 0.16 to 0.12 (decrease by 19.01%), with an increase in drying temperature from 50 °C to 70 °C, and with an increase in power from 240 W to 480 W in VMD, the concentration decreased from 0.18 to 0.15 (decrease by 14.29%). Sanchez et al. [8] in research on sweet basil also reported this dependence. As far as the CPD-VMFD method is concerned, the losses of volatile compounds compared to other drying methods were highest, as a 48% decrease in the volatile compounds in essential oil was observed. Consequently, this method is not a recommended drying method because it does not improve the aroma quality of dried hemp flowers. A similar situation was documented during research into drying shiitake mushrooms [55], where the combined drying method proved to be the least effective. This shows that VMD is a better method for drying cannabis flowers if the aim is to preserve as much essential oil as possible.

It is also worth noting the individual compounds of the 11 main ingredients. For example, to preserve the main fragrance compounds in the cannabis flower such as β-myrcene and β-(E)-caryophyllene, CD at 70 °C (for β-myrcene) and CD at 50 °C (for β-(E)-caryophyllene) proved to be the best drying methods.

It is also important to note the increase of α-pinene from 2.16% to 11.13% (CPD-VMFD) and caryophyllene oxide from 3.79% to 12.16% (VMD at 480 W), as well as the decrease of β-myrcene from 26.66% to 7.54% (VMD at 240 W). The direction of percentage changes of individual compounds resulted from their different susceptibility to the thermal degradation as an effect to an irreversible oxidation process and volatilization enhanced by water evaporation, leading to a decrease of the dry matter content. This shows that all drying protocols had a considerable impact on the final ratio of the

mass of individual components with different retention ability to the reduced mass of the dry matter in the hemp flower.

3.5. Sensory Value of Cannabis sativa Flowers According to Various Drying Methods

Only odor descriptors (perception of volatile compounds with the product outside the mouth) were evaluated, and the protocol followed was similar to that described previously to other dried herbs [7] and mushrooms [31]. Six descriptors related to fresh plant material (hemp ID: aromatics associated with fresh hemp flowers, and fresh vegetables: aromatics associated with fresh but non-identified vegetables) were assayed together with five that were mostly related to the drying process (e.g., hay-woody and burnt). The nine samples of hemp dried flowers were analyzed in three sessions in which three samples per session were randomly presented to the panel. The results presented in Table 5 clearly showed that the sample that kept most of the intensity of the fresh material was that obtained using VMD at 240 W, followed by that prepared using the combined method at 50 °C.

Table 5. Sensory profile of dried hemp flowers.

Aroma Description	Drying Method						
	CD 50 °C	CD 60 °C	CD 70 °C	VMD 240 W	VMD 360 W	VMD 480 W	CPD-VMFD
Hemp ID	3.0 ^{c,†}	2.0 ^{c,d}	1.0 ^{d}	7.0 ^{a}	2.0 ^{c,d}	2.5 ^{c,d}	3.0 ^{c}
Fresh vegetable	4.5 ^{c}	3.5 ^{c}	1.0 ^{d,e}	7.5 ^{a}	2.0 ^{d}	4.0 ^{c}	3.5 ^{c}
Citrus	3.5 ^{b,c}	2.0 ^{d}	1.0 ^{e}	5.5 ^{a}	2.0 ^{d}	3.0 ^{c}	2.5 ^{c,d}
Balsamic (rosemary)	2.5 ^{c}	2.0 ^{c,d}	1.5 ^{d,e}	5.5 ^{a}	1.5 ^{d,e}	2.5 ^{c}	2.0 ^{c,d}
Spicy (black pepper)	2.0 ^{b,c}	1.5 ^{c,d}	1.0 ^{d}	3.5 ^{a}	1.5 ^{c,d}	1.5 ^{c}	1.5 ^{c,d}
Anise	2.5 ^{b}	2.5 ^{b}	1.5 ^{c}	4.5 ^{a}	1.5 ^{c}	2.0 ^{b,c}	1.0 ^{c,d}
Cooked	0 ^{c}	0 ^{c}	0 ^{c}	0 ^{c}	0 ^{c}	0 ^{c}	0.5 ^{b}
Hay-woody	1.0 ^{c}	2.5 ^{b}	2.5 ^{b}	1.0 ^{c}	3.0 ^{b}	2.0 ^{b,c}	2.0 ^{b,c}
Camomile	1.0 ^{c}	2.5 ^{a,b}	2.5 ^{a,b}	1.0 ^{c}	2.5 ^{a,b}	2.0 ^{b}	2.0 ^{b}
Earthy	0	0	0	0	0	0	0
Burnt	0 ^{b}	0 ^{b}	0 ^{b}	0 ^{b}	0 ^{b}	0 ^{b}	0 ^{b}

† Mean values followed by the same letter within the same row were not significantly different ($p > 0.05$) according to the HSD Tukey's least significance difference test.

In general, with the increasing strength of the drying process, i.e., the temperature in the CD and the power in the VMD, the sensory quality of the dried samples deteriorates due to significant losses of key odor compounds related to the fresh plant material. The sensory results agreed with previously discussed trends for the total content of volatile compounds.

4. Conclusions

The results of the study revealed that the drying kinetics of hemp flowers treated by convective drying (CD), vacuum–microwave drying (VMD), and combined drying composed of convective pre-drying followed by vacuum–microwave finish drying (CPD-VMFD) can be satisfactory described using a two-term empirical model. During the drying process, we found loses in 93 analyzed volatiles from 48% for CPD-VMFD to 15% for VMD at 240 W, which was finally chosen as optimal for retention of aroma-active compounds. In that variant, a significant decrease of β-myrcene was observed. Taking drying time into consideration, the shortest dehydration operation was VMD at 480 W (40 min) in contrast to CD at 50 °C (840 min), although the loses of compounds were around 30%. From a sensory point of view, the best drying treatment was VMD at 240 W, because it produced dried samples most resembling the fresh material, with high intensities of key sensory descriptors such as hemp flower ID, fresh vegetables, citrus, balsamic, and anise. Unfortunately, that process produced the most changes in flower color. Although combined drying (CPD-VMFD) could be advantageous from a practical point of view, too much degradation of the chemical composition identified in the raw material prevents this method to be applied to a greater extent. Taking into account the influence of individual drying conditions on the drying time and quality parameters of the dried product, VMD at 240 W can be recommended by the industry as the best option for hemp flower drying.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/8/1118/s1, the Supplementary Materials contain chromatograms from the GC–MS analysis of volatile compounds content of hemp flowers, Figure S1: Fresh hemp flower—GC-MS volatile profile analysis; Figure S2: Dried hemp flower at 240W—GC-MS volatile profile analysis.

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Review

Comparison of Traditional and Novel Drying Techniques and Its Effect on Quality of Fruits, Vegetables and Aromatic Herbs

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Abstract: Drying is known as the best method to preserve fruits, vegetables, and herbs, decreasing not only the raw material volume but also its weight. This results in cheaper transportation and increments the product shelf life, limiting the food waste. Drying involves the application of energy in order to vaporize and mobilize the moisture content within the porous products. During this process, the heat and mass transfer occurs simultaneously. The quality of dehydrated fruits, vegetables, and aromatic herbs is a key problem closely related to the development and optimization of novel drying techniques. This review reports the weaknesses of common drying methods applied for fruits, vegetables, and aromatic herbs and the possible options to improve the quality of dried products using different drying techniques or their combination. The quality parameters under study include color, bulk density, porosity, shrinkage, phytochemicals, antioxidant capacity, sugars, proteins, volatile compounds, and sensory attributes. In general, drying leads to reduction in all studied parameters. However, the behavior of each plant material is different. On the whole, the optimal drying technique is different for each of the materials studied and specific conditions must be recommended after a proper evaluation of the drying protocols. However, a novel or combined technique must assure a high quality of dried products. Furthermore, the term quality must englobe the energy efficiency and the environmental impact leading to production of sustainable dried products.

Keywords: drying trends; drying techniques; dehydration; combined drying; food properties

1. Introduction

Drying is an ancient and unparalleled physical procedure of food conservation used for direct preparation of food products as well as for further processing in the food industry. It has always been a valuable and common practice of conservation, ensuring the availability of food and medicinal products all year long. Drying used to be natural and simple as the process was driven by solar energy. Nowadays, it became more sophisticated and complex as it uses a lot of equipment and the drying parameters are carefully examined and optimized at every stage of the process. Emerging new methods have been extensively studied in terms of chemical and biochemical changes in the product during the dehydration process. Drying not only preserves the product but also can have a positive

impact on materials quality e.g., in spices, medicinal plants, herbs, bioactive enzymes, and nuts that can generate value-added compounds during drying [1,2].

Drying is the process of water removal, usually driven by heat, from solid and liquid products resulting in solid-dried products. Within a fresh foodstuff exist two types of moisture, firstly the bound moisture characterized by the liquid retained in the microstructure of the solid part and secondly the unbounded moisture represented by the excess of the bounded water. The bound moisture is represented by a liquid solution retained into a solid matrix. This leads to the coexistence of complex processes during the thermal drying of fresh food products. First, energy is transferred from the hot drying agent to the fresh product. Secondly, an evaporation of the unbounded moisture (free water) occurs, and eventually, water particles bounded within the cellular structure, subjected to diffusion and thus migration, are transferred to the surface of the product, where the water is finally evaporated [3]. Removing the moisture from the fresh product inhibits the bacteria growth and its proliferation increasing the product shelf-life. Moreover, the enzymatic activity, sensory properties, and microbial growth are also affected by the drying process [4]. As explained above, drying mechanism consists of unbound moisture removal (constant rate period) followed by the internal moisture elimination (falling rate period). Even though the surface evaporation occurs, it is crucial to vaporize also bounded water, as only after falling rate period the process results in secure, dried product [5].

Drying is known as the best method to preserve fruits, vegetables, and herbs, decreasing their volume and weight thus, reducing the packaging, storage, and transportation costs. Moreover, flavor and texture properties are modified, obtaining a new generation of products such as snacks that can be a healthier alternative to other commercial products such as sweets [6]. Besides, water removal prevents microorganism evolution and harmful chemical reactions and leads to longer storage time [7]. This is often achieved when the water activity is smaller than 0.3. Thus, depending on the method of water removal, there are different types of drying processes such as (i) thermal drying which is divided into air drying, low air environment drying and modified atmosphere drying; (ii) osmotic dehydration in which a solution is used in order to remove the water and finally (iii) mechanical dewatering which uses physical force for drying [1].

The most important objectives of drying are: (i) preservation of fresh products, making them available whole year (ii) conversion of the fresh product into a dry one while maintaining or improving its final quality; (iii) reduction of the volume and weight of the product for an easier transportation and storage; and last but not least (iv) sustainable processing as the most popular drying methods use enormous quantities of energy at low efficiency [8]. Thus, the new drying techniques should provide advantages such as higher energy efficiency, better product quality, cost reduction, and lower environmental impact.

The energy efficiency, food quality, and drying time are the main parameters to be optimized within the ongoing studies. All these drying techniques aim to develop food products with various characteristics. Although plenty of drying machines (around 50 different types of dryers) have already been designed, proved, and used, not all are suitable to be used in the food industry [9].

The most popular techniques used for moisture removal in fruits, vegetables, and herbs are briefly summarized in this paper. Also, new methods that emerged by modification of existing ones or by using known techniques previously unapplied in the food drying are described below. This review reports the weaknesses of common drying methods applied for fruits, vegetables, and aromatic herbs and the possible options to improve the quality of dried products using different drying techniques or their combination. A detailed evaluation of selected methods in terms of its effect on physiochemical, functional, and sensory properties of the dried product is also provided.

2. Drying Techniques in Fruits, Vegetable, and Herbs Preservation

Numerous drying techniques have been developed and used for dehydration of vegetal products over the years. In this section, the most relevant drying techniques, such as convective drying (CD), spray drying (SD), freeze-drying (FD), and osmotic dehydration (OD) are reviewed and their characteristics provided (Table 1). However, these broadly applied methods are not without some drawbacks and

therefore extensive research has been carried out to limit these negative aspects as well as minimize the energy consumption throughout the process (Figure 1). According to other authors [10] novel drying technologies that might be accepted by the food industry include energy saving solutions such as dryers with the use of heat pumps, combination of existing technologies in order to optimize the cost and quality of dried products, and every method that allows better control over process conditions and food quality. Hence, these new technologies are described and compared with existing, conventional methods in this section.

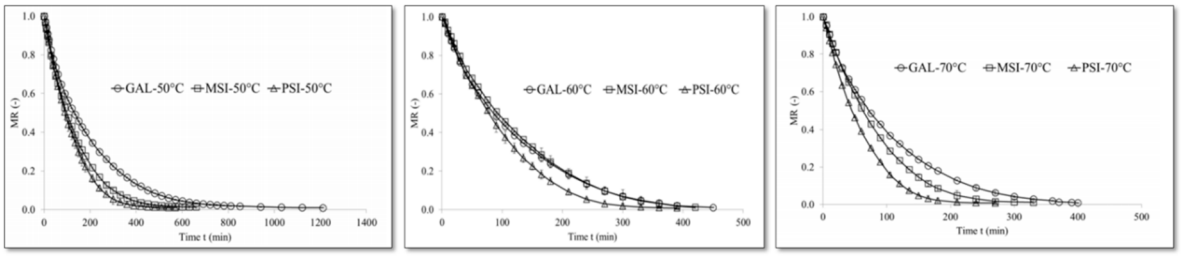

Figure 1. Convective drying of different jujube cultivars at different temperatures [11].

2.1. Heat Pump Drying (HP)

Energy losses during the conventional hot air drying are quite significant. Therefore, many methods have been designed to focus on recovering of the exhausted air in the process [12]. However, those methods could only recover sensible heat from the exhaust losing the rest of the heat in form of the latent heat of steam. To avoid this phenomenon, heat pump dryer was designed. In this type of dryer, a refrigerator is used in order to recover the latent heat by water condensation. In fact, this dryer is an improved convection dryer with refrigeration system, which contributes to energy efficiency and improves the product quality limiting negative environmental impact. In the process, dry heated air is supplied to the product as a result releasing humid air. The air travels to the heat pump evaporator, where it is condensed, allowing the latent heat of vaporization to be reused for reheating of the drying air. The advantage of this drying method is the reduction of time and temperature due to the relative humidity decline when compared to the conventional hot air dryer [1,13].

An alternative to the compression heat pump is a chemical heat pump. This method is considered as one of the most energy efficient and consists of a solar collector, storage tank, and a drying chamber. The chemical heat pump stores misused heat from the dryer exhaust or solar energy in the form of chemical energy and release it at different temperatures during the drying process. This method works by utilizing the reversible chemical reaction needed to change the temperature level of the thermal energy stored by the chemical substances. The chemical substances such as metal hydrides are very important in absorbing and eliminating heat. This method has the advantages of reducing energy consumption and is designed for a continuous operation [13].

2.2. Electromagnetic Radiation Techniques

Many conventional drying methods use hot air obtained through electric heater or flue gas to enforce heat transfer between the hot air and the material principally through convection. However, there are plenty of other methods that use electromagnetic wavelength spectrum as energy. Electromagnetic waves of a certain length reach out to the product generating in this way heat, which increases the drying rate [1]. This method works by indirect electro heating because the electrical energy is first converted to electromagnetic radiation to later be transformed into heat in the food product [14]. Some of the drying techniques using this mechanism are described below:

2.2.1. Microwave Drying (MD)

Microwave drying is based on the volumetric heating occurring when electromagnetic waves pass through the material causing a molecules oscillation. This oscillation generates thermal energy that is then used to remove water from the wet material. Microwave radiation is included in the

electromagnetic spectrum, and its wavelengths range from 1 mm to 1 m. The most used frequencies within the food drying are 915 and 2450 MHz. This drying method is able to obtain high quality dried products with reduced costs and high energy efficiency due to the volumetric heating that is spread through the whole sample reducing the time of drying when compared to the conventional methods (hot air-based ones e.g., convective drying described in Table 1). However, it is considered to cause product damage due to improper heat control and mass transfer during the process [15]. Hence, researchers recommend combining this technique with other techniques e.g., by combining the use of microwaves with reduced pressure (microwave-vacuum drying described in the next section).

2.2.2. Infrared Drying (ID)

The infrared drying occurs by the exposure of the fresh product to electromagnetic radiation in the wavelength range of 0.8–1000 μm. The infrared radiation energy is transferred from the heating source to the product surface. However, the surrounded air is not heated in the process. This method is one of the most appropriate to be used in combination with conventional drying methods due to the equipment simplicity and energy savings. In addition, it is considered to produce a quick and efficient heat transfer obtaining in this way a better organoleptic and nutritional value of the product with a uniform heating and lower final costs [16].

2.2.3. Radio Frequency Drying (FR)

This technology can be used not only for wireless communication but also in food processing. Radio frequency heating consists of the interaction between electromagnetic field, which is produced by radio frequency generator, and the molecular species in the product. Thus, the food product is situated between two electrodes displayed to an electric field which alternates around 40,000,000 times per second. The electric fields alternate and so do the polar molecules from the food product creating friction, which heats the whole product. As the water is naturally bipolar it gets heated leading to the evaporation in the process [5]. Radiofrequency has been widely studied as an alternative to conventional hot air-drying process (convective drying) in different horticultural products such as apple slices and snack foods [14].

2.2.4. Refractance Window Drying (RW)

This drying technique includes three types of heat transfer mechanisms, (i) convection, (ii) conduction and (iii) radiation. All these heat transfer modes are needed in order to obtain an energy efficient drying method. The product submitted at refractance window must be of liquid or semiliquid texture. The material is applied to a conveyer belt surface, normally an infrared transparent plastic which floats on the area of heated circulating water. This method works by refractive principle of the water surface that creates a window when infrared energy crosses by. The infrared window is formed at the contact between the wet material and the transparent plastic and permits direct infrared energy transfer to the material. Studies on pure pumpkin concluded that the drying time for this method is very short [17]. Refractance window dehydration is conducted under atmospheric pressure and lower temperatures (~30 °C), being a good option for heat-sensitive foods. This method has emerged as a new low-cost possibility for dehydration of vegetal material such as mango, avocado, and herbs [18].

2.3. Explosion Puffing Drying (EPD)

This method is usually applied in an intermediate phase of the drying process and is caused by the product bounded water vaporization and its expansion due to an abrupt pressure decrease or temperature increase. In this moment, the released vapor is used for both development of an internal structure or expansion and/or breaking of an existing one by producing a porous structure, saving time and energy. There are different methods of puffing, such as high-temperature and short-time air puffing and superheated steam puffing. Explosion puffing drying (EPD) system consists of a puffing chamber, vacuum chamber, vacuum pump, decompression valve steam generator, and air compressor. This method is usually combined with CD and FD and is used as a cheaper alternative to FD products [19,20].

2.4. Low-Pressure Superheated Steam Drying (LPSSD)

The benefits of low-pressure superheated steam drying (LPSSD) results from the reduction of operation temperature due to lowered pressure and completely lack of oxygen as the drying agent is a steam instead of hot air, which is commonly used for heat and mass exchange in traditional drying methods. The process of dehydration using superheated steam takes place in insulated drying chamber under reduced pressure maintained by vacuum pump. A steam trap is installed to reduce the excess steam condensation in the reservoir, which receives the drying agent from the boiler. With the use of a heater equipped with the temperature control system the initial steam condensation during the start-up period is reduced considerably. A variable-speed electric fan is used to disperse steam throughout the drying chamber.

2.5. Combined Drying Methods

Combined drying methods represent the next group of novel drying techniques that overcome the shortcomings mentioned before by combining the advantages of selected methods and reducing negative aspects occurring when only one technique is applied. There are several combined drying methods. However, some of them deserve special attention due to their applicability in the food industry.

2.5.1. Microwave-Assisted Convective Drying (CD-MD)

Hot air is an effective drying medium to produce dried fruits, vegetables, and aromatic herbs. However, as presented in the Table 1. Convective drying has some weaknesses i.e., long time of drying and the crust formation on the product surface due to the high temperatures. These issues can be diminished if microwave-assisted convective drying is applied. Therefore, the hot air reduces the products surface unbound moisture while the microwave energy eliminates the bound moisture from the inside of the product via volumetric heating. Nevertheless, there is still the need for further development regarding the time at which microwaves should be optimally incorporated into the process, whether when the drying rate starts to fall or when the drying rate is already falling or maybe even at very low moisture content [21].

2.5.2. Vacuum-Microwave Drying (VMD)

Vacuum-microwave drying is a modern technique that might overcome the conventional drying weaknesses having an ability to enhance the quality of the dried products. In general, vacuum-microwave drying (VMD) gathers the four most important requirements for food drying: high operational speed, high energetic efficiency, low operational costs, and high quality of the dried product. The process involves the use of vacuum, which assures a quick mass transfer and low temperature and is combined with the microwave heating which guarantees an accelerated energy transfer. Thus, all together result in prompt, low temperature drying process. In addition, the absence of air prevents the product oxidation. This system is not yet very common in the food industry; however, there is a large number of scientific studies that have successfully applied this method to obtain better quality products (including nutritional and sensory properties), such as fruits, vegetables, and aromatic herbs. However, one of its major limitations is the non-uniformity of the microwave radiation, which induces over-heating in borders and corners of the sample [13,22–24]. In recent years, several studies have been focused on using VMD, because of the shorter drying time and lower temperature in comparison with microwave drying. Several vegetal materials have been studied under this technique such as apple, blackcurrant, blueberry, pomegranate, garlic, strawberries, cranberries, and tomatoes [2,23,25–27].

2.5.3. Convective Drying Followed by Vacuum Microwave Drying (CD-VMD)

Combining convective drying with vacuum-microwave drying leads to obtaining products with improved quality with lower cost of the process as well as lower energy consumption. The process consists of two stages: in the first stage, the fresh product is subjected to a convective pre-drying followed by the second stage in which a vacuum microwave finishing drying is applied to the product.

The convective pre-drying diminishes considerably the unbound moisture of the fresh material without affecting their bioactive compounds. Later the vacuum-microwave finishing drying brings the moisture content to the desired level. These two combined drying processes have been reported to be more effective than either of the methods applied separately. The positive impact on the quality was observed in large number of fruits and herbs: sour cherries, jujube, orange peel, beetroot, blackcurrant, pumpkin, plums, and hemp [2,28–30] (Figure 2).

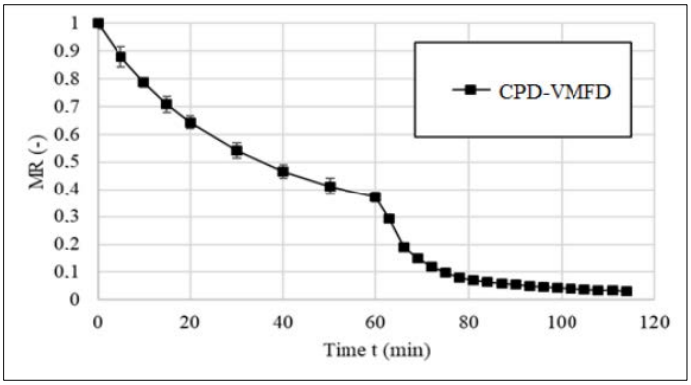

Figure 2. Combined drying consisting on convective pre-drying (CPD) at 60 °C and vacuum microwave finishing drying (VMFD) at 360 W of hemp [30].

2.5.4. Fluidized Bed Drying (FBD)-Assisted by Microwaves, Far Infrared Rays, and Ultrasounds

Traditional fluidized bed drying can be assisted by microwave energy in a similar way to convective drying. However, this method requires different steps of drying and especially further research on the usefulness for various products. Also, the initial system costs are immense.

Far infrared rays are found at the farthest side of the visible rays and have the purpose to raise the temperature of the food product to the above wavelength, which coincides with the vibration of the molecules. As a difference to the microwave-assisted fluidized bed drier this method can be applied in any stage to control the matter components quality. For instance, it was possible to control the influence of allicin (the organosulfur compound of garlic) in the drying process when far infrared rays assisted fluidized bed was applied in the first stage of drying. In addition, functional components such as amino acids were not affected by both individual fluidized bed, or combined with far infrared rays methods.

It is considered that high power ultrasound application to the heat-sensitive horticultural products can raise drying rate by accelerating the mass transfer process contributing to a high quality of dried product. These important aspects can be achieved due to the lower temperatures and times needed for the drying treatment. It was found that using ultrasound methods for fruits and vegetables could avoid negative effects such as shrinkage, color darkening, cracking, or nutritional changes due to the low temperatures needed within this method. Besides, it is considered not only to have an intuitive mechanism of utilization but also to be low-cost and energy efficient method [31].

2.5.5. Intermittent Drying (IMD) of Food Products Assisted by Temperature, Pressure, Humidity, Convection, Radiation, and Microwave

As previously mentioned, drying is probably the most energy process of the major industrial process. Intermittent drying has been considered as one of the most energy efficient drying processes. Intermittent drying is a drying method where drying conditions are changed with time. It can be achieved by varying drying air temperature, humidity, pressure or even mode of heat input [32]. Intermittent drying can be accomplished by controlling the supply of thermal energy, which can be achieved by varying the airflow rate, air temperature, humidity, or operating pressure. One can also vary the mode of energy input (e.g., convection, conduction, radiation, or microwave) to achieve intermittency [32].

Regarding the food quality, authors reported that the intermittent drying can reduce the browning effects, the hydro-thermo-mechanical stress inside sample and the chemical reactions which help to protect the bioactive compounds of the product [33]. Intermittent drying is a technique specially developed to overcome essential limitations of convective drying which are longer time and energy consumption, case hardening and low-quality products [33]. Thus, is an effective method developed to improve the drying kinetics, enhancing product quality, and reducing energy consumption.

Intermittent microwave convective drying (IMCD) significantly reduces drying time and improves product quality compared to convection drying and overcomes the problem of overheating that persist in continuous microwave convective drying. Moreover, the non-uniformity of temperature distribution is one of the major drawbacks of microwave drying which can be minimized by supplying microwave power intermittently [34].

This method (IMCD), it has been already used in drying of thermolabile plant-based food products due to its soft processing conditions which protect the sample from overheating and product deterioration [33]. For instance, during the drying period the superficial moisture is evaporated, and the inner moisture is carried to the surface; this repeated process of rewetting help to reduce the overheating maintaining stable thermolabile product characteristics such as color, pigments, browning, etc. Moreover, the reduction in oxidation, hydrolytic enzymes, and microorganism can be also reduced due to the short time of heat during IMD which can inactivate them without damaging the heat-sensitive bioactive compounds and enhance the product shelf life [33].

Authors reported that using the optimum level microwave power and intermittency could significantly enhance the preservation of nutrient contents, microstructure, and color of the dried sample. For instance, using IMCD at 1:4 power ratio in kiwifruit, was the optimum condition with the highest ascorbic acid retention, the lowest color change and with a porous structure resembling the fresh sample; however, using higher microwave density a (1:3) the highest polyphenol content was maintained [35].

Finally, a study on the effect of IMCD on a heat-sensitive fruit such as Red Flesh papaya (cv. Red Hill) was also reported [36]. The authors developed an IMCD model describing simultaneous heat and mass transfers, together with microwave volumetric heating (for temperature of 60 °C, 100 W and 1:3, 1:4, and 1:5 power ratios) to predict the distribution of moisture and temperature and its effect product quality. They concluded that power rate had a key role in quality attributes as was also above-mentioned. For instance, around 70% of the ascorbic acid was degraded using 1:3 power rate, but these losses were decreased when the power rate was reduced to 1:4 or 1:5. Additionally, the total phenolic content, was reported to be degraded significantly during the early stages (first 60 min) but was stabilized at later stages.

3. Product Quality Parameters Affected by Drying Methods

Food drying usually results in product deterioration not only from a sensorial point of view but also from a physicochemical and nutritional one. As explained in the previous sections, the conventional methods of drying are more susceptible to physical and chemical degradation in the final product. For this reason, it is essential to use an appropriate drying method for each product and select the adequate conditions that will reduce possible changes to a minimum. In the following section, some of the physical parameters influenced by the drying methods are described. Moreover, Tables 1 and 2 summarizes all the drying methods and their effect on quality parameters discussed in this section.

Table 1. Characteristics of selected conventional drying methods.

Drying Method	Drying Agent	Feed Type	Mechanism	Advantages	Disadvantages	Application	References
Convective drying (CD)	hot drying air	Solids—fruits, vegetables, fruit and vegetable pomace	Moisture exchange between the food product and the hot air flowing through the drying chamber	Long shelf-life, simple design; Easy operation; Low cost	High inlet gas temperature or very dry gas; Long drying time, exposure to oxidation; Generates off flavors; Crust formation on the product surface due to the high temperatures	Food industry; Vegetable and fruit dry products; Pomace processing—functional ingredients production	[31,37–40]
Spray drying (SD)	hot drying gas (usually air)	Liquid—i.e., juices, purée, solutions, vegetable milk	Transformation of liquid product into dry powder form in one-step processing operation	Low moisture content and high-quality products; Long shelf-life; Similar size and shape of dried material; Continuous operation Lower cost than freeze-drying	Might lead to bioactive compounds loss and stickiness due to the high temperature, equipment size, products with large fat content require a defat process, high installation cost	Powder production; Microencapsulation; Production of instant powders	[41–44]
Freeze-drying (FD)		All types of food	Two steps process: (1) freezing the water from the raw material; (2) heating of the frozen solid to induce the moisture sublimation	Prevents oxidation damages; Minimize chemical compounds changes; Minimal shrinkage and shift of soluble solids; Retention of volatile compounds; Maintenance of porous structure	Very high facilities cost; Slow and expensive process	Production of heat-sensitive compounds i.e., vitamins, microbial cultures, and antibiotics; Production of high-quality products with high final cost: exotic fruits, vegetables, soup ingredients, mushrooms, and juices	[1,2]
Osmotic dehydration (OD)	sugar, salt (sodium chloride) solutions, concentrate juices, polyols solutions	Fruits, vegetables	Moisture reduction by immersion of the raw material in a high osmotic pressure solution → moisture transfer from the food to the solution driven by the difference in osmotic pressure	Maintenance of the physicochemical and sensory parameters; When carried out in concentrated juices might enhance product quality	High final moisture content; Usually needs further drying; High content of sugar or salt in the product when dehydrated in this type of solution; Difficulty in predicting final chemical composition when dehydrated in concentrated juices	Fruit chips production; Production of dried fruits i.e., plums as a pre-treatment before further drying	[45–48]
Intermittent drying	hot air, microwave power, vacuum and infrared	Fruits, vegetables	Intermittent microwave heating is led by applying microwave energy as sequential pulses, where power ratio has an important role in drying kinetics	Protect bioactive compounds, color, texture; reduce the browning effects and enhance the shelf life.	Higher power ratio can damage important compounds such as ascorbic acid.	Plant-based food material; Fruits: kiwi, papaya, banana, guava, carrot, etc.	[33,35,36]

Table 2. Effects of drying methods on the quality of dried materials.

Drying Method	Color	Structural Properties	Polyphenols Content	Antioxidant Activity	Volatile Compounds	Essential Oil (EO) Content	Sensory
Convective drying (CD)	color changes, generally darkening of the product (blueberries, black mulberries) improved color in case of blackcurrant powder	product hardening, high shrinkage, dense structure, low porosity, high bulk density; when combined with ultrasounds-higher capacity of dehydration in mushrooms, Brussel sprouts, cauliflowers	reduction of TPC in i.e., chokecherries, chokeberry, chokeberries, moringa leaves, and mango cubes	high reduction of antioxidant activity in many products (chokecherries, blueberries, chokeberries, mango cubes)	generally high loss of volatiles; higher content than for other methods on the studies on shitake mushrooms and chanterelle	higher yield of essential oil than during MD of herbs (rosemary and basil)	generate off flavors, decrease of fresh, floral, herbaceous attributes
Microwave drying (MD)	better preservation of color than CD	high porous materials in the studies in potato and carrots decreased porosity in the studies on apple and banana	retention of polyphenols in moringa leaves	retention of antioxidants in moringa leaves	high loss of volatiles, but lower than CD	better yield and preservation in basil and coriander	-
Vacuum drying (VD)	-	high porosity in the studies on apple and banana low porosity on the studies of potato and carrot	retention of polyphenols in moringa leaves	retention of antioxidants in moringa leaves	-	-	-
Vacuum-microwave drying (VMD)	Improved color in case of blackcurrant powder	low shrinkage in comparison to CD but higher than FD, porous structure, better than CD in the studies on chokeberries, faster reconstitution, lower bulk density than CD	higher than CD in the studies on sour cherries	higher than CD in the studies on sour cherries	higher loss of some compounds than CD	increased EO yield in garlic, higher loss of EO than CD of rosemary	decrease of fresh, floral, herbaceous attributes, increase of sweetness, bitterness and adhesiveness
combined convective drying followed by vacuum-microwave drying (CD-VMD)	Slight degradation of color (better than CD and VMD); Improved color in case of blackcurrant powder	lower bulk density than CD	higher than CD and VMD in the studies on sour cherries, chokeberries,	higher than CD and VMD in the studies on sour cherries, Saskatoon berries, chokeberries	higher retention in the studies on chanterelle than other drying methods	increased EO yield in thyme, oregano, and rosemary	-
Freeze-drying (FD)	good preservation of natural color in many studies (i.e., black mulberries)	no shrinkage, no collapse, highest porosity, loss of elasticity, viscous material, lower bulk density than CD	preservation of TPC (black mulberry, chokeberries)	preservation of antioxidants	major loss in drying of parsley, low loss of flavor and aroma	preservation of most EOs	-

Table 2. *Cont.*

Drying Method	Color	Structural Properties	Polyphenols Content	Antioxidant Activity	Volatile Compounds	Essential Oil (EO) Content	Sensory
Osmotic dehydration (OD)	good preservation of color, change of color due to the osmotic solution properties (when concentrated juice is used as osmotic solution)	when combined with FD—strengthen the material structure when used as a pre-treatment before CD or CD-VMD—increase porosity lower bulk density than CD	increase when dehydrated in chokeberry, sour cherry solution, degradation in the studies on sour cherries	degradation in the studies on sour cherries increase when carried out in concentrated pomegranate and chokeberry juices	-	-	-
Heat pump drying (HP)	improved color in rosemary and parsley brown areas when applied on nuts	good preservation of the structure in some herbs than other drying methods	good preservation of polyphenols in drying of herbs	good preservation of polyphenols in drying of herbs	volatiles retention on the studies on ginger	-	-
Fluidized bed drying (FB)	good color retention	-	no significant reduction in kafir leaves	no significant reduction in kafir leaves	-	-	-
Refractance window drying (RW)	decreased browning reaction in pomegranate leather	positively affected	retention of polyphenols high content in pestil pomegranate	retention or improved antioxidant activity in the studies on asparagus, sweet corn and tomatoes high content in pestil pomegranate	-	-	-
Intermittent drying	reduce the color degradation	maintain the product microstructure obtaining a porous structure similar to the fresh sample	Retention of polyphenols	retention of ascorbic acid, carotenoids, and so increasing in the antioxidant activity	retains the lower volatile compounds (due to microwave energy penetration which accelerates the disruption of the cell membranes that ultimately releases the volatile compounds faster)	-	protect cells from oxidative injury, providing better sensory quality

3.1. Color Changes during Dehydration

Maintaining of natural color in dried food products is very important as the visual appearance is one of the first judgments made by consumers. Color together with size, gloss, shape, etc. form the appearance and represent a valuable indicative parameter used in quality control. In the food industry, color modification has enforced enhancing of the visual appearance by adding coloring agents, stimulating high consumer acceptance in foods, particularly dried fruits, and vegetables. One of the most challenging aspects for drying methods is to perform the process in a way that will result in an attractive color for the final product.

Most of the time color variations during drying are related to browning reactions which can be caused both by enzymatic and non-enzymatic reactions. The enzymatic browning occurs in fruits and vegetables due to the phenolic compounds oxidation by polyphenols oxidase, which starts the generation of the brown pigments (melanins) called *o*-quinones. However, there are alternatives to prevent this degradation such as the previous inhibition of enzymatic system by using sulphites [39]. On the other hand, the non-enzymatic browning is produced by different types of non-enzymatic reactions such as Maillard, caramelization, and ascorbic acid oxidation, and is influenced by water activity, temperature, pH, and product composition. Browning is accelerated when the level of water content is intermediate and decreases at very low or very high levels of moisture content. Consequently, browning is harsh close to the end of drying process due to the low levels of moisture remaining in the product. In carbohydrate dried foods (especially fruits and vegetables), changes of color after dehydration are linked with the presence of high amount of reducing sugars such as glucose and fructose. These compounds interact with amino groups from proteins and undergo Maillard reaction during the exposition to air at high temperatures, long drying times and the amount of water. In addition, color changes appear due the degradation of some thermosensitive compounds such as anthocyanins and carotenes, which results in a loss of functionality and color. A quick dehydration of the product to 15–20% moisture content can reduce Maillard reaction time. For this reason, the drying methods are designed to accelerate the drying time in order to achieve the desired moisture content while reducing the browning time [49,50].

Since the color can be affected by several factors (raw material properties, drying methods, additives), it is important to discuss the changes with the total color differences (ΔE). Colorimeter is used in order to determine the color of the product by measuring CIE L^*, a^*, b^* color coordinates values for each sample. To calculate ΔE authors used the following equation:

$$\Delta E = \left[(L - L^*)^2 + (a - a^*)^2 + (b - b^*)^2\right]^{0.5} \quad (1)$$

In which L, a, b, represents the blank sample while the L^*, a^*, b^* are the values obtained for the dried samples. The ΔE indicates the degree of total color change while the L^*, a^* and b^* coordinates refer to: L^* (whiteness-darkness), a^* (red-green) and b^* (yellow-blue) values. Authors have found that if the ΔE is between 0 and 1.0, the color difference cannot be distinguished from a sensorial point of view [51,52].

Degradation of product original color appears at long drying time exposure and high temperatures. According to many studies in this area conventional techniques, such as convective drying, are considered to cause significant losses in color, while the novel technologies based on combined drying methods (i.e., convective and microwave techniques) are limiting the greatest color alterations by minimal heat exposure or by using high temperatures combined with short time and pH adjustment.

Color changes by different drying methods have been already demonstrated for numerous plant materials (Table 3), i.e., blackcurrant [53], pomegranate [54], soya [55], *Piper borbonense* [56], sour cherries [57], blueberry [27], black mulberry [20], and, chokeberry [58].

In the studies on basil leaves microwave drying, convective drying and freeze-drying were compared. Microwave drying prove to be the best method in terms of color retention as it led to fewer changes than convective drying [59].

Wojdyło et al. [52] studied the bioactivity of sour cherry dried by three methods of drying: freeze-drying, convective drying and vacuum-microwave drying. The authors confirmed that using low drying temperatures, short time, and narrow exposure of the raw material to the oxygen present in air are essential to maintain the bioactivity of the product. For this reason, the VMD was found to be recommended in order to assure the abovementioned conditions rather than the CD as this method resulted in similar results to the FD method which was considered as the control. The best parameters in this study were VMD at 480 W until 1 kg/kg dm moisture content followed by drying at 120 W until the end. These parameters assured an attractive final product color linked to a proper anthocyanin content along with a higher polyphenols content and antioxidant capacity [52]. Moreover, Šumic et al. [60] worked on the vacuum-drying process optimization for frozen sour cherries and concluded that the higher product quality can be obtained if it is performed at temperature of 54.03 °C together with a vacuum pressure of 148.16 mbars. The quality in this study was described by the maximum antioxidant activity in dried cherries along with the maximum amount of total phenolic content, Vitamin C and anthocyanins assuring in this way the minimal color change and the desired sample texture [60]. Finally, Horuz et al. [57] also studied sour cherry drying subjected to both convective drying (50 °C, 60 °C, 70 °C) and combined convective-microwave drying (120 W, 150 W, 180 W coupled with CD 50 °C, 60 °C, 70 °C). The authors concluded that the sour cherries color parameters were similar for both CD and CD-MD. However, it should be noted that the drying time was reduced for CD-MD when compared with the CD method. Consequently, CD-MD increased not only the final product quality and its rehydration capacity but also the energy efficiency which is essential for environmental impact and costs [57].

Zielinska et al. [27] studied the CD (60 °C and 90 °C), VMD and their combination CD (60 °C and 90 °C)-VMD in blueberry drying process. As explained above, degradation of anthocyanins results in color degradation due to the location of these compounds in the material as they are mainly situated in the blueberry outer layer. The authors have found that the content of anthocyanins was higher if the samples were dried with VMD when compared with CD. However, when comparing all the methods used in this study, the CD at 90 °C combined with VMD was found to be the most suitable drying method regarding the retention of anthocyanin in blueberries and color preservation. Nevertheless, if compared with the fresh frozen blueberries the anthocyanin content was reduced to 30% in dried samples for the combined CD (90 °C)-VMD. This agrees with other authors that had worked with Saskatoon berries dried with combined CD-VMD and showed an anthocyanin content reduction to 38% [27,61].

In studies about black mulberries using 4 different drying methods such as CD, FD, CD, combined with EPD and FD combined with EPD, the most suitable drying process after FD was found to be FD-EPD. Black mulberry dried with this method was found to present the best color and so to retain the most anthocyanin content when compared to both CD and CD-EPD. This happened due to the vacuum environment which helped to retain more pigment. On the other hand, the thermal process of CD contributed to pigment degradation resulting in less positive color alterations [20].

Samoticha et al. [58] studied drying of chokeberries using different methods. For this reason, FD, vacuum drying (VD), CD, VMD and combined convection drying followed by vacuum-microwave drying methods (CD-VMD) have been used. The authors concluded that FD resulted in maintaining the most of bioactive compounds content in dried fruits when compared with other methods. In addition, the color correlated with the anthocyanin content along with other functional components such as phenolic compounds and antioxidant capacity and was found to deteriorate due to the air temperature increment during CD method as well as the raise in the material temperature for VMD. Furthermore, the combined method CD-VMD (especially with the following parameters: CD at 70 °C for 2 h followed by vacuum-microwave with 360 W reduced to 240 W) was found to result in the highest quality final product when compared to the CD and MVD [58].

Furthermore, an osmotic dehydration pre-treatment can improve the color attributes in plant materials as confirmed by Cano-Lamadrid et al. [62] in their study on pomegranate arils. In this study,

they have used osmotic dehydration followed by a combined CD-VMD to improve the quality of the *Mollar de Elche* pomegranate arils. They observed that using osmotic dehydration as a pre-treatment depending on the osmotic solution (in this case, the juice obtained from the *Wonderful* variety of pomegranate, which is characterized by an attractive color) considerably increased the dried arils color due to the anthocyanin present in the osmotic solution [62] There are also many studies regarding drying of powders. Authors such as Michalska et al. [53] studied the effect of different drying techniques (FD, CD 50–90 °C, MVD 120–240–360 W, CD-MVD) on color of blackcurrant pomace powder. Within this study, it was observed that the drying process improved the powder color when compared to the raw material. In general, the red components were faster released when using VMD or FD methods due to the lower pressure administered. In addition, chroma value (C*), which represents the degree of saturation and is an important marker of product color intensity as kept by human eye, also showed a color enhancement when drying methods were used. However, the color results of powder obtained using CD, MVD and CD-MVD were similar to those obtained by using FD which means that color parameter of blackcurrant powders is not affected by processing temperatures, even though is recommended to be evaluated for processing time and costs [53].

Table 3. Color changes of some dried fruits and vegetables by drying methods.

Drying Method ±	Conditions	Vegetal Material	Parameter Affected	Reference
CD	50–90 °C	Blackcurrant pomace powder	*L**, *a**, *b**, *C**	[53]
	50–70 °C	Soya	*L**, h	[55]
	60 °C	Piper borbonense	*L**, *a**, *b**	[56]
	50–60 °C	Pepper	*L**, *a**, *b**	[63]
	30–70 °C	Pumpkin and green pepper	*C**, h	[64]
	50–70 °C	Sour Cherries	*L**, *a**, *b**	[57]
	50–90 °C	Chokeberry	*L**, *a**, *b**	[58]
	50–70 °C	Pomegranate	*L**, *a**, *b**	[55]
	60 °C	Apples	*L**, *b**	[65]
VD	240–480 W	Chokeberry	*L**, *a**, *b**	[58]
VMD	240–480 W	Blackcurrant pomace powder	*L**, *a**, *b**, *C**	[53]
	2.5, 1.9, and 1.3 W/g	Carrots	*L**, *a**, *b**	[66]
	240–480 W	Chokeberry	*L**, *a**, *b**	[58]
	320–120 W	Apples	*L**, *b**	[65]
CD-VMD	50–90 °C/480 W	Blackcurrant pomace powder	*L**, *a**, *b**, *C**	[53]
	300 W/40 °C	Herbs: basil, lovage, mint, oregano, parsley and rocket	*a**, *b**	[67]
	120–180 W/50–70 °C	Sour Cherries	*L**, *a**, *b**	[57]
	60 °C/320–120 W	Apples	*L**, *b**	[65]
	50–70 °C/90–180 W	Pomegranate	*L**, *a**, *b**	[54]
	50–70 °C/360–120 W	Chokeberry	*L**, *a**, *b**	[58]
CD-EPD	CD 70 °C-EP 80 °C-CD 70 °C	Black mulberry	*L**, *a**, *b**	[20]
RWD	90–98 °C	Pomegranate	*L**, *a**, *b**	[54]

± CD: hot air convective drying; VD: vacuum drying; VMD: vacuum microwave drying; CD-VMD: vacuum microwave drying after hot air convective drying; EPD: explosion puffin drying; RWD: reflectance window drying.

Tontul et al. [54] have studied the color changes in pomegranate leather (pestil) dried by different drying methods: CD at 50 °C, 60 °C or 70 °C, combined CD-MD (90 W, 25 W or 180 W) at 50 °C, 60 °C or 70 °C and RW drying at 90 °C, 95 °C and 98 °C. The authors concluded that the RW technique decreased the browning reaction providing better color to the final product. Besides, the textural properties, as well as the functional ones, were also positively affected by this method of drying [54].

In herbs such as rosemary, parsley, etc., the color and aroma were improved when heat pump drying was used, while Iranian Saffron considered one of the most expensive spices is highly recommended to be dried with this method due to its sensitivity to heat. However, some dried nuts presented brown areas at elevated temperatures [1,13].

Soy okara is a byproduct resulting from the soymilk and tofu production with a great nutritive value. In order to avoid food waste, the industry uses different conservation methods and one of them is drying. However, consumer acceptance of dried okara is disturbed due to the darker color when yellow one is expected in the final product. For this reason, authors studied the possibility of preserving the desired color by using the CD method at different drying temperatures (50 °C, 60 °C and 70 °C) and processing time. In this study, authors observed that the browning increment was directly proportional to

the processing time. Hence, the longer the time of hot air exposure the higher the color degradation. The study recommended using CD at 50 °C as the best option in order to maintain the nutritional quality, even though the L^*, a^*, and b^* values were similar at the end of the process for all temperatures. In addition, they affirmed that when okara was dried in a jet spouted-bed dryer the browning was reduced [55].

Drying impact on *Piper borbonese* was also studied and the changes in the color of the dried pepper by using convective drying at different temperatures (60 °C, 75 °C, 100 °C) were reported. Those changes can be controlled by applying different pre-treatments prior to drying such as blanching and sweating which included maintaining of the samples in a climatic chamber at 35 °C and 99% relative humidity for 24 h. Moreover, optimization of drying process including pre-treatments would definitely improve color of dried pepper. For instance, they concluded that correcting the blanching parameters would help to reduce not only the drying time but also the enzymatic browning [56].

3.2. Physical Properties of Dried Fruit

3.2.1. Structure

The structure of food materials can be characterized by density (apparent and true), porosity, pore size distribution, specific volume, particle density, and shrinkage, etc. Among these, bulk density, porosity, and shrinkage are the most common structural properties reported in the literature [68]. The physical properties of dried fruits are very important mostly in terms of rehydration characterized by the ability of the dried product to return to its initial features. It is well-known that many dried fruits and vegetables along with other ingredients are used in breakfast, ready-to-eat meals, or soups and therefore a proper rehydration is necessary. Rehydration depends on various factors such as type of pre-treatment, moisture content, processing method, and drying conditions. As water is removed from the matrix during the drying process significant changes in structural properties can be observed. Thus, in order to develop high quality dried products besides retaining its color and other functional properties it is also necessary to assure a proper rehydration. Drying methods influence the density, porosity, and sorption features of the product thus, the election of drying methods is essential in order to produce high quality product both dried and rehydrated. Consequently, reduced hydrophilic properties are developed due to the cellular rupture, which is irreversible and results in an integrity loss and dense structure formed by broken down and shrunken capillaries. All of this obstruct the water absorbance and so the rehydration. During rehydration, the dried product suffers numerous internal structure changes i.e., moisture, porosity, volume etc. changes. Thus, the ability of the dried product to regain the form from before the drying depends mostly on the internal structure of the dried particle and the degree of deterioration during the drying process of water holding chemical components such as proteins or starch. CD with a higher drying rate at the beginning of the drying process may lead to product hardening, which results in lower reconstitution capacity. Studies on asparagus dried with both CD and FD concluded that higher quality (faster reconstitution and smooth texture) was obtained when hot water blanching pre-treatment was combined with FD. Usually, the products dried with CD method have a dense structure and a high shrinkage contrary to those dried with microwave, which are characterized by a porous structure that leads to faster reconstitution. Microwaves facilitate obtaining porous products due to drying mechanism that uses volumetric heating to evaporate bounded water producing high internal vapor pressure. Combined CD with the use of ultrasounds is recommended in order to control the internal structure of the dried product. The ultrasounds have a mechanical effect on the drying product, contribute to higher capacity of rehydration without significant overheating. The rehydration depends on the damage of the matrix cell and the structural rupture occurring within the drying product. For instance, ultrasound-assisted CD helped to improve the rehydration capacity of the dried product when compared with those dried without ultrasound power. In studies on mushrooms, Brussel sprouts or cauliflowers dried at 80 °C the rehydration capacity was improved when ultrasound pre-treatment was applied at low acoustic intensity (0.5 W cm^{-2}) in the shortest time (3 min) [69,70]. When VMD is applied the nutrient and sensory (color) properties are preserved during

a longer period. With this method, the energy administered is right to the product molecules and then it is spread to the whole food product instead of only surface. Hence, this method also influences the product texture creating in this way a porous product.

Usually the internal structure of solids or microstructure of semisolids products is analyzed by using sensory panels or by analytical methods by measuring the physical properties, such as texture with a texture analyzer. However, lately 3D image analysis was found to be helpful in terms of internal structure by giving valuable information on size, volume fraction, wall thickness etc. X-ray microtomography (XMT) is a relatively new technique that provides a non-destructive 3D visualization of the internal structure of objects. In recent years, much attention has been given to expanding this imaging technique to food science in order to help in the study of food microstructure, including fresh and dried products. Recently, XMT has been successfully applied for highly porous products to determine the shrinkage, cracking, and the internal moisture of different products such as dried banana, carrots, chokeberries, apples etc. [71–73] (Figure 3).

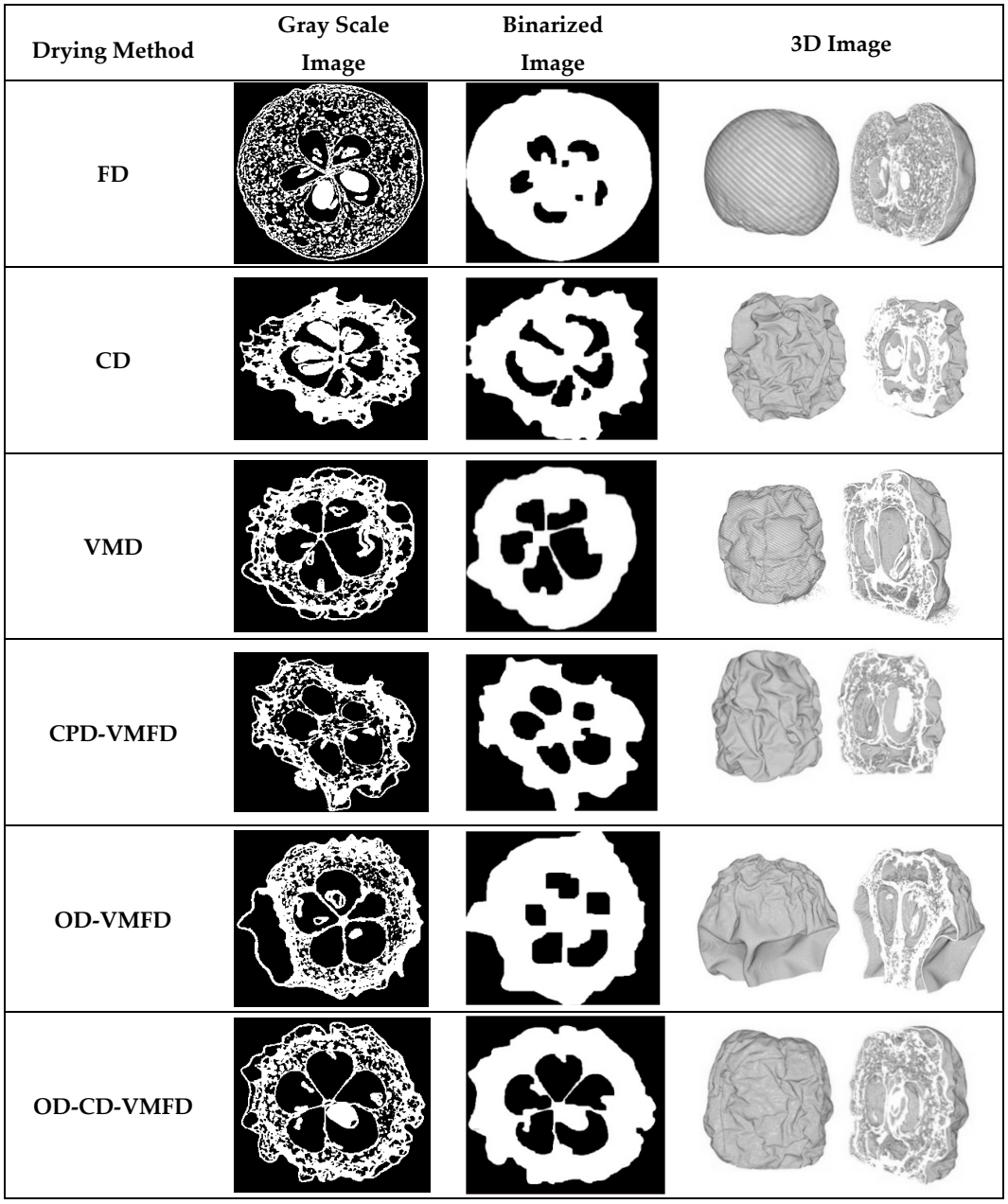

Figure 3. X ray reconstructed images of black chokeberry samples dried using different methods [72].

3.2.2. Porosity

Porosity is one of the most important features used to describe the texture of dried fruits and to characterize its open structure. Porosity (ε) is a measure of empty spaces in the material and it is usually calculated with the apparent density (ρ_α) and true density (ρ_p) of the product by using the equation provided below:

$$\varepsilon = 1 - \rho_\alpha / \rho_\rho \quad (2)$$

The apparent density refers to the density of the material, including the pores and it is calculated as the mass of the material divided by its apparent volume while the true density is calculated with the mass of the material divided by its true volume and it refers to the density of the material without the pores. This physical parameter depends on the drying system and a well-suited one would produce materials with a high degree of porosity. Different drying methods have been compared in terms of porosity and FD was found to produce the higher porosity materials (80–90%). However, freeze-dried products were found to have a sensible structure during rehydration, which leads to a loss of elasticity and more viscous material. For this reason, combined techniques are needed in order to reduce this issue. Osmotic pre-treatment combined with FD was found to strengthen the dried product structure. Microwave drying can also produce high porous levels depending on the drying product. For instance, the porosity of microwave dried potato and carrot was around 75%, while for microwave dried apple and banana lower porosity was obtained (60% and 25%, respectively). The same phenomenon was found for vacuum drying technique in which dried banana and apple obtained high porosity (70%), while for vacuum dried carrot and potato porosity values were lower (50% and 25%) [69,72].

Recently, Calín-Sánchez et al. [72] performed a comparison of dried chokeberries dehydrated by several methods. The porosity values oscillated from 18.4 up to 76.3% during convective drying and freeze-drying, respectively. When comparing convective drying with vacuum-microwave drying for the last one, an important increment in porosity (39%) was found. Products in which the osmotic dehydration was used as pre-treatment and combined with convective drying and vacuum-microwave drying have significantly increased their porosity (42.3%) compared to those only dried with CD (18.4%), VMD (38.6%) and combined dried (CD-VMD) chokeberries (28.4%). However, this increment was higher when before VMD an osmotic dehydration pre-treatment was applied leading to values around 45–49% respectively. Consequently, these authors also agreed that freeze-drying achieved higher porosity samples when compared to the other individual drying techniques (CD and VMD). In addition, they have concluded that the other combined techniques (CD-VMD) could enhance these parameters only when osmotic dehydration pre-treatment was applied leading to good results regarding porosity [72]. Porosity as well as the total pore volume were assessed by XMT and reconstructed X-ray images. Porosity and the total pore volume of dried samples were determined from the binarized images; the steps employed for this purpose were as follows: (i) calculation of the solid volume (V_S) from the black and white images and (ii) calculation of the total volume (V) after filling the samples without considering the volume of the chokeberry seed (Figure 4). Porosity was calculated using Equation (3) and results were expressed as percentage, and the total pore volume was calculated using Equation (4) and results were reported in mm^3.

$$\varepsilon = (1 - \frac{V_s}{V}) \times 100 \quad (3)$$

$$V_p = V - V_s \quad (4)$$

Other authors reported that using low-pressure superheated steam drying (LPSSD) helped to improve the porosity degree of the dried products when compared to conventional hot air or vacuum drying. This is possible due to the expansion of the material cells produced by the high-pressure gradient within the product. However, this process is quite slow, so a combined LPSSD with far infrared radiation (FIR) should be applied in order to accelerate the process. Besides process acceleration, FIR also contributes to drying time reduction, increasing the product quality [73].

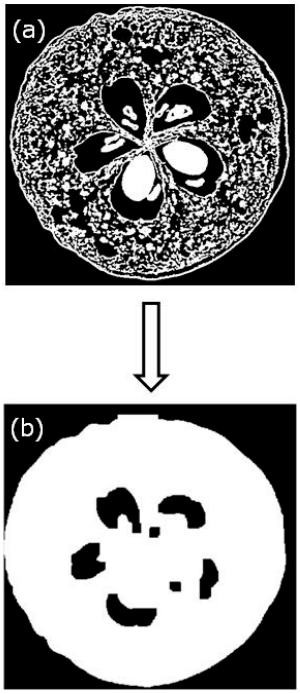

Figure 4. Images used to calculate (**a**) solid volume and (**b**) total volume [72].

3.2.3. Bulk Density of the Dried Material

Bulk density (p_b) applies to powdered and porous materials, completely occupying the volume of the container (bulk volume) in which they are located. It is defined as the mass of the dried sample (m) divided by its bulk volume (V_b) and is calculated with the following formula:

$$p_b = \frac{m}{V_b} \tag{5}$$

Low bulk density of the dried products obtained due to the "puffing" effect is mostly desired in order to increase the sensory aspects resulting in higher consumer acceptance. Many factors influence bulk density of the dried products and the most important, besides the porosity that has already been mentioned, is the drying method with its parameters (temperature, microwave power and time). The drying conditions affect not only the apparent volume, which characterizes the individual particles but also the shape of these particles and thus the bulk volume which, in turn depends on the degree of packing of the material. In this way, studies on dried chokeberry showed that both FD and MVD (360 W) were found to be the most efficient methods in terms of bulk density reducing it to around 50% when compared to the CD. In addition, the combined CD-VMD, developed to prevent the sample overheating, was also found to be a good option in order to reduce the bulk density of the dried products up to 55% with respect to the convective dried products. This could be due to volumetric heating occurring when microwaves are applied, which results in puffing of the material. The application of osmotic dehydration improved the values of bulk density when compared to convective dried chokeberries; however, these results were as good as vacuum-microwave dried samples and comparable to freeze-dried samples. Moreover, bulk density has been deeply studied in many other dried fruits; for instance, Yemmireddy et al. [74] studied blueberries (rabbiteye blueberries) and reported that the highest values of bulk density were observed for the samples dried with hot air. Van Arsdel et al. [75] showed that the bulk density of the food product dried to the same moisture content depends on the rate of shrinkage. This statement is strongly affected by the drying method and the drying conditions, such as temperature or time. Longer drying times might have resulted in internal cell destruction and excessive shrinkage of blueberries and hence high bulk density [76].

The shrinkage can also be related to the preservation of foods, the rehydration rate and the biochemical reactions that occur during further storage [72,74,75,77].

3.2.4. Shrinkage

Considerable changes in the physical structure of the product, such as reduction in volume and decrease in internal porosity (apparent porosity) can occur during the drying process. Shape and size changes during drying modify not only the dimensions and transport properties of individual particles but also the thickness and porosity of the packed bed in the dryer. The shrinkage results from the reduction of particles porosity, while dehydration increases the bulk density of the material. Experimental data on shrinkage of fruits during hot air-drying reported in previous studies showed products with higher shrinkage and dense structure. Constant porosity and minimal shrinkage are often stated as key assumptions in the model for dryer design. Freeze-dried fruits and vegetables are usually characterized by minimal shrinkage, while the product quality of hot air-dried products is relatively low, mainly due to the hard texture of the products dried by this method [69].

Shrinkage of vacuum-microwave dried products is significantly lower, the rate of drying is noticeably higher, and the duration of the drying process is considerably shorter in comparison to convective drying. Puffing phenomenon associated with a fast dehydration is responsible for a porous structure of vacuum-microwave dried products; however, the number of pores is smaller, and the size of pores is bigger when compared to freeze-drying products. Consequently, the shrinkage of vacuum-microwave dried products is slightly higher than freeze-dried ones but significantly lower in comparison to hot air-dried product. Freeze-drying has been found to minimally affect the structure of dried material resulting in no noticeable shrinkage or collapsing in the studies on chokecherries [78]. Combined drying consisting of the osmotic dehydration and the vacuum microwave drying (OD-VMD) might additionally improve the physical properties, e.g., the texture of the dried product. Application of osmotic drying using sucrose reduced the shrinkage and improved the rehydration capacity of vacuum microwave dried pineapple circular discs providing a softer texture and less hardened surface. Torringa et al. [79] reported that the increase in a concentration of the osmotic solution decreased the shrinkage of the mushroom finish dried by combined microwave hot air drying method. This relationship can be explained by increased maximum temperature of vacuum microwave drying at final stage resulting from increased osmotic solution concentration. New techniques ensure a lower shrinkage than traditional methods due to the puffing phenomenon, which is enhanced by a high inner pressure associated with the temperature of the dried material [29,69,79,80].

4. Effects of Drying on Functional Properties and Nutritional Quality of Food Products

4.1. Changes in Phytochemicals Compounds

The presence of phytochemicals, including carotenoids, polyphenols, and vitamins (ascorbic acid), minerals, etc. have been attributed to protective action on degenerative illness. Table 4 shows a recompilation of the main phytochemical compounds (carotenoids, polyphenols, and vitamins) with widely reported antioxidant capacity, affected by drying methods on several plant materials (Table 4).

The variation of content of these phytochemicals can be affected by temperature, exposure time, levels of oxygen and the presence of light. Several studies indicate that the phytochemicals content in dried products obtained by low temperatures is higher than in dried products obtained by high temperatures [81]. Alternatively, reduction of oxygen levels at the absence of light in microwave, refractance window, low pressure superheated steam, and vacuum drying methods [50] can increase the retention of these compounds. In studies on dried leaves, a higher retention of lipophilic vitamins was found when FD or microwave drying was applied, while the opposite phenomenon happened for the convective dried samples. In addition, when fluidized bed drier (FB) was used to dry kaffir lime leaves, no significant reduction of Vitamin C and A was observed. Regarding the mineral content of moringa dried leaves the temperature had a significant impact on many elements except magnesium and FD was also found to be the most efficient, followed by air drying and oven drying [5].

Table 4. Recompilation of different phytochemicals (carotenoids, polyphenols, and vitamins) affected by drying methods.

Drying Method ±	Conditions ±	Vegetal Material	Phytochemicals	Reference
CD	55–62 °C	Sweet potato	β-carotene Vitamin C	[39]
	50–70 °C	Jujube	Flavonoids Vitamin C	[82]
	65–73 °C	Avocado	Flavonoids Phenolic acids	[83] [40]
	50–70 °C	Pomegranate	Anthocyanins	[84]
	50–60 °C	Strawberry		[85]
	55–65 °C	Cauliflower	Vitamin C	[86]
VMD	120 W–480 W	Jujube	Flavonoids Vitamin C	[40] [82]
CD-VMD	60 °C/480–120 W	Jujube	Flavonoids Vitamin C	[40] [82]
SD	110–130 °C	Tomato pulp	Licopene	[87]
	120 °C	Grapefruit	Flavonoids Phenolic acids	[83]

± CD: hot air convective drying; VMD: vacuum microwave drying; CD-VMD: vacuum microwave drying after hot air convective drying; drying; SD: spray-drying.

Pomegranate pestil exhibited a high content of phenolic compounds. When combined, drying method was used and it was observed that microwave-assisted drying provides a high content of polyphenols while refractance window ensures a higher anthocyanin and ascorbic acid content in the dried material [54]. Furthermore, carotenoids were found to be less sensitive to time of drying than to temperature while ascorbic acid was reduced within the prolonged drying time. On the other hand, lycopene content was found to be higher when combined drying methods were applied than individual drying. For instance, the tomatoes dried by osmotic-vacuum drying were found to have a higher lycopene content than those dried only with vacuum or convective method. This phenomenon is due to heat and oxygen exposure [81].

4.2. Changes in Antioxidant Capacity as a Result of Dehydration

The stability of compounds with antioxidant activity is influenced by many factors, mainly raw material, temperature, and process time. Even though the antioxidants are mostly retained during drying, it is essential to know the retention of the antioxidant capacity for each drying technique in order to choose the right one that leads to high-quality dried products.

Considerable scientific evidence of the effect on antioxidant capacity and the total polyphenolic content by drying treatments are shown in Table 5. Since many of bioactive compounds degrade during drying, the optimization of the drying process is the key component to obtain the best quality dried product. Studies on antioxidant capacity after drying of asparagus with novel methods such as RW or combined microwave and spouted bed affirmed that the bioactive compounds retention improved when these methods were applied compared to CD. During hot air drying the loss in antioxidant capacity was due to the product exposure to oxygen. In an oxygen-free environment, it is highly recommended to use low temperatures necessary to avoid loss of phenolic compounds and antioxidant capacity due to the thermal and oxidative degradation. This was proved in the studies on mango cubes. On the other hand, RW drying rapidly heats the product which results in a faster release of the phenolic compounds in cell material. In addition, the water loss is severe during the first minute of the operation while the intense vapor pressure produced by the moisture evaporation reduces the partial pressure close to the product and prevents the phenolic compounds from oxidation. This argument was stated by other authors in studies on sweet corn or tomatoes who observed an increment in antioxidant capacity due to the heating process.

Agreeing that even the natural antioxidants are lost during the process, the antioxidant capacity is enhanced due to the production of new antioxidants. Sehrawat et al. [6] in their study about the production of nutritive dried mango cubes snack found that the low pressure superheated drying method at 70 °C is the most suitable method when compared with vacuum drying and hot air drying, even though this two methods also showed good results at 60 °C [6,88]. Finally, osmotic dehydration pre-treatment in pomegranate and chokeberry juices of pomegranate arils increase not only the antioxidant capacity but also other functional compounds in the studies on pomegranate arils [62].

Table 5. Recompilation of antioxidant capacity (DPPH, ABTS, and FRAP) and total polyphenolic compounds (TPC) affected by drying methods.

Drying Method ±	Conditions ±	Vegetal Material	AC Affected	Reference
CD	40 °C	Date fiber	DPPH TPC	[89]
	50–70 °C	Pomegranate	DPPH	[25]
	50–90 °C	Blackcurrant	ABTS TPC	[53]
	50–70 °C	Jujube	ABTS FRAP	[82]
	55–62 °C	Sweet potato	TPC	[39]
	40–60 °C	Cocao bean	TPC	[90]
VMD	240–480 W	Pomegranate	DPPH TPC	[25]
	120 W–480 W	Blackcurrant	ABTS TPC	[53]
	120 W–480 W	Jujube	ABTS FRAP	[82]
CD-VMD	50–90 °C/480 W	Blackcurrant	ABTS TPC	[53]
	50–90 °C/480 W 60 °C/480–120 W	Jujube	ABTS FRAP	[82]
SD	120 °C	Grapefruit	DPPH	[83]

± CD: hot air convective drying; VD: vacuum drying; MVD: vacuum microwave drying; CD-VMD: vacuum microwave drying after hot air convective drying; EPD: explosion puffin drying; RWD: reflectance window drying; SP: spray-drying.

4.3. Changes in Nutriotional Quality of Dehydrated Food Products

The reduction of the content of sugars in plant material after drying is due to the browning reactions, especially Maillard reactions. A clear example is given: an increase of temperature and time during convective drying of pomegranate arils caused a reduction of some characteristic sugars (fructose and glucose) [25]. These sugars create conjugates with amino group from proteins. Therefore, the functional properties of proteins can be influenced by drying method, especially, lysine is used as an indicator for protein quality deterioration [83]. A visible example is the change of amino acid profile (mainly, arginine and lysine) of chickpea proteins concentrates among convective drying [84]. As explained in the color section, the Maillard reactions produce changes in the color of the product which results in a darkening and browning usually considered as a negative aspect. However, some authors affirmed that the antioxidant capacity of the product could be improved by Maillard reaction due to the production of melanoidins [88].

5. Changes in the Volatile Compounds or Essential Oils during Dehydration of Fruits, Vegetables, and Aromatic Herbs

The effect of dehydration on essential oils and/or volatile compounds has been reported mostly in aromatic herbs. The two basic forms of consuming culinary herbs are fresh and dried. Fresh herbs

cannot be supplied in a profitable way to all worldwide locations. The essential oil flavor composition of aromatic herbs has been the subject of considerable research in recent years. It is well-known that the presence of essential oils and their composition determine the specific aroma of plants and the flavor of the resulting condiments.

In general, the drying process leads to significant losses of volatile compounds. Among the drying treatments, some authors have reported higher losses of volatiles in convective hot air drying, and microwave drying as the air temperature and the wattage increased [72,91–93]. However, these statements were not observed in Hungarian thyme. In this particular case, as the air temperature increased, the losses decreased. This behavior was attributed to the longer processing at lower temperatures compared to the shorter times required for higher temperatures [88]. Volatile compounds retention was observed in studies on ginger when heat pump drying was applied. This is a positive effect with respect to other driers that usually loose the volatile compounds of the product [1,13].

Some authors have considered an initial hypothesis regarding the improvement produced by the microwave drying compared with traditional convective hot air drying. This improvement was reported by many authors in dried marjoram, thyme, oregano and basil [25,28,72,92,93]. However, other authors reported that rosemary and basil samples dried by convective drying showed higher yield of essential oils [91,94]. All these authors agree in the fact that the essential oil yield varies considerably from one species to another. The important losses of volatile compounds might be diminished by using assisted and combined drying techniques. For instance, Figiel [28] suggested that microwave drying assisted by vacuum increased the yield of essential oils in garlic, while others reported that the higher the vacuum intensity in the drying system for a specific microwave power and the higher the power intensity, the lower the concentration of total volatiles [28,95]. Besides, a convective pre-drying followed by microwave finishing drying increased the content of essential oils in samples of thyme, oregano, and rosemary [28,91,96]. Therefore, assisted techniques as vacuum-microwave drying and the combined techniques, such as convective pre-drying followed by vacuum-microwave finishing drying seem to be very promising options to dry aromatic herbs.

Some authors have recently reported the effect of dehydration on the volatile compounds in different food products such as oyster mushrooms, shitake and chanterelle suggesting that the total concentration of volatiles of fresh mushrooms was drastically reduced by all drying treatments; although, the highest contents were found using (i) convective pre-drying (50 °C)-vacuum microwave finishing drying (480 W), and (ii) vacuum microwave drying at 480 W. Regarding dried shitake mushrooms the most recommended drying method in order to retain the highest content of volatile compounds was CD at 80 °C. Finally, for dried chanterelle the higher retention of volatile compounds content was registered when (i) convective drying at 80 °C, (ii) convective pre-drying followed by vacuum microwave finishing drying at 70 °C/480/240 W and (iii) convective pre-drying followed by vacuum microwave finishing drying at 80 °C/480/240 W were applied [97–99]. These new findings confirm that combined techniques tend to retain higher content of volatile compounds when compared to traditional (hot air convective drying) or modern techniques (vacuum-microwave drying) applied as a single treatment, improving the quality of some dried products and obtaining a reduction in the costs of the processing [28,29].

6. Sensory Properties of Dried Fruits, Vegetables, and Aromatic Herbs

Descriptive sensory analysis (DSA) is used to quantitatively determine the intensities of the main sensory properties and attributes of food. Such analysis requires the use of a trained panel. The proper number of panelists ranges between 7 and 12 of highly trained panelists [100]. DSA has been previously applied in the description of the main attributes of fruits, vegetables, but especially in aromatic herbs such as marjoram, thyme, basil, parsley, bay leaf, spearmint, and rosemary [76,91,92,95,96]. In general, dried samples of aromatic herbs have been typified by significant increases in the intensities of attributes such as spicy, hay-like, sweet, earthy, woody, and infusion. Modern drying techniques led to reduction of typical attributes of dried herbs. At the same time, an increase in the hot air temperature or microwave power led to decrease of some attributes such as fresh, floral and herbaceous [96].

Regarding DSA of dried fruits, some of the most affected attributes were sweetness, bitterness, adhesiveness, and caramel flavor. All these attributes are increased when the drying temperature and wattage also increased [72,96,101,102]. Recently, other authors applied osmotic dehydration as first steps during drying of pomegranate arils and highlighted that this technique with different solutions (pomegranate, chokeberry, and apple) provides samples without measurable off flavors. This finding justified that the solutions had high content of sugars (40%) and an attractive color [62].

Product quality, consequently sensory properties, of dehydrated fruits is a key feature in innovation of future drying technology (Figure 5), which is closely related to: (i) development of novel drying techniques and (ii) process optimization [103]. Sensory evaluation will be a perfect tool to determine whether the effects of a particular drying technique will lead to high quality products, with elevated guarantee of being accepted by consumers.

Figure 5. Effect of different drying techniques on sensory characteristics of loquat cultivar '*Algar*' [102].

7. Conclusions

Research, development, and innovation are carried out in the food processing, especially in fruits, vegetables and aromatic herbs. Nowadays, consumers are demanding novel and healthier ready-to-eat products with long shelf-life and dehydrated products meet these criteria. Moreover, their functional properties and quality characteristics should be as close as possible to those of the fresh vegetal material. For these reasons, the food industry supports the research in both quality characteristics and processing techniques. In this sense, dehydration of agricultural products seems to be an extremely important matter in order to assure the physical, chemical and sensory quality of the final products.

Recent studies concerning dehydration of agricultural products using vacuum-microwave technique showed that the most promising method is combined drying which ensures high quality at the lowest possible energy consumption. However, the optimization of novel drying techniques as well as the combination of them still requires additional studies in order to comprise other forms of treatments necessary to improve the texture, retention of valuable compounds (phytochemical and volatile compounds), health-promoting properties and an attractive sensory property.

Finally, freeze-drying is one of the most recommended methods in terms of functional quality retention. However, from a physical point of view, the product can lose elasticity and become viscous when rehydrating. In order to avoid this issue, an osmotic pre-treatment is recommended. Thus, this phenomenon happens with many of the individual drying techniques, and for this reason, a combined technique is highly recommended depending on the kind of raw material. As it can be stated, all the techniques have strengths and weaknesses but the most important for a combined or novel technique is to reduce the costs, to be environmentally friendly, and to assure high quality of the dried products from a functional, physical, and sensorial point of view.

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An HS-GC-IMS Method for the Quality Classification of Virgin Olive Oils as Screening Support for the Panel Test

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Abstract: Sensory evaluation, carried out by panel tests, is essential for quality classification of virgin olive oils (VOOs), but is time consuming and costly when many samples need to be assessed; sensory evaluation could be assisted by the application of screening methods. Rapid instrumental methods based on the analysis of volatile molecules might be considered interesting to assist the panel test through fast pre-classification of samples with a known level of probability, thus increasing the efficiency of quality control. With this objective, a headspace gas chromatography-ion mobility spectrometer (HS-GC-IMS) was used to analyze 198 commercial VOOs (extra virgin, virgin and lampante) by a semi-targeted approach. Different partial least squares-discriminant analysis (PLS-DA) chemometric models were then built by data matrices composed of 15 volatile compounds, which were previously selected as markers: a first approach was proposed to classify samples according to their quality grade and a second based on the presence of sensory defects. The performance (intra-day and inter-day repeatability, linearity) of the method was evaluated. The average percentages of correctly classified samples obtained from the two models were satisfactory, namely 77% (prediction of the quality grades) and 64% (prediction of the presence of three defects) in external validation, thus demonstrating that this easy-to-use screening instrumental approach is promising to support the work carried out by panel tests.

Keywords: virgin olive oil; HS-GC-IMS; volatile compounds; chemometric analysis; sensory analysis

1. Introduction

Thanks to their unique sensory attributes and their compositional uniqueness, extra virgin olive oils (EVOOs) and virgin olive oils (VOOs) are usually marketed at a higher price than other vegetable oils [1], frequently rendering them the object of fraudulent practices. EVOOs and VOOs can be destined for human consumption; however, lampante olive oil (LOO) is not edible and therefore not marketable. Therefore, it is very important to classify each product in the proper commercial category, and to verify that the quality degree as reported in the label corresponds to the product contained in the related recipient, in order to not mislead consumers.

Sensory analysis carried out by a specific methodology, the panel test, plays a crucial role in classification of VOOs, together with chemical-physical analytical determinations. Its main objective is to define a sample to a specific quality grade by identifying and quantifying the intensity of eventual most perceived defect and positive attribute of fruity [2,3]. The origin of positive and negative sensory characteristics in VOOs, perceived by both orthonasal and retronasal olfaction, is due to the presence of volatile molecules that depend on many factors, such as the variety of the olives and cultivation area, as well as environmental, agronomic and technological variables [4–6]. The qualitative-quantitative combination of six carbon atom compounds (C6) as well as five carbon atom (C5) molecules deriving from the lipoxygenase (LOX) pathway is responsible, together with others such as terpenes, for the positive notes of fruitiness and resemble characteristic secondary attributes, e.g., grass, artichoke [5]. However, in addition to these molecules, other volatile compounds may originate from fermentative and degradative microbial processes affecting sugars and proteins, as well as lipid oxidation [4]. These latter molecules have been correlated with the presence of specific negative sensory attributes and, depending on their concentration and the perceived intensity of the defect, determine a lower quality of the product, which can no longer be marketed as "extra virgin". For this reason, the identification and quantification of volatile compounds in the aroma of VOOs are of great importance to assess its quality [7]. For this purpose, numerous analytical procedures have been adopted [8,9], among which gas chromatography (GC) is the most widely used separative technique. The combination of the results obtained from sensory and instrumental analysis can allow rapid screening of samples, increasing the number of controls and supporting sensory evaluation [10–12]. In recent years, alternative instrumental techniques have been developed based on different principles that emulate the responses of the human nose, tongue, and eyes [13]. In this context, HS-GC-IMS (headspace gas chromatography-ion mobility spectrometry) is an interesting screening tool. This technique is able to realize a digital fingerprint of the aroma for possible discrimination of samples in a relatively simple, rapid, and cost-effective way [14]. HS-GC-IMS was recently used in several investigations for the analysis of volatile compounds in VOOs for the determination of geographical origin [15,16] and for discrimination of quality grades [17–19].

In this work, a new semi-targeted analytical approach has been developed by focusing on 15 volatile compounds that were previously selected from analytical investigations within the European project Horizon 2020 OLEUM "Advanced solutions for assuring the authenticity and quality of olive oil at a global scale", grant agreement no. 635690, and known to be associated with positive and negative sensory attributes in VOOs [8,20]. In particular, HS-GC-IMS analysis was performed on a set of 198 VOO samples and followed by development of two-category PLS-DA (partial least squares-discriminant analysis) discrimination models of which one was adopted for the first time in the classification of samples on the basis of the presence of sensory defects. Furthermore, most of the samples were evaluated by 6 different sensory panels using the decision tree developed within the OLEUM project [21]. The goal of this investigation was to establish a semi-targeted screening methodology that can support the panel test with the aim of being successfully used by olive oil companies in the future, as well as in laboratories for routine quality control analyses.

2. Materials and Methods

2.1. Virgin Olive Oil (VOO) Samples and Sensory Evaluation

A set of 198 VOO samples was analyzed. Specifically, 153 samples, collected from olive oil companies in 2018 within the European H2020 OLEUM project, were evaluated by 6 different sensory panels involved as partners in the project; based on the sensory results elaborated according to a decision tree [21], samples were classified into three quality grades according to Regulation (EU) 2019/1604: EVOO (69 samples), VOO (51 samples), and LOO (33 samples). The remaining 45 samples were evaluated sensorially by the professional committee of VOO tasters of the University of Bologna: 14 were classified as EVOO, 18 as VOO, and 13 as LOO. All samples were stored in a freezer at −18 °C until analysis, thawing them for an adequate time—until no solid phase was observable—at room

temperature and shaken carefully before use. The oil recipients were kept open only for a short time and the headspace volume was always minimized.

2.2. Headspace Gas Chromatography-Ion Mobility Spectrometry (HS-GC-IMS): Instrumental Equipment

The analysis was performed using a GC-IMS Flavourspec® instrument (G.A.S. Dortmund, Dortmund Germany) connected to a nitrogen generator for carrier/drift gas production (Microprogel, Pordenone, Italy). For injection, 100 µL of each sample headspace was withdrawn using a 2.5 mL Hamilton syringe with a 51 mm needle, through an autosampler unit, HT2000H (HTA s.r.l., Brescia, Italy), and introduced in a splitless heated injector (2 mm ID, 6.5 mm OD × 78.5 mm fused quartz glass). The analytes passed into a low polar column FS-SE-54-CB-0.5, 30 m, 0.32 mm ID, film thickness 0.5 µm (94% methyl-5% phenyl-1% vinylsilicone) for a first separation. The eluate was subjected to a second separation by IMS equipped with a tritium ionizing radioactive source at 5000 V and a 9.8 cm long drift tube (Gesellschaft für Analytische Sensorsysteme mbH, G.A.S.; Dortmund, Germany).

2.3. Selected Volatile Compounds

In this study, 15 volatile compounds were analyzed as two different standard mixtures (SM), coded as SMA and SMB: 3-methyl-1-butanol (purity ≥ 98.5%), propanoic acid (≥99.8%), 6-methyl-5-hepten-2-one (≥97.0%), ethyl acetate (≥99.8%), (*E*)-2-heptenal (≥95.0%), ethyl propanoate (≥99.7%), (*E*,*E*)-2,4-hexadienal (≥95.0%) (compounds present in the SMA) and ethanol (≥99.9%), acetic acid (≥ 99.8%), 1-octen-3-ol (≥98.0%), hexanal (≥98.0%), nonanal (≥95.0%), (*E*)-2-hexenal (≥97.0%), (*Z*)-3-hexenyl acetate (≥98.0%), 1-hexanol (≥99.9%) (compounds present in the SMB). All these reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA). The aforementioned volatile standards were dissolved in fresh refined olive oil to be analyzed both individually, at a concentration of 50 mg kg^{-1}, and within the two SMs (at a concentration range: 0.05–50 mg kg^{-1}).

2.4. HS-GC-IMS Analysis of Volatile Compounds Mixtures

Mixtures of individual volatile compounds were prepared from stock solutions of pure standards prepared by dissolving each standard in fresh refined olive oil at approximately 5000 mg kg^{-1}. A rapid preparation at controlled room temperature was carried out to avoid evaporation of standards. By 1:100 dilution (*w*/*w*), individual volatile compounds mixtures were prepared at about 50 mg kg^{-1}, in a 20 mL headspace glass vial, weighing approximately 2 g. Next, the vial was hermetically closed with polytetrafluoroethylene septum (PTFE). The sample was incubated at 40 °C for 8 min and 100 µL of headspace was injected using a heated syringe (80 °C) into the injector (set at 80 °C). The carrier gas (nitrogen gas with inlet pressure of 4 bar) passed through the GC-IMS injector transferring the sample into the GC column, using a flow ramp set as follows: the flow was initially set at 2 mL min^{-1} (default) for 2 min, then increased to 17 mL min^{-1} for the next 8 min (70% of maximum flow) and maintained at this flow for another 20 min. Finally, the flow was reduced for the next 2 min to the predefined value (2 mL min^{-1}); end of the program was set at 32 min. The analytes were separated in isothermal mode at 40 °C and introduced into the ionization chamber of the IMS where the tritium source (5000 V) ionized compounds eluting from the GC column and the ions reached the drift tube of the IMS through the shutter grid. The drift tube was maintained at a constant temperature of 45 °C. The gas flow rate of nitrogen introduced in the opposite direction of the sample into the IMS (drift gas) was 150 mL min^{-1}.

In addition to being analyzed individually, the 15 volatile compounds were also determined within two different standard mixtures (SM), coded as SMA and SMB (see Section 2.3), both prepared at approximately 50 mg kg^{-1}. In this way it was possible to identify each single compound in the two SMs, obtaining the advantage of processing the SMA and SMB results to evaluate the performance of the method (see Section 2.6) rather than the data of the 15 volatile compounds obtained individually, with a significant advantage in terms of time needed to perform the analysis. The 15 volatile compounds were individually identified and quantified in chromatograms.

2.5. HS-GC-IMS Analysis of Virgin Olive Oil Samples

We weighed 2 g of each VOO in a 20 mL headspace glass vial that was hermetically closed. Subsequently, samples were analyzed following the same method reported in Section 2.4.

For each sample, a heat map (3D chromatogram) was obtained: only the 15 selected volatile compounds were considered (see Sections 2.3 and 2.4), thus highlighting their respective signals present in the form of a monomer and/or dimer in the chromatogram, using VOCal software (Gesellschaft für Analytische Sensorsysteme mbH, G.A.S.; Dortmund, Germany). Using a specific function of the software, it was possible to export the results to a data matrix that was used to develop the discrimination models (see Section 2.7).

2.6. Performance of the Method

To evaluate the performance of the method, the following parameters were taken into consideration: linearity of the 15 volatile compounds, expressed in terms of range and determination coefficient (R^2); intra and inter-day repeatability, as relative standard deviation percentage (RSD%) values, calculated on the maximum intensity value of two specific volatile compounds. In this latter case, three samples, corresponding to three quality grades, were evaluated.

2.6.1. Linearity

The linearity of the 15 selected volatile compounds was evaluated by developing calibration curves for each analyte built through analysis of the two standard mixtures SMA and SMB as described in Section 2.3. The starting stock solutions at approximately 10,000 mg kg^{-1} for these two mixtures were prepared by weighing each volatile standard (10 compounds for SMA and 8 for SMB) in fresh refined olive oil. For the low concentration mixture (A), the following 12 dilutions were prepared: 0.05, 0.10, 0.15, 0.20, 0.25, 0.5, 1.00, 1.50, 2.00, 2.50, 5.00, 10.00 mg kg^{-1}. For the high concentration mixture (B), it was necessary to prepare 15 dilutions: 0.05, 0.10, 0.15, 0.20, 0.25, 0.5, 1.00, 1.50, 2.00, 2.50, 5.00, 10.00, 15.00, 20.00, 25.00 mg kg^{-1}.

2.6.2. Intra-Day and Inter-Day Repeatability

Three samples of the 198 oils (see Section 2.1) were selected to be representative for each quality grade. These were an EVOO with a median of the fruity attribute of 3.0, and a VOO and a LOO with medians of the most perceived defects of 1.7 (winey-vinegary) and 6.6 (fusty/muddy sediment), respectively. Furthermore, for each sample, two specific volatile compounds were chosen for the repeatability study: (*E*)-2-hexenal and hexanal (typical of the fruity positive attribute, in the case of hexanal when it is at low-medium concentration) for EVOO; ethanol and ethyl acetate (typical of the winey-vinegary defect) for VOO; ethyl propanoate and 3-methyl-1-butanol (typical of the fusty/muddy sediment defect) for LOO. The selection of these markers was based on the previous literature [8,20], considering the high values of their determination coefficient values (see Section 3.2.1).

Intra-day repeatability was determined based on the average RSD% values of the maximum intensity (expressed in mV), calculated on the areas of the signals related to the two volatile compounds dimers in each of the three samples selected for each quality grades, analyzing them in 7 replicates on the same day.

For inter-day repeatability, the same procedure was followed but calculating the average RSD% values on the maximum intensity of the two volatile compounds dimers in each of the three samples selected for each quality grades, analyzing them for each day for one week (7 days).

2.7. Data Analysis

From the HS-GC-IMS analysis, a 3D chromatogram (heat map) was obtained. Each point in the heat map is characterized by the GC retention time measured in seconds, by the IMS drift time in milliseconds, and by the intensity of the ion current signal in millivolts (mV). The raw 3D data [22]

were normalized on the reactant ion peak (RIP). The RIP corresponds to the reactant ions or hydrated protons, which are generated in the ion source of the employed IMS device. The analytes interact with the RIP to generate protonated species by the displacement of water [19]. Subsequently, the maximum intensity of the areas (monomer and dimer) belonging to the 15 volatile compounds were selected and used to develop the chemometric models (normalized values). Not all 15 volatile markers had both the monomer and the dimer in the heat map. For this reason, a total of 25 signals were used rather than 30.

Principal component analysis (PCA) was used as an explorative technique to evaluate the relationships between variables and to visualize the data according to the quality grade.

Different PLS-DA models were built: a first approach was used to classify the sample according to quality grades, and a second to classify samples on the basis of the presence of defects (negative sensorial attribute). For the latter, only VOOs and LOOs were considered (115 samples, of which 49% with fusty/muddy sediment defect, 29% musty-humid-earthy and 44% rancid). PLS-DA models were developed using the PLS Toolbox for Matlab; volatile compound signals were used as variable X (mean center pretreatment), while the quality grade or presence of defects were implemented as variable Y (binary variables, 0–1).

For the quality grades, 4 classification models were built, EVOO vs. no-EVOO followed by VOO vs. LOO, and LOO vs. no-LOO following by EVOO vs. VOO; for the presence of defects, 3 models were developed based on the 3 main perceived defects in the VOO and LOO samples: musty, rancid and fusty/muddy sediment.

In all cases, the sample data set were split into a calibration/cross validation set (75% of the sample) and external validation set (25% of the sample) using the Kennard-Stone method [23]. Samples for the cross validation were selected using the venetian blind method (number of data split: 10). The threshold value useful to define the category of each sample was defined using a probabilistic approach based on Bayes's rule.

2.8. Set-Up of Analytical Conditions

In order to obtain the most information in the shortest time, several analytical parameters were investigated to optimize the headspace extraction and repeatability of the analysis.

Sample conditioning: a comparison between three different settings in terms of conditioning time and temperature was carried out: (i) 40 °C/20 min, according with previous investigations dealing with a similar rapid chromatographic separation [24]; (ii) 60 °C/8 min, adopting the same conditions applied by Contreras et al. [19]; (iii) 40 °C/8 min, to take advantage of both a shorter analytical time and temperature, as in (i), more similar to the real tasting experience in the panel test procedure. Comparison of heat maps obtained from the analysis of VOO samples injected after conditioning at 40 °C/8 min and 40 °C/20 min, no differences were observed in terms of either coordinates (retention time/drift time) or intensity of the spots. For this reason, the condition 40 °C/8 min was chosen to take advantage of a temperature closer to the oral cavity (about 37 °C), through which the retro-olfactory evaluation of the VOOs takes place, and of the shorter analysis time. Using a temperature that was 20 °C higher, for the same short time (60 °C/8 min), an increase in the intensity of the spots of all the volatile compounds, both associated with positive and negative attributes, was seen. These conditions improved the sensitivity of the analysis, but a higher temperature also led to variations in the chemical-physical balance between volatile compounds of the headspace, moving away from the quali- and quantitative equilibrium occurring in the mouth. Therefore, with the aim of establishing a rapid screening procedure to support the panel test, it was decided to adopt the temperature (40 °C) that was closest to that of organoleptic evaluation, while taking advantage of the short analysis time (8 min) proposed by Contreras et al. [19].

Gas carrier flow: constant flow (isobaric analysis) and flow ramp were compared. The former has the advantage of being extremely simple even for inexperienced operators, while the second improved the separation of spots obtained in heat maps, showing better resolution. The flow ramp conditions are described in detail in Section 2.4.

GC column temperature: a comparison between 40 °C and 55 °C [19] was carried out; it was decided to adopt a temperature of 40 °C, as an evident compression of the heat map in terms of retention time was observed at 55 °C, contrasting the positive effect of the flow ramp mentioned above.

3. Results and Discussion

3.1. Selected Volatile Compounds

One of the main objectives of the H2020 EU OLEUM project is to develop instrumental methods that support the panel test [10]. Many analytical efforts have been addressed by the research institutions involved to select a list of volatile compounds, focusing on the most relevant ones, that can define sensory characteristics, both fruity and defects. Finally, 18 volatile compounds were identified as the most relevant markers: it was also decided to split these selected compounds into two mixtures (SMA and SMB), depending mainly on the presence of each one at lower or higher concentrations in VOOs. Three markers of the 18 were excluded when performing this investigation, namely octane, pentanoic acid, and (E)-2-decenal. This was due to the chemical ionization of these analytes in the IMS region that occurs if the proton affinity of the analyte is greater than that with water [25]. The alkanes, to which octane belongs, have a proton affinity less than that with water: this means that these compounds will be more difficult to ionize, consequently causing low sensitivity of the GC-IMS towards them. (E)-2-decenal was also not considered due to the low sensitivity of the instrument towards it as well as its long retention time (51 min), which is not within the working range (0–32 min); an increase of the analysis time would make this analytical approach less attractive for screening purposes. Similar considerations also apply to pentanoic acid. This semi-targeted approach also made it possible to facilitate data elaboration due to the lower amount of raw data to be processed compared to an untargeted method.

3.2. Performance of the Method

3.2.1. Linearity

Table 1 shows that the linear range in the standard matrixes of almost all the 15 volatile compounds is narrower than the ranges discussed above. 6-methyl-5-hepten-2-one and propanoic acid showed a linear response for the entire concentration range considered for the SMA (0.05–10 mg kg^{-1}). The same was observed for 1-hexanol in the SMB (0.05–25 mg kg^{-1}). All other volatile compounds had smaller linear ranges; in particular, this was highlighted for ethyl acetate, ethyl propanoate, and ethanol (0.05–0.5 mg kg^{-1}). This behavior should be further investigated in the future, as especially in LOOs it is well known that some of these compounds are present even at much higher concentrations [8]. Nonetheless, it should be underlined that quantification of these molecules was not one of the main objectives of this method, as it is proposed for a semi-targeted screening. Despite this, the possibility to use this instrument for quantification purposes, with the use of an internal standard and as an alternative to other techniques (e.g., SPME-GC–MS), would be interesting to investigate.

Table 1. Parameters considered for evaluation of the linearity of the volatile compounds in standard mixtures SMA (from compound 1 to compound 7) and SMB (from compound 8 to compound 15). The compounds are arranged by retention time in the respective SMA and SMB.

Volatile Compounds	Rt [a] (s)	Dt [b] (ms)	Calibration Curve Equation	Linearity Range (mg kg^{-1})	(R^2) [c]
1. Ethyl acetate	170	10.908	y = 672.5x + 70.5	0.05–0.5	0.980
2. Ethyl propanoate	230	11.844	y = 549.7x + 9.6	0.05–0.5	0.978
3. Propanoic acid	218	9.102	y = 15.3x + 68.4	0.05–10	0.932
4. 3-methyl-1-butanol	259	12.203	y = 279.9x + 43.6	0.05–1.5	0.986
5. (*E*,*E*)-2,4-hexadienal	522	11.827	y = 87.3x + 27.8	1.5–10	0.982
6. (*E*)-2-heptenal	639	13.71	y = 18.4x + 175.6	1.5–10	0.969
7. 6-methyl-5-hepten-2-one	749	9.588	y = 72.2x + 162.5	0.05–10	0.994
8. Ethanol	121	9.255	y = 345.4x + 150.4	0.05–0.5	0.980
9. Acetic acid	149	9.434	y = 14.5x + 42.7	0.10–25	0.982
10. Hexanal	317	12.723	y = 198.3x + 23.3	0.05–1.5	0.991
11. (*E*)-2-hexenal	404	12.358	y = 47.3x + 7.3	0.10–10	0.989
12. 1-hexanol	450	13.415	y = 32.9x + 83.8	0.05–25	0.988
13. 1-octen-3-ol	733	9.451	y = 33.0x + 176.2	0.05–20	0.996
14. (*Z*)-3-hexenyl acetate	846	14.908	y = 6.9x + 281.7	5.0–25	0.989
15. Nonanal	1554	12.128	y = 5.1x + 138.0	0.05–15	0.990

[a] retention time; [b] drift time; [c] determination coefficient.

3.2.2. Intra-Day and Inter-Day Repeatability

Figure 1 shows the signals corresponding to the selected volatile compounds described in Section 2.6.2. The RSD% values for intra-day repeatability, calculated on the maximum intensity of the compound areas selected for the three quality grades, ranged from 1.0 to 1.7, with the only exception being hexanal, which had a higher value of 5.0. In the case of inter-day repeatability, the RSD% intervals were similar to those obtained in the intra-day experiment, with lower repeatability for ethyl propanoate (3.3) and hexanal (6.7). In any case, all these values are widely acceptable and comparable with those found in the literature [17,19,26,27]. From a recent study by Contreras et al. [28], it was observed that, working with the HS-GC-IMS in isothermal mode, the ethanol dimer signal (shown in Figure 1B) partly co-eluted with a ghost signal in the Rt and Dt dimensions [1]. For this reason, in this investigation a distinction between the ethanol signal and the ghost signal was difficult; therefore, the area considered for ethanol was given by the sum of the dimer signal plus the ghost signal.

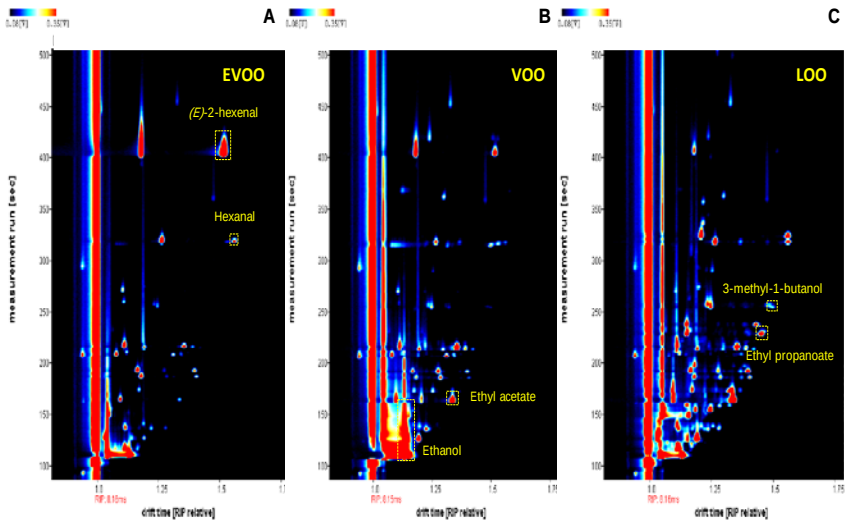

Figure 1. Heat maps in which the signals corresponding to the volatile compounds selected for the evaluation of intra- and inter-day repeatability have been indicated. (**A**) extra virgin olive oil (EVOO) sample with highlighted signals of (*E*)-2-hexenal and hexanal; (**B**) virgin olive oil (VOO) sample with highlighted signals of ethyl acetate and ethanol; (**C**) lampante olive oil (LOO) sample with highlighted signals of 3-methyl-1-butanol and ethyl propanoate.

3.3. Results of the Semi-Targeted Chemometric Models for the Quality Grade Classification and on the Presence of the Defects

The score plot of the first two PCs (35.71, and 13.36%) obtained by the PCA is shown in Figure 2A. Clear separation between the EVOO and LOO samples can be seen, while the VOOs are dispersed among the EVOOs and LOOs. The effect of the variables on each component and according to the contribution in the group separation were evaluated by a loading plot (Figure 2B). For the PC1, the greater contribution is due to the (*E*)-2-hexenal, acetic acid, 3-methyl-1-butanol and ethyl propanoate, while PC2 was strongly influenced by hexanal and ethyl acetate.

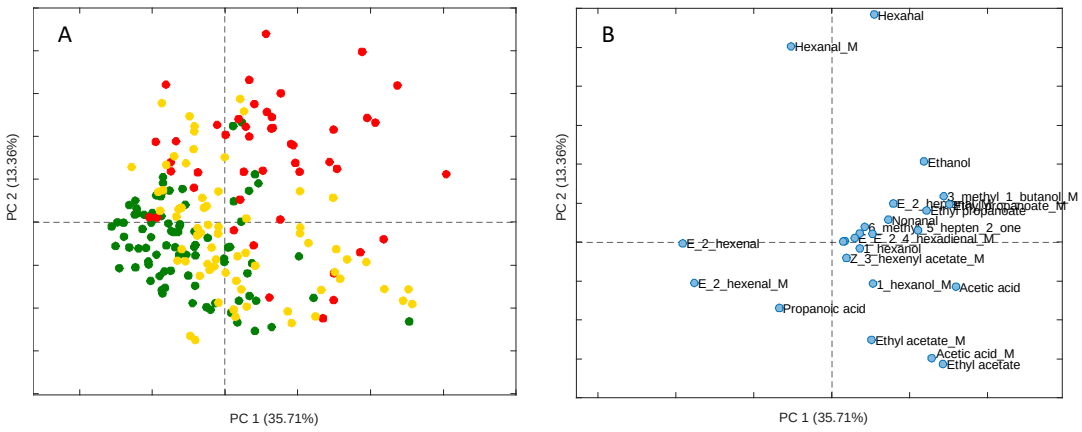

Figure 2. Score plot (**A**): green (EVOO), yellow (VOO), red (LOO); loading plot (**B**) obtained by principal component analysis (PCA).

Concerning the PLS–DA results, the values of the estimated Y variable (quality grades) obtained by the model in cross and external validation are shown in Figure 3A,B. The dotted line identifies the threshold value used to define the categorization of samples to different classes. In particular, the examples of two PLS–DA models are shown: Figure 2A represents the EVOO vs. no-EVOO model, while Figure 2B shows the LOO vs. no-LOO model.

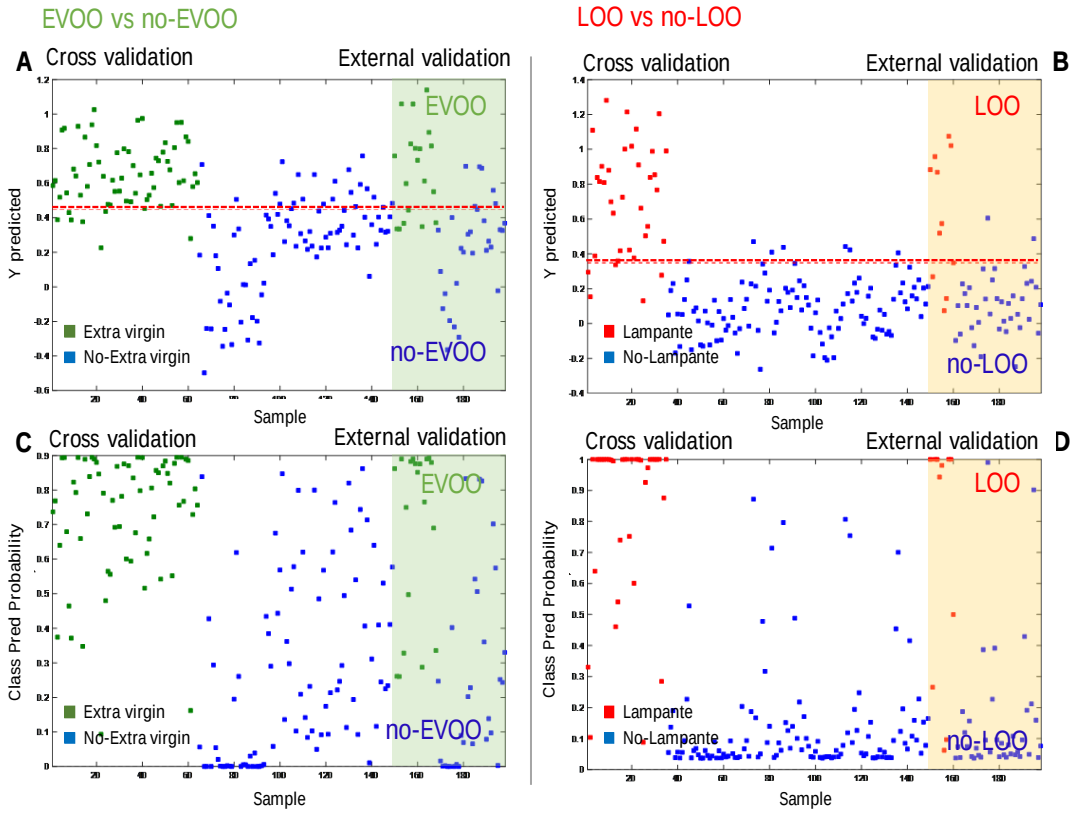

Figure 3. Graphical results obtained from 2 of the 4 partial least squares—discriminant analysis (PLS–DA) models for prediction of quality grade of virgin olive oils (VOOs). (**A**,**B**): values of the estimated Y variable by the model, extra virgin olive oil (EVOO) vs. no-EVOO (**A**) and lampante olive oil (LOO) vs. no-LOO (**B**), in cross and external validation. (**C**,**D**): values of the class prediction probability by the model, EVOO vs. no-EVOO (**C**) and LOO vs. no-LOO (**D**), in cross and external validation.

The results, in terms of percentage of correctly classified samples, are reported in Table 2; the percentages ranged from 67% to 95%. Considering the external validation data, the best result in terms of prediction was obtained for the LOO vs. no-LOO model (95%), while the worst was the EVOO vs. VOO model (67%). This is likely due to the fact that some of the VOO samples could be considered as borderline compared to EVOOs since they have similar profile patterns of volatile compounds, and are more difficult to be discriminated by the EVOO vs. VOO model. The results are comparable with those found in similar studies [11,19]. In the targeted approach by Contreras et al. 2019 [19], the results, in terms of prediction obtained by the models, are in agreement with those reported herein. In particular, the highest percentages of correctly classified samples are obtained for the LOO vs. no-LOO model. Similar results (84% of samples correctly classified, calculated as mean % among the three commercial categories) have also been obtained from PLS-DA models based on the SPME-GC-MS analysis, as in the study by Quintanilla-Casas et al. 2020 [11] where an EVOO vs. no-EVOO followed by VOO vs. LOO approach was applied.

Table 2. Percentages of correctly classified samples by the 4 PLS–DA models for the quality grade classification of VOOs (EVOO vs. no-EVOO; LOO vs. no-LOO; VOO vs. LOO; EVOO vs. VOO).

Category	Calibration	Cross Validation	External Validation
EVOO	91%	89%	74%
no-EVOO	84%	75%	77%
LOO	89%	86%	73%
no-LOO	94%	94%	95%
VOO	92%	91%	87%
LOO	83%	76%	77%
EVOO	74%	73%	70%
VOO	80%	80%	67%

For all PLS-DA models, sensitivity (number of samples predicted as in the class divided by number actually in the class) and specificity (number of samples predicted as not in the class divided by actual number not in the class) were evaluated by receiver operating characteristic (ROC) curves (Figure 4). For each model, the sensitivity and 1-specificity are marked by a red circle. The area under the curve (AUC) identifies the degree of discrimination. The best discrimination was achieved for the LOO vs. no-LOO model (AUC = 0.9083), while the worst was observed for the EVOO vs. VOO model (AUC = 0.7733) as confirmed by the classification percentage.

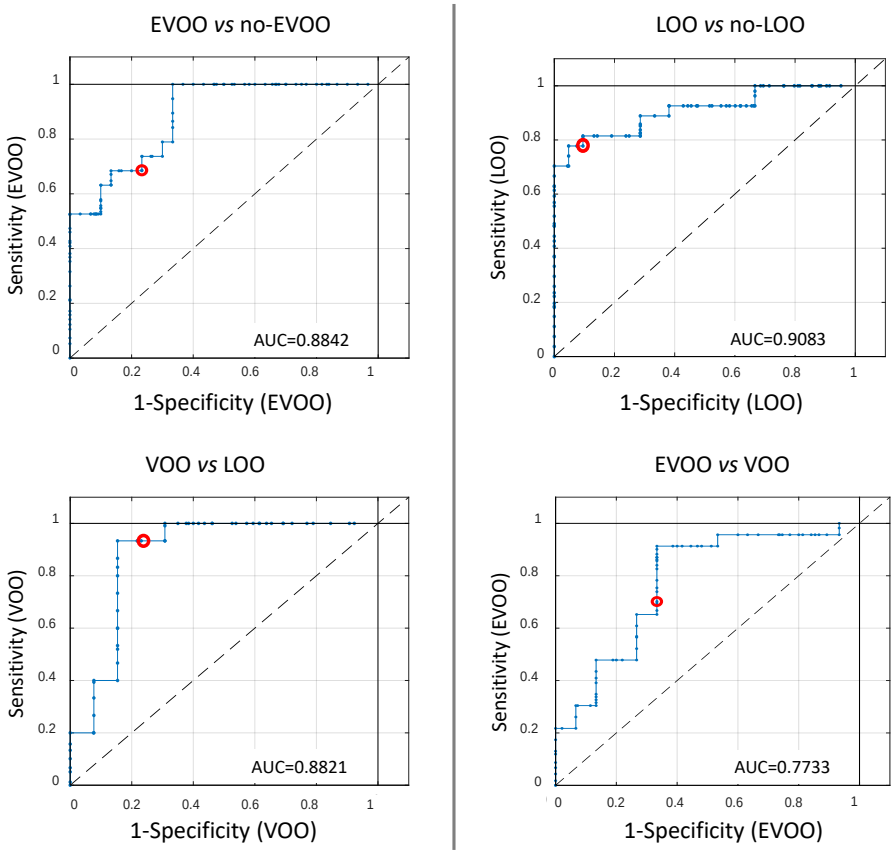

Figure 4. Receiver operating characteristic (ROC) curves of PLS-DA models used to discriminate samples according to quality grade. The red circle identifies selected sensitivity and 1-specificity values for the prediction model.

The VIP (variable importance in projection) score obtained by the PLS-DA models shows that the volatile compounds with the highest contribution to sample discrimination, as shown in Figure 5, are (E)-2-hexenal and hexanal for EVOOs, while they also include 3-methyl-1-butanol, ethyl propanoate,

and propanoic acid for LOOs, in agreement with those evaluated by PCA. In reality, these molecules are well-known markers associated with the fruity attribute or with sensory defects [8,20].

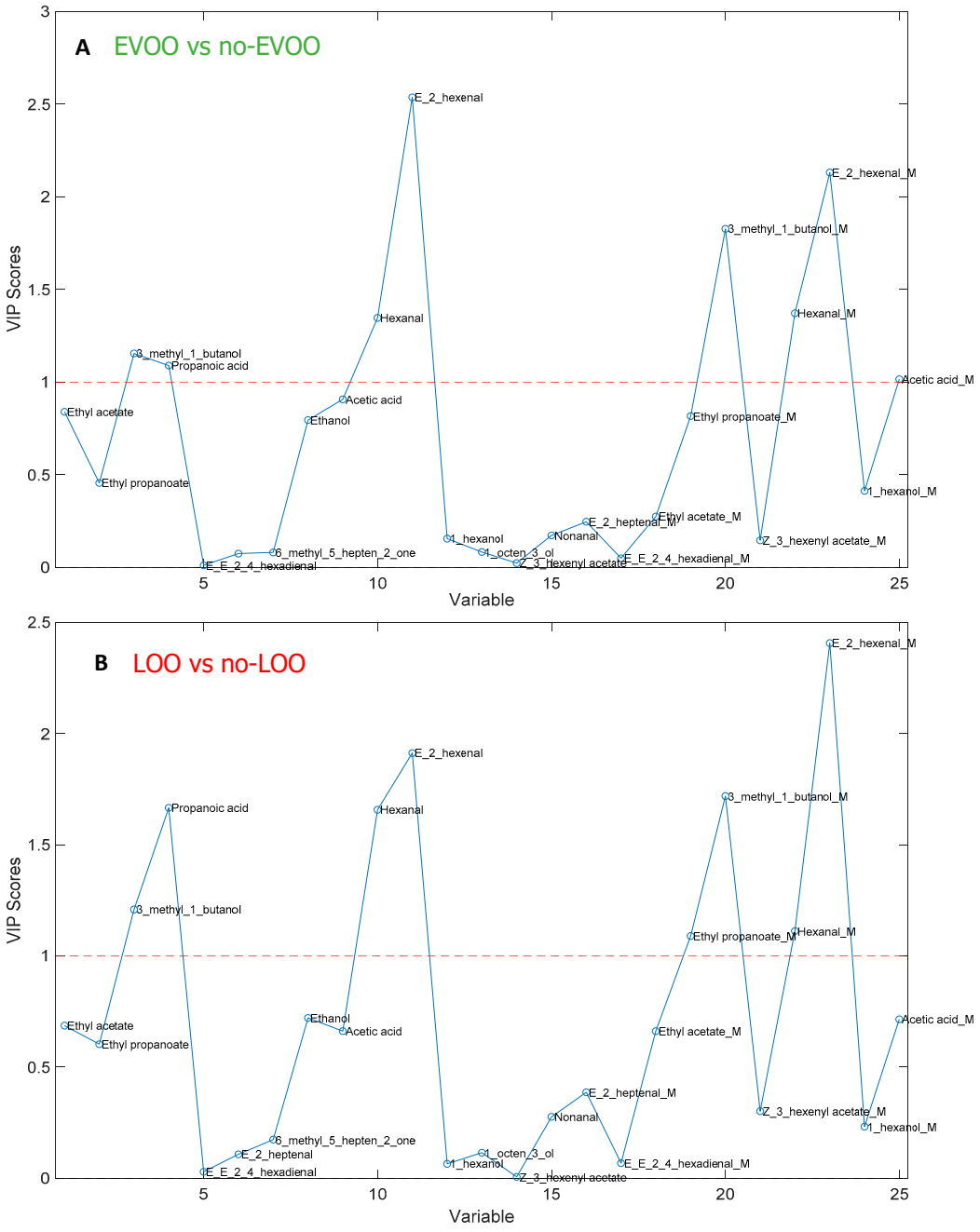

Figure 5. (**A**) Variable importance in projection (VIP) score obtained by the EVOO vs. no-EVOO model. (**B**) Variable importance in projection (VIP) score obtained by the LOO vs. no-LOO model.

The results in terms of probability in belonging to the different categories are shown in Figure 3C,D. Figure 3C refers to the category EVOO, while Figure 3D refers to category LOO: the higher a sample is placed in the graph, the higher the probability for which it is classified accordingly to quality grade. As a consequence, samples classified as no-EVOO for Figure 3C and no-LOO for Figure 3D are located in the bottom area of the graph. In Figure 3C, it can be seen that 63% of EVOO samples and 70% of no-EVOO are classified with a probability higher than 70%. For the LOO and no-LOO samples, the corresponding percentages were 63% and 87%, respectively (Figure 3D).

The percentage values of correctly classified samples, obtained from the PLS-DA models based on the presence of 3 sensory defects (musty, rancid, fusty/muddy sediment), are shown in Table 3. The percentages ranged from 48% to 80%. The best result was obtained for the musty vs. no-musty model, even if the percentages (both in cross and external validation) for this model are not entirely satisfactory. The prediction of the presence/absence of a defect in VOO samples is very challenging. The complexity is also due to the fact that each defective sample analyzed was often characterized by more than one defect, as commonly occurs in VOOs. Future studies will aim to improve this issue by analyzing a greater number of defective samples.

Table 3. Percentages of correctly classified samples by the 3 PLS–DA models to determine the presence of defects in virgin olive oils (musty vs. no-musty; rancid vs. no-rancid; fusty/muddy sediment vs. no-fusty/muddy sediment).

Defects	Calibration	Cross Validation	External Validation
Musty	71%	63%	60%
No-musty	81%	80%	80%
Rancid	81%	78%	62%
No-rancid	69%	64%	64%
Fusty/muddy sediment	82%	79%	67%
No-fusty/muddy sediment	67%	58%	48%

4. Conclusions

The panel test is fundamental to discriminate the quality grade of EVOOs and to distinguish them from the virgin and lampante categories, which is relevant since the latter is not edible and must be subjected to refining.

This sensory analysis is strategic during both blending and bottling of VOOs and EVOOs carried out by olive oil companies, and within the quality control performed by official bodies. In all these cases, thousands of samples must be evaluated sensorially over the course of a year. To speed up this bottleneck, the proposed HS-GC-IMS method consists in a screening to pre-classify samples, before the panel test, into different clusters: (a) those with a probability of belonging to a commercial category greater than an established threshold (to be defined by each olive oil company, laboratory, or other user); (b) others (not reaching this threshold) that must be treated as insufficiently robustly classified. For the former, the execution of the panel test is less urgent than for the latter. In both cases, the result obtained in terms of prediction must be confirmed—or disconfirmed—by the panel test outcomes, which has legal value. An alternative or complementary use of the prediction result, in terms of confirmation or disconfirmation, can be in case of discordant classifications by different panels, where it can work as an additional information.

The promising models developed herein to predict the quality grade and presence of three sensory defects (musty, rancid, fusty/muddy sediment) provided percentages of correctly classified samples in external validation from 67% to 95%, for the quality grade prediction model, and from 48% to 80%, for the presence of each of the aforementioned defects.

Moreover, the method showed good results in terms of linearity and intra- and inter-day repeatability, although additional investigations are needed before it can be implemented commercially; furthermore, to test the performance of this approach, inter-laboratory tests involving independent laboratories will be carried out in the future.

For routine quality control, we suggest dividing the classification in two phases, firstly clustering LOO vs. no-LOO to identify non-edible samples (LOO) before being assessed by panelists, and then classifying EVOO vs. VOO. The reliability of the model can be improved upon by increasing the number of the samples to be included in the calibration, as long as they are robustly classified sensorially, e.g., by more panels with a decision tree, such as in the present paper. Furthermore, to establish its own predictive model, each laboratory could also select an internal threshold probability to

discriminate between samples with acceptable and uncertain classification, and integrate this analytical information into their respective traceability systems. The possibility to use a common prediction model in different laboratories, using the same analytical conditions, can also be explored in the future, depending on the reproducibility of the signals (to be evaluated in the upcoming inter-laboratory tests) and, secondly, given the effective availability and willingness of each laboratory to share their data with others. A calibration data sharing, e.g., in a databank that could be effectively used by official control bodies or to favor harmonization and proficiency of countries that apply the same standards to olive oil.

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Chemical Markers to Distinguish the Homo- and Heterozygous Bitter Genotype in Sweet Almond Kernels

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Abstract: Bitterness in almonds is controlled by a single gene (*Sk* dominant for sweet kernel, *sk* recessive for bitter kernel) and the proportions of the offspring genotypes (*SkSk*, *Sksk*, *sksk*) depend on the progenitors' genotype. Currently, the latter is deduced after crossing by recording the phenotype of their descendants through kernel tasting. Chemical markers to early identify parental genotypes related to bitter traits can significantly enhance the efficiency of almond breeding programs. On this basis, volatile metabolites related to almond bitterness were investigated by Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry coupled to univariate and multivariate statistics on 244 homo- and heterozygous samples from 42 different cultivars. This study evidenced the association between sweet almonds' genotype and some volatile metabolites, in particular benzaldehyde, and provided for the first time chemical markers to discriminate between homo- and heterozygous sweet almond genotypes. Furthermore, a multivariate approach based on independent variables was developed to increase the reliability of almond classification. The Partial Least Square-Discriminant Analysis classification model built with selected volatile metabolites that showed discrimination capacity allowed a 98.0% correct classification. The metabolites identified, in particular benzaldehyde, become suitable markers for the early genotype identification in almonds, while a DNA molecular marker is not yet available.

Keywords: *Prunus dulcis*; *Prunus amygdalus*; breeding; almond kernel; bitterness; genotype; benzaldehyde; chemical marker

1. Introduction

Almond (*Prunus dulcis* (Mill.), D. A. Webb; syn. *P. amygdalus*, Batsch.) is the main nut tree worldwide and almonds have an important commercial value, with an annual world production exceeding 3,000,000 tons in shell [1]. Sweet almond kernels are widely consumed raw or minimally processed, as well as used as an ingredient in food products. Genetic improvement programs for almonds in different countries such as Spain, Australia and the United States, have been selecting and

releasing cultivars with the best agronomic and industrial characteristics [2–6]. One of the important aspects in the manufacturing of almond products is bitterness, since the presence of bitter almonds in sweet almond batches is detrimental to the quality of the final product. Bitterness in almonds kernels is due to the presence of the cyanogenic glucoside amygdalin, which undergoes enzymatic hydrolysis by β-glucosidases upon disruption of tissues, to form glucose, hydrogen cyanide and benzaldehyde [7]. This enzymatic breakdown and the concomitant liberation of hydrogen cyanide and benzaldehyde are responsible for the marzipan-like and bitter taste of some kernels [8–10]. The precursor prunasin is produced in plant mother tissues and translocated into the developing kernel, where it is transformed in amygdalin [7]. Thus, the genotype of the mother plant controls kernel bitterness, which is the same for all the kernels of a tree [11–13].

A single gene controls the bitter character in almond with a sweet allele (*Sweet Kernel*, *Sk*) that is dominant over the bitter one (*sk*) [14–16]. The gene *Sk* has been mapped in linkage group five of the almond genome [17] and its chromosome 5 position and function were recently revealed [18,19]. After crossing, three possible genotypes are expected: homozygous *SkSk* (sweet), *sksk* (bitter), and heterozygous *Sksk* (sweet or semi-bitter). There is no genetic distinction between sweet and semi-bitter cultivars, but Dicenta and García [12] suggested that semi-bitter forms correspond to heterozygous trees (*Sksk*) in which the recessive allele may induce some slightly bitter taste. All the semi-bitter forms are heterozygous, but not all the heterozygous forms are semi-bitter.

Almond is an outcrossing species, mostly self-incompatible, that has been made self-compatible through domestication and breeding. Commercially, there are orchards of self-incompatible cultivars in USA and Australia and self-compatible (self-fertile) cultivars mainly in the Mediterranean region. Usually, cross- or self-pollination, respectively, is favored using beehives in the orchards, which are open-pollinated. In almond scion breeding programs, many cultivars used as progenitors are heterozygous, and homozygous bitter progenitors can sometimes be advantageous to introduce some favorable agronomic traits in the progeny [16]. After crossing, the ratio of each genotype (*SkSk, Sksk, sksk*) in the offspring depends on the progenitors' genotype. When one of the progenitors has a dominant homozygous genotype (*SkSk*) the entire progeny shows a sweet phenotype, but the descendants with bitter phenotype are around 25% when crossing two heterozygous cultivars and around 50% when crossing heterozygous with recessive homozygous ones [12]. As seedlings with bitter phenotype must be discarded during the selection process, the efficiency of the genetic improvement programs can be significantly enhanced by reducing the crossing of heterozygous individuals among them. With this scope, a classification of the genitor cultivars into homozygous or heterozygous for the sweet character is necessary. At present, the genotype of almond cultivars and selections is deduced after crossing by recording the phenotype of their descendants through kernel tasting, and quantifying the seedlings with sweet and bitter kernel [12,20], because molecular markers are not well developed yet to be useful [17,21]. This implies a long waiting time until cropping (3–4 years). All semi-bitter descendants can be classified as heterozygous (*Sksk*) according to Dicenta and García [12], but their differentiation from sweet ones is difficult and requires a trained sensory panel, and this criterion would not consider the rest of heterozygous cases presenting a completely sweet kernel.

Some efforts have been made to find chemical markers for early genotype identification in almond cultivars and selections used in breeding programs. With this aim, the content of amygdalin in almond kernels has been monitored as a function of the phenotype and genotype of several almond cultivars [22,23]. Although a clear difference was evidenced in the content of amygdalin between sweet and bitter almond kernels, a high variability was observed within the sweet phenotype. In fact, amygdalin in bitter cultivars ranged from 2000 to 60,000 mg/kg, while in semi-bitter and sweet cultivars it ranged from 20 to 1772 mg/kg and from not detectable (n.d.) to more than 200 mg/kg, respectively [9,24], thus presenting overlapping ranges of amygdalin concentration. In particular, the amygdalin content did not allow a clear distinction between sweet kernelled heterozygotes (*Sksk*) and sweet kernelled homozygotes (*SkSk*), in which it fluctuated from 18.7 to 80.2 mg/kg and from n.d. to 55 mg/kg, respectively [7,22]. These results suggest that even though amygdalin has a clear correlation

with bitterness, this marker is not completely effective in predicting slight differences in bitterness such as those existing between sweet and semi-bitter kernels, and even less effective in detecting possible differences between sweet homo- and heterozygotes. This could be due to the performances of the analytical methods applied for the determination of the cyanogenic glucoside, or to the existence of secondary factors linked to the recessive allele affecting the production of benzaldehyde or other compounds causing bitterness perception.

According to Wirthensohn et al. [10] the overlap of the concentration ranges in sweet and semi-bitter kernel indicates that amygdalin may not be the only compound defining the marzipan-like flavor in sweet almonds. Some authors have pointed out the close correlation between bitter marzipan-like flavor and benzaldehyde, one of the amygdalin catabolites, even at low bitterness intensities assessed in sweet almond cultivars [25]. In addition, other almond volatile compounds, such as benzyl alcohol, revealed higher values in bitter almonds than in sweet almonds [26], and their levels tend to be higher in almonds with higher levels of benzaldehyde [25,27].

On this basis, the concentration of benzaldehyde and other related volatile compounds was monitored in 42 homozygous and heterozygous almond cultivars and selections, with the aim of identifying suitable chemical markers to classify sweet kernel almonds according to their genotype (homozygotes or heterozygotes). With this aim, a Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS) method was optimized and applied to 244 almond samples obtained from 124 different trees.

2. Materials and Methods

2.1. Samples

Almonds (*Prunus dulcis* (Mill.), D. A. Webb; syn. *P. amygdalus*, Batsch.) of 41 different cultivars and selections and one feral tree were studied. For 37 of these, their genotypes were previously reported in the literature [12,17,20,28] or determined by IRTA's almond breeding program. In agreement with these sources, the 42 cultivars and selections consisted of 22 homozygous and 14 heterozygous sweet kernel cultivars, five selections without known genotype and one reference bitter feral tree. Few of these heterozygous cultivars ('Tuono', 'Guara', 'Genco') are described as semi-bitter, although no precise and objective criteria have been set for this classification. Hereinafter, all the samples except the bitter one will be considered as sweet kernel almonds. A total of 244 almond samples were obtained from 124 different trees (Table 1). These samples were produced in 2012 and 2015 in different geographical areas: Constantí and Gandesa in Tarragona and Les Borges Blanques in Lleida (Catalonia, Spain). Out of the 42 cultivars, 10 (8 homozygous and 2 heterozygous) were analysed both in 2012 and 2015. Almonds were collected, shelled and blanched by hand, then packed under vacuum, stored at 2–8 °C, and analysed within three months.

Table 1. Almond samples' pedigrees, harvesting year, tree and sample number, *Sk* genotype and bitterness.

	Cultivar/Selection	2012 (*n*)		2015 (*n*)		Genotype [a]	Ref.	Bitterness [b] (0–10)
		Trees	Samples	Trees	Samples			
1	IRTA-7 (Lauranne × OP [c])	8	17			unknown	[d]	na [e]
2	IRTA-9 (Masbovera × Lauranne)	2	4			*SkSk*	[d]	na
3	IRTA-4 (A-202 × FGFP092)	3	5	1	2	*SkSk*	[d]	na
4	IRTA-10 (4-665 × Lauranne)	2	4			*SkSk*	[d]	0
5	IRTA-12 (4-665 × Lauranne)	3	6			*SkSk*	[d]	na
6	IRTA-11 (Primorskyi × Cristomorto) × IRTA-7)	2	4			unknown	[d]	na
7	IRTA-8 (Anxaneta × IRTA-4)	2	4			*SkSk*	[d]	0.7
8	Belona (Blanquerna × Belle d'Aurons)	2	4			*SkSk*	[d]	na
9	Cambra (Ferragnes × Tuono [f])	2	4			unknown		na
10	Constantí (FGFD2 × OP)	7	14			*SkSk*	[d]	na
11	Desmayo Largueta (Spanish local)	2	4			*Sksk*	[20]	1.8
12	Felisia (Titan × Tuono)	2	4			unknown	[d]	na
13	Ferragnes (Cristomorto × Aï)	2	4	1	2	*SkSk*	[12,20]	0.5

Table 1. *cont.*

	Cultivar/Selection	2012 (*n*)		2015 (*n*)		Genotype [a]		Ref.	Bitterness [b] (0–10)
		Trees	Samples	Trees	Samples				
14	Francolí (Cristomorto × Tuono)	7	13	1	2	*SkSk*		[20]	0
15	Glorieta (Primorskiy × Cristomorto)	6	14	1	2	*SkSk*		[20]	0
16	Guara (syn. Tuono)	6	11	1	2		*Sksk*	[28]	2.8
17	Lauranne (Ferragnes × Tuono)	2	4			*SkSk*		[20]	0.3
18	Marcona (Spanish local)	2	4	1	2		*Sksk*	[20]	0.3
19	Marinada (Lauranne × Glorieta)	7	13	1	2	*SkSk*		[d]	0.2
20	Marta (Ferragnes × Tuono)	3	5				*Sksk*	[17]	na
21	Masbovera (Primorskiy × Cristomorto)	6	11	1	2	*SkSk*		[20]	0.3
22	Nonpareil (Californian reference)	2	4				*Sksk*	[17]	1.1
23	Soleta (Blanquerna × Belle d'Aurons)	5	10				unknown		0.4
24	Tarraco (FLTU18 × Anxaneta)	6	12	1	2	*SkSk*		[d]	0.6
25	Vairo (4-665 × Lauranne)	6	12	1	2	*SkSk*		[d]	0.3
26	IRTA-2 (A-60 × A-192)			1	2	*SkSk*		[d]	0.6
27	IRTA-1 (Wawona × Lauranne)			1	2		*Sksk*	[d]	0.3
28	IRTA-3 (4-665 × Lauranne)			1	1	*SkSk*		[d]	0.6
29	4-665 (Primorskiy × Cristomorto)			1	2	*SkSk*		[20]	0
30	Cristomorto (Italian local)			1	2	*SkSk*		[20]	0.4
31	Falsa Barese (Italian local)			1	2		*Sksk*	[20]	1.3
32	FGFP092 (Ferragnes × Filippo Ceo)			1	2		*Sksk*	[20]	0
33	FGTR13 (Ferragnes × Troito)			1	2		*Sksk*	[20]	2.1
34	FLTU18 (Ferralise × Tuono)			1	2		*Sksk*	[20]	0.3
35	Gabaix (Spanish local)			1	2		*Sksk*	[20]	0.3
36	Garbí (Cristomorto × OP)			1	2	*SkSk*		[20]	0.4
37	Genco (Italian local)			1	2		*Sksk*	[12,20]	3.5
38	Primorskiy (Princess × Nikitskiy)			1	2	*SkSk*		[12,20]	0.3
39	Ramillete (Spanish local)			1	2	*SkSk*		[12,20]	0.4
40	Stelliete (Ferragnes × Tuono)			1	2		*Sksk*	[20]	1.8
41	Tuono [f] (Italian local)			1	2		*Sksk*	[12,20]	0.7
42	Bitter almond (Spanish feral)			1	2		*sksk*	[d]	10

[a]: Genotype: *SkSk*, sweet homozygous; *Sksk*, sweet heterozygous; *sksk*, bitter homozygous. [b]: Bitterness: intensity on a 0–10 sensory scale, assessed by IRTA panel and obtained by averaging data of 1, 2 or 3 harvest years (unpublished data); [c]: OP, open pollinated; [d]: IRTA's breeding records, unpublished; [e]: na: not available; [f]: Tuono (syns. Troito, Mazzeto and Guara).

2.2. Chemical Reagents

4-Methyl-2-pentanol, ethyl acetate, hexanal, 1-Penten-3-ol, 3-Methylbutan-1-ol, 1-Hexanol, nonanal, 1-Heptanol, benzaldehyde, phenylethyl alcohol and benzyl alcohol were from Sigma-Aldrich Co (St. Louis, MO, USA). Ultrapure water (Milli-Q Millipore Corporation, Billerica, MA, USA) was used.

2.3. Sample Preparation and Solid Phase Microextraction (SPME) Conditions

The SPME fiber divinylbenzene/carboxen/polydimethylsiloxane fiber (50/30 µm, 2 cm long from Supelco Ltd., Bellefonte, PA, USA) was selected as being the most suitable for compounds with a wide range of molecular weight and polarity. The extraction of volatiles was performed on a suspension of ground almonds in aqueous solution on the basis of preliminary results obtained by comparing the uptake of volatiles obtained from ground almonds (1 g) and from ground almonds in aqueous suspension (1 g in 2 mL of ultrapure water). A multilevel factorial experiment was then applied to optimize the rest of the parameters affecting the extraction of volatile compounds: extraction temperature (40, 50, 60 °C), extraction time (20, 30, 40 min), sample amount (1, 1.5 g) and pH of the suspension (3.5, 7). The optimized factorial design consisted of 20 experiments performed in duplicate and randomized (Supplementary Table S1). The dependent variables were the GC-MS responses of 12 representative compounds of the volatile profile, belonging to different chemical families (Table 2). The influence of the different factors was evaluated by means of a normalized Pareto diagram, elaborated with the chromatographic responses of each analyte in the different extraction conditions. The optimal value of each factor involved in the extraction was statistically calculated and the best extraction conditions were chosen for the analysis.

Table 2. Results of the factorial design: optimal extraction conditions based on the regression models for the factors that significantly influenced extraction ($p < 0.05$).

RT [a] (min)	Compound	T [b] (°C)	T [c] (min)	pH [d]	Sample [e] (g)
6.36	hexanal	60	40	7	ns [f]
7.30	2-Methy-1-propanol	40	ns	ns	1
7.98	2-Pentanol	40	ns	ns	ns
10.17	1-Penten-3-ol	ns	40	ns	ns
12.01	3-Methyl-1-butanol	ns	40	ns	1
16.68	2-Methyl-3-buten-1-ol	60	40	7	ns
17.95	1-Hexanol	60	40	7	ns
19.23	nonanal	60	40	ns	ns
21.92	1-Heptanol	60	ns	ns	1.5
24.10	benzaldehyde	60	40	ns	ns
32.69	benzyl alcohol	60	40	ns	ns
33.26	phenylethyl alcohol	60	40	ns	ns

[a]: RT, retention time; [b]: T, temperature (40; 50; 60 °C); [c]: t, time (20, 30, 40 min); [d]: pH, 3.5; 7; [e]: Sample weight, 1 g; 1.5 g; [f]: ns, not significant.

Finally, almond samples were analysed as follows: 10 g of skinless almonds were ground during 1 min using a domestic grinder (Iberica Group, Barcelona, Spain), then 1 g of the sample was suspended in 2 mL of ultrapure water (pH 7) in a 10 mL vial. The sample was spiked with 4-methyl-2-pentanol (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.5 μg/g of almonds and sealed with a PTFE-silicone septum. The vial was placed in a water bath at 60 °C under magnetic stirring, and the SPME fiber was maintained for 40 min in the sample headspace. The volatile compounds of the fiber were desorbed for 1 min at 260 °C in the gas chromatograph injection port.

Intra-day repeatability was assessed by analyzing the same almond sample five times and calculating the percent relative standard deviation (Supplementary Table S2).

2.4. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analyses were performed in 2012 on a Thermo Scientific Trace GC Ultra coupled to a quadrupole mass selective spectrometer DSQ II (Thermo Scientific, Bremen, Germany) and in 2015 on an Agilent GC 6890N coupled to a quadrupole mass selective spectrometer 5973 (Agilent Technology, Palo Alto, CA, USA). Both were equipped with a split-splitless injection port. Helium was the gas carrier, at linear velocity of 1 mL/min. The separation of the volatiles was performed by a column Supelcowax-10 (30 m × 0.25 mm i.d., 0.25 μm film thickness), purchased from Supelco Ltd (Bellefonte, PA, USA). The temperature of the column was held at 40 °C for 5 min and increased to 250 °C at 6 °C/min. Electron impact mass spectra were recorded at 70 eV ionization energy in the 35–250 *m/z* range, 2 scan/s.

Volatile compounds were identified by comparison of their mass spectra and retention times with those of standard compounds or tentatively identified by comparing their mass spectra with the reference mass spectra of the Wiley 6.0 library and their linear retention indices with those reported in the literature. For quantitative analysis, relative amounts of volatile compounds were calculated by using the internal standard method. The compounds were quantified by considering the relative response factor to be 1 and were expressed as micrograms per gram equivalents of 4-Methyl-2-pentanol.

2.5. Statistical Analysis

Statistical elaboration for the optimization of the SPME conditions was carried out using Statgraphics Plus 5.1© (Statgraphic Technologies Inc., The Plains, VA, USA). Four factors were tested at three or two levels, as previously described. The factorial design consisted of 20 experiments performed in duplicate. The normalized results of the experimental design, evaluated at a significance level of 5%, were analysed using a standardized Pareto diagram, which shows a frequency histogram

where the length of each bar in the graph is proportional to the absolute value of its standardized effect. The significance of the factors studied and the optimal values for each factor were established by means of an ANOVA and a regression analysis of the model, respectively. The results were considered significant with values of $p < 0.05$.

Univariate statistical analysis was performed with SPSS software v25© (IBM Corp., NY, USA). Student's t-test was applied to compare homo- and heterozygous groups, and bilateral Pearson correlations were assessed between benzaldehyde and the compounds presenting significant differences by the t-test, and between benzaldehyde and bitterness. In all cases, $p < 0.05$ was considered significant. Analysis of variance by General Linear Model (GLM) of SPSS was carried out according to the harvest year and geographical production area.

Multivariate analysis was carried out with SIMCA software v13.0© (Umetrics AB, Sweden). With the variables selected by univariate statistics (6 variables) and after data pre-processing (scaling to unit variance), a Principal Component Analysis (PCA) was developed to explore the natural clustering of samples and detect potential outliers (according to Hotelling's T2 range and distance to the model parameters). A Partial Least Square-Discriminant Analysis (PLS-DA) classification model was then built with the same variables to classify the samples into homo- or heterozygous categories.

3. Results and Discussion

In almonds, individuals with sweet kernel phenotype can present homozygous (*SkSk*) or heterozygous (*Sksk*) genotype. To classify them according to this genotype, suitable metabolic markers were investigated after optimizing a proper analytical method.

3.1. Optimization of SPME-GC-MS Method for the Assessment of Volatile Compounds

A 29% increase in total chromatographic area was observed by analyzing ground almonds in suspension in comparison to dry extraction (Supplementary Table S3). This greater efficiency is justified by a better mass transfer due to a greater exposure of the surface of the almond particles compared to direct extraction, in which these particles tend to agglomerate. The presence of water could also favor enzymatic reactions leading to some volatiles related to almond bitterness [26].

Table 2 shows the optimal values for the extraction variables that were found to significantly influence the extraction of each volatile compound. The temperature and the extraction time were the parameters with the highest influence on volatiles uptake. As expected, for most compounds an increased chromatographic response was observed at 60 °C and 40 min. The compounds whose uptake was significantly influenced by pH showed a better extraction at pH 7. The amount of sample only showed a significant effect on few volatile compounds, and it was maintained at 1 g to favor a proper stirring during the extraction.

3.2. Univariate Statistical Analysis of Raw Almond Volatile Components

Thirty compounds were detected in the headspace of the samples under study (Supplementary Table S2), most of which were previously described in almonds [29,30]. To identify metabolites whose biogenesis could be related to the almond genotype (*SkSk, Sksk*), we focused on the compounds that presented significant differences between homo- and heterozygous almonds when assessed by univariate analysis (Table 3). Benzaldehyde, benzyl alcohol and 1-penten-3-ol presented significantly higher concentrations in kernels from heterozygous (*Sksk*) cultivars, while branched aldehydes 2- and 3-methylbutanal, and branched alcohols 2-Methylpropan-1-ol, 3-Methylbutan-1-ol, 3-Methyl-3-buten-1-ol and 3-Methyl-2-buten-1-ol were more abundant in homozygous (*SkSk*) ones. A relationship with the recessive allele could be hypothesized for those of them that presented clear trends according to the genotype: *SkSk<Sksk<sksk*, such as benzaldehyde and benzyl alcohol; or *SkSk>Sksk>sksk*, such as branched alcohols 2-Methylpropan-1-ol, 3-Methylbutan-1-ol, 3-Methyl-3-buten-1-ol and 3-Methyl-2-buten-1-ol (Table 3). All these compounds' results significantly correlated with benzaldehyde in all the sweet almond phenotypes (Table 3). On the contrary, branched

aldehydes and 1-Penten-3-ol did not follow any of these trends, and they did not significantly correlate with benzaldehyde, suggesting that their formation could be driven by varietal factors unrelated to the kernel bitterness. For this reason, they were not further considered as possible genotype markers in sweet almonds. Although the harvest year and the production area influenced the concentration of the selected volatiles (Supplementary Table S4), the differences between *SkSk* and *Sksk* groups were high enough to allow the differentiation of these genotypes in spite of the annual and geographical variability.

Table 3. Occurrence of main volatile compounds showing significant differences between genotypes (*SkSk, Sksk*) by Student's t-test, presented as mean ± standard deviation. Correlation of volatiles with benzaldehyde (in *SkSk, Sksk* samples) are also shown.

Compound	Concentration [a]			t-Test [b]	Pearson Correlation [c]	
	SkSk **(*n* = 153)**	***Sksk*** **(*n* = 150)**	***sksk*** **(*n* = 2)**	***p***	**r**	***p***
2-Methylbutanal	0.015 ± 0.011	0.007 ± 0.005	0.030 ± 0.000	<0.001	-	-
3-Methylbutanal	0.031 ± 0.019	0.013 ± 0.010	0.037 ± 0.004	<0.001	-	-
2-Methylpropanol	0.16 ± 0.15	0.070 ± 0.089	0.009 ± 0.000	<0.001	−0.236	<0.001
1-Penten-3-ol	0.092 ± 0.098	0.15 ± 0.15	0.011 ± 0.002	<0.001	-	-
3-Methylbutan-1-ol	0.91 ± 0.51	0.50 ± 0.44	0.034 ± 0.008	<0.001	−0.290	<0.001
3-Methyl-3-buten-1-ol	0.33 ± 0.15	0.23 ± 0.17	0.009 ± 0.001	<0.001	−0.213	<0.01
3-Methyl-2-buten-1-ol	0.29 ± 0.14	0.20 ± 0.13	0.012 ± 0.001	<0.001	−0.165	<0.01
benzaldehyde	0.88 ± 1.06	26.3 ± 10.7	129.7 ± 4.7	<0.001	1	-
benzyl alcohol	0.45 ± 0.31	1.29 ± 1.28	33.2 ± 2.5	<0.001	0.767	<0.001

[a]: mean concentration, expressed as µg equivalents of 4-Methyl-2-pentanol (IS)/g of almond; [b]: significance of the difference between *SkSk* and *Sksk* means as resulted by Student's t-test; [c]: bilateral Pearson correlation of compounds with benzaldehyde. Only significant correlations are reported.

While bitterness and marzipan-like flavor had been previously related to benzaldehyde and benzyl alcohol in semi-bitter and bitter almonds [10,25,31], no data were available about the occurrence of these compounds in sweet almonds according to their genotype. While benzaldehyde is known to proceed from amygdalin catabolism [7–9], the biosynthesis of benzyl alcohol in almonds has not been elucidated. Kwak et al. [26] documented that it is formed in bitter almond kernel by enzymatic reactions, which may consist of the reversible enzymatic reduction of benzaldehydes as described in other plants [32]. This would substantiate the association of benzyl alcohol with benzaldehyde and almonds' bitter character. In the same way, the enzymatic formation of branched alcohols was predominant in sweet rather than in bitter almond kernels [26], but it was unknown that these compounds were also predominant in homozygous sweet almond genotypes compared to heterozygotes.

Box-and-whisker plots were built to explore the concentration ranges of the selected compounds and their capacity to differentiate homo- and heterozygous sweet genotypes (Figure 1). While most of the compounds presented certain overlap in the ranges of homo- and heterozygous groups, benzaldehyde levels allowed a neat distinction between these groups. We report for the first time a discrimination between homo- and heterozygous sweet almond genotypes based on a chemical marker, which resulted from the analysis of more than 200 samples from 36 distinct cultivars. These results indicate that benzaldehyde performed better than reported for amygdalin to differentiate homo- and heterozygous sweet almond kernels [7,22]. This could be the consequence of a higher sensitivity in the detection of benzaldehyde, which led to differentiation even between kernels of very low bitterness. This was sustained by the significant correlation (Pearson correlation = 0.787, $p < 0.001$) between benzaldehyde and the mean bitterness intensity of the sweet cultivars under study, assessed by IRTA's almond sensory panel on samples from previous harvest years (Table 1). In addition, we could hypothesize that the accumulation of amygdalin in the kernel is not the only effect of the recessive bitter allele in heterozygotes, and that the latter could influence other enzymatic reactions

such as the catabolic routes yielding benzaldehyde and related compounds, as well as the synthesis of branched alcohols.

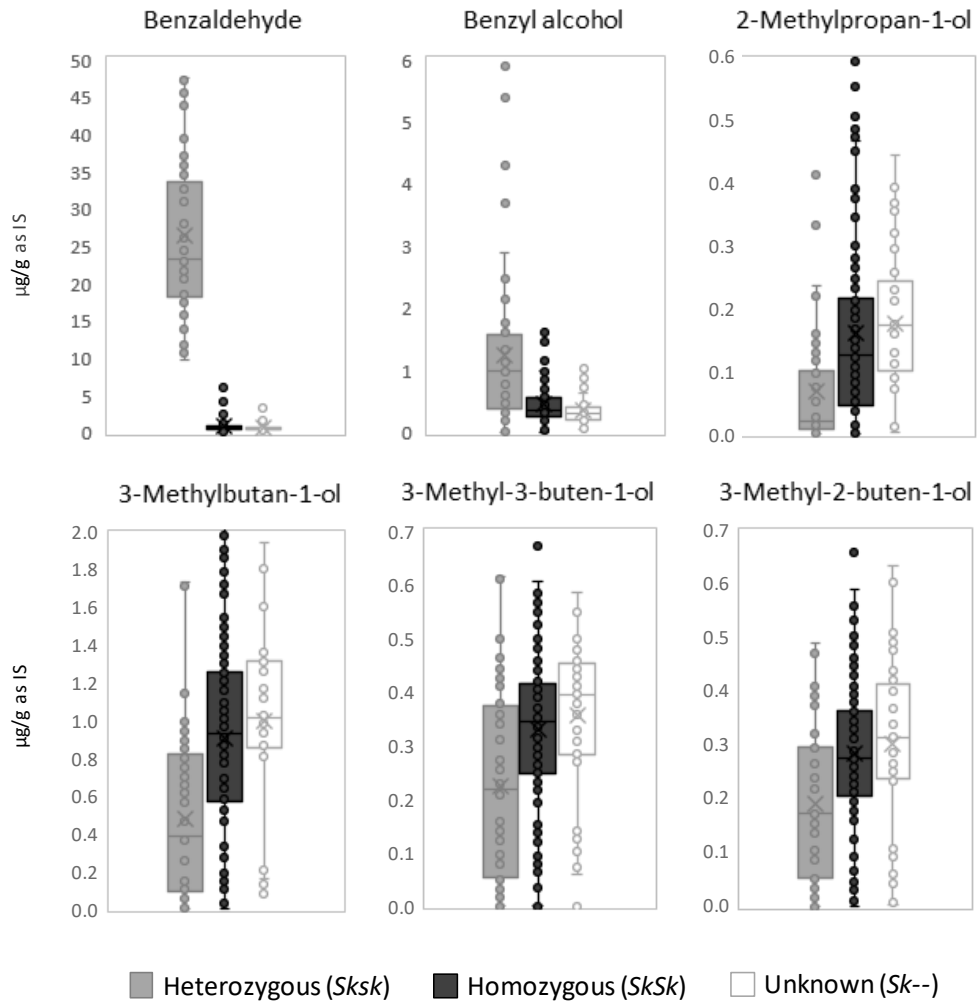

Figure 1. Box-and-whisker plots obtained for the selected variables in each group of sweet almond samples: heterozygous (*Sksk*), homozygous (*Sksk*) and samples with unknown (*Sk*–) genotype.

Benzaldehyde could represent a suitable chemical marker for the early genotype identification in almond cultivars and selections used in breeding programs. In this regard, samples from the five sweet almond selections without known genotype (IRTA-7, IRTA-11, 'Cambra', 'Felisia' and 'Soleta') were classified as homozygous cultivars (Figure 1). This classification may be verified once the bitter character segregation data are available in the progeny of these cultivars.

Although the homo- and heterozygous sweet almonds considered in the present study could be discriminated directly by their levels of benzaldehyde, all the metabolites whose biogenesis seemed to be linked to the almond genotype could be useful to support this classification as confirmation parameters or in multivariate models.

3.3. Multivariate Statistical Analysis of Raw Almond Volatile Components

A multivariate statistical approach based on various potential genotype markers was carried out to support the differentiation allowed by benzaldehyde with the aim of providing a more reliable classification tool. PCA was carried out with the biomarkers previously selected by univariate analysis (3 Principal Components (PCs) accounted for 94.7% of the total variance explained, no outliers detected). While PC1 seemed to depend on varietal characteristics not linked to the bitter allele

(data not shown), the scores and loadings plots corresponding to PC2 and PC3 confirmed that a clear differentiation between hetero- and homozygous individuals (Figure 2a) was driven by benzaldehyde and benzyl alcohol, and branched alcohols, respectively (Figure 2b). PC2 was the component that mainly contributed to the differentiation between hetero- and homozygous individuals (19.6% of explained variance). As expected, benzaldehyde was the variable that mainly contributed positively to this component, followed by benzyl alcohol (PC2 loadings 0.744 and 0.503, respectively), while 2-methyl propanol, 3-Methylbutan-ol, 3-Methyl-3-buten-1-ol and 3-Methyl-2-buten-1-ol were the ones mainly contributing negatively to this PC (PC2 loadings −0.310, −0.252, −0.140 and −0.113, respectively).

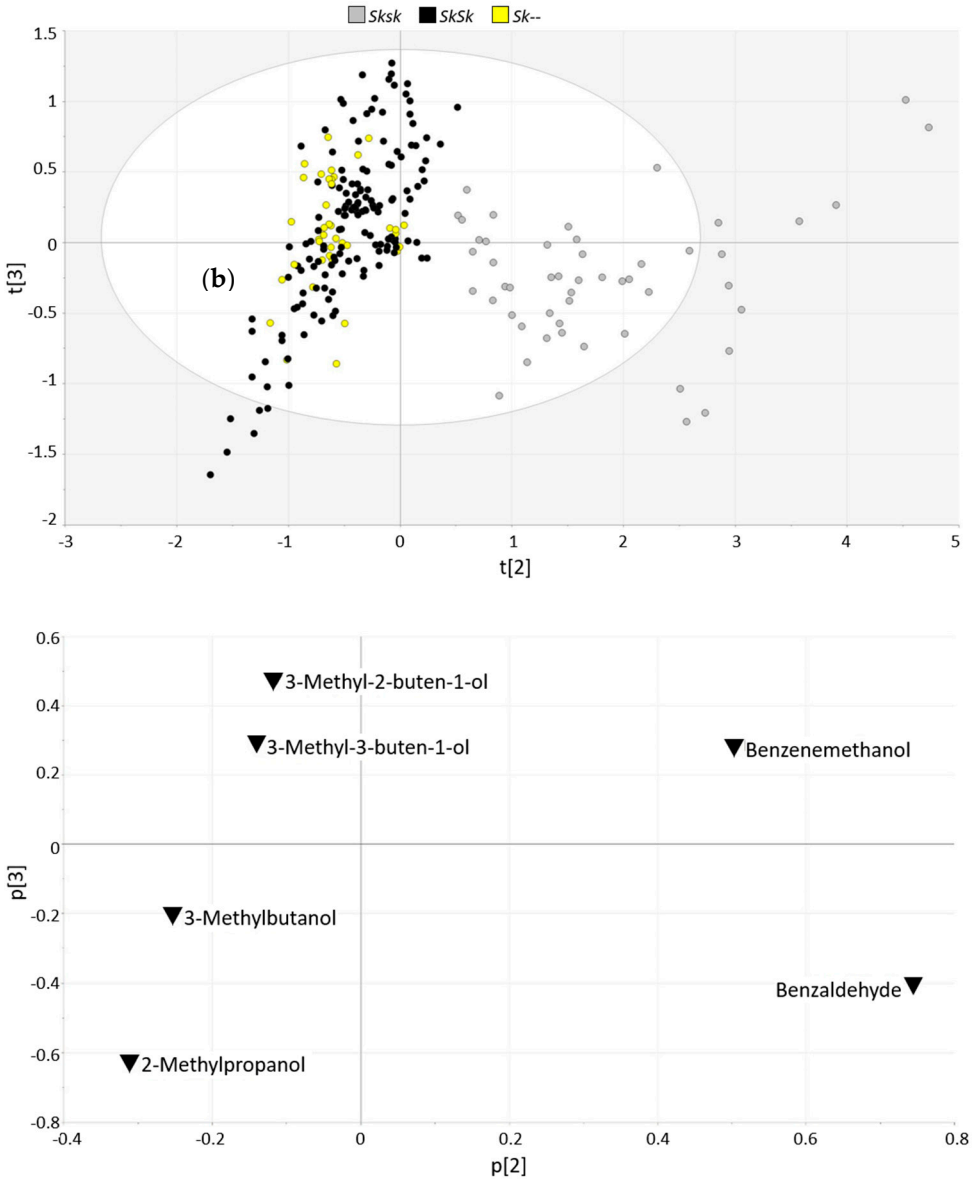

Figure 2. Principal Component Analysis (PCA) (n = 242, 6 variables, scaled to unit variance, 3 PC, 94.7% total variance explained). (**a**) Scores plot and (**b**) loadings plot corresponding to PC2 and PC3 (19.6% and 4.8% total variance explained, respectively).

On this basis, to dispose of a classification tool for sweet almonds based on all volatile compounds whose biogenesis seemed to be linked to their genotype, a supervised discriminant technique was applied to find the maximum correlation between the data and each of the categories of interest (heterozygous vs homozygous). A PLS-DA classification model developed according to the almond

genotype and based on the previously selected variables provided a 98.0% correct classification, as obtained by leave-10%-out cross-validation (Table 4). The corresponding predicted values are reported in Supplementary Table S5. The permutation test (n = 20) indicated that the model was not over-fitted according to the Q^2 scores (Model's Q^2 = 0.81, permutation models' Q^2 < 0). Moreover, PLS-DA regression coefficients confirmed the major role of benzaldehyde in the classification model and evidenced the lower but significant contribution of some branched alcohols (Figure 3).

Table 4. Classification results of the classification model (PLS-DA) developed to discriminate between homo- and heterozygous sweet almond categories (n = 203, 6 variables, scaling to unit variance; 3 latent variables), cross-validated by leave 10%-out.

	n	**Correct Classification**	***SkSk***	***Sksk***
SkSk (homozygous)	153	100%	153	0
Sksk (heterozygous)	50	92%	4	46
Total	203	98.03%	157	46

n = 203, Q^2 = 0.808, RMSEcv = 0.189, ANOVA p-value < 0.05.

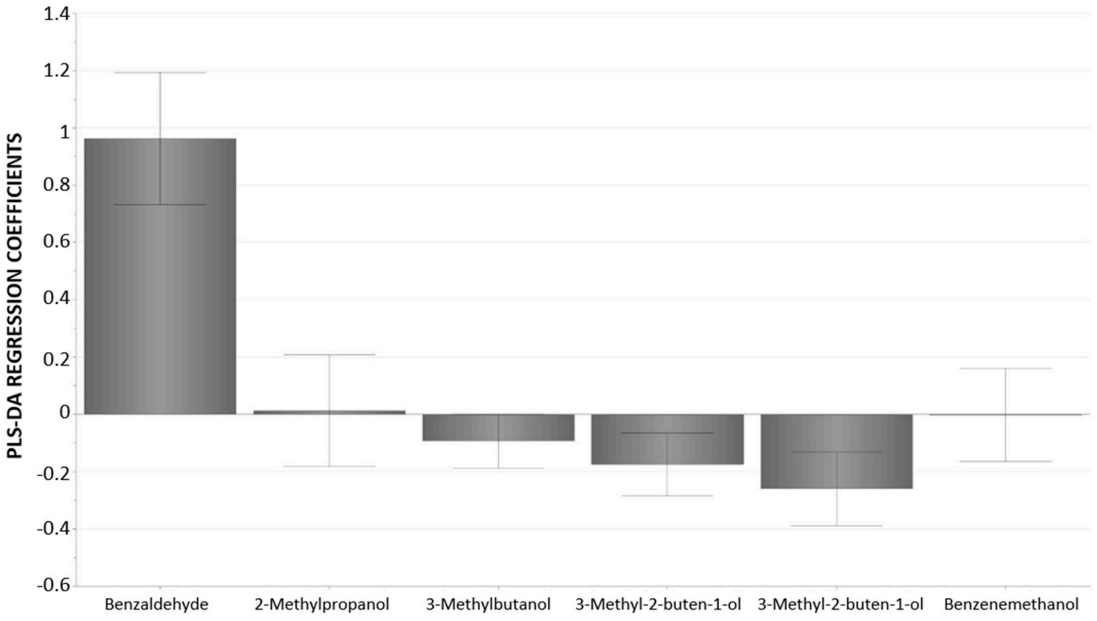

Figure 3. Partial Least Square-Discriminant Analysis (PLS-DA) regression coefficients for the heterozygous (*Sksk*) category, with confidence intervals derived from jack-knifing.

Four heterozygous samples out of 203 were misclassified by the PLS-DA, three 'Nonpareil' and one 'FGFP092'. Other samples from these cultivars were correctly classified by the model. All the samples from these cultivars could be well distinguished from homozygous samples by considering only the benzaldehyde content (Figure 1). The slight reduction in the classification efficiency observed by PLS-DA was compensated by a higher classification reliability, given by the application of an approach based on various independent variables.

According to the PLS-DA model, and in agreement with the benzaldehyde content, all the samples belonging to the five cultivars with unknown genotype were classified as homozygous, according to their predicted values (Supplementary Table S6). Such classification was feasible according to their genealogy.

4. Conclusions

In conclusion, the results obtained in this work evidenced the association between sweet almonds' genotype and some volatile metabolites and provided for the first time chemical markers to discriminate between homo- and heterozygous sweet almonds. In particular, the amount of benzaldehyde, assessed by a simple, rapid, automatable and affordable technique such as SPME-GC-MS allowed to differentiate between the homo- and heterozygous samples analyzed in the study (n = 203) and to tentatively classify almond kernels with unknown genotype (n = 39). Moreover, the PLS-DA classification model built with selected independent metabolites that had discrimination capacity and were thus more likely to provide a greater reliability to the classification, allowed 98.0% of correct category assignment. The selected metabolites, and in particular benzaldehyde, represent suitable chemical markers for the early genotype identification in almond cultivars and selections used in breeding programs. While a DNA molecular marker is not available, this technique can be used to distinguish homo- and heterozygous bitter genotypes in sweet almond and thus it is useful both to determine genotypes of parents for further breeding or screening unwanted seedlings derived from crosses when breeding.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/6/747/s1, Table S1: Experiments performed to develop the SPME method, after optimizing the experimental design, Table S2: Volatile compounds identified in the homozygous and heterozygous cultivars and selections under study, Table S3: comparison of chromatographic areas obtained by dry extraction and extraction in aqueous suspension, Table S4: influence of harvest year and production area on the concentration of the selected volatiles obtained by analysis of variance, S5: samples from cultivars with known genotype and their predicted values as the *SkSk* (homozygous) and *Sksk* (heterozygous) class of the PLS-DA model, Table S6: samples from cultivars and selections with unknown genotype, and their predicted values as the *SkSk* (homozygous) class of the PLS-DA model.

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Renewing Traditions: A Sensory and Chemical Characterisation of Mexican Pigmented Corn Beers

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Abstract: This study was undertaken to explore how the use of pigmented corn as brewing ingredient influences the sensory profile of craft beers, by using both sensory and chemical analyses. Six pigmented corn and barley beers were brewed and then analysed to obtain their sensory characteristics, volatile composition and non-volatile (alcohol, bitterness, anthocyanins and polyphenol content) composition. ANOVAs, Principal Component Analysis (PCA) and Multiple Factor Analysis (MFA) were used to visualise these data for exploring the differences between beers based on the type of malt and to characterise corn beers considering the relationships between their sensory characteristics and their chemical parameters. The sensory attributes such as fermented fruits, cooked vegetables, tortillas, bread, dried fruits and dried chili characterised beers made 100% with pigmented corn. Over 100 volatiles were identified by head space-solid phase micro-extraction coupled with gas chromatography-mass spectrometry (HS-SPME/GC-MS). Among them, phenols and terpenes were the groups of volatiles that better characterised beers containing corn. The content of anthocyanins in corn beers provide the 'amber-red-cooper' colours in beers and may prevent the development of off-aromas and tastes. The use of pigmented corn seems to be a good option to renew the traditional 'Sendechó' while preserving some of its sensory attributes.

Keywords: *Zea mays*; Sendechó; volatiles; anthocyanins; HS-SPME; GC-MS; sensory profile

1. Introduction

Corn (*Zea mays* L.), a cereal native to Mexico, has been the most important cultivated and domesticated crop from ancient times until today [1]. It comes in a great variety of pigmented grains, with colours that range from white and yellow to purple, red, blue and even black [2,3]. In Mexico, ancient civilizations consumed this cereal as the basis of their diet [1]. They developed several fermented beverages based on specific types of pigmented corn, widely referred to as "corn beers" [4–6].

Sendechó is one of these typical fermented beverages made by the Mazahuas population in the Valley of Mexico, whose method of production is very similar to the beer process. It is produced with regional ingredients such as blue pigmented corn that goes through a malting process and Guajillo chili [6], which is a traditional ingredient in Mexican cuisine [7]. But as for most of the traditional beverages, the consumption of Sendechó has gradually declined due to changes in eating habits and urbanisation.

In order to rescue this beverage and preserve some of its sensory properties, we transferred its main ingredients (pigmented corns and guajillo chili) to develop a more modern and consumed beverage, e.g., beer. Therefore, we considered that the use of native varieties of pigmented corn from Mexico for brewing purposes will give added value to both corn grains and beer. Moreover, these types of corn could be used as an alternative cereal in the brewing industry.

Beer is defined as a fermented beverage generally made with four main ingredients: water, malt, hop and yeast [8–10]. Traditionally, barley malt is the most common cereal used in the brewing process [9,10]. Nowadays, as a result of the increased consumption of craft beers, the use of alternative cereals and non-traditional ingredients in the brewing process has increased [11–14]. This allow brewers to create new and different beer styles with a variety of innovative sensory characteristics [9,15].

Several studies of beers have focused on the partial replacement of barley using alternative cereals like wheat [11], rice [12] oats [13] and sorghum [14]. While corn has been considered an economical source of starch [9,16], typically used as an adjunct, authors like Diakabana et al. [17] and Eneje et al. [16] have studied the potential of corn (yellow and white varieties) to produce malt for brewing purposes. Furthermore, in a previous work from our research group, Flores-Calderon et al. [5] developed some beer styles using blue corn malt as the main ingredient. Nevertheless, the use of native varieties of pigmented corn from Mexico has not received similar attention to date.

Since the use of pigmented corn malt as a main ingredient is relatively new to the brewing process [4,5], it is essential to understand the influence of this ingredient on both sensory and chemical composition of these types of beers. Considered as one of the most complex features, beer flavour is generally used in the brewing industry to determine the sensory quality of the beverage. Beer flavour, comprising aromas and tastes, is the result of the combination and interaction of a wide diversity of volatile and non-volatile compounds, originating from the raw ingredients and the brewing process [10,18]. Sensory characteristics of beer are deeply influenced by its chemical profile. Volatile compounds play a key role in the overall aromatic profile of beer. In addition, other non-volatile compounds such as anthocyanins and phenolic components have a significant impact on the sensory attributes such as taste, mouthfeel and colour. Altogether, they serve as a quality indicator and have great importance as they might drive the consumer's acceptance or rejection of this beverage [9]. Although there are many studies regarding sensory and chemical properties of beer [8,11,18,19], little information could be found on beers made with different varieties of corn [4,5]. Moreover, there are no references of the sensory characteristics and volatile compounds of these type of beers.

Thus, in this study we applied both sensory and chemical approaches, combined with an appropriate statistical methodology, to obtain a complete characterisation of beers, and information about those properties that discriminate between samples and explore the associations between the sensory and chemical properties [20,21]. Specifically, the use of multivariate tools like principal component analysis (PCA) and multiple factor analysis (MFA) to analyse sensory and chemical data at the same time can provide a better overview of the sensory characteristics of the 'pigmented corn beers' and chemical components (volatiles and non-volatiles) that can be used as indicators of the use of corn malt.

The main objective of this study was to understand how the use of pigmented corn malt influences the chemical composition and sensory characteristics of beers. To this end, we focused on: (1) characterising the sensory properties of beers made with pigmented corn malt, (2) characterising the volatile composition and non-volatile parameters of the beers (3) identifying sensory attributes that could be influenced by the volatiles and non-volatiles parameters and (4) identifying components (sensory, volatiles and non-volatiles) that can be used as indicators of the use of pigmented corn malt.

2. Materials and Methods

In this work, six beers were brewed using different proportions of pigmented corn malt and barley malt (Table 1), hops, water and yeast under an Ale fermentation process. The corn malt was obtained by malting two varieties of pigmented corn (red and blue) and two types of commercial barley malt (base

and caramel) were used. In addition, Guajillo chili (*Capsicum annuum*) was used as an adjunct to preserve the main ingredients of the typical Sendechó beverage. Thereafter, chemical properties of each beer were determined by analysing volatile composition (VoC), alcohol content (ABV), international bitterness units (IBU), total anthocyanins content (TAC) and total polyphenol content (TPC). Moreover, sensory analysis was performed to assess the attributes of the six beers. Finally, a correlation between chemical and sensory data was made to understand the contribution of corn malt to the beer sensory properties.

Table 1. Beer formulations.

Prototype	Abbreviation	Beer Formulation
1	BC	100% Blue corn malt
2	RC	100% Red corn malt
3	RBC	50% Red corn malt, 50% blue corn malt
4	Ba	85% Barley base malt, 15 % caramel barley malt
5	BCBa	50% Blue corn malt, 35% base barley malt, 15% caramel barley malt
6	RCBa	50% Red corn malt, 35% base barley malt, 15% caramel barley malt

2.1. Corn Malting Procedure

Two Chalqueño varieties of red and blue pigmented corn were purchased locally in Milpa Alta, Mexico City. Each variety of corn was used for the preparation of corn malt. The two varieties of pigmented corn were manually cleaned to remove impurities and then were subjected to a micro-malting procedure as described in Mexico Patent No. 365,910 [22]. The red and blue corn grains were soaked for 12 to 24 h respectively, after which the grains were germinated for three days. Green malt was dried afterwards in a kiln at 50 °C for two days to obtain the base corn malt.

2.2. Beer Formulation and Brewing Process

Based on a mixture design, six beers (Table 1) were produced using different proportions of corn and barley malts and brewed under the same conditions. Two batches of each beer (15 L) were produced in a microbrewery pilot plant (30 L) at Universidad Autonoma Metropolitana. For all beers, mashing, brewing, fermentation and maturation procedures were performed according to the procedure described in Mexico Patent No. 365,910 (2014) [22]. Hops (Saaz, 3–5 α-acids and Magnum, 12–15 α-acids, HopUnion LLC, US) were added during boiling of mash to achieve 30 International Bitterness Units (IBU). Guajillo chili (*Capsicum annuum*) was also added during this step. Fermentation of wort by a dry top-fermenting yeast *Saccharomyces cerevisiae* (Safale US-05, Fermentis, Marcq-en-Baroeul Cedex, France) took place in a 20 L fermentation tank at 15 °C for 10 days. The green beer obtained was conditioned by adding sucrose (2 g/L) and immediately packed in amber bottles (355 mL) where maturation was carried out at 5 ± 1 °C for three months.

2.3. Analysis of Non-Volatile Components

2.3.1. Alcohol by Volume (ABV)

The volume of alcohol was determined following the ASBC method for Beer-4B, where beer and distillate were measured gravimetrically [23]. Alcohol content was expressed as percentage of alcohol by volume (ABV) and was determined by measuring the specific gravity of the distillate (at 20 °C) and referring to its value in tables.

2.3.2. International Bitterness Units (IBU)

IBU is a standard system used to quantify and express hop bitterness in beer due to the amount of iso-alpha acids. The higher the value, the greater the level of bitterness due to the hops [24,25].

Determination of IBU was estimated following the ASBC method Beer-23A [26]. Aliquots of beer previously degassed were transferred into a 50 mL centrifuge tube and 0.5 mL of 3 M HCl and 10 mL of

2,2,4-trimethylpentane were added. Consequently, samples were shaken and centrifuged at 2500 rpm for 10 min. The absorbance was measured at 275 nm. IBU was obtained by multiplying the absorbance value by a factor of 50.

2.3.3. Total Anthocyanin Content (TAC)

The pH differential method was used to quantify total anthocyanins content (TAC) [27]. Results were expressed as mg cyanidin-3-glucoside per litter (C3G/L) for beers based on a molar extinction coefficient (ε) of 26,900 $M^{-1}cm^{-1}$.

2.3.4. Total Polyphenols Content (TPC)

The Folin-Ciocalteu spectrophotometric method developed by Singleton and Rossi [28] was used for the determination of total polyphenols content (TPC) in the beer samples. The measurement was compared with a standard calibration curve of a gallic acid solution over the range 50–1000 mg/L. Results were expressed as mg of gallic acid equivalents per litre (mg GAE/L).

2.4. Analysis of Volatile Compounds (VoC)

The volatile composition of beers was analysed by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC) with mass spectrometry (MS). The extraction and concentration of the volatile compounds were performed using the HS-SPME technique using a 1-cm-long divinylbenzene/carboxen/polidimethylsiloxane (50/30 µm DVB/CAR/PDMS) fibre (Supelco, Mexico). The DVB/CAR/PDMS fibre is the most appropriate for flavour volatile analysis as it covers a wide range of groups of volatile compounds, as has been proved by Dong et al. [29]; Riu-Aumatell et al. [30]. The fibre was heated at 250 °C for 15 min between each analysis to prevent contamination from previous injections.

For the HS-SPME procedure, 10 mL of degassed content from each beer were enclosed in 20-mL glass vials containing 2 g of NaCl. Vials were sealed with a polyethylene and silicone septum cap. The sample was magnetically stirred for 10 min at 20 °C ± 1 for sample/headspace equilibration. After this period, the fibre was exposed to the headspace for 35 min with oscillation at 45 °C; this temperature was maintained throughout the extraction step using a heated circulating bath.

After the extraction of volatile compounds, the fibre was immediately desorbed into GC injection port at 250 °C for 10 min to ensure total desorption. For each sample, the analysis was undertaken in duplicate, taking one sample of each batch, and the results were averaged.

The extracted analytes were analysed in a 7890B/5977A GC-MSD chromatographic system (Agilent Technologies, Palo Alto, CA, USA). Elution and separation of compounds were carried out in a HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness, 19091S-433UI). Splitless mode was operated in the injector, and helium was used as the carrier gas at a flow rate of 1.3 mL/min. Oven temperature was set to 40 °C, held for 3 min, raised to 190 °C with a heating rate of 5 °C/min, then raised 15 °C/min to 250 °C and held for 20 min. In the GC-MS system the rate of gas carrier was 1.3 mL/min for 37.5 min, raised 0.5 mL/min to 1.8 mL/min and held until the end of the run.

The 5977A MSD (Agilent Technologies, Palo Alto, CA, USA) detector was at 250 °C and the quadrupole was operated in the electron-impact mode at 70 eV and in the scan range (*m*/*z*) from 29 to 300, with an ion source temperature of 230 °C.

Data was collected with Mass Hunter GC/MS software (B.07.02.1938). Volatiles were identified by comparing their mass spectrum and their retention times with 36 pure commercial standards. Additionally, all identities were confirmed by comparison of their mass spectra with those of the NIST14 MS library database. In addition, linear retention indices (LRI) were determined with reference to a homologous series of aliphatic hydrocarbons and compared with those reported in literature (Table 2). Since one of the aims of the study was to identify whole volatile compounds that characterise each of the six beer samples, no attempts were made to determine the actual concentration of all

identified compounds. The chromatographic peak area was used as an approach of the abundance of each volatile compound in beers and was expressed as arbitrary units (peak area counts × 10^6) (Table 2).

2.5. Sensory Analysis

In this study, we obtained the sensory profile of the six beers using a conventional descriptive method based on the quantitative descriptive analysis [31,32]. Thirteen judges (students from the Autonomous Metropolitan University) were screened and selected based on their sensory acuity to identify and differentiate between the samples, and their potential to describe and communicate sensory perceptions [31].

Panel members were trained in the descriptive language of beer category [33]. First, the panel was familiarised with a wide range of commercial beers. Then, panellists generated a list of attributes pertaining to appearance, odour (nasal), aroma (retronasal), taste and mouthfeel of the beer samples. The panel reached a consensus definition of the terms best describing the attributes of barley and corn beers. Physical references were given in order to develop a common and unified understanding of each attribute. Sensory attributes together with their definitions and physical references used by the panel are shown in Table 3. The attributes were followed by an "Ap", "O", "A", "T", "M", in case these pertained to appearance, odour, aroma, taste or mouthfeel category respectively. Panellist training was accomplished during twelve 1h-working sessions, which involved learning, associating and rating the intensity of the specific beer attributes developed before. Performance of the panel was assessed by measuring its repeatability between sessions, agreement between panellists and consensus, and the discriminative ability of the panel (Supplementary Table S1) [31].

The trained panel subsequently assessed six samples of beers. Three samples of beer were evaluated per session using a balanced sample presentation design. Each beer sample was evaluated in duplicate by each judge. Samples were coded with a randomly selected three-digit number and were presented in monadic form. All samples were kept refrigerated before being served, and 50 mL were presented in a glass at a range of temperature between 5 to 8 °C. A time-out of 5 min between samples was implemented to minimise fatigue, and water and crackers were provided for palate cleansing.

The panellists were instructed to rate the intensity of each attribute using a 15-cm unstructured line scales anchored by "minimum" to "maximum". The evaluation of the colour attribute was done following the instructions of the 'Beer Judge Certification Program (BJCP) Color Guide' [34]. This guide is designed to allow a beer panellist to quickly estimate the colour of a beer sample in Standard Reference Method (SRM) units. The SRM is a numerical scale developed by the American Society of Brewing Chemist (ASBC) to describe beer colour [34]. The scale ranges from 1 SMR (straw) to 40+ SMR (black). All evaluations were performed in an individual sensory evaluation booth equipped with the electronic data-capturing Fizz® system (version 2.5; Biosystems, Courtenon, France), and all sessions were conducted in the Spanish language.

Table 2. Volatile compounds identified in all beer samples.

No	Compound Name	LRI [1]	LRI [2]	Peak Area Counts × 10^6						ID [3]	Flavour [4]
				BC	RC	RBC	Ba	BCBa	RCBa		
				Alcohols							
1	Ethanol	668	527	508.6 ± 132.5	510.5 ± 6.7	354.8 ± 107.4	672.2 ± 14.9	654.2 ± 18.7	548.3 ± 10.8	MS,S	Sweet [6]
2	1-Propanol	536	605	8.8 ± 1.4	2.8 ± 1.6	6.1 ± 4.7	15.0 ± 10.5	6.5 ± 0.2	5.8 ± 0.1	MS,S	Sour [6]
3	2-Methyl-1-propanol	647	644	16.2 ± 11.8	10.4 ± 0.2	16.2 ± 7.4	38.4 ± 28.6	18.1 ± 2.1	26.2 ± 6.7	MS,S	Wine, solvent, bitter [6]
4	3-Methyl-1-butanol	736	750	237.9 ± 118.0	216.4 ± 40.0	171.2 ± 51.3	353.1 ± 22.1	164.7 ± 32.4	287.1 ± 85.8	MS	Whiskey, malt, burnt [6]
5	2-Methyl-1-butanol	755	754	32.9 ± 13.3	32.8 ± 1.2	28.8 ± 9.7	76.6 ± 40.5	34.1 ± 4.4	60.5 ± 4.7	MS	Fermented [6]
6	2,3-Butanediol	769	809	2.0 ± 0.3	2.4 ± 0.2	0.8 ± 0.3	2.32 ± 0.3	0.99 ± 0.2	0.2 ± 0	MS,S	Fruity [6]
7	4-Methyl-1-pentanol	840	835	n.d	n.d	n.d	0.8 ± 0.6	0.3 ± 0.1	0.4 ± 0.1	MS	
8	2-Furanmethanol	851	846	n.d	n.d	n.d	5.7 ± 5.5	2 ± 1.8	1.1 ± 0.3	MS,S	Burnt [6]
9	1-Hexanol	851	854	1.2 ± 1.3	2.4 ± 0.4	6.4 ± 1.8	5.8 ± 4.0	2.7 ± 0.2	3.8 ± 0.8	MS,S	Resin, flower, green [6]
10	3-Methyl-1-hexanol	895	898	n.d	n.d	n.d	1.7 ± 0.8	0.5 ± 0.1	1.2 ± 0.3	MS	
11	1-Heptanol	962	986	1.3 ± 1.3	2.8 ± 1.1	3.2 ± 0.6	2.6 ± 1.7	2.6 ± 0.6	2.3 ± 0.7	MS,S	Chemical, green [6]
12	1-Octen-3-ol	982	1004	n.d	n.d	1.3 ± 0.3	1.2 ± 0.6	1.2 ± 0.2	1.6 ± 0.2	MS,S	Mushroom, earthy [6]
13	2-Ethyl-1-hexanol	1032	1054	3.3 ± 4.3	27.8 ± 3.2	38.7 ± 2.8	52.3 ± 22.0	43.8 ± 14.4	35.7 ± 11.6	MS	Rose, green [6]
14	1-Octanol	1072	1096	5.4 ± 7.1	6.2 ± 2.3	4.7 ± 0.8	6.9 ± 3.6	10.4 ± 3.2	8.7 ± 2	MS,S	Chemical, metal [6]
15	Phenylethyl alcohol	1118	1139	80.1 ± 72.9	36.6 ± 11.7	43.7 ± 5.1	154.4 ± 58.0	112.6 ± 67.7	112.5 ± 32.4	MS	Honey, spice, rose [6]
16	(Z)-3-Nonen-1-ol	1152	1181	n.d	n.d	0.5 ± 0.001	0.8 ± 0.4	7.8 ± 2.1	1.5 ± 0.6	MS	Waxy, green, melon [7]
17	1-Nonanol	1154	1198	n.d	2.0 ± 0.3	n.d	n.d	n.d	4.0 ± 1	MS	Fat, green [6]
18	2-Decanol	1186	1229	14.3 ± 16.8	2.9 ± 2.8	n.d	n.d	n.d	n.d	MS	
19	Citronellol	1233	1255	19.6 ± 22.0	21.1 ± 9.7	3.6 ± 0.1	39.1 ± 20.2	16.8 ± 11.2	9.9 ± 3.1	MS,S	Rose [6]
20	Iso-geraniol	1254	1262	n.d	n.d	n.d	2.0 ± 1.4	1.9 ± 1.7	1.0 ± 0.4	MS	Rose [6]
21	1,9-Nonanediol	-	1292	n.d	1.7 ± 1.3	1.0 ± 0.001	n.d	0.9 ± 0.1	1.0 ± 0.4	MS	
22	1-Decanol	1263	1300	n.d	5.3 ± 3.5	2.2 ± 2.4	13.7 ± 8.6	5 ± 3.7	3.5 ± 1.2	MS	Fat [6]
23	2-Undecanol	1294	1330	8.8 ± 9.1	2.2 ± 1.0	1.0 ± 0.3	5.7 ± 3.8	2.7 ± 1.7	1.5 ± 0.7	MS	
24	Caryophyllenyl alcohol	1568	1608	0.9 ± 0.5	0.3 ± 0.01	n.d	1.2 ± 0.8	n.d	n.d	MS	
				Aldehydes							
25	Acetaldehyde	427	503	0.3 ± 0.3	n.d	n.d	0.5 ± 0.2	n.d	n.d	MS,S	Pungent, ether [6]
26	Benzeneacetaldehyde	1044	1068	n.d	n.d	n.d	1.1 ± 0.3	0.5 ± 0.1	0.8 ± 0.2	MS	
27	Nonanal	1104	1130	3.7 ± 4.1	2.9 ± 1.9	1.5 ± 0.001	2.7 ± 1.1	5.4 ± 1.6	2.2 ± 0.8	MS	Fat, citrus, green [6]
28	Decanal	1209	1233	8.2 ± 9.5	1.9 ± 1.7	2.7 ± 0.2	n.d	n.d	2.8 ± 1.6	MS	Soap, orange peel, tallow [6]
				Aliphatic hydrocarbons							
29	Tetradecane	1400	1430	2.2 ± 2.2	0.7 ± 0.3	1.2 ± 0.001	3.0 ± 1.4	n.d	n.d	MS	Waxy [5]
30	Pentadecane	1500	1530	2.6 ± 2.9	0.6 ± 0.4	0.8 ± 0.4	3.0 ± 1.5	n.d	n.d	MS	Waxy [5]
				Carboxylic acids							
31	Acetic acid	600	619	11.7 ± 0.001	1.5 ± 0.5	n.d	n.d	1.2 ± 1.5	8.5 ± 1.2	MS,S	Sour [6]
32	2-Methyl-propanoic acid	752	779	3.5 ± 0.7	2.4 ± 0.2	7.2 ± 2.3	3.1 ± 0.3	n.d	n.d	MS	Rancid butter [5]
33	3-Methyl-butanoic acid	877	839	1.4 ± 0.001	1.8 ± 1.3	6.9 ± 5.3	3.4 ± 0.001	0.3 ± 0.1	0.8 ± 0.1	MS	Sweat, acid, rancid [5]
34	2-Methyl-hexanoic acid	-	844	n.d	n.d	0.2 ± 0.1	n.d	1 ± 0.8	0.9 ± 0.5	MS	
35	2-Methyl-butanoic acid	896	845	n.d	n.d	0.5 ± 0.1	1.3 ± 0.2	0.7 ± 0.3	n.d	MS	
36	Hexanoic acid	1019	1010	n.d	2.2 ± 0.1	21.4 ± 4.4	17.8 ± 0.2	n.d	13.0 ± 0.6	MS,S	Fatty, sour, sweat, cheese [6]
37	Heptanoic acid	1078	1103	n.d	1.5 ± 1.6	n.d	2.6 ± 2.4	1.0 ± 0.4	1.3 ± 0.7	MS	Cheesy, waxy, sweaty [5]
38	2-Ethyl-hexanoic acid	1116	1167	0.9 ± 0.8	1.0 ± 0.5	n.d	3.8 ± 1.3	n.d	0.6 ± 0.9	MS	

Table 2. *Cont.*

				Peak Area Counts × 10^6							
No	**Compound Name**	**LRI** [1]	**LRI** [2]	**BC**	**RC**	**RBC**	**Ba**	**BCBa**	**RCBa**	**ID** [3]	**Flavour** [4]
39	Octanoic acid	1279	1209	608.8 ± 5.3	194.3 ± 4.5	217.3 ± 1.6	411.8 ± 16.4	12 ± 0.4	458.1 ± 30.7	MS,S	Sweat, cheese [6]
40	9-Decenoic acid	1358	1392	35.8 ± 31.8	8.6 ± 6.8	10.9 ± 0.9	n.d	7.4 ± 0.6	n.d	MS	
41	Decanoic acid	1373	1399	51.0 ± 45.7	n.d	23.5 ± 7.0	68.0 ± 54.7	7.8 ± 0.5	10.9 ± 2.9	MS,S	Rancid, fat [6]
42	Hexadecanoic acid	1984	2000	n.d	3.8 ± 4.0	3.3 ± 3.9	1.6 ± 1.7	n.d	n.d	MS,S	Oily [6]
						Esters					
43	Ethyl acetate	628	635	25.0 ± 12.7	16.6 ± 9.6	27.1 ± 1.9	57.7 ± 34.8	42.5 ± 6.5	46.0 ± 16.3	MS,S	Pineapple [6]
44	Ethyl propanoate	713	728	n.d	n.d	2.1 ± 1.6	2.7 ± 1.4	2 ± 0.1	2.4 ± 0.3	MS	Fruit [6]
45	Propyl acetate	720	731	n.d	n.d	n.d	1.0 ± 0.2	n.d	0.4 ± 0.1	MS	Sweet, fruity, caramel [7]
46	Ethyl isobutanoate	756	780	n.d	n.d	n.d	1.3 ± 0.4	1.2 ± 0.1	2.8 ± 0.8	MS	Sweet, rubber [6]
47	Isobutyl acetate	776	798	1.8 ± 0.2	1.4 ± 0.3	1.7 ± 1.3	1.4 ± 0.7	2.3 ± 0.2	2.3 ± 0.8	MS	Fruit, apple, banana [6]
48	Ethyl butanoate	804	814	2.2 ± 0.3	2.1 ± 0.5	1.9 ± 0.1	4.5 ± 2.7	9 ± 0.3	5.8 ± 1.8	MS	Apple [6]
49	3-Methylbutyl acetate	877	859	31.7 ± 18.5	38.7 ± 12.7	20.3 ± 7.9	47.9 ± 25.6	113 ± 2.6	66.6 ± 19.7	MS	Fresh, banana, sweet [5]
50	2-Methylbutyl acetate	876	861	2.0 ± 1.2	3.0 ± 1.1	1.2 ± 0.7	2.7 ± 1.5	5.2 ± 0.7	5.6 ± 1.5	MS	Herbal, fermented fruity [5]
51	Ethyl pentanoate	900	875	n.d	n.d	n.d	1.1 ± 0.6	1.5 ± 0.1	2.2 ± 0.5	MS	Yeast, fruit [7]
52	Ethyl iso-hexanoate	-	974	n.d	n.d	n.d	0.8 ± 0.4	1.3 ± 0.7	1.4 ± 0.5	MS,T	Sweet, fruity, tropical, green, apple [7]
53	Methylbutyl propanoate	-	992	0.7 ± 0.5	1.2 ± 0.8	n.d	n.d	0.9 ± 0.4	n.d	MS,T	
54	Ethyl hexanoate	1002	1025	8.9 ± 7.5	14.8 ± 8.8	10.0 ± 2.2	27.7 ± 13.9	151.2 ± 89.3	139.8 ± 41.5	MS,S	Apple peel, fruit [6]
55	Hexyl acetate	1014	1039	2.1 ± 1.4	3.6 ± 2.3	1.8 ± 0.6	1.8 ± 0.4	3.3 ± 1.2	1.3 ± 0.4	MS	Fruity, spicy, herbal, sweet wine, rubbery [7]
56	2-Metylbutyl isobutanoate	1014	1042	5.0 ± 2.9	15.7 ± 10.2	n.d	n.d	1.1 ± 0.4	n.d	MS	Fruity, ethereal [7]
57	Ethyl 5-methylhexanoate	-	1088	n.d	1.1 ± 0.5	n.d	0.9 ± 0.3	6.7 ± 3.5	4.3 ± 1.2	MS,T	
58	Ethyl benzoate	1185	1197	n.d	n.d	n.d	4.0 ± 2.2	2.7± 1.7	n.d	MS	Chamomile, flower [6]
59	Ethyl octanoate	1198	1225	67.1 ± 59.9	63.5 ± 36.1	51.8 ± 6.2	n.d	724.7 ± 45.0	3.9 ± 1.3	MS,S	Fruit, fat [6]
60	Ethyl phenylacetate	1252	1273	n.d	n.d	0.8 ± 0.2	2.8 ± 1.3	2.2 ± 1.3	1.4 ± 0.7	MS	Fruit, sweet [7]
61	Phenethyl acetate	1265	1285	14.5 ± 13.9	5.4 ± 2.4	5.8 ± 0.8	20.6 ± 9.3	15.2 ± 8.3	8.5 ± 2.8	MS	Rose, floral [7]
62	Ethyl nonanoate	1295	1326	1.8 ± 1.5	n.d	0.7 ± 0.00	2.0 ± 1.1	3.2 ± 1.9	1.9 ± 0.9	MS	Fruity, rose [6]
63	Methyl geranoate	1323	1354	6.6 ± 6.1	3.0 ± 1.6	3.3 ± 0.7	3.4 ± 1.6	2.2 ±1.5	0.7 ± 0.3	MS	Floral [6]
64	Ethyl benzenepropanoate	1390	1379	n.d	n.d	n.d	1.7 ± 1.0	1.5 ± 1	n.d	MS	
65	Ethyl (*E*)-4-decenoate	-	1408	4.3 ± 3.0	1.7 ± 0.8	n.d	6.9 ± 1.0	3.6 ±1.2	3.7 ± 1.1	MS,T	
66	Ethyl 9-decenoate	1387	1417	15.2 ± 1.2	13.2 ± 7.0	12.7 ± 3.2	15.5 ± 6.3	59.6 ± 18.7	22.7 ± 6.4	MS	
67	Ethyl decanoate	1397	1426	20.3 ± 14.0	12.5 ± 5.9	13.5 ± 4.6	50.1 ± 17.1	24.3 ± 5.9	31.6 ± 10.6	MS,S	Grape, fruit [6]
68	Isoamyl octanoate	-	1478	0.7 ± 0.6	n.d	0.3 ± 0.1	1.5 ± 0.5	0.7 ± 0.3	1.1 ± 0.4	MS,T	
69	Ethyl dodecanoate	1494	1628	9.9 ± 4.3	3.1 ± 1.3	1.6 ± 0.7	8.7 ± 4.4	n.d	n.d	MS	Leaf [6]
70	Dibutyl maleate	-	1571	1.6 ± 1.1	n.d	0.4 ± 0.001	0.8 ± 0.6	n.d	n.d	MS,T	
71	Ethyl *cis*-9-pentadecenoate	-	1622	6.4 ± 4.3	0.9 ± 0.5	0.4 ± 0.1	n.d	n.d	n.d	MS,T	
72	Ethyl tetradecanoate	1793	1832	1.5 ± 0.6	0.7 ± 0.1	0.5 ± 0.1	1.7 ± 1.0	4.8 ± 1.6	n.d	MS,S	Oily, violet [6]
73	2-Ethylhexyl salicylate	1816	1847	1.3 ± 0.7	2.9 ± 3.7	1.6 ± 0.5	5.2 ± 6.4	0.9 ± 0.5	1.2 ± 0	MS	
74	Ethyl 9-hexadecenoate	-	2015	n.d	0.9 ± 0.5	1.1 ± 1.0	0.9 ± 0.6	0.3 ± 0.1	n.d	MS,T	
75	Ethyl hexadecanoate	1991	2038	1.1 ± 0.2	1.1 ± 0.4	1.9 ± 0.9	2.1 ± 1.5	0.3 ± 0.2	n.d	MS	Waxy [6]
76	Isopropyl palmitate	-	2070	n.d	n.d	2.5 ± 2.5	2.2 ±2.6	n.d	n.d	MS,T	
77	1-Propylpentyl dodecanoate	-	2152	0.6 ± 0.2	3.5 ± 3.9	2.8 ± 2.5	4.5 ± 5.2	n.d	n.d	MS,T	

Table 2. *Cont.*

No	Compound Name	LRI [1]	LRI [2]	Peak Area Counts × 10^6						ID [3]	Flavour [4]
				BC	RC	RBC	Ba	BCBa	RCBa		
				Furans							
78	Acetylfuran	893	881	n.d	n.d	n.d	1.8 ± 1.5	0.3 ± 0.1	n.d	MS,S	Balsamic [6]
79	3-Methyl-2,3-dihydro-1-benzofuran	-	1178	n.d	n.d	0.5 ± 0.1	n.d	1.3 ± 1.0	n.d	MS,T	
80	2,3-Dihydro-benzofuran	-	1246	2.5 ± 2.7	3.9 ± 0.5	n.d	4.3 ± 2.0	n.d	3.9 ± 1.1	MS,T	
81	Dihydro-5-pentyl-2(3H)-furanone	-	1392	n.d	n.d	n.d	16.7 ± 7.7	5.6 ± 1.6	7.8 ± 2.8	MS,T	
				Aromatic hydrocarbons							
82	Styrene	893	867	33.2 ± 18.7	32.3 ± 19.7	25.7 ± 0.4	56.8 ± 25.3	52.9 ± 13.1	78.0 ± 27.4	MS	Balsamic, gasoline [6]
83	1,4-Dichloro-benzene	1015	1035	29.0 ± 28.7	13.0 ± 0.5	23.5 ± 3.3	29.7 ± 11.4	18.0 ± 1.1	15.7 ± 2.7	MS	Mothball-like [5]
84	Squalene	2833	2881	2.6 ± 0.3	16.4 ± 16.2	18.7 ± 21.0	12.9 ± 14.8	n.d	n.d	MS	
				Ketones							
85	2-Pentanone	636	708	n.d	n.d	n.d	0.6 ± 0.4	0.6 ± 0	n.d	MS	Ether [6]
86	3-Methyl-2-pentanone	759	777	n.d	1.1 ± 1.2	n.d	n.d	n.d	n.d	MS	
87	Acetophenone	1041	1091	n.d	n.d	0.4 ± 0.1	0.7 ± 0.2	n.d	n.d	MS,S	Must, flower, almond [6]
88	2-Nonanone	1091	1118	n.d	1.3 ± 0.8	n.d	n.d	0.9 ± 0.3	n.d	MS	Fruity, sweet, waxy, soapy, herbaceous, coconut [5]
89	β-Damascenone	1386	1386	n.d	n.d	n.d	n.d	7.8 ± 2.7	6.7 ± 1.6	MS	
90	β-Ionone	1493	1526	1.2 ± 0.1	0.8 ± 0.2	n.d	n.d	0.6 ± 0.3	0.4 ± 0.2	MS,S	Seaweed, violet, flower, raspberry [6]
				Miscellaneous							
91	Methoxy-phenyl-oxime	-	883	9.1 ± 6.9	4.1 ± 1.3	3.5 ± 0.4	8.2 ± 7.4	n.d	0.5 ± 0.3	MS,T	
92	Geranyl vinyl ether	-	1259	3.0 ± 3.0	1.3 ± 1.0	n.d	n.d	n.d	n.d	MS,T	
93	9-Decen-1-ol methyl ether	-	1312	7.5 ± 7.8	n.d	0.7 ± 0.2	n.d	1.6 ± 0.01	n.d	MS,T	
				Phenols							
94	Phenol	980	1007	n.d	n.d	5.7 ± 0.001	4.6 ± 1.5	n.d	1.0 ± 0.5	MS	Phenolic, medicinal [6]
95	2-Methoxy-phenol	1089	1115	3.3 ± 3.6	n.d	2.3 ± 0.3	n.d	2.4 ± 1.9	0.4 ± 0.1	MS,S	Smoke, sweet, medicine [6]
96	4-Ethyl-phenol	1287	1193	4.4 ± 4.9	0.8 ± 0.1	41.4 ± 4.0	1.5 ± 0.1	0.7 ± 0.3	1.0 ± 0.4	MS,S	Spice, clove [6]
97	4-Ethyl-2-methoxy-phenol	-	1308	8.0 ± 7.9	1.7 ± 0.8	22.2 ± 4.0	n.d	2.3 ± 2	2.0 ± 0.8	MS,T	Spice, smoke, clove, medicinal [5]
98	2-Methyl-5-(1-methylethyl)-phenol	1307	1323	4.4 ± 4.9	0.7 ± 0.5	0.5 ± 0.2	2.0 ± 1.8	n.d	n.d	MS	Spicy, cooling, thymol-like, herbal and camphoreous [5]
99	2-Methoxy-4-vinylphenol	1315	1344	1.8 ± 1.6	21.2 ± 4.7	23.0 ± 4.2	15.4 ± 7.8	2.5 ± 1.5	2.6 ± 0.6	MS	Smoky, bacon [5]
100	2,6-Di-tert-butylphenol	1444	1502	2.0 ± 2.0	n.d	2.8 ± 0.001	n.d	n.d	n.d	MS,T	Phenolic [5]
				Pyrrole and pyrazine							
101	2-Acetylpyrrole	1045	1086	n.d	n.d	n.d	1.3 ± 0.5	0.5 ± 0.2	n.d	MS	Nut, walnut, bread [6]
102	Tetramethyl-pyrazine	-	1122	n.d	n.d	n.d	1.2 ± 0.3	1.6 ± 0.6	0.7 ± 0.1	MS,T	Nutty [7]
				Sulphur compounds							
103	Dimethyl sulfide	505	569	3.8 ± 2.6	3.2 ± 1.6	3.8 ± 0.4	4.7 ± 1.2	2.7 ± 0.4	2.4 ± 0	MS	Cabbage, sulphur, gasoline [6]
				Terpenes							
104	β-Myrcene	992	1016	10.7 ± 11.9	21.9 ± 17.8	n.d	n.d	4.7 ± 1.7	9.6 ± 4.7	MS,S	Balsamic, must, spice [6]
105	Limonene	1033	1056	42.6 ± 57.6	11.3 ± 5.8	1.2 ± 0.5	0.7 ± 0.5	n.d	n.d	MS,S	Lemon, orange [6]
106	Linalool	1100	1126	40.7 ± 46.9	37.0 ± 14.1	8.7 ± 0.4	22.3 ± 9.9	28.3 ± 11.9	18.5 ± 4.8	MS,S	Flower, lavender [6]

Table 2. *Cont.*

				Peak Area Counts × 10^6							
No	**Compound Name**	**LRI** [1]	**LRI** [2]	**BC**	**RC**	**RBC**	**Ba**	**BCBa**	**RCBa**	**ID** [3]	**Flavour** [4]
107	Camphor	1139	1171	n.d	n.d	0.6 ± 0.1	1.3 ± 0.8	1 ± 0.9	n.d	MS,S	Camphor [6]
108	Geraniol	1276	1283	12.0 ± 13.1	3.8 ± 2.1	2.2 ± 0.2	13.8 ± 7.9	3.6 ± 2.2	1.9 ± 0.8	MS,S	Rose, geranium [6]
109	Caryophyllene	1467	1454	2.4 ± 1.8	1.5 ± 0.4	n.d	1.5 ± 0.4	0.8 ± 0.2	2.7 ± 1	MS,S	Wood, spice [6]
110	Humulene	1467	1489	11.3 ± 5.1	7.4 ± 2.5	0.7 ± 0.1	6.5 ± 2.2	1 ± 0.4	11.6 ± 4.5	MS,S	Wood [6]
111	3-Methoxy-2-naphthalenol	-	1518	n.d	n.d	n.d	2.7 ± 1.3	0.3 ± 0.2	0.4 ± 0.2	MS,T	
112	δ-Cadinene	1519	1559	0.7 ± 0.3	n.d	n.d	1.2 ± 0.3	n.d	0.5 ± 0.2	MS	Thyme, medicine, wood [6]
113	*E*-Nerolidol	1539	1597	2.7 ± 1.8	n.d	0.6 ± 0.1	3.6 ± 1.8	1.2 ± 0.6	0.5 ± 0.2	MS	Wood, flower, wax [6]
114	Caryophyllene oxide	1573	1612	1.4 ± 1.3	n.d	n.d	1.7 ± 1.1	0.8 ± 0.4	0.7 ± 0.1	MS,T	Herb, sweet, spice [6]
115	Humulene oxide	1642	1641	1.3 ± 1.0	0.9 ± 0.3	0.3 ± 0.1	9.8 ± 5.3	0.4 ± 0.2	n.d	MS,S	Herb [6]
116	Cubenol	1645	1666	1.2 ± 0.7	0.9 ± 0.5	0.4 ± 0.2	1.2 ± 0.4	0.4 ± 0.2	n.d	MS	Spice, herb, green tea [6]
117	Di-epi-1,10-cubenol	1613	1669	2.0 ± 1.2	n.d	n.d	3.3 ± 1.7	0.3 ± 0.1	n.d	MS	
118	Calarene epoxide	-	1672	7.9 ± 4.9	n.d	n.d	n.d	1.1 ± 0.6	0.7 ± 0.1	MS,T	Woody [5]
119	τ-Cadinol	1635	1679	4.3 ± 2.8	n.d	n.d	4.5 ± 3.0	n.d	n.d	MS	Herb, weak spice [6]
120	δ-Cadinol	1674	1689	0.5 ± 0.6	n.d	n.d	1.1 ± 0.1	0.7 ± 0.3	n.d	MS	Herb [6]
121	α-Cadinol	1676	1695	2.8 ± 1.8	n.d	n.d	1.9 ± 1.9	0.3 ± 0.1	n.d	MS	Herb, wood [6]

Chromatographic peak area (peak area counts × 10^6) of the flavour volatile compounds. Results are expressed as mean ± standard deviation (n = 2); BC = 100% blue corn, RC = 100% red corn, RBC = 50:50 red and blue corn, Ba = 100% barley, BCBa = 50:50 blue corn and barley, RCBa = 50:50 red corn and barley. [1] LRI = Linear retention index (NIST values (http://webbook.nist.gov/chemistry/name-ser.html). [2] LRI = Linear retention index on HP-5MS column (Agilent Technologies), calculated via duplicated averaged alkanes, and found to be comparable with NIST values (http://webbook.nist.gov/chemistry/name-ser.html). [3] ID = Identification used as confirmation of compounds per: MS = library match; S = standards; T = tentative. [4] Flavour descriptors according to [5] The Good Scents Company (http://www.thegoodscentscompany.com/), [6] Flavornet (http://www.flavornet.org/flavornet.html) and [7] Pherobase (http://www.pherobase.com/). n.d. no detected.

Table 3. Sensory attributes, description and physical references used in this study (attributes pertaining to appearance, odour, aroma, taste and mouthfeel are designated by an "Ap", "O", "A", "T", "M" respectively after the attribute).

Attributes	Abbreviation	Description	Reference
Colour	Colour-Ap	Refers to the colour of the beer	Standard Reference Method (SRM) scale
Turbidity	Turbidity-Ap	Refers to the haziness of the beer	Range of different beer samples
Banana	Banana-O	Sweet gum flavoured banana	Isoamyl acetate (Siebel® kit)
Fruity	Fruity-O	A mix of fruits as pear, strawberry and grapefruit	Linalool (Siebel® kit)
Apple	Apple-O	Green apple	Acetaldehyde (Siebel® kit)
Cooked corn	Cook.corn-O	"Esquites odour"	Dimethyl sulfide (Siebel® kit)
Fermented fruits	Ferm fruits-O	Traditional fermented beverage made of a mix of fruits as pineapple, guava and apple	"Tepache"
Dried fruits	Dried fruits-O	Raisins, prunes, plum	Firmenich® reference
Dried chili	Dried chili-O	Odour of the Guajillo chili	Guajillo chili (6 g/L)
Pineapple	Pineapple-O	Ripe pineapple	Firmenich® reference
Hoppy	Hoppy-O	Pine -herbaceous odour	Tea made of Saaz and Magnum hops (0.5 g/L)
Bread	Bread-O	Fresh bread recently cooked	Firmenich® reference
Caramel	Caramel-O	Associated to caramel	Firmenich® reference
Brown sugar	Brown sugar-O	Product elaborated from raw brown sugar	"Piloncillo"
Olive	Olive-O	Vinegar-like	Acetic acid (Siebel® kit)
Floral	Floral-O	Flowers-like, roses	Geraniol (Siebel® kit)
Hoppy	Hoppy.A	Pine -herbaceous aroma	Tea made of Saaz and Magnum hops (0.5 g/L)
Malty	Malty.A	Malty-like	Firmenich® reference
Alcohol	Alcohol-A	A warming sensation in the mouth and throat	Firmenich® reference
Cooked vegetables	Cook.veg-A	Mix of cooked vegetables	Dimethyl sulfide (Siebel® kit)
Burnt tortillas	Tortillas-A	Aroma related to tortillas after being heated	Burnt tortillas
Sweet	Sweet-T	Associate with sugar taste	Sucrose 7.5 g/L
Bitter	Bitter-T	Associate with bitter taste	Isolone (Siebel® kit)
Sour	Sour-T	Associate with acid taste	Lactic acid (Siebel® kit)
Oxidised	Oxidised-M	Papery, cardboard	trans-2-nonenal (Siebel® kit)
Spicy	Spicy-M	Pungent sensation in the tongue caused by chili	Guajillo chili (6 g/L)
Metallic	Metallic-M	Metal-like	Ferrous sulfate (Siebel® kit)
Astringent	Astringent-M	Sensation of dryness in the tongue and mouth	Tannic acid (0.6 g/L)
Carbonatation	Carbonatation-M	Sensation tingle in the tongue related to CO_2	Peñafiel mineral water
Fullness	Fullness-M	Refers to the perceived density while it is being consumed	Range of different beer samples

2.6. Statistical Analysis

Data relative to the peak chromatographic areas of the identified volatile compounds were reported as the average of the two independent replicates ± standard deviation (six beer samples, each one by duplicate).

Analysis of variance (ANOVA) was performed on the sensory and non-volatile data (ABV, IBU, TAC, TPC) to ascertain significant differences among all six beer samples. A post-hoc Tukey's test was carried out when a significant difference ($p < 0.05$) was detected among samples.

To explore the sensory differences among the beer samples a Principal Component Analysis (PCA) with Pearson correlation coefficients was performed on the table beers x attributes (6 rows × 30 columns) containing the mean intensity scores obtained by each beer for each sensory attribute (calculated over the panellist and the repetitions). No rotation option was applied.

Multiple factor analysis (MFA) is a useful statistical method to analyse the similarities and discrepancies between a set of observations explained by data tables of different groups of variables. It can also be used to show correlation between those sets of variables [21,35].

In this study, MFA was conducted on the data matrices of sensory and chemical (volatile and non-volatiles) variables. More specifically the sensory matrix was divided into two matrices of respectively 19 'odour-aroma' variables (14 odour attributes and 5 aroma attributes) and 7 'taste-mouthfeel' variables (3 taste attributes and 4 mouthfeel attributes). The goal of this separation was to provide a better representation of the chemical data contributions on the odour-aroma and taste-mouthfeel attributes.

Therefore, the MFA was computed on four data tables consisting of: 19 odour-aroma attributes, 7 taste-mouthfeel attributes, 121 volatiles and 4 non-volatile parameters. Additionally, attributes namely colour, turbidity, carbonatation and fullness, which are important for beer characterisation but are not directly influenced by volatile components, were used as supplementary (non-active) variables in the analysis.

All statistical analyses were performed using XLSTAT (version 2018.7, XLSTAT-Sensory package, Addinsoft, Paris, France).

3. Results and Discussion

3.1. Analysis of Non-volatile Parameters

Results for the non-volatile analyses of beers are shown in Table 4. We can see significant differences ($p < 0.05$) among all samples on every parameter analysed (ABV, IBU, TAC and TPC).

Table 4. Mean score of beer samples for each non-volatile parameter.

Parameter		Beer					
		BC	**RC**	**RBC**	**Ba**	**BCBa**	**RCBa**
Alcohol (%, v/v)	ABV	3.71 c	2.98 cd	1.93 d	7.01 ab	5.45 b	7.21 a
International bitterness units	IBU	14.57 b	19.05 a	19.62 a	18.45 a	18.92 a	15.72 b
Anthocyanins (mg/L)	TAC	14.45 a	8.84 b	14.60 a	0.00 e	3.90 d	6.17 c
Polyphenols (mg GAE/L)	TPC	750.0 ab	331.0 c	367.5 c	398.5 c	849.5 a	721 b

Values with different letters across a row are significantly different ($p < 0.05$) according to the Tukey post-hoc test. BC = 100% blue corn, RC = 100% red corn, RBC = 50:50 red and blue corn, Ba = 100% barley, BCBa = 50:50 blue corn and barley, RCBa = 50:50 red corn and barley.

The content of alcohol (ABV) was significantly higher in beers that contained barley malt than in those made only with corn malt. This might be explained as corn has shown a low diastatic power compared to barley [5,9], which leads to wort contained less fermentable sugars and thus, less alcohol content. As the brewing process remained under the same conditions for all beers, it was surprising to find that the bitterness unit (IBU) in beers were significantly different only for the blended beer made of red corn and barley malt (15.72 IBU) and the blue corn beer (14.57 IBU). These beers showed

lowest IBU than the rest of beers (ranged between 15.7 to 19.6). The IBU is a measurement of how much iso-α-acids (1 IBU = 1 ppm iso-humulone) is in the final product, but it does not always really tell if a beer is bitter or not [24]. The amount of iso-α-acids in the beer depends on the time and temperature the hops spend in the boiling step [25]. Thus, minor changes in temperature or time the hops are added to the wort could change the amount of iso- α-acids in beer. Additionally, some authors have reported the susceptibility of this method to the interference from other compounds present in beer, such as polyphenols, that absorb light at the wavelength of measurement (275 nm). Therefore, minor contributions from compounds unrelated to bitterness can be detected (oxidised fatty acids), whereas others contributing to bitterness are not detected [36]. Moreover, coloured beers absorb light which directly decrease the emission intensity and result in lower IBU values [37]. Despite limitations, the IBU method is widely used as an indicator of bitterness in quality control [24,25].

Beers containing only corn malt showed a higher content of anthocyanins (TAC) than those blended beer made of barley and corn malt. The anthocyanins value for beers made of blue corn and red corn malt varied from 14.6 to 8.84 mg C3G/L respectively. These results are in agreement with Flores-Calderón et al. [5] who assessed different styles of blue corn beer and reported values that ranged from 13.2 to 18.7 mg C3G/L. A significantly higher difference between beers made of blue corn malt than the one made of red corn malt is expected as a greater amount of anthocyanins has been reported in varieties of blue corn than in the red corn variety [38]. Also, as was expected, the beer made of 100% barley malt did not show presence of anthocyanins. Red and blue corn contain anthocyanins, such as pelargonidin-3-glucoside and cyanidin-3-glucoside, which are responsible of the colour of the grains. Additionally, these anthocyanins have been reported to have various biological activities, such as antioxidant, antimicrobial, antimutagenic and anticancer effects [3,38]. Regarding sensory profile, presence of anthocyanins in beer not only has an effect on colour (ranging from amber-red-cooper) but also on taste and mouthfeel as these compounds could contribute with bitterness and astringency attributes. Thus, the presence of anthocyanins in pigmented corn beers could improve the quality of these beverages.

Finally, all the beers showed considerable amounts of total phenolic content (TPC). The main polyphenols present in a typical barley beer are hydroxybenzoic, cinnamic and ferulic acids. Malt is the main source of polyphenol compounds, providing 70 to 80% of them. Also, a small proportion is originated from hops (20–30%), such as α- and β- acids and their isomeric forms [36,38,39]. In beers made of pigmented corn malt, the presence of polyphenols is also expected. Blue and red corn also have shown the presence of phenolic compounds such as cyanidin-3-glucoside and pelargonidin-3-glucoside, respectively. In addition, ferulic acid and *p*-coumaric acid could be found in these varieties of corn [5]. The results showed significant difference between beers. Higher quantities of TPC were found in those beers made of blue corn malt (BC) and the blended beers made of red and blue corn and barley (RCBa, BCBa). The value of polyphenols ranged between 398.5 to 750 mg GAE/L. Other studies have shown similar results for beers made of blue corn (342 to 560 mg GAE/L) [5,16] and traditional beers made of barley malt (152.0 to 339.12 mg GAE/L) [40]. The differences of the total content of polyphenols may be explained by the variation in the quantity and quality of raw material, the brewing process and the storage conditions during ageing. Polyphenols provide beer with bitterness and astringency but also improve its functionality in terms of foamability, oxidative stability and heat stability which help to preserve the beverage during storage and ageing [39,41].

3.2. Volatile Composition

One hundred and twenty-one volatile compounds were identified in beer samples by HS-SPME/GC-MS. The chromatographic data of the volatile compounds of each beer is summarised in Table 2. Compounds were classified into 12 groups of which, the most abundant include esters, representing ~29% of the volatiles, followed by alcohols (~20%), terpenes (~15%) and phenols (~6%). These compounds, particularly alcohols and esters have been the most reported volatiles in

barley beers [42]. The major volatiles detected in this study were consistent with those of previously published studies [11,18,42,43].

As mentioned before, esters were the largest group found in all beer samples. Esters are the most common compounds in the majority of beers and these volatiles are considered desirable as they act in synergy with other compounds and contribute with most of the pleasant fruity-floral aromas in beer [44,45]. According to our results, it seems that beers made with barley malt contain higher number of esters than the beers made with corn malt (Table 2). For instance, ethyl propanoate, ethyl isobutanoate, ethyl pentanoate, ethyl isohexanoate, ethyl benzoate and isopropyl palmitate were only found in beers made with barley (Ba, BCBa, RCBa). It is well known that the presence of alcohols leads the production of esters [44]. Thus, the presence of a greater number of esters in barley beers could be attributed to their content of alcohols, which are precursors of these compounds.

Esters such as ethyl acetate, 3-methylbutyl acetate, ethyl hexanoate, phenethyl acetate, ethyl 9-decenoate and ethyl decanoate were found in all samples in higher abundance than the rest of the esters.

Ethyl octanoate, a product of fermentation by *Saccharomyces* yeast, was detected in all five beers that contain corn malt except in the one made of 100% barley malt. Conversely, octanoic acid was more abundant in the barley beer than in the corn beers, which is consistent with Saerens et al. [45] who found that higher levels of unsaturated fatty acids in beers, like in the corn beer samples, result in a decrease in ethyl ester production. The contrary effect can be seen for ethyl hexanoate and hexanoic acid, where in samples that exhibited a higher peak area of the ester, the presence of the acid seems to be reduced (BCBa and RCBa).

Alcohols were the second largest group of volatiles found in beers. We identified 24 alcohols and some of them were found in all six beer samples such as ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-ethyl-1-hexanol, phenylethyl alcohol and citronellol. These alcohols come mainly from alcoholic fermentation while others such as citronellol and phenylethyl alcohol come from the essential oils of hops. According to Lyu et al. [12] and Dong et al. [29] aromas like sweet alcohol, rough, whiskey, fruity and rose could be attributed to these compounds.

In addition, some alcohols such as 2-furanmethanol, 4-methyl-1-pentanol, 3-methyl-1-hexanol and iso-geraniol were only found in those beers that contain barley malt (Ba, BCBa and RCBa). Of them, 2-furanmethanol is a product of Maillard reactions that occur during the roasting process of malt, especially in the production of 'dark' and 'caramel' malts; hence the caramel malt used in Ba, RCBa and BCBa beers could be the source of this volatile [30]. Interestingly, to our knowledge, there are no reports of iso-geraniol in beers. This compound is the result of the partial oxidation of geraniol. It was previously identified in some flowers, fruits (grapes) and the essential oil of lemon, imparting a pleasant rose odour [46].

In beers, terpenic compounds are generally derived from the hop essential oils, which are added to the wort during the boiling process. These compounds have been related to pleasant aromas like citrus, floweryand lilac [29,30]. We identified 18 terpenes in the beer samples, most of them have previously been reported in barley beers [30,47]. Only linalool, geraniol and humulene, associated with flower, geranium and wood aromas respectively, were detected in all six samples of beer. In turn, limonene and β-myrcene were found in beers made 100% with corn malt (RC, BC). In addition, these beers (RC, BC) showed more abundance of limonene and linalool than the other samples of beer. Interestingly, δ-cadinol and α-cadinol were found in those beers made with blue corn malt but (BC and BCBa) and 3-methoxy-2-naphthalenol was found only in those that contain barley malt (Ba, BCBa, RCBa). Among these terpenes, limonene have been previously reported in corn starch and corn products [48,49].

Seven phenol volatile compounds were identified among the beer samples. These compounds contribute to clove and spice aromas in beers, which are desirable in some Belgian styles (amber and Trappist beers) and wheat beers [50]. For instance, 4-ethyl-2-methoxy-phenol was detected in all beers containing red and/or blue corn malt, but not in barley beer. Buttery and Ling [49] reported that

4-ethyl-2-methoxy-phenol is one of the major components in products like corn tortillas and tortilla chips. Furthermore, 2-methoxy-phenol was found only in beers containing blue corn malt. Even though 4-ethyl-phenol and 2-methoxy-4-vinylphenol were found in all beers, these compounds exhibited a higher peak area in those beers that contain both red and blue corn malt (RBC) than in the other beers. Of those, 4-ethyl-phenol is usually found in beers made of wheat malt. This molecule is formed from the biodegradation of hydroxycinnamic acids, such as ferulic and coumaric acid, during wort boiling. In high concentrations it imparts unpleasant aromas like medicinal, phenolic, clove-like, or smoky. However, in some beer styles such Belgian wheat and German Weizen these aromas are appreciated [51,52]. 2-methoxy-4-vinylphenol and 4-vinylphenol (the precursor of 4-ethyl-phenol) have been reported as major components of sweet corn products such as tortillas [53].

Styrene was the most abundant hydrocarbon found in all beer samples. This compound usually comes from the malt and it derives from the metabolism of cinnamic acid in barley malt by top-fermenting yeast [18]. Its presence in the corn beers is explained as its formation occurs in parallel to the formation of 2-methoxy-4-vinylphenol and 4-vinylphenol. Styrene has been described as a "sweet-smelling colourless fluid" [54].

Interestingly, dimethyl sulfide (DMS) exhibited a higher peak area in the beer made 100% with barley malt (Ba), followed by those made with corn malt (BC, RC, RBC). DMS is usually lost during the kilning of malt and the boiling of the wort, however its presence in the beer depends on the type of malt used. This sulphur compound has been reported in barley beers. Its presence is desirable in some styles of beers, like some lagers, while in others is not desirable as it adds sweet corn aroma to the beer [10]. In addition, DMS has been identified as an important contributor to the aroma of corn products [48,49].

Additionally, β-ionone was only found in beers made with blue and red corn malt (BC, RC, BCBa, RCBa) with the exception of RBC. This ketone has been previously reported as potential contributor of hop aroma. It has been identified in tortillas and corn dough [49], in late-hopped and dry-hopped beers [55] and in samples of whiskey made with corn [56].

3.3. Descriptive Sensory Analysis

The sensory panel developed a list of 30 attributes to describe the appearance, odour, taste, aroma and mouthfeel characteristics perceived in all beer samples (Table 3). The panel was asked to be as specific as possible in identifying attributes. Some terms and references were similar to those defined in the "beer flavour wheel", developed by Meilgaard [33], but others were unique attributes related to the presence of pigmented corn and chili.

The mean scores of the attributes were plotted in a radial diagram (except for the colour attribute) (Figure 1). Significant differences ($p < 0.05$) were found in 17 out the 30 attributes across the samples (Supplementary Table S1). In order to have a complete description of all sensory characteristics of the beers, all attributes were kept and used in the subsequent analysis. We can see that the non-significant attributes were mainly those pertaining to the odour category. These odour characteristics are common to most of the commercial beers and some of them are the result of the volatile compounds developed during the fermentation process (e.g., banana, apple, floral, fruity). Thus, as all steps in the brewing process remained the same, we can expect some similarities between beers.

All beers in this study exhibited a range of sensory characteristics commonly found in most of the commercial beer samples, however some characteristics such as 'dried fruits-O', 'dried-chili-O', 'brown sugar-O', 'tortillas-A' and 'spicy-M' are not in the common lexicon of beers [33]. Thus, the pigmented corn malt and the chili used in these beers appear to contribute to the development of these attributes. Despite the fact that cooked vegetable-A and cooked corn-O are usually associated with off-aromas in barley beers, we could expect that the pigmented corn beers develop these characteristics as they are sensory attributes found in the 'Sendechó' beverage [4] and in many corn-derived products [48,49].

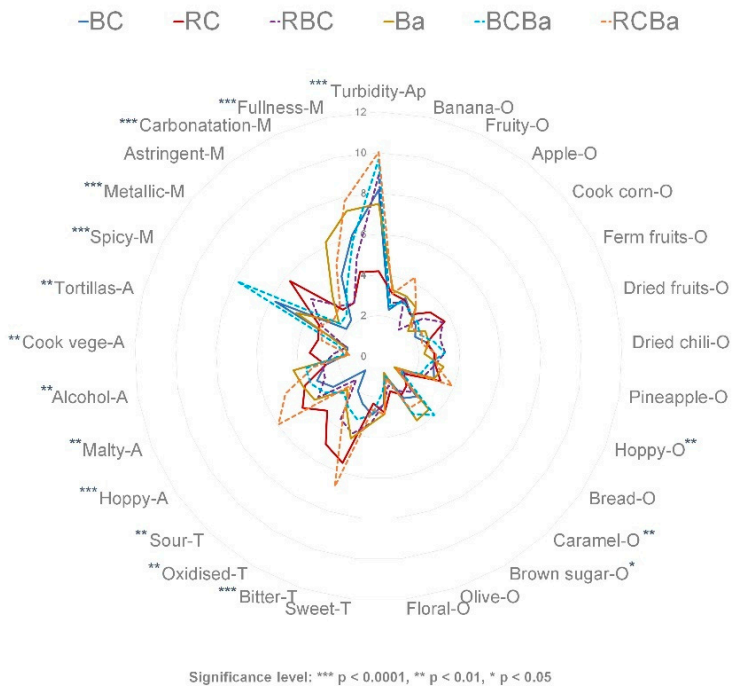

Figure 1. Sensory profile of the six beers. BC = 100% blue corn, RC = 100% red corn, RBC = 50:50 red and blue corn, Ba = 100% barley, BCBa = 50:50 blue corn and barley, RCBa = 50:50 red corn and barley.

The beer made 100% with barley malt (Ba) had a significantly higher intensity of brown sugar and caramel attributes than the other beers, which was expected as the caramel malt used in this beer contributes with the development of these aromas. Furthermore, alcohol aroma was higher in barley beer (Ba) than in the others, this is reasonable as barley malt contributes more to the formation of fermentable sugars than corn and therefore barley beers had higher alcohol content than the beers made with corn malt (see Table 4).

RC and RCBa, both containing red corn malt, were rated higher in bitter taste, as compared to the other beers. In general, those beers containing red corn malt (RC and RCBa) were characterised by higher intensity of aroma attributes such as cooked vegetables and tortillas, related to the type of corn used. In addition, sour taste, oxidised and metallic sensations were scored high in the RC beer. The latter attributes are usually associated to an ageing effect [32].

Despite the fact that Guajillo chili was added to all the beers in the same proportion and conditions during the brewing process, the perception of spicy attribute was different in all the beers. For instance, the beer made of blue corn and barley (BCBa) was rated significantly higher in spicy mouthfeel than the rest of beers, followed by blue corn beer (BC). The perception of the 'spicy' or 'pungent' sensation elicited by the capsaicin (the active ingredient of the Guajillo chili) may be influenced by factors such as the temperature, acidity and carbonatation of the beverage [57]. In addition, phenolic compounds that evoke an oral irritation [39,41] might increase the perception of this sensation. Thus, the content of polyphenols in BC and BCBa might contribute to the increase perception of the attribute spicy. Beers made with barley (Ba, BCBA, RCBa) had a higher carbonatation sensation than those beers made with pigmented corn malt (BC, RC, RBC). The perception of the fullness, which is associated with the body of the beer, was higher in the beers that contain blue corn and/or barley malts (BC, Ba, RCBa and BCBa) than in the ones made with red corn malt (RC and RCBa). The fullness palate sensation is related to the unfermentable sugars namely dextrins, developed during the mashing process. These compounds contribute to the body of the beer without imparting sweetness [10].

The assessment of a beer's appearance includes its colour, which according to the SMR colour chart it can range from straw to black. All beer samples analysed are in the range of the colours that goes from 10 SMR to 15 SMR units. Significant difference can be observed (Supplementary Table S1)

between the RC beer with a 'medium amber' colour (9 SMR), the BC beer with a 'light brown-reddish' colour (15 SMR) and the rest of the beers with a 'cooper-red' colour (12–13 SMR). It is well known that malt has the greatest impact on beer colour because of its content of melanoidins and Maillard compounds, which add colours that range from yellow, orange to red and brown [58]. In this case, the anthocyanins in the pigmented corn beers contribute to develop of these 'amber–red-cooper' colours, especially in those beers made 100% with red and blue corn malt. In acidic solutions such as beer, anthocyanins are chemically stable and turns their colours to reddish tones [3].

With the aim of illustrating the differences among beers produced by different types of malt (red corn, blue corn and barley), a PCA was applied on the total data set of 30 attributes. The biplot obtained is shown in Figure 2. The first two components (PC) explained 72.58% of the total variation in the samples with contributions of 40.39% by PC1 and 32.19% by PC2, where most of the attributes contributed considerably to samples discrimination.

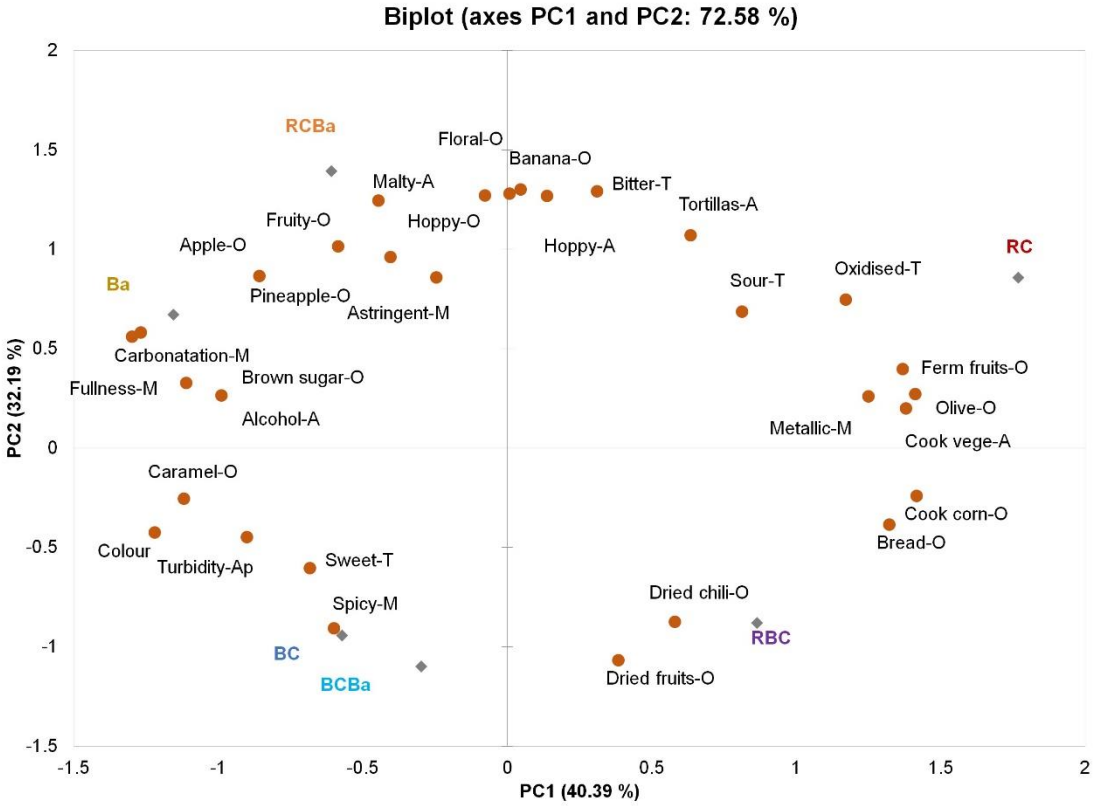

Figure 2. Principal Component Analysis (PCA) bi-plot of variables and individuals of descriptive sensory data. BC = 100% blue corn, RC = 100% red corn, RBC = 50:50 red and blue corn, Ba = 100% barley, BCBa = 50:50 blue corn and barley, RCBa = 50:50 red corn and barley.

PCA permitted a clear-cut separation of the samples based on the type of malt used.

PC1 opposed the beers made with barley malt like Ba, RCBa and BCBa (on the left) to the RC and RBC beer (on the right). On the other hand, PC2 opposed beers made of red corn malt (positive side) to beers made of blue corn malt (negative side). The RC beer was characterised by attributes such as fermented fruits-O, olive-O, tortillas-A, cooked vegetables-A, metallic-M and oxidised-M. On the contrary, BC and BCBa were characterised by spicy-M, sweet-T, Turbidity-Ap.

The beer made of 100% barley malt (Ba) was discriminated along PC1 (at the negative side) and was characterised by brown sugar-O, apple-O, alcohol-A, carbonatation-M and fullness-M.

Blended beer made of both type of corn malt (RBC) was placed in between red corn beer (RC) and blue corn beer (BC), sharing attributes of both malts used such as bread-O, cooked corn-O and dried chili-O and dried fruits-O. This behaviour was also shown in blended beer made of red corn

and barley malt (RCBa), preserving the sensory characteristics of both 100% barley (Ba) and 100% red corn (RC) beers such as apple-A, fruity-A, banana-A, malty-A and floral-A, attributes that are more common in typical barley beers.

These sensory data showed that by adding corn malt to the beer formulation, the sensory profile of the typical barley beer can be reached easily, while preserving at the same time odours and aromas of corn products, especially those of the Sendechó beverage such as corn and spicy and dried chili [4].

3.4. MFA of Sensory Attributes and Chemical Data

In this study, MFA was used to explore the differences and similarities between beers due to the type of malt used in brewing. In addition, MFA helped to identify associations between sensory and chemical datasets that brought us to know those components (sensory and chemical) that can be used as markers of beers made with pigmented corn malt.

The first two dimensions (Dim 1 and Dim 2) in Figure 3 accounted for 56.31% of the total variation with contributions of 31.19% by Dim 1 and 25.12% by Dim 2.

First, the variable plot (Figure 3b) shows that Dim 1 separates samples based on the sensory 'odour-aroma' attributes (in green; 34.05% of the variance) and the 'non-volatile' components (in pink; 34.53% of the variance). For Dim 2, the groups of variables 'volatiles' (in orange) and 'taste-mouthfeel' (in blue) are those that contribute the most to the dimension with 22.41% and 44.91% of variance respectively. The plot of the individuals (Figure 3a) allows us to visualise the global resemblance between beers by considering the information of all variables (sensory and chemical). It clearly showed that Dim 2 separated the samples based on the type of malt used, with beers made with pigmented corn (red and blue) on the top of the plot, and the beers that contain barley malt plotted on the bottom (Figure 3a).

Second, the RV coefficients (Table 5) show the relationship between the data matrices, the closer the RV coefficient to 1, the more similar the matrices [21,35]. According to the RV, a good correlation can be observed between the 'odour-aroma' and 'non-volatile' variables (0.740). Moreover, a better correlation between 'volatiles' and 'taste-mouthfeel' variables (0.649) than for 'odour-aroma' and 'volatiles' data matrices (0.509).

Table 5. RV coefficients between odour-aroma, taste-mouthfeel, volatiles, non-volatiles and supplementary data matrices of the MFA.

	Odour-Aroma	Taste-Mouthfeel	Volatiles	Non-Volatiles	Supplementary	MFA
Odour-Aroma	1.000	0.403	0.509	0.740	0.741	0.846
Taste-mouthfeel	0.403	1.000	0.649	0.374	0.307	0.755
Volatiles	0.509	0.649	1.000	0.364	0.428	0.793
Non-volatiles	0.740	0.374	0.364	1.000	0.321	0.779
Supplementary	0.741	0.307	0.428	0.321	1.000	0.579
MFA	0.846	0.755	0.793	0.779	0.579	1.000

A deeper analysis of Figure 3 allows detailing these relations between the different types of variables that strengthen the characterisation of the beers. On the negative side of Dim 1 of the variable plot (Figure 3b), it can be observed that the sensory attributes floral-O, hoppy-O and pineapple-O are positively correlated mainly with esters (i.e., ethyl butanoate (48), phenylethyl acetate (61), ethyl (E)-4-decenoate (65), ethyl decanoate (67), isoamyl octanoate (68), terpenes (i.e., geraniol (108), δ-cadinene (112), humulene oxide (115), δ-cadinol (120), and alcohols (i.e., phenylethyl alcohol (15), citronellol (19) and 1-decanol (22)). Numbers correspond to those on Table 2. Esters and alcohols are well known for their floral and fruity contribution to the beers, and terpenes are more likely associated with herb and green odours-aromas, which are consistent with the description of the hoppy odour. These correlations between the sensory attributes and the volatiles compounds strengthen the aromatic profile of the barley beer (Ba). Also compounds such as 2-nonanone (88), heptanoic acid (37), 2-ethylhexanoic acid (38) and acetaldehyde (25) were also correlated with the sensory attributes

mentioned before. The positive correlation of these fruity and floral sensory attributes with carboxylic acid compounds could suggest that the presence of esters, even in low levels, might reduce the perception of off-aromas like sweat and rancid, caused by octanoic acid [59].

On the positive side of Dim 1, RBC (Figure 3a) can be separated from the other beers mainly by the presence of phenol volatile compounds. Among them, phenol (94), 2-methoxyphenol (95), 4-ethylphenol (96) and 4-ethyl-methoxy-phenol (97) showed association with the sensory attributes related to the presence of pigmented corn malt such as cooked vegetables-A, cooked corn-A, olive-O and fermented fruits-O (Figure 3b). These compounds and the sensory attributes allow us to differentiate between the beers made 100% with corn malt, suggesting that these phenol compounds could be use as indicators of the use of pigmented corn in the brewing process.

On the negative side of Dim 2, we found positive correlations between attributes such as malty-A banana-O, brown sugar-O, tortillas-A and fruity-O and the compounds 2-furanmethanol (8), ethyl propanoate (44), propyl acetate (45), ethyl pentanoate (51), ethyl isohexanoate (52), ethyl hexanoate (54), iso-geraniol (20), acetophenone (87), 2-acetylpyrrol (101) and tetramethyl-pyrazine (102). The presence of these compounds, characterised by fruity, bready, brown sugar and caramel aromas [45,60], is consistent with the use of roasted malts (caramel malt) in the beers associated to these compounds (RCBa and Ba). Furthermore, on Dim2 (negative side), a weak correlation was also found for benzeneacetaldehyde (26) with astringent, which is consistent with the results obtained by Owusu et al. [61], where the presence of this compound has been associated with the astringent mouthfeel in products as cocoa and dark chocolates.

The positive side of Dim 2 is positively correlated with beers made from red corn malt (RC) and blue corn malt (BC) (Figure 3a, top side). These beers are well characterised by compounds such as linalool (106), limonene (105), β-ionone (90) and 4-ethyl-2-methoxy-phenol (90). These volatile compounds have been found in other corn products such as tortillas and pop-corn [49,60] and especially limonene and β-ionone have also been reported in samples of whiskey made with corn [56]. Thus, these compounds could also be used as markers of the presence of corn in beers.

In addition, the spicy attribute was strongly correlated with 2-methyl-5-(1-methylethyl)-phenol (98) well known as carvacrol -a key aroma compound in oregano spice- that is concordant with the pungent mouthfeel associated with this compound [60]. Unexpectedly, dimethyl sulfide (103) which usually imparts cooked vegetable off-aroma also showed a positive correlation with the spicy attribute. This behaviour could be attributed to the high abundance of phenylethyl alcohol (15) that could suppressed the perception of this compound [8].

Correlations between the non-volatiles variables (ABV, IBU, TPC, TAC) and the sensory and volatile data were also studied. For instance, a positive correlation was found between alcohol sensory attribute and alcohol content (ABV). Regarding the total polyphenol content (TPC), a negative correlation was observed between TPC and metallic and oxidised sensory attributes, confirming that polyphenols help to retard the development of these attributes in beer [54]. Moreover, TPC showed a positive correlation with carvacrol volatile (98). According to Lee et al. [62] carvacrol is a volatile compound that has exhibited potent antioxidant activity.

It is well known that anthocyanins do not impart aromas, but sometimes these compounds have been related to an astringent or bitter taste [41]. Even though, no obvious correlations were found between TAC and bitter or astringent attributes. The results showed a positive correlation between TAC and phenol compounds such as phenol (94), 2-methoxy-phenol (95), 4-ethyl-phenol (96) and 4-ethyl-2-methoxy-phenol. This could suggest an interaction between the anthocyanins that comes from corn malt and those phenol volatile compounds. According to Dufour and Sauvaitre [63] and Ruta and Farcasanu [64], interactions between anthocyanins and some aroma compounds such as phenol and 2-methoxy-phenol, lead the formation of copigments, which improve the stability of the anthocyanins and hence the colour stability of the beverage.

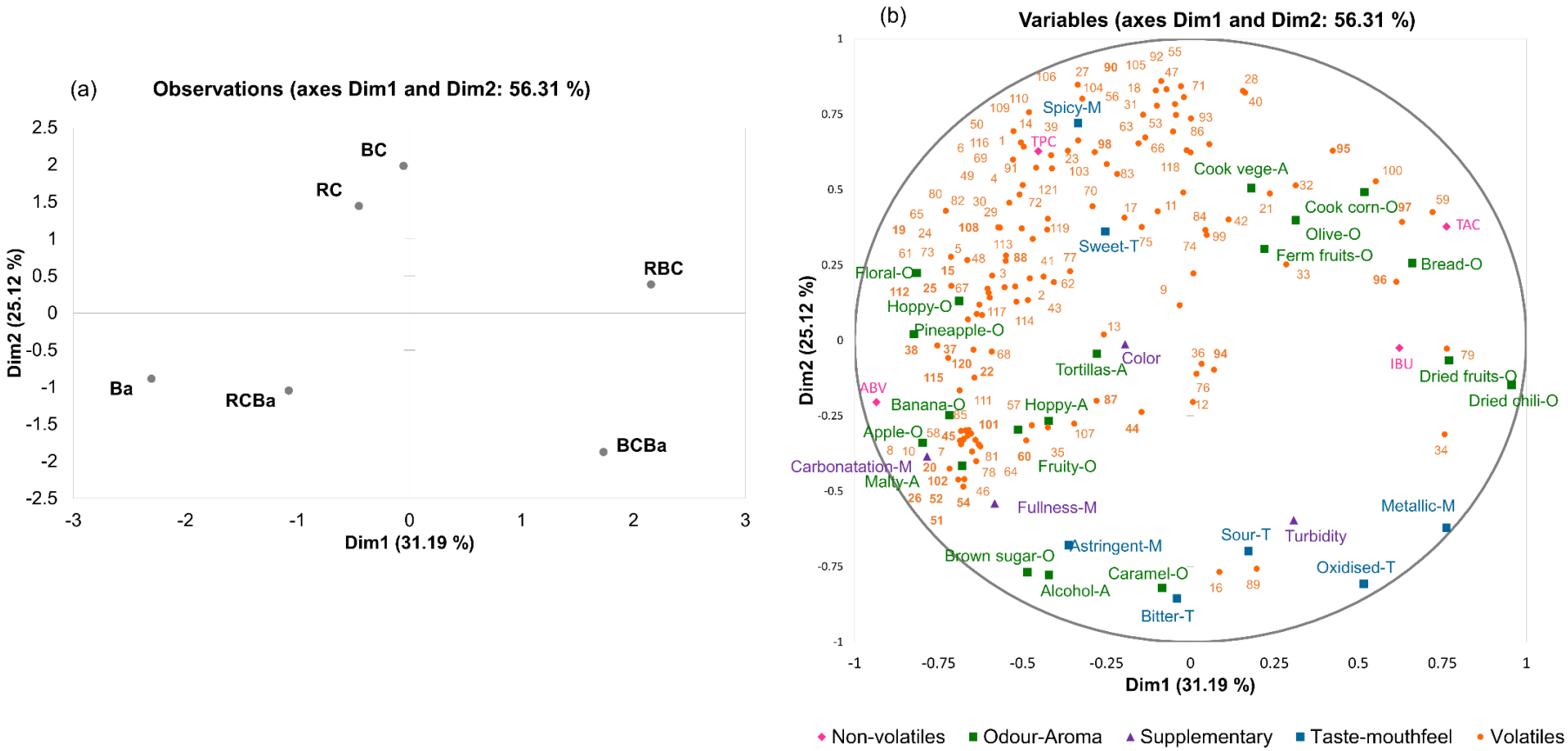

Figure 3. Multiple Factor Analysis (MFA) of descriptive sensory and chemical data of the six beer samples. (**a**) Observations plot of MFA, (**b**) Variables plot of MFA. Numbers correspond to the compounds listened in Table 2. BC = 100% blue corn, RC = 100% red corn, RBC = 50:50 red and blue corn, Ba = 100% barley, BCBa = 50:50 blue corn and barley, RCBa = 50:50 red corn and barley.

Apparently, no positive correlation was found between IBU parameter and bitter sensory attribute. However, there are other components that could contribute to the perception of bitterness such as the Maillard products formed during the kilning and roasting process of caramel and dark malts [57,65]. In addition, bitterness can be masked by sweetness due to sugars (residual sugar) that remain after the fermentation process. As has been mentioned before, IBU measures a beer's bitterness due to the α-acids of the hops, which gives an approximate idea of beer bitterness but there are other compounds that could impart or mask the bitter taste. Thus, it is not possible to directly correlate IBU to the perceived sensory bitterness [41].

Finally, the different groups of variables (sensory and chemical) had different influences in each beer. The major difference was found for the BCBa and RCB which were mainly described based on taste-mouthfeel attributes and non-volatile parameters respectively. Beers Ba and RCBa were mainly described based on the odour-aroma attributes and volatile compounds. For beers made 100% with pigmented corn (RC and BC) the group of volatiles had more influence in their characterisation. Overall, the volatile composition also separates beers depending on the presence of corn, supporting the fact that the use of corn as an ingredient clearly alters the sensory profile of beers.

4. Conclusions

It is well known that sensory evaluation plays an important role when new products needs to be characterised, but it is also an important quality factor used to control the brewing process. In this study, sensory evaluation enabled the complete description of the corn beers.

Beers made with these specific types of pigmented corn (red and blue) are mainly characterised by fermented fruits, cooked vegetables odours, tortillas, bread, dried fruits and dried chili.

We evidenced for the first time that among the groups of volatile compounds, ketone (β-ionone), terpenes (limonene, linalool) and phenol volatiles (2-methoxy-penol, 4-ethyl-phenol and 2-methoxy-4-vinylphenol, 4-ethyl-2-methoxy-phenol, 4-ethyl-2-methoxy-phenol), as well as the presence of anthocyanins appear as relevant criteria for corn beers differentiation. The latter can also be used as indicators to determine whether a beer is made with pigmented corn malt or not and therefore be used as a quality parameter in further studies. Moreover, the study of the relationship between the sensory attributes and the chemical parameters by MFA allowed to elucidate the effect of each type of malt (red corn, blue corn and barley malt) on the chemical parameters (VOC, ABV, IBU, TAC, TPC) and the association with the sensory attributes.

Both varieties of corn malt showed a clear influence in all parameters measured, especially in their sensory profiles. However, the blended beers (RCBa and BCBa) show the closest resemblance to a typical barley beer, while preserving those traditional aromas and tastes of the 'Sendechó' beverage. Additionally, the use of pigmented corn malt could help to prevent the development of off-aromas (e.g., oxidised), which could extend the shelf life of the beer.

This study will enable the Mexican brewing industry to gain an insight into the use of alternative and native cereals, which could renew and preserve autochthonal beverages in a modern way. Whether the sensory characteristics of these beers may carry the acceptance or rejection of consumers needs to be further investigated.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/7/886/s1, Table S1: Sensory attribute score means of beer samples.

Author Contributions: A.R.-M., H.B.E.-B., J.R.V.-C. and M.L.-D., conceptualization and design; A.R.-M., H.B.E.-B., J.R.V.-C. and M.E.-E., methodology, acquisition, formal analysis; A.R.-M., H.B.E.-B., data interpretation and writing-original draft; A.R.-M., H.B.E.-B., J.R.V.-C., M.E.-E. and M.L.-D., review and editing. All authors have read and agreed to the published version of the manuscript.

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How a Spanish Group of Millennial Generation Perceives the Commercial Novel Smoothies?

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Abstract: The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) constantly emphasize the importance of increasing fruit and vegetable consumption; these natural products help in the prevention of major diseases. Smoothies are a simple and convenient way of doing so; thus, their demand is constantly growing and their market is becoming important for the food industry. Therefore, the objective of this research was to determine Millennial consumer opinion towards novel fruit- and vegetable-smoothies available on the retail market. Napping®, descriptive sensory analysis, and consumer studies were conducted. Napping® results group samples into four clusters of smoothies; the main grouping factor was the type of fruit and the percentage of vegetables. Penalty analysis showed that smoothies need improvement mainly dealing with sweetness, bitterness, and vegetable flavors. Millennial consumers formed a homogeneous sensory group in which the overall liking was negatively correlated with the level of sweetness, and earthy, carrot, beetroot, and pear flavors. The key liking drivers were sourness and notes of mango, banana, and peach flavors. This research is a new insight into the perception of smoothies, provides comprehensive knowledge for the food industry, and can guide the design of new healthy smoothies.

Keywords: Napping®; descriptive sensory analysis; JAR; PLS; drivers of liking; penalty analysis

1. Introduction

Fruits and vegetables intake is highly recommended and necessary in a healthy diet due to their important nutrients and bioactive constituents (phenolic compounds, vitamin C, anthocyanins, among others) that was demonstrated in several studies to diminish the risk of chronic non-communicable diseases [1]. The Mediterranean diet, which has been widely studied regarding its effect on the prevention of diabetes and low-density lipoprotein oxidation, includes fruits and vegetables (tomato, broccoli, carrot, etc.) with high content of phytochemicals (carotenoids, phenolic compounds, vitamins, foliates, minerals, etc.) [2]. Nutritional recommendations point to the consumption of fruit and vegetables as part of a balanced diet, although with a reduction in the consumption of juices and smoothies due to the possible excessive supply of energy from sugars. In addition, these products

have a moderate or high glycemic index and, as a consequence, can cause a rapid increase in glucose and insulin levels. However, smoothies are rich in soluble fiber (pectin, mucus) and insoluble fiber (cellulose and lignin), which delays the absorption of monosaccharides into the bloodstream and thus effectively regulates glucose-insulin homeostasis [3].

The World Health Organization (WHO) is still alerting that 71% of all deaths worldwide are caused by non-communicable diseases (NCDs), and every year 15 million people die prematurely, i.e., between the ages of 30 and 69 years, from NCD. Unfortunately, there is still a lack of public health actions about fruits and vegetables consumption (e.g., health education and health program promotion); this is true for both developing countries but also for developed countries due to the current lifestyle [4,5]. The recommended fruit and vegetables intake is 400 g per day. To encourage, to maintain and/or to increase their intake, the food industry comes with an alternative to raw fruits and vegetable intake, developing new products, easy-to-eat, and with longer shelf-life than regular fruits, such as smoothies [1]. In 2018, although consumption average of raw fruits and vegetables per person in Spain was 412 g (247 g and 155 g for fruits and vegetables, respectively) [6], part of the Spanish population are below the recommended intake (no available information about the percentage of people who are consuming less than the recommended level).

The word "smoothie" comes from the English term "smooth" (tender, creamy), and defines a creamy non-alcoholic drink with a thick texture similar to that of milkshakes. This beverage includes only natural ingredients such as puree fruit with fruit juice, and possibly dairy products or/and crushed ice cubes [5,7]. Their preparation is based on the use of the entire fruit, which is processed from pulp to puree, with only the seeds and peel being removed. To develop different flavors and to obtain the appropriate texture of the final product, the juice from different fruits is used, and it is important to highlight that they are prepared without adding preservatives, stabilizers, or chemical correctors of pH and acidity [8]. This type of product is the easiest form to eat fruits and vegetables and to increase its consumption between consumers with the current lifestyle [5].

The smoothies market can be divided into several segments, attending to different criteria (e.g., product type, distribution channel, and geographical location). Based on product type, the market is segmented into fruit-based, vegetal-based, dairy-based, and others. The fruit-based smoothies segment accounts for the largest market and is expected to be the fastest growing segment in the healthy beverage market. The global smoothies market were worth $12.1 billion in 2020 and is projected to reach a compound annual growth rate (CAGR) of 8% over the next five years, to reach a value of $17 billion in 2025 [9]. North America dominates the smoothie market, while the Asia-Pacific region is expected to be the fastest growing area. It is worth mentioning that, although an increase in smoothies' consumption exists in those areas, people's health have worsened during recent years, basically increasing overweight problems. On the other hand, despite the health-promoting potential of smoothies, in Spain, it is still a sub-segment. However, consumer interest is growing, driven by health and wellness trends. Therefore, it is essential to test their sensory quality to improve their consumption [10].

Nowadays, several research studies about the food habits of the Millennial generation (those born between 1980 and 2000) have been carried out and now there is information about their needs and demands. The literature shows the uniqueness of Millennials and this is why current food sensory studies are focusing on this specific population [11]. On the other hand, although sensory analysis has been previously applied to smoothies [12–17], there is a lack of information about the opinion and preference of commercial smoothies by consumers, including Millennial ones (generation Y) on smoothies; this is relevant because this generation constitutes the largest group consuming healthy minimally processed foods. In a previous consumer study, Polish consumers indicated that smoothies containing cranberry, black currant, dog rose, and bilberry purees were more acceptable than other fruits [18].

Descriptive sensory analysis allows defining the food product in the context of its appearance, aroma, taste and texture, and also gives the opportunity to link these descriptive data with consumer

preferences. Several studies about smoothies used sensory descriptive analysis to characterize them. For instance, a recent study of different cultivars of pomegranate indicated that the smoothies prepared with *Mollar de Elche* pomegranate juice were characterized by having high intensity of pear odor/aroma and consistency, and the *Wonderful* smoothies were characterized by lower consistency and more intense pomegranate aroma and sourness [17]. Therefore, the aim of this study was to determine a Spanish group of Millennial consumers preference towards novel fruit and vegetable smoothies available on the retail market. Napping®, descriptive sensory analysis, and consumer studies were conducted. The research will provide a new insight into the perception of smoothie products and comprehensive knowledge for the food industry and can guide the design of new foods, including functional and healthy fruit-based products.

2. Materials and Methods

2.1. Experimental Design

The research consisted of two parts:

- In the first part of the study (session 1), a Napping® test was conducted focusing on the smell and taste of the 14 fruit- and vegetable-based smoothies; 100 consumers participated in this session. The objective of this part was to narrow down the selection of samples for second part of the study.
- In the second part of the study, a descriptive sensory evaluation by a trained panel and a consumer study (session 2) was conducted using only randomly selected smoothies by researchers, representing the clusters shown in the napping study. The consumers panelist for consumer study was the same people as the Napping test. The objective of this part was to know the consumer's liking drivers of types of smoothies (clusters).

With this design, the number of smoothie products in the first stage was 14, while in the second one, 4 samples were used. Two weeks passed between Session 1 and Session 2, data collection and statistic were needed before Session 2 for the selection of the appropriated sample of each detected cluster.

The sensory tests were carried out in a special tasting room with individual booths (controlled temperature of 21 ± 1 °C and combined natural/artificial light), and ballot charts were used to collect panelists' evaluations. The samples were presented according to a randomized block design to avoid biases. Samples were served chilled at 4 °C into odor-free, disposable 90 mL covered plastic cups, at room temperature and were coded using 3-digit numbers. In each vessel, 40 mL was added in all sensory tests carried out. Trained panelists and consumers were instructed to drink the served portion of the sample for doing descriptive sensory analysis, Napping test, and consumer study, respectively. Unsalted crackers and water were provided to panelists to clean their palates between samples.

All panelists (descriptive test) and consumers (Napping and affective tests) gave their informed consent for inclusion before they participated in the study. Universidad Miguel Hernández de Elche automatically exempts "general taste tests", including descriptive sensory tests from needing ethical approval, based on European Union guidelines. However, the study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Escuela Politécnica Superior de Orihuela, Universidad Miguel Hernández de Elche.

2.2. Smoothie Samples

Fruit- and vegetable-based smoothies were purchased on the retail market (supermarkets and grocery stores) in the province of Alicante (Spain) in 2018. The products were stored in their original unit packets under refrigerated conditions (as indicated on packaging: 4 °C) until analysis. Each smoothie came from 1 batch, and the general sample destined for analysis consisted of 4 primary samples. The compositions of smoothies declared by the producers are given in Table 1.

Table 1. Composition of commercial smoothies used in this study.

Ingredient	Smoothie (%)													
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**
Fruit Purée														
Apple	†	†	0	0	0	3 1/2 ‡	0	1 1/2 ‡	0	1 1/2 ‡	0	0	0	0
Apple puree	0	0	0	0	0	0	0	0	11	0	0	0	22	0
Apple sauce	0	0	0	0	21	0	0	0	0	0	0	0	0	0
Banana	†	0	0	0	0	1 1/2 ‡	0	0	0	2 ‡	0	0	0	0
Banana puree	0	0	20	15	0	0	0	3/4 ‡	0	0	0	5	0	10
Blackberry puree	0	0	8	0	0	0	0	0	0	0	0		0	0
Blackcurrant	0	0	0	0	0	0	0	9 ‡	0	17 ‡	0	0	0	0
Blueberry puree	0	0	0	0	0	0	6	0	0	0	0	5	0	0
Cherries	0	0	0	0	0	0	0	4 ‡	0	0	0	0	0	0
Currant puree	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Guarana	0	0	0	0	0	0	0	†	0	0	0	0	0	0
Kiwi	†	0	0	0	0	0	0	0	0	0	0	0	0	0
Lime	0	0	0	0	0	†	0	0	0	0	0	0	0	0
Mango	7.7	0	0	0	0	17	0	0	0	0	0	0	0	0
Mango puree	0	0	0	16	0	0	0	0	0	0	0	0	0	15
Orange pulp	0	0	6	0	0	0	0	0	0	0	0	0	0	0
Passion fruit	0	0.2	0	0	0	1/4 ‡	0	0	0	0	0	0	0	0
Peach puree	0	0	0	2	0	†	0	0	0	0	0	0	0	0
Pear puree	0	0	0	0	0	0	0	0	0	0	17	0	0	0
Pineapple	10.5	11	0	0	0	0	0	0	0	0	0	0	0	0
Raspberry puree	0	0	8	0	0	0	6	0	0	0	0	17	0	0
Red grapes	0	0	0	0	0	0	0	14 ‡	0	0	0	0	0	0
Strawberry	0	0	0	0	0	0	0	0	0	22 ‡	0	0	0	0
Strawberry puree	0	0	9	0	0	0	4	3 1/2 ‡	0	0	0	20	0	0
White grape	0	0	0	0	0	0	0	0	0	41 ‡	0	0	0	0
Fruit and Vegetable Juices/Concentrated														
Apple juice	0	0	39	52	43	0	57	0	41	0	0	53	43	71.5
Apple juice from concentrate	†	0	0	0	0	0	0	0	0	0	0	0	0	0
Beet juice	0	0	0	0	0	0	0	†	0	0	0	0	0	0
Cucumber juice	0	0	0	0	0	0	0	0	0	0	10	0	0	0
Ginger juice	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Grape juice	0	0	10	0	0	0	0	0	0	0	0	0	0	0
Lemon juice	0	0	0	†	4	0	0	0	6	0	4.5	0	2	0
Orange juice	0	39.5	0	10	0	1‡	15	0	0	1/4 ‡	0	0	0	0
Passion fruit juice	0	0	0	4	0	0	0	0	0	0	0	0	0	3.5
Pear juice	0	0	0	0	0	0	0	0	0	0	28	0	0	0
Pineapple juice	24	0	0	0	0	0	0	0	10	0	18	0	0	0
Pomegranate juice	0	0	0	0	0	0	5	0	0	0	0	0	0	0
Romaine lettuce juice	0	0	0	0	0	0	0	0	0	0	3.5	0	0	0
Vegetables Purée														
Aloe vera puree	0	0	0	0	0	0	7	0	0	0	0	0	0	0
Beet puree	0	0	0	0	14.5	0	0	0	0	0	0	0	0	0
Carrot	†	15	0	0	0	0	0	0	0	0	0	0	0	0
Carrot puree	0	0	0	0	17	0	0	0	17	0	0	0	0	0
Celery	0	0	0	0	0	0	0	0	0	0	13	0	0	0
Celery puree	0	0	0	0	0	0	0	0	0	0	0	0	8	0
Cucumber puree	0	0	0	0	0	0	0	0	0	0	0	0	14	0
Iceberg lettuce	6	0	0	0	0	0	0	0	0	0	0	0	0	0
Kale puree	0	0	0	0	0	0	0	0	0	0	0	0	5	0
Ginger puree	0	0	0	0	<0.1	0	0	0	0.1	0	0	0	0	0
Sweet corn	†	0	0	0	0	0	0	0	0	0	0	0	0	0
Pumpkin	0	7	0	0	0	0	0	0	0	0	0	0	0	0
Pumpkin puree	0	0	0	0	0	0	0	0	14	0	0	0	0	0
Spinach puree	0	0	0	0	0	0	0	0	0	0	5	0	5	0
Others														
Agave syrup	0	†	0	0	0	0	0	0	0	0	0	0	0	0
Ascorbic acid	0	0	0	0	0	0	†	0	0	0	†	0	0	0
Cinnamon	0	0	0	0	0	0	0	0	<0.1	0	0	0	0	0
Citric fiber	†	†	0	0	0	0	0	0	0	0	0	0	0	0
Coconut milk	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Ground flax seeds	0	0	0	0	0	0	0	†	0	0	0	0	0	0
Hemp seeds	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0
Natural aroma	†	0	0	0	0	0	0	0	0	0	0	0	0	0
Nettles	0	0	0	0	0	0	0	0	0	0	†	0	0	0
Vitamin infusion	0	0	0	0	0	0	0	†	0	0	0	0	0	0

† Presence but quantity was not indicated on ingredient list. ‡ ingredient expressed as fruit pieces and not as %.

2.3. Consumer Information

A Spanish group of Millennial consumers (n = 100) were recruited among the students of Pharmacy Degree at the Miguel Hernández University (UMH). The ages ranged from 20 to 35 years old (born between 1985 and 1999), being 56% females and 44% males. First, they participated in the napping test (session 1) and later in a consumer study with the 4 selected smoothies (session 2).

2.4. Napping® Technique

Napping® is a relatively new and rapid sensory profiling technique based on positioning products on a paper sheet (from French "nappe"—tablecloth) to collect sensory distance perceives among products. Data are processed using multivariate techniques such as Generalized Procrustes Analysis (GPA) and Multiple Factor Analysis (MFA). Finally, a Napping® test creates sample clusters according to their specific organoleptic attributes and the own definitions given by the panelists.

The napping test was performed focusing on the flavor of the 14 fruit- and vegetable-based smoothies in session 1. Explanations on how to perform the test was provided to participants at the beginning. Each consumer was provided with a 60 cm × 40 cm blank paper, which was approximately A2 size (the napping sheet), a pen and a tray with the 14 samples. The sample order on the individual trays was randomized to avoid the effect of order of presentation and the carryover effect of a preceding sample over a series [19], even though the napping methodology allows and requires subjects to go back and forth among samples. Samples were coded with 3-digit random numbers.

Consumers were instructed to evaluate samples according to similarities or dissimilarities in flavor attributes by placing similar samples close to each other and more dissimilar samples further apart on the napping sheet. They were asked to complete the task using their own criteria and they were told that there were no right or wrong answers. After they had reached a final configuration, consumers wrote down appropriate descriptors for flavor notes of the samples, which were moved around the napping sheet, when needed. This procedure is known as ultra-flash profiling and is commonly used to add a descriptive dimension to a napping task [20]. When all the samples had been placed on the paper, they replaced samples with an X and noted the sample codes and the smoothie descriptors next to the X. Re-tastings and spitting out the smoothies were allowed. Water and crackers were used as palate cleansers.

From the 4 clusters generated in this part of the study, 1 representative model smoothie of each cluster was selected for the second part of this research (session 2).

2.5. Descriptive Sensory Analysis

Eight trained panelists (aged 25 to 55 years; 4 females and 4 males) with more than 600 h of training in sensory testing from the department of Agro-Food Technology (UMH) participated in this study. The panel was selected and trained following the ISO standard 8586-1 (1993), and it is specialized in descriptive sensory evaluation of fruit products [17,21–23]. For the present study, the panel worked during 2 orientation sessions (90 min for each one) discussing the main organoleptic characteristics of commercial smoothies. The lexicon used was based on the previous ones developed by other authors [17,22,24–27] (Table 2). Lexicons were adapted for smoothies during the orientation sessions. Samples were assessed using sensory attributes of basic tastes, flavor notes and texture and somatic sensations (n = 22), and the order process was (i) flavor notes, (ii) basic tastes, and (iii) texture and somatic sensations. References were chosen and prepared according to previous publications using similar attributes [17,22,24–27], and then provided to panelists. The scale to be used was range from 0 (no intensity) to 10 (extremely high intensity) with 0.5 increments.

Table 2. Lexicon of the sensory descriptors.

Attribute	Definition	References	Previous Publications
After taste	Longevity of key attributes intensity after swallow the sample	5 s = 1.0; 30 s = 4.0; 60 s = 8.0	[17,22]
Bitter	The basic taste associated with a caffeine solution	0.008% caffeine solution = 1.0 0.15% citric acid solution = 2.0	[17,22,26]
Sour	The taste factor associated with some organic acid, specifically citric acid	0.043% citric acid solution = 2.0 0.064% citric acid solution = 3.0 0.120% citric acid solution = 5.0 0.168% citric acid solution = 7.0	[17,22]
Sweet	The basic taste associated with a sucrose solution	3% sucrose solution = 2.0 6% sucrose solution = 4.0 12% sucrose solution = 8.0	[17,22]
Aloe	Aromatic associated with aloe	Diluted aloe juice (1:1) = 5 Fresh aloe juice = 10	[26]
Apple	Aromatic compounds associated with processed apple juice and cooked apples	Hacendado mango–apple nectar = 5.5	[17]
Banana	Aromatic associated with bananas	Fresh peeled banana = 10	[26]
Beetroot	The damp, musty/earthy, slightly sweet aromatics commonly associated with canned/cooked beets	Diluted kroger canned beet juice (1:2) = 4.0	[24]
Carrot	The aromatics commonly associated with canned, cooked carrots	Del monte sliced canned carrots = 7.0	[26]
Citric	Volatile compounds associated with lemon or lime	Fresh-squeezed orange juice = 8 Fresh-squeezed orange juice diluted 1:1 = 4	[22]
Earthy	Musty, somewhat sweet, full aromatics commonly associated with decaying vegetative matter and damp black soil	Geosmin (4,000 ppm) = 9.0	[27]
Green-vinery	Green, fresh aromatics associated with green vegetables and newly cut vines and stems; related to cucumber	Trans-2-hexen-1-ol 5000 ppm = 4.0 Heinz tomato ketchup (vinegar) = 4.5 Freshly sliced tomatoes = 10.0	[24]
Mango	A sweet, fruity aromatic associated with mango	Fresh peeled mango = 10	[26]
Orange	The aromatics associated with oranges; including juice, pulp and peel	Fresh-squeezed orange juice = 9	[26]
Passion fruit	A sweet, fruity aromatic associated with passionfruit	Fresh passionfruit = 10	[26]
Peach	Aromatic compounds from ripe peach	Fresh peeled peach = 10	[26]
Pear	Sweet, slightly musty, floral, honey/caramel-like, fruity aromatic associated with ripe pears	Hacendado pear nectar = 6.5	[17]
Lumpiness	The perception of large particles that are not dissolved in the product	Yoplait Strawberry Yogurt = 4.0	[24,25]
Mouth coating	The amount of film left on the mouth surfaces	Whipped cream = 6 Pureed potato = 10	
Pulpy	A soft moist residue	Del Monte slices peaches = 2	
Tooth etch	A sensation of abrasion and drying of the surface of the teeth.	Welch's grape juice diluted (1:1) = 6.0 Distilled water = 1	
Viscosity	The force required to move the product across the tongue	Condensed milk diluted (1:1) = 5 Condensed milk =10	

2.6. Consumer Studies

The consumer study was carried out in the tasting rooms of UMH. Each consumer tasted all 4 selected samples in a single session (session 2). Consumers were asked about their overall liking on a nine-point hedonic scale (1 =dislike extremely, 5 = neither like nor dislike, and 9 = like extremely) followed by questions about the appearance, flavor, and texture attributes. Additionally, a nine-point scale was used for the Just About Right (JAR) questions (1, 2, and 3 mean deficit of each attribute, too low in Penalty Analysis; 4, 5 and 6 mean "Just About Right", JAR in Penalty Analysis; and, 7, 8 and 9 mean excess, too high in Penalty Analysis) to determine possible improvements of the attributes: color, bitterness, sourness, sweetness, fruity, vegetal, and viscosity. These results provide direction of improving, but it does not provide information on degree of intensity change and ingredients adjustment.

2.7. Statistical Analysis

Two consecutive tests were performed: (i) one-way analysis of variance (ANOVA), and (ii) Tukey's multiple range test. Homogenous groups and the least significant difference (LSD) were determined at a significance level of $p < 0.05$ (95% of confidence level). Data were subjected to ANOVA, after checking the normality and homogeneity of the variance, and later to Tukey's multiple-range test to compare the means. Overall, liking data (y: dependent variable) versus descriptive sensory analysis (x: independent variables) were used to perform partial least squares regression (PLS). In addition, penalty analysis was conducted to provide extra information about the possible improvements of some samples. All statistical analyses were performed using StatGraphics Plus 5.0 software (The Plains, VA, USA) except Napping, Penalty Analysis, PLS, and dendrogram were prepared using XLSTAT Premium 2016 software (2016, París, Frane).

3. Results and Discussion

3.1. Results of Napping®

Appearance, including the color and texture of fruits-based drinks, is the first quality parameter evaluated by consumers and is a key tool for product selection and, then, acceptance or rejection. However, taste is more strongly correlated with the overall assessment of products and is the most relevant acceptability feature used by consumers to evaluate fruit and vegetable products [1,28]. Therefore, in the napping® test, consumers evaluated only "flavor" attributes of 14 smoothies. The terms and their frequencies of mentions for each sample (Table 3) were added as supplementary variable in the Multiple Factor Analysis (MFA). The terms were, thus, not included in the construction of the MFA factors but projected in the product space [19].

Consumers described the taste and aroma (perception of volatile compounds with the product within the mouth) using a total of 34 different sensory descriptors. Each of the smoothies was described with an average of 310 terms (average of number of terms of each smoothie, see Table 3) which were used and repeated by consumers depending on the product. Nevertheless, the most frequently used terms were fruity, citric, tropical fruit, sweetness, and sourness (from 273 to 382 repetitions for each of these terms for the 100 consumers). The average number of mentions of these attributes per product was above 20. The terms caramel, artificial, persistent, unpleasant, aromatic, unknown, and soft were used with a lower frequency (maximum 36 repetitions of these terms in whole task).

The frequency and number of sensory descriptors for each smoothie were subjected to multiple factor analysis and the outcomes are shown on the MFA biplot (Figure 1). The first two dimensions (F1 and F2) explained 70.49% of the total variance (54.61% and 15.88%, respectively) (p-value < 0.01, alpha = 0.05). Additionally, the results were supported by a dendrogram using Pearson's correlation based on the unweighted average (Figure 1). Millennial consumers created four representative clusters composed of the following smoothies and the most characteristic attributes:

1. Group 1: Smoothies E, K and M were described using the attributes vegetal, vegetable, herbal, earthy, spicy, cooked notes, beetroot, bitterness, and unpleasant, among others. These products had a high content in forms of juices or/and purees of carrot, beet, celery, cucumber, spinach and kale, and small additions of ginger and lemon.
2. Group 2: Smoothies G, I, and L were defined as liquid, sour, citric and astringent. These smoothies contained high percentages of orange, strawberry, raspberry, blueberry, pomegranate, carrot, pumpkin, lemon, pineapple in forms of juices or purees, and spices (ginger, cinnamon), among others.
3. Group 3: Smoothies A and B created the smallest group and were described by the terms viscosity, graininess, chalkiness, and aromatic. Smoothies were characterized by a high content of pineapple, orange, mango, carrot, pumpkin and ingredients unusual for other tested smoothies such as hemp seeds, citric fiber, and agave syrup, among others.
4. Group 4: This cluster was which included the most number of smoothies: C, D, F, H, J, and N. Consumers described them as familiar to them and probably, therefore, they used the largest number of attributes, including fruity, fresh fruity, overripe fruity, tropical fruit, and sweetness, among others. These products mainly contained banana, mango, grape, berries, orange, passion fruit, and peach, in crushed forms, purees and juices, as well as lemon and lime, among others.

Table 3. Terms used by Millennial consumers to describe commercial smoothies in the Napping® test and number of mentions for each of the samples, considering that 100 consumers participated in the test.

Term	Smoothie														Number of Term Repetitions
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	
Fruity	16	17	30	32	6	34	16	22	22	26	0	23	1	28	273
Fresh Fruity	9	9	11	16	3	20	18	19	18	16	2	19	2	11	173
Overripe fruity	16	12	16	19	6	19	10	15	7	19	1	9	1	20	170
Citric	5	26	21	15	1	14	43	19	52	16	0	46	0	23	281
Berries	1	3	11	1	1	0	7	9	3	10	0	9	0	0	55
Tropical fruit	18	17	28	39	3	38	22	30	19	29	0	22	0	33	298
Stone fruit	3	7	5	12	1	12	3	5	2	5	0	0	1	6	62
Caramel	0	2	4	3	2	4	1	6	2	6	0	2	0	4	36
Cooked notes	6	5	3	2	10	1	4	5	0	3	9	1	8	2	59
Earthy	16	8	4	1	24	0	1	1	3	1	26	0	22	2	109
Herbal	15	3	2	2	29	1	2	1	3	2	41	2	41	2	146
Vegetal	16	8	3	2	53	2	8	2	6	2	61	0	59	5	227
Spicy	7	4	2	2	10	1	4	2	0	2	14	2	14	3	67
Beetroot	3	0	0	1	21	1	5	1	4	2	19	3	19	0	79
Familiar	8	8	9	21	3	19	9	13	10	19	3	14	3	17	156
Unknown	5	2	2	0	5	0	1	1	4	1	8	0	7	0	36
Apple	4	4	12	6	1	6	2	12	2	9	0	6	0	1	65
Vegetable	16	5	2	1	41	0	4	2	10	1	48	1	47	3	181
After-taste	16	11	10	8	11	8	13	10	16	6	19	13	17	13	171
Artificial	2	2	2	1	2	0	1	1	1	0	4	0	4	3	23
Sweetness	30	16	29	63	10	59	13	34	14	44	1	18	2	50	383
Sourness	6	19	32	15	3	15	48	27	53	21	2	57	2	22	322
Bitterness	8	12	7	3	23	2	12	7	8	2	28	8	30	3	153
Astringency	3	7	5	0	3	1	9	7	8	4	4	8	5	2	66
Chalkiness	9	17	13	4	8	7	7	12	6	11	8	5	8	6	121
Grainy	22	27	15	9	3	8	7	10	8	18	9	9	5	10	160
Viscosity	25	25	17	11	6	10	8	15	10	18	5	10	5	9	174
Liquid	1	7	8	11	10	13	22	11	21	5	7	19	6	17	158
Persistent	1	1	1	1	1	0	0	0	1	1	2	1	1	0	11
Flat	4	6	3	2	6	1	2	1	2	2	2	1	4	4	40
Unpleasant	2	1	0	0	4	0	1	0	0	0	7	0	7	1	23
Dense	8	6	4	3	1	4	0	3	4	3	2	1	1	2	42
Aromatic	2	1	0	1	1	1	0	1	0	0	0	0	0	2	9
Soft	1	0	2	1	1	2	1	3	0	2	0	1	0	2	16
Number of Terms of Each Sample	304	298	313	308	313	303	304	307	319	306	332	310	322	306	

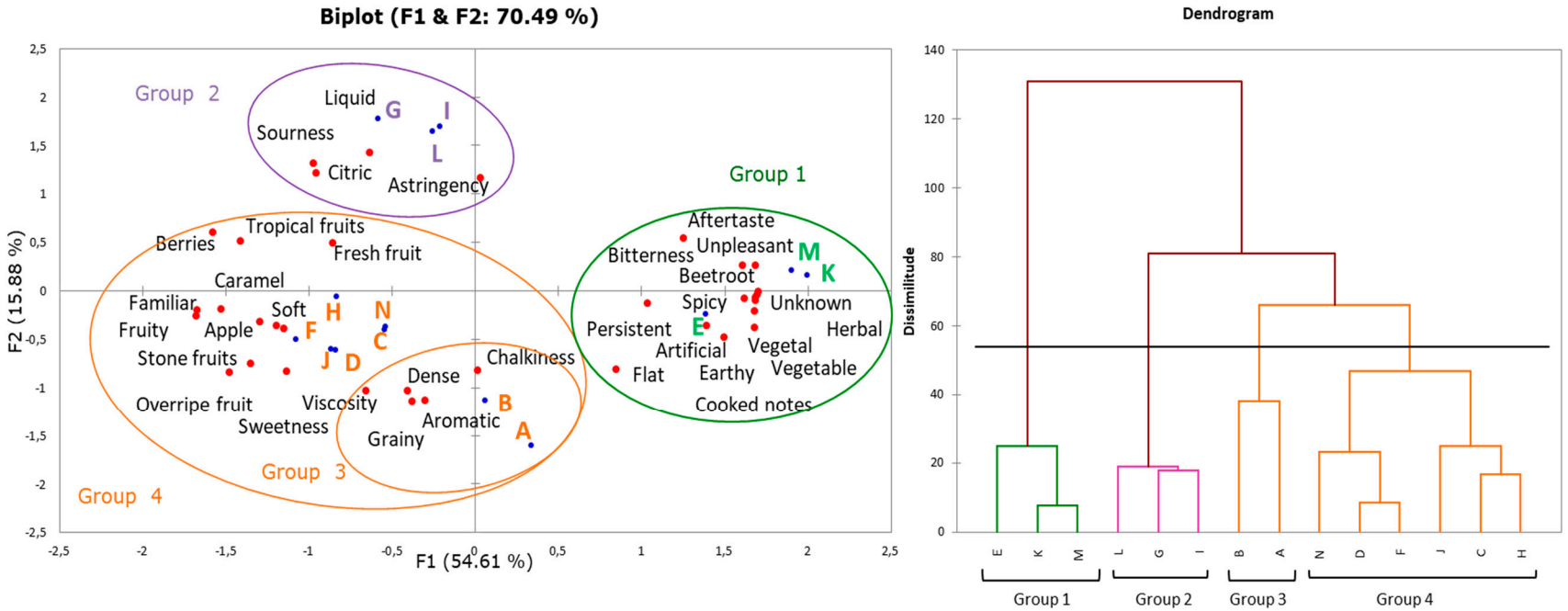

Figure 1. Multiple factor analysis biplot of terms used by a Spanish group of Millennial consumers for commercial smoothies in the Napping® test and dendrogram using Pearson's correlation based on the unweighted average.

For the F1 dimension, the highest % of contributions were bitterness, fruity, earthy, spicy, and beetroot variables (>4%), while, for the F2 dimension, citric, sourness and liquid were the highest contributors (>8%).

The terms sweetness, chalkiness, grainy, viscosity, dense, and aromatic were common to smoothies from Groups 3 and 4. Moreover, a clear differentiation among fruit smoothies and vegetable smoothies was found. The use of vegetable ingredients, such as carrot, beetroot, pumpkin, celery, cucumber, spinach, and kale, in a total amount of 31% to 33% for smoothies I, E, K, and M influenced the clearly perceptible vegetable, earthy, herbal, and astringency flavors, as well as spicy except for smoothie I. Terms for these vegetable smoothies were also associated with aftertaste, cooked notes, unknown and unpleasant flavors. As for cooked notes, it can be said that mild-heat treatment should have been applied during the smoothie processing as pasteurization (stored at refrigeration temperature), and could be the reason this flavor was detected by consumers.

Smoothies A and B were defined as dense, which could result not from the fruit and vegetable composition but from the presence of citric fiber with high water holding capacity and apparent viscosity. At this stage, the napping® test showed no difference among sensory perception of smoothies with conventional and organic fruits and vegetables.

Finally, four smoothies representing each of these four clusters created by consumers in the Napping® task were subjected to descriptive sensory analysis and consumer study (session 2) to obtained the improvements needed and the buying drivers: group 1: smoothie E, group 2: smoothie G, group 3: smoothie B, and group 4: smoothie D.

3.2. Descriptive Sensory Analysis

Napping® itself does not characterize products; thus, there is a need of a descriptive method to get a full profile of the products [19]. Descriptive sensory analysis was carried out to assess the sensory profile of commercial smoothies and to check significant differences among the four groups; the main differences are shown in Table 4.

The study showed variability in the intensity of the main sensory descriptors. The sweetness of the smoothies was relatively low (maximum 4.5 points), and it can be observed that D samples was significantly higher than G and E samples. Smoothies B and G had the highest sour score (8.0 and 7.5, respectively). The presence of tropical fruits (mango, banana) was linked with higher scores of sweetness, while orange, pineapple, and berries with a higher intensity of sour taste. The results were in line with research on smoothies by other authors [29], where strawberries were linked with a sour flavor, and banana pulp with a high sweetness perception.

Product B, besides being sour, was characterized by the highest bitter notes and the longest aftertaste. Bitter notes were significantly lower in other smoothies and did not differ statistically ($p > 0.05$). A high value of aftertaste was also found in sample E, being both based on vegetable ingredients.

The detailed taste was determined based on descriptors of direct flavor of specific fruits and vegetables. Thus, the flavor of smoothie E with carrot (17%) and beet purees (14.5%) was defined as the highest scores of beetroot, earthy, and carrot (8.0, 9.0, and 6.0, respectively). The taste of this product was the most complex and described by seven attributes. Only this smoothie defined with low pear flavor notes. Smoothie B was described with only two flavor attributes, as orange and carrot, with the predominance of the first (scores 6.5 and 3.5, respectively).

In the smoothie G, only 7% aloe vera puree caused a medium intense flavor of this plant (score 5.0) and a slightly perceptible green-vinery hint. Additionally, low values of apple, orange, and citric flavors were detected. In product D, flavors in the decreasing intensity were mango > passion fruit > peach > apple and orange> banana were observed. The intensity of apple flavor reached up to 2.0, despite its 57% in the studied smoothies. Thus, apple seems to be a good base for creating and modulating flavor bouquets and as a carrier of ingredient flavors in fruit and vegetable smoothies without masking the minor ingredients. In other studies, apple improved the sensory acceptance of smoothies [15,18].

Table 4. Descriptive sensory analysis of the selected commercial smoothies. The scale was range from 0 (no intensity) to 10 (extremely high intensity) with 0.5 increments.

Attribute	ANOVA †	Smoothie			
		E	B	G	D
		Basic taste			
After taste	**	7.0 ‡ ab	7.5 a	6.0 b	5.5 b
Bitter	*	0.5 b	1.5 a	0.5 b	0.5 b
Sour	***	3.0 c	8.0 a	7.5 a	5.5 b
Sweet	*	2.5 b	3.5 ab	3.0 b	4.5 a
		Flavor			
Aloe	***	0.0 b	0.0 b	5.0 a	0.0 b
Apple	**	2.0 a	0.0 b	1.0 ab	2.0 a
Banana	*	0.0 b	0.0 b	0.0 b	1.5 a
Beetroot	***	8.0 a	0.0 b	0.0 b	0.0 b
Carrot	***	6.0 a	3.5 b	0.0 c	0.0 c
Citric	***	1.5 b	0.0 c	2.5 a	0.0 c
Earthy	***	9.0 a	0.0 b	0.0 b	0.0 b
Green-vinery	*	0.0 b	0.0 b	2.0 a	0.0 b
Mango	***	0.0 b	0.0 b	0.0 b	5.0 a
Orange	***	1.0 b	6.5 a	2.0 b	2.0 b
Passion fruit	***	0.0 b	0.0 b	0.0 b	4.0 a
Peach	**	0.0 b	0.0 b	0.0 b	3.5 a
Pear	*	1.5 a	0.0 b	0.0 b	0.0 b
		Texture and somatic sensations			
Lumpiness	***	0.0 b	5.5 a	0.0 b	0.0 b
Mouth coating	*	1.0 ab	0.5 b	1.5 a	1.0 ab
Pulpy	**	0.0 b	3.5 a	0.0 b	0.0 b
Tooth etch	***	0.0 b	0.0 b	4.5 a	0.0 b
Viscosity	***	1.5 b	4.0 a	1.0 b	1.5 b

† *, **, and *** significant at $p < 0.05$, 0.01, and 0.001, respectively. ‡ Values (mean of eight trained panelists) followed by the same letter within the same row were not significantly different according to Tukey's least significant difference test. Scale used ranged from 0 (no intensity) to 10 (extremely strong intensity).

Commercial smoothies were not characterized by high values of the texture and somatic sensations attributes. Lumpiness was found only for smoothie B, and, in turn, tooth etch only for smoothie G. Pulpy residue and viscosity were higher in product B than the other smoothies. The evaluation of these attributes should consider not only the raw materials but also fineness/particle size (juice, puree, pulp, chopped pieces) and processing (cold, heat and enzymatic treatment).

3.3. Consumer Acceptability

3.3.1. Hedonic Rating

Mean scores for liking of color, appearance, flavor notes (fruity, vegetal), basic tastes (sweetness, sourness), viscosity and overall liking of samples for a Spanish group of Millennial consumers are shown in Table 5.

In general, not all selected smoothies were considered acceptable, and can be ranked in decreasing order of overall liking D ≈ G > B > E, although smoothies D and G received the highest scores (6.4 and 5.6 points, respectively). For these products, the highest scores were found for fruity notes, sweetness, and sourness, which in turn were linked with the presence of mango, orange, banana, berries, passion fruit, and peach.

Clearly earthy flavors (e.g., beetroot and carrot) were not accepted by consumers. Therefore, smoothie E was rated overall very disliked (score 2.1), which resulted from low ratings for flavor notes and basic taste (below 4.5).

Table 5. Mean scores and ANOVA for liking degree of a Spanish group of Millennial consumers for color, appearance, flavor notes (fruity, vegetal), basic tastes (sweetness, sourness), viscosity and overall liking of selected commercial smoothies.

Attribute	ANOVA †	Smoothie's			
		E	B	G	D
Appearance	***	6.8 ‡ a	6.5 a	6.1 a	4.8 b
Color	***	7.6 a	7.3 a	6.3 b	5.0 c
Bitterness	***	4.2 b	5.2 a	5.1 a	5.9 a
Sourness	***	4.5 c	5.4 b	5.6 ab	6.2 a
Sweetness	***	4.1 c	5.7 ab	5.5 b	6.5 a
Fruity	***	3.7 c	6.0 b	6.1 b	7.0 a
Vegetal	***	3.8 b	5.9 a	5.8 a	6.3 a
Viscosity	***	5.6 ab	2.4 c	6.3 a	5.3 b
Overall liking	***	2.1 c	4.8 b	5.6 a	6.4 a

† *** significant at $p < 0.001$. ‡ Values (mean of 100 consumers) followed by the same letter within the same row were not significantly different according to Tukey's least significant difference test. Scale used from 1 (dislike it extremely) to 9 (like it extremely).

Color and appearance liking values were rated as the highest for smoothies E, B, and G. Fruit flavors were more liked than vegetables, and smoothie D was the best rated (score 7.0). However, no significant differences were found in the acceptance of vegetable flavors and bitterness (scores above 5.0), except for smoothie E (both attributes defined as dislike slightly). Sweetness and acidity were most liked in smoothie D. Furthermore, viscosity did not have a strong impact on the overall rating of the smoothies. This attribute was ranged between 2.4 (smoothie B previously defined as thick) to 6.3 (smoothies G).

Although previous studies examined consumer preferences for various fruits and vegetables for juices and smoothies, their results cannot be used directly as a reference to this paper. In this way, the sensory evaluation of smoothies was closely related to the set of products tasted, the profile of consumers, and their nutritional knowledge, economic and cultural factors, and others [30,31]. Literature data, however, indicates some recurring trends, which are also reflected in this study. Consumer preferences strongly correlate with the sensation of sweetness, intense fruit flavors (orange and tropical fruits), and balanced sweet and sour tastes [32–34].

3.3.2. Penalty Analysis (PA)

In addition to the overall liking and the satisfaction degree for color, appearance, flavor notes, basic tastes, and viscosity, Just About Right (JAR) questions were asked during the consumer study among Spanish Millennial consumers under study, for all attributes except appearance and overall liking. The questions were done to determine levels of adequacy and identify possible improvements to these specific attributes in four selected smoothies. For better understanding of the relationships between JAR results and consumer acceptance, Penalty Analysis was conducted. "Too low" intensity of attributes was indicated by the symbol "−", and "too high" intensity by the symbol "+" (Figure 2). Critical corners were set to highlight attributes with the greatest negative impact on liking. The attributes impacting 20% of Millennial consumers under study and causing a drop of at least one point were included in the critical corners.

Specific food products are related to particular color attributes. Colors that diverge from these preferences for the product suggest a lack of ripeness or loss of freshness and may reduce consumer desire [34]. Smoothies were not penalized for the color. However, it was noted that smoothies with carrot, beet, pumpkin, and berries, containing high content of natural pigments (carotenoids and anthocyanins) were better rated in terms of color. The intensity of the fruity flavor needed improvement in product E, for which at the same time the vegetal taste was too intense. Interestingly, despite the low acceptance of vegetable smoothies, Millennial consumers under study rated the flavors of product D as not vegetal enough. Smoothies, except for D, need a sweetness improvement. In turn, the sour taste should be corrected in smoothie G. The blend of orange and berries caused too intense feelings of

sourness and bitterness, in contrast to root vegetable combinations. Too high viscosity was determined for smoothie D. In summary, the color intensity and perception of the fruity taste were the best rated. It is essential to highlight that fruity notes were clearly driving liking as in the following section. This tendency does not occur in all fruit-based products. For instance, in pomegranate-based jelly, fruit notes were not a good driver, with color and brightness being the most important drivers [21], but, in pomegranate dried products, both fruity and color attributes were part of the consumer drivers [23]. Four of the seven sensory attributes were found to be improvable in smoothie E, a model of the group 1 cluster.

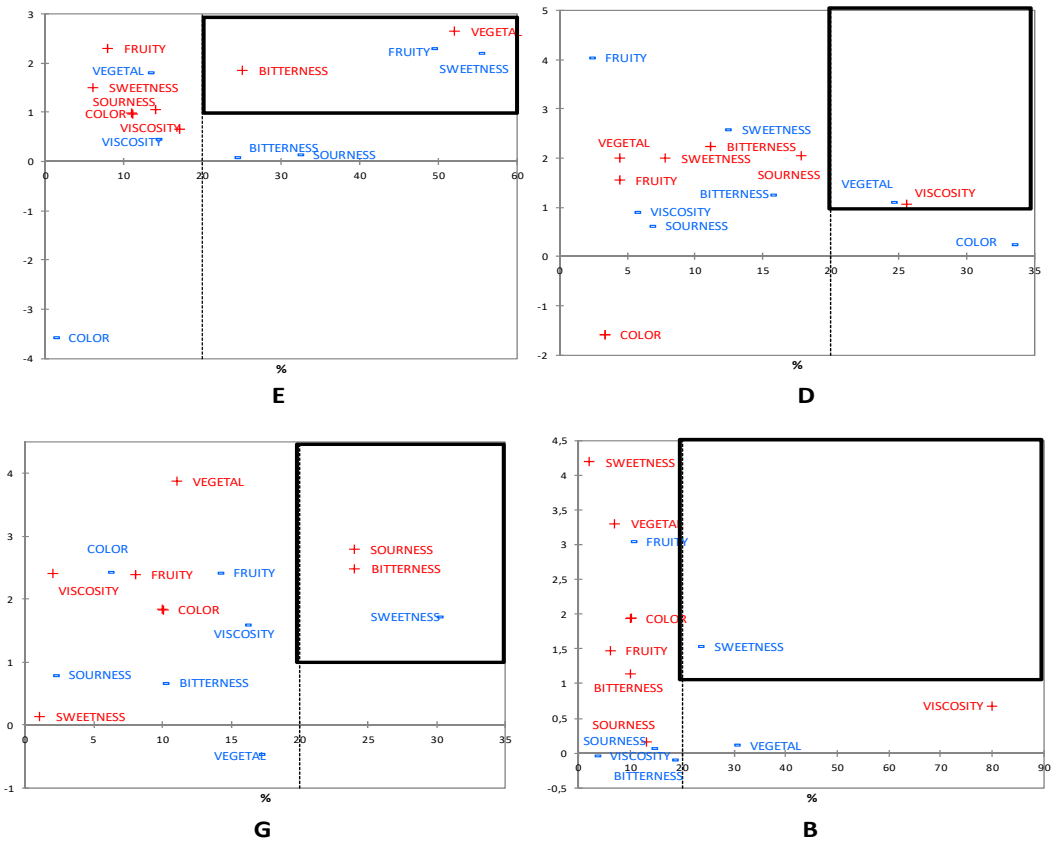

Figure 2. Penalty analysis of attribute intensity assessed by consumers of selected commercial smoothies (sample code indicated on the top right of each figure; "too low intensity" is indicated by the symbol "−", and "too high intensity" is indicated by the symbol "+").

3.4. Driving Sensory Attributes

Partial least squares regression (PLS) analysis was conducted to determine the liking drivers for the selected commercial smoothies. Figure 3 shows the consumer overall liking and the relationship with specific sensory descriptors (contained in Table 3).

The preference map revealed one large group of consumers grouped near mango, peach, and banana flavors. Similar results were obtained previously [35–37] in the analysis of smoothies, where blends of papaya, mango, and pineapple as well as mango and grape were highly desired by panelists. However, contrary to their results, a combination of banana with fruits with high acidity were clearly accepted by Millennial consumers.

The current study showed that the lowest acceptance of smoothies was associated with earthy, carrot, beetroot, and pear flavors.

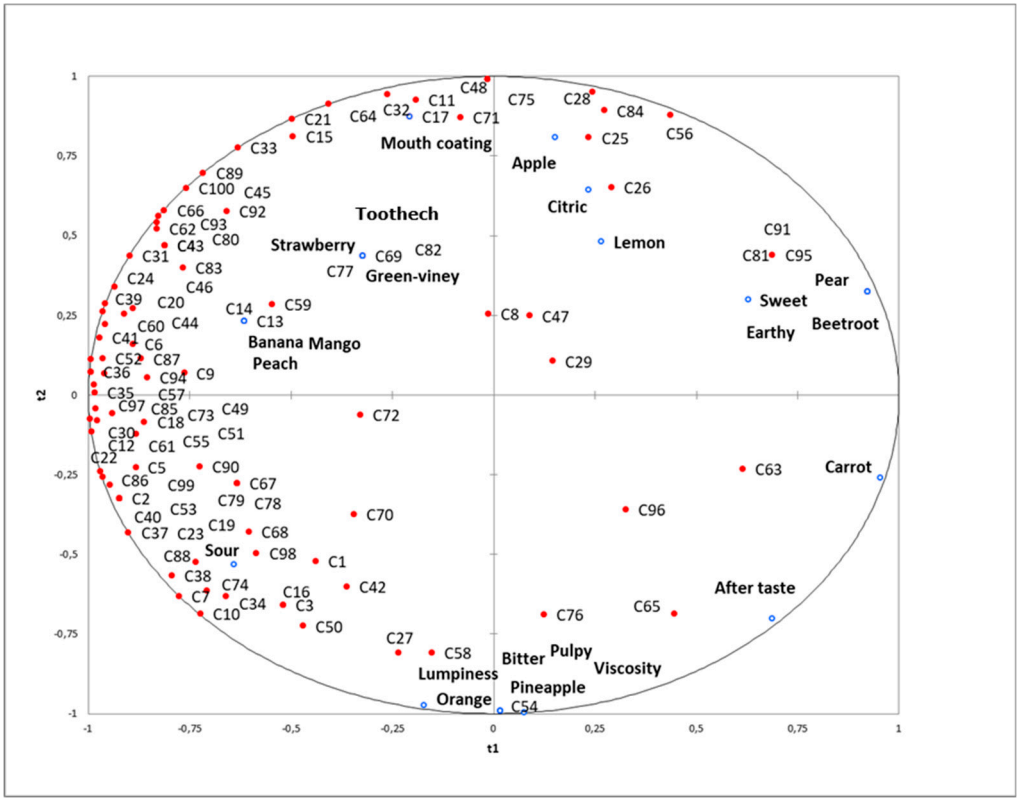

Figure 3. Partial least squares regression (PLS) of the descriptive sensory profile (Y) and consumer overall liking (X) of selected commercial smoothies.

Considering the basic tastes, sour was the main liking driver, while sweet, bitter, and aftertaste seemed to be less important. These results were in line with the penalty analysis data, in which sweetness and bitterness required correction. Interestingly, previous studies on fresh fruits, including pineapples [37], strawberries [38], nectarines and peaches [32], and apples [30,39] showed sweetness as a positive attribute, often even as the main sensory preference driver. Other research into fruit products such as oranges juice [33], pear fruit leather [40], dried pomegranate arils [23], as well as jelly candies from pomegranate juice [21], indicated a strong correlation between general acceptability and sensation of sweetness. However, another study reported sourness as a key apple acceptance driver [39], although it depends on the apple cultivar (sour, and sweet ones).

Lumpiness, viscosity, and pulpy were not appreciated by Millennial consumers under study. Forde and Delahunty [31] proved that young panelists can be strongly influenced by the predominant chemosensory attributes such as taste and aroma level [31], while older assessors placed more emphasis on the mouthfeel and irritant properties. Moreover, the homogeneity of the sample is preferred by younger consumers as compared to older ones; this situation was observed in the current study. Therefore, it should be emphasized that the main liking drivers for consumers of smoothies are sourness and tropical fruit flavors, and earthy and heterogeneous textures must be avoided.

4. Conclusions

This research evaluated sensory properties of commercial smoothies and identified liking drivers among the Spanish group of Millennial consumers under study. At the beginning of this conclusion, it is worth mentioning the limitation of this research to help and to make future research better. The sample size of consumers is one of the most important limitations observed, and the number of consumers should be higher to reach the global Millennial generation. It is also important to highlight the potential interactions based on the flavor and basic tastes that can occur; therefore, samples with

specific flavor (e.g., mango) could be scored sweetener by consumers (never by trained panel) due to their expectations.

Descriptive sensory analysis and consumer studies preceded by the napping® test seem to be an appropriate combination to optimize the formulation of novel fruit- and vegetable-smoothies. It was found that the key attributes controlling overall liking were: adequate intensity of sour taste and notes of mango, banana, and peach. Nevertheless, it should strive to improve recipes of smoothies to increase the consumption of fruits and vegetables in this form, which is considered a simple supplement in a balanced diet. Results of the penalty analysis can give a good direction to optimize this type of smoothies by avoiding vegetable ingredients with earthy or strong vegetal notes (e.g., beetroot, carrot). Research provides a series of practical tips for the food industry to understand consumer preferences, select raw materials, and improve marketing strategies. Knowing the separate clusters of smoothies available on the market and the drivers of their preferences, highly acceptable products can be developed targeting a specific consumer profile.

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