foods

Recent Developments in Identification of Genuine Odor- and Taste-Active Compounds in Foods

Edited by
Remedios Castro-Mejías and Enrique Durán-Guerrero

Printed Edition of the Special Issue Published in *Foods*

www.mdpi.com/journal/foods

MDPI

Recent Developments in Identification of Genuine Odor- and Taste-Active Compounds in Foods

Recent Developments in Identification of Genuine Odor- and Taste-Active Compounds in Foods

Editors

Remedios Castro-Mejías
Enrique Durán-Guerrero

MDPI • Basel • Beijing • Wuhan • Barcelona • Belgrade • Manchester • Tokyo • Cluj • Tianjin

Editors

Remedios Castro-Mejías
Analytical Chemistry
Universidad de Cádiz
Puerto Real
Spain

Enrique Durán-Guerrero
Analytical Chemistry
Department
University of Cadiz
Puerto Real
Spain

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Foods* (ISSN 2304-8158) (available at: www.mdpi.com/journal/foods/special_issues/Recent_Developments_Identification_Genuine_Odor-_Taste-Active_Compounds_Foods).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

LastName, A.A.; LastName, B.B.; LastName, C.C. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-0365-1668-4 (Hbk)
ISBN 978-3-0365-1667-7 (PDF)

Contents

About the Editors

Remedios Castro-Mejías

Prof. Dr. Remedios Castro Mejías is an associate professor of the Analytical Chemistry Department of the University of Cadiz.

Her current research interests are focused on wine, vinegar, oenological products, beer, polyphenols, volatile compounds, chromatography, food analysis, food quality, sensory analysis, extraction, among others.

Enrique Durán-Guerrero

Prof. Dr. Enrique Durán Guerrero is an associate professor of the Analytical Chemistry Department of the University of Cádiz.

His current research interests are focused on wine, vinegar, oenological products, polyphenols, volatile compounds, chromatography, food analysis, food quality, sensory analysis, extraction, among others.

Preface to "Recent Developments in Identification of Genuine Odor- and Taste-Active Compounds in Foods"

Both aroma and taste are important quality criteria for foods, having a great influence on our consumption behaviours. It is due to this that the accurate quantification of the compounds responsible for these qualities is a crucial need nowadays.

In recent years, a significant increase in the identification of these compounds has been observed thanks to the development of more and more sensitive and selective analytical methods. However, as the concentration of these compounds is usually low, in most cases, highly efficient preconcentration approaches must be employed for their later determination by instrumental techniques (gas or liquid chromatography and/or sensory approaches).

This book is focused on some recent analytical developments for the identification of some compounds responsible for odor and taste in foods, including methodologies for isolating volatile compounds from complex food matrices and possible relationships between flavor compounds and industrial processing or cultivar-dependent conditions.

The following authors have contributed to the scientific content of the book:

Ana M. Roldán (University of Cadiz, Spain)
Ana Sayago (University of Huelva)
Ángeles Fernández-Recamales (University of Huelva)
Carmelo G. Barroso (University of Cadiz, Spain)
Ceferino Carrera (University of Cadiz, Spain)
Cristina Cebrián-Tarancón (University of Castilla-La Mancha)
Dominico A. Guillén-Sánchez (University of Cadiz, Spain)
Enrique Durán-Guerrero (University of Cadiz, Spain)
Estrella Espada-Bellido (University of Cadiz, Spain)
Eva-María Rivas (Complutense University of Madrid)
Fabian Weber (University of Bonn)
Fini Sánchez-García (University of Cadiz, Spain)
Gerardo F. Barbero (University of Cadiz, Spain)
Gonzalo L. Alonso (University of Castilla-La Mancha)
Ikram Akhatou (University of Huelva)
Jesús Ayuso (University of Cadiz, Spain)
José Ángel Álvarez (University of Cadiz, Spain)
José Antonio Jarillo (University of Cadiz, Spain)
José E. Ruvalcaba (Autonomic University of San Luis Potosí, Mexico)
José Oliva (University of Murcia)
Luis Pérez-Rodríguez (University of Cadiz, Spain)
Manuel J. Valcárcel-Muñoz (Bodegas Fundador SLU, Spain)
María de Valme García-Moreno (University of Cadiz, Spain)
María del Carmen Rodríguez-Dodero (University of Cadiz, Spain)
María Guerrero-Chanivet (Bodegas Fundador SLU, Spain)
María José Aliaño-González (University of Cadiz, Spain)

María Rosario Salinas (University of Castilla-La Mancha)
María Isabel de Silóniz (Complutense University of Madrid)
Marta Ferreiro-González (University of Cadiz, Spain)
Miguel Ángel Cámara (University of Murcia)
Miguel Palma (University of Cadiz, Spain)
Mónica Schwarz (University of Cadiz, Spain)
Peter Winterhalter (Institute of Food Chemistry, Technical University of Braunschweig)
Petra Wrent (Complutense University of Madrid)
Raúl González-Domínguez (University of Huelva)
Remedios Castro (University of Cadiz, Spain)
Víctor Manuel Palacios (University of Cadiz, Spain).

We also would like to acknowledge the technical support prodvided by MDPI Editorial for the creation of this book.

Remedios Castro-Mejías, Enrique Durán-Guerrero
Editors

foods

MDPI

Article

Influence of Different Vinification Techniques on Volatile Compounds and the Aromatic Profile of Palomino Fino Wines

Ana M. Roldán *, Fini Sánchez-García, Luis Pérez-Rodríguez and Víctor M. Palacios

Department of Chemical Engineering and Food Technology, Faculty of Sciences, University of Cadiz, Box 40, 11510 Puerto Real, Cadiz, Spain; fini.sanchez@uca.es (F.S.-G.); luis.perez@uca.es (L.P.-R.); victor.palacios@uca.es (V.M.P.)

* Correspondence: ana.roldan@uca.es; Tel.: +34-9-5601-6376

Abstract: The aim of this study was to evaluate the influence of vinification techniques on volatile compounds and sensory profiles in young Palomino fino white wines. Four winemaking techniques (pellicular maceration, supra-extraction and use of commercial yeast strains and of β-glycosidase enzymes) were implemented to enhance the aromatic quality of wines elaborated from this neutral variety of grape. Volatile compound content, aromatic profile (OAVs) and sensorial analysis were determined. The results showed that all the vinification techniques studied led to an increase in volatile compounds compared to the control wine. Likewise, an influence of the vineyard and must extraction method on these compounds was observed. However, the greatest changes in aroma activity and sensory profile were a result of the pellicular maceration and supra-extraction techniques. The latter was differentiated by the highest content of terpenes and, consequently, the highest odour activity values of floral series. In addition, the supra-extraction was a very selective technique since it extracted terpenes and aromatic precursors, but not the acids responsible for the fatty characteristic, such as octanoic acid. In terms of sensory profile, the supra-extraction technique improved the intensity of the Palomino fino white wine and its aromatic quality with a previously not-determined floral character.

Keywords: pellicular maceration; supra-extraction; β-glycosidase; enzymes; yeasts; volatile compounds; sensory analysis

Citation: Roldán, A.M.; Sánchez-García, F.; Pérez-Rodríguez, L.; Palacios, V.M. Influence of Different Vinification Techniques on Volatile Compounds and the Aromatic Profile of Palomino Fino Wines. *Foods* **2021**, *10*, 453. https://doi.org/10.3390/foods10020453

Academic Editors: Joaquín Bautista-Gallego and Nuno Mateus

Received: 28 December 2020
Accepted: 17 February 2021
Published: 19 February 2021

1. Introduction

Palomino fino is the undisputed leading grape variety in the Jerez-Xeres-Sherry D.O. wine production area (Andalusia, Spain) and is considered a key element in the production of dry and sweet sherry wines by biological and oxidative aging [1]. This grape variety is adapted to the warm conditions of this south-western Spanish region [2], and although it is characterized by its high yield, no remarkable aromatic attributes have been found [3]. In fact, the Palomino fino grape variety is considered a "neutral" grape with low content of aroma precursors [2]. This "neutral" characteristic makes this grape variety ideal for sherry wine production. This is because its chemical composition, aroma and sensory characteristics are determined by biological and chemical processes that take place during aging [4] (during the dynamic phase in the "solera" system), but not for fresh and fruity white wines. In addition, the low total acidity of these wines produced in a warm climate contributes to the minimal aromatic intensity and also to the lack of freshness [5].

In order to diversify traditional production by adapting it to current needs or demands, in recent years, the use of the Palomino fino variety has been promoted for the production of young white wines. Therefore, it is necessary to implement alternative cultivation and technological practices in order to intensify the aromatic potential of these wines. It is remarkable that the aroma of wine constitutes an important factor in the wine quality and price as well as a preference attribute for consumers [6,7]. The variety of grape employed in making a particular wine, in many cases, completely determines the aroma

of that wine [8–10]. However, it is well known that other factors such as climate, region, viticultural practices, degree of grape maturation, yeast and winemaking techniques, including aging, influence the wine aroma [11–18]. Among them, pre-fermentative cold maceration (cold soaking or cryomaceration) and the application of selected yeasts and pectinases are examples of techniques used by oenologists to improve the aromatic quality and sensory characteristics of white wines. The first technique leads to the greater extraction of aromatic compounds and precursors from the skins by means of maceration in the must before the fermentation [19]. The use of yeasts and enzymes with glycosidase activity favour the release of the aromatic compounds that are found in the grape combined with sugars in non-aromatic form [20,21].

Some studies have been published on the influence of certain winemaking techniques on the volatile compounds of Palomino fino wines and other warm-climate grape varieties (Jerez region, Andalusia, Spain) and comparisons between them. These winemaking techniques include cold soaking [8], addition of glycosidase enzymes [5,8], co-inoculation of non-*Saccharomyces* and *Saccharomyces cerevisiae* yeast [2] and the use of bee pollen as a fermentative activator [22]. However, although some authors considered this variety to have good potential for producing new styles of wines [2], it is not clear if the aroma and quality of the Palomino fino wine is improved or which of the winemaking techniques used offer the best results. Therefore, the aim of this study was to assess the effect of various vinification techniques (pellicular maceration, supra-extraction (freezing and thawing of grapes before pressing) and the use of different types of yeast strains and β-glycosidase enzymes on volatile compounds and sensory profiles in white wines elaborated from an autochthonous grape variety: Palomino fino.

2. Materials and Methods

2.1. Winemaking Techniques

Palomino fino grapes and must from vineyards and wineries, respectively, belonging to the Jerez area (Cádiz, Spain, coordinates: 36.6866, −6.13717) and from two vintages (2006 and 2007) were used to perform the trials.

2.1.1. Pellicular Maceration and Supra-Extraction Trials

Palomino fino grapes for pellicular maceration (PM) and supra-extraction (SUPRA) were from the "CIFA Rancho La Merced" in Jerez de la Frontera (Cádiz, Spain). Grapes were transported in 25 kg food-material boxes to the laboratory, where they were separated by weight into 5 equal fractions of 10 kg each. One fraction of whole grapes was frozen at −18 °C until its use in SUPRA trials, and the rest was destemmed and ground and must and skins were separated. Subsequently, 4 mixtures of 1:3 must and skins were prepared. Each mixture was kept in a refrigerated tank of 5 L capacity where macerations were performed at different maceration times: 0 h (control), 4 h (PM4), 8 h (PM8) and 12 h (PM12) and at a controlled temperature (15 °C). A total of 8 tanks were used to carry out the trials in duplicate. After maceration, pressing of each tank was performed and potassium metabisulfite (50 mg/L) and tartaric acid were added to the musts to correct their pH to a value of 3.5–3.6 (both Agrovin, Ciudad Real, Spain). The sulphited grape musts were then subjected to static settling for 24 h at a low temperature (10 °C). The clarified grape musts were then racked to glass tanks with cooling jackets (V = 5 L) for directed alcoholic fermentation at a controlled temperature of 18 °C using a commercial active dry wine yeast (ADWY) strain of *Saccharomyces cerevisiae* Fermivin 7013 (DSM Food Specialties Spain, S.L., Barcelona, Spain), at a dose of 20 g/hL. This ADWY is recommended for white and red wines and is characterized by neutrality and fast fermentation. Once the alcoholic fermentation was complete (stable density measurement and the residual reducing sugars below 2 g/L), the wines were chilled (6 °C) for 7 days and subsequently treated with gelatin (4 g/hL) and bentonite (40 g/hL), filtered (sterilizing filter plates SA-990) (Papeleras del Besós Placas filtrantes, S.L., Barcelona, Spain) and bottled using nitrogen pressure.

For the SUPRA trials, once the grapes were thawed and pressed, the same winemaking protocol was followed as for the PM.

2.1.2. Yeast Strains and Enzyme Trials

The Palomino fino grape must, without the addition of sulphurous anhydride and pH correction, was obtained from the Grupo Osborne winery in El Puerto de Santa María (Cádiz, Spain). Sulphurous addition, pH correction and settling of the musts were carried out in the same way as in the PM and SUPRA trials. Once the grape must was clarified, it was separated into 4 fractions and racked to the fermentation tanks. A total of 8 tanks were used to carry out the trials in duplicate. Four yeast strains (3 commercial and 1 selected native) were used to carry out the alcoholic fermentation by inoculating each tank with each yeast strain. A total of 8 tanks were used to carry out the trials in duplicate. In addition, Fermivin (DSM Food Specialties Spain, S.L., Barcelona) (F) at a dose of 20 g/hL (used as reference), ENSIS-L5 (Ensis Sciences, Barcelona, Spain) at a dose of 10 g/hL (ENSIS) and CK S-102 (Enolviz, S.L., Bilbao, Spain) (CK) at a dose of 15 g/hL were used. The fourth strain was an autochthonous strain of *S. cerevisiae* isolated by the winery Domecq S.L. in Jerez de la Frontera (Cádiz, Spain) and commonly used as "pie de cuba" (PC) in the elaboration of fino-type wines.

After complete fermentation, each type of wine (elaborated by each yeast strain) was separated into 3 new fractions. One fraction was clarified, filtered and bottled following the same process as the PM and SUPRA wines obtaining the PC, F, ENSIS and CK wines. The second and third fractions of each wine were kept in clarification tanks for 2 weeks at 20 °C after the addition of a commercial enzyme extract with high β-glucosidase activity to each fraction. The enzyme extracts were Novoferm 12G (Novo Nordisk Pharma S.A., Madrid, Spain) (12G) and Rapidase AR-2000 (DSM Food Specialties Spain, S.L., Barcelona, Spain) (AR) at a dose of 4 and 2.5 g/hL, respectively. The β-glucosidase activity under the assay conditions was determined, resulting in 33.7 and 38.6 units/g of extract for 12G and AR, respectively. After enzymatic treatment, the wines were clarified, filtered and bottled, obtaining 8 types of wine (PC-12G, PC-AR, F-12G, F-AR, ENSIS-12G, ENSIS-AR, CK-12G and CK-AR).

2.2. Volatile Compounds

Higher alcohols, acetaldehyde, ethyl acetate and methanol were analysed using a GC-FID HP 5890 Series II system (Agilent Technologies, California, USA) equipped with a Carbowax 20 M column (50 m, 0.25 mm ID, 0.25 μm). The operation conditions of the GC were: injector and detector temperatures, 250 °C; oven temperature, 35 °C for 10 min (followed by a ramp of 4 °C/min until 200 °C); sample volume, 20 μL (distilled sample for alcoholic strength determination) in split mode (split ratio 1/20); and carrier gas, H (1 mL/min). The identification and quantification of major volatile compounds was carried out using pure standard compounds (Sigma–Aldrich Química, S.A., Madrid, Spain) and 4-methyl-2-pentanol (783 mg/L) as the internal standard.

Semiquantitative GC–MS analyses, after solid-phase extraction (SPE), were used to determine minor volatile aroma compounds. The method described by Di Stefano [23] was followed for the extraction using DSC-18 of 1 g (6 mL) cartridges (SUPELCO, Bellefonte, PA, USA). GC–MS analysis was performed on a Voyager® system (Termoquest, Milan, Italy), equipped with a Supelcowax-10 column (60 m, 0.32 mm ID, 0.5 μm). The injector and detector temperature was 200 °C, using He (1 mL/min) as the carrier gas. The GC oven program was as follows: held at 40 °C for 5 min, then ramped at 2 °C/min to 200 °C and held for 5 min. A direct injection of 2 μL in splitless mode (40 s) of sample was carried out. The electronic impact mode (EI+) with an electron energy value of 70 eV was applied. The initial and interface temperatures were 220 °C and 320 °C, respectively. The MS collected data at a scan index of 1 scan/s and mass acquisition range of 45–400 m/z. The procedure was also described in [22]. Peak identification was carried out using the Xcalibur v.1.1. Library Browser (Thermo Fisher Scientific, Waltham, MA, USA) by analogy of mass spectra

(90% minimum matching level), some of them confirmed by retention times of standards from Sigma Aldrich (St. Louis, MO, USA). Semiquantitative analyses were carried out, assuming a response factor equal to one. All determinations were carried out in duplicate. Total volatile compounds were determined by the sum of the compounds identified and quantified.

2.3. Odour Activity Values

According to Francis et al. [24], odour activity values (OAV) were calculated as the ratio between the mean concentration of each volatile aroma compound and its odour threshold value (OTV), as reported by other authors [25–30]. To estimate the overall wine aroma, the odour descriptors were grouped into different aromatic series and every compound was assigned to one aromatic series based on the main odour descriptors. The total intensities for each aromatic series were calculated as the ΣOAV of each of the compounds assigned to this series. The odorant series used in this study (fruity, sweet, fatty, floral, grassy, spicy, earthy and mushroom, chemical and dried fruit) represented the main constituents of the aromatic profile used by Amores-Arrocha et al. [22] for the wines made using the same grape variety. An organoleptic profile of the wines was obtained through the relationship between quantitative results derived from chemical and sensory analyses [31].

2.4. Sensory Analysis

The differences between the sensorial profiles of wines made using different winemaking techniques and the control wine were evaluated by a panel of 10 trained tasters, both men and women between 30 and 55 years of age. All wines were evaluated between three and five days after bottling. The tasting panel used individual booths with controlled illumination, located in the tasting room of the Institute of Viticulture and Agri-Food Research (IVAGRO, Puerto Real, Cádiz); wines were presented in standard tasting glasses [32] and covered with watch-glasses to minimize the evaporation of volatile compounds. The wines were served to each taster (50 mL) at room temperature (20 ± 2 °C). A 5-point scale (from 0 to 5 according to increasing intensity) was used to evaluate the general acceptability of wines and their visual (intensity and quality), olfactory (quality and intensity) and gustatory (quality and intensity) characteristics. Some aspects of interest such as characteristic odour (floral, fruity, vegetable, spice, balsamic and dried fruit), flavours (acidity, salinity, sweetness, bitterness and warmness) and mouthfeel (smoothness, persistence and aftertaste) were also considered following the procedure by Amores-Arrocha [22]. The tasting descriptors used were selected based on those for white wines defined by Jackson [33].

2.5. Statistical Analysis

A two-way analysis of variance (ANOVA) was performed to identify statistically significant differences between samples using the statistical package GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA). Statistically significant differences between samples according to Bonferroni's multiple range (BSD) test was defined as $p < 0.05$. A principal component analysis (PCA) was performed to determine the influence of the vinification techniques on the aromatic profile of wines. The statistical computer package SPSS 23.0 (SPSS Inc., Chicago, IL, USA) and the factor extraction method of rotated component matrix loadings were used, with "quartimax" Kaiser normalization.

3. Results and Discussion

3.1. Influence of Pre-Fermentative and Aroma-Release Treatments on Volatile Compounds of Wines

Total volatile compounds of the Palomino fino control wine and those obtained by the different techniques are shown in Figure 1. As can be observed, the PM and SUPRA techniques led to an increase of the total volatile compounds similar to those produced by most yeast strains or glycosidase enzymes.

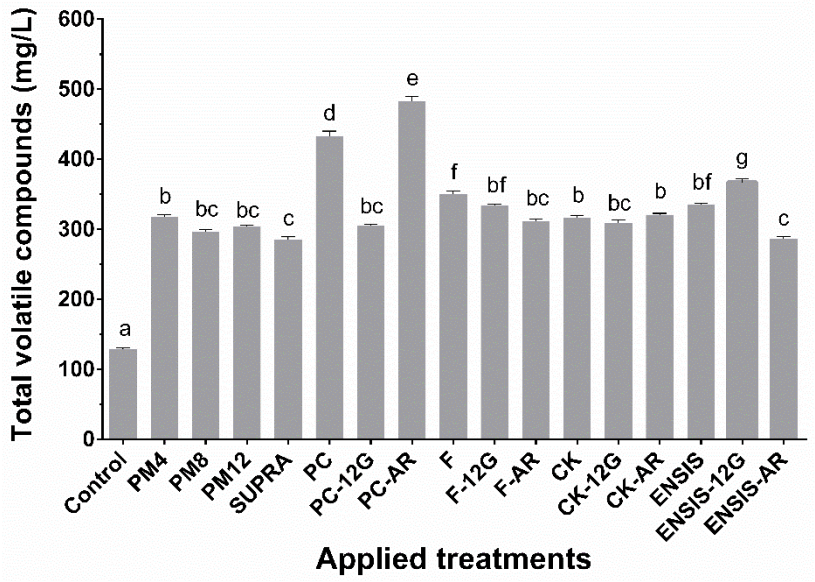


Figure 1. Total volatile compounds in Palomino fino wine samples with application of extractive and aroma-release techniques. Lowercase superscript letters show statistical significance ($p < 0.05$). PM4, PM8 and PM12: pellicular maceration for 4, 8 and 12 h, respectively; SUPRA: supra-extraction wine; PC, F, CK and ENSIS: wine elaborated by different yeast strains; 12G and AR: correspond to enzymatic extracts used. PC: "pie de cuba"; F: Fermivin; CK: CK S-102; ENSIS: ENSIS-L5; 12G: Novoferm 12G; AR: Rapidase AR-2000.

It is necessary to highlight that the control and F wines were made with the same yeast strain and under the same conditions, but the grapes and must came from different vineyards (Jerez and El Puerto de Santa María, respectively) and were obtained with different pressing systems (horizontal membrane presses and horizontal plate presses, respectively). The differences observed in the total volatile content between both were conditioned by both factors. Several authors [10,34] have shown the influence of terroir and grape ripening [10,12] on volatile compounds, as well as influence of technological practices [8,13]. Also, it has been reported that the greatest extraction of grape solids is obtained by application of a high degree of pressure, which modifies the physicochemical and sensory composition of the wines [35,36]. However, to date, no studies have been carried out about the influence of press type on volatile compounds. According to these results, horizontal membrane presses lead to lower total volatile compounds than horizontal plate presses, most likely due to less extraction from the solid parts, particularly aromatic compounds present in the grapes. On the one hand, the PM and SUPRA favoured this extraction, increasing total concentrations of most aromatic components in the final wine [37]. According to Ribereau-Gayón et al. [38], supra-extraction causes changes in the ultrastructure of the grape tissues, producing an effect comparable to that of skin maceration and releasing aromas and aromatic precursors more easily from the grape. Therefore, winemaking practices that favour grape crushing and extraction of components from the skins (including pressing) lead to wines with a higher content of volatile compounds.

On the other hand, the total content of the volatile compounds found in Palomino fino wine was also dependent on the yeast strain used. The maximum proportion of volatile compounds was yielded by the PC strain, followed by the F strain, whereas similar levels of volatiles were found in the CK and ENSIS wines. The PC strain is an autochthonous yeast strain used in sherry wine elaboration characterized by higher alcohol and acetaldehyde production, hence the higher production of volatiles.

Regarding β-glycosidase enzymes, an increase of total volatile compounds was observed with respect to the control. However, their effects were reflected less clearly, being dependent on the characteristics of the wine after fermentation with a specific yeast strain.

The 46 volatile compounds positively identified and quantified in Palomino fino white wines obtained with pre-fermentative and aroma-release treatments are shown in Tables 1 and 2, respectively. The compounds are grouped according to their chemical structure, including higher alcohols, methanol, acids, C6 alcohols, alcohols, terpenes, esters, aldehydes, thiols and phenols. As can be seen, the higher alcohols, methanol, esters and acetaldehyde produced an enrichment in the volatile compounds of wines made with different vinification techniques.

3.1.1. Higher Alcohols and Methanol

As can be seen from the results in Tables 1 and 2, higher alcohols were the largest group of volatile compounds in the evaluated Palomino fino wines, in agreement with results reported by other authors for this and other white wine varieties [6,22,39]. Among them, the isoamyl alcohol was quantitatively the main compound, reaching levels above 200 mg/L in yeast strain- and enzyme-treated wines regardless of the yeast strain and β-glycosidase enzyme used. It was reported that the greatest amounts of higher alcohols are obtained from wines fermented with the highest level of solids [40], which could explain these results.

The pellicular maceration and supra-extraction favoured the formation of higher alcohols, with a higher influence of the processes themselves (Table 1) on this increase than the time of maceration, which could be related to the amino acid content on which the pellicular maceration exerts the same effect [41].

As can be seen in Table 2, the relative concentrations of total alcohols in yeast strain- and enzymatically treated wines were significantly higher than those of the control and similar between them. They are the main yeast-synthesised aroma substances of the fermentation bouquet by sugar catabolism or decarboxylation and deamination of amino acids in yeast [42], which would explain the differences between the yeast strains used.

Similarly, the methanol content of all wines also increased, generally reaching levels twice that of the control. This increase could be due to the presence of the high pectin content in must. Pectic residues, mainly cellulose and pectins, are released from the cellular tissue due to the damages, breaks and maceration. The yeast could solubilize and metabolize pectin to produce methanol during alcoholic fermentation by enzymatic hydrolysis [43]. In terms of alcohol production and food safety, pellicular maceration may not be recommended. However, the levels produced by this technique are well below the limits established by the International Organisation of Vine and Wine (OIV) for white wines (250 mg/L) in its resolution No. OENO 19/2004 [44].

3.1.2. Acids

A total of six acids were identified in the volatile fraction of Palomino fino wines. However, in yeast strain- and enzyme-treated wines, only hexanoic, octanoic and *n*-decanoic acids were detected and quantified. The average total concentration of these acids ranged between 0.4 and 1.9 mg/L, reaching the highest values in the control and PM wines. Therefore, they could contribute to the quality of the wine by increasing aroma complexity [6].

On the one hand, the octanoic acid, followed by hexanoic acid, showed the highest contribution to the total acid content (Tables 1 and 2). The *n*-decanoic, 9-decenoic and benzoic acids were present in the control, PM and SUPRA wines. The first two generally increased with maceration process and time, while benzoic acid decreased until 50 µg/L and was not detected in SUPRA wines (Table 1).

Table 1. Volatile compound concentrations (μg/L) of Palomino fino wines made using extractive techniques.

Volatile Compounds	ID	Control	PM4	PM8	PM12	SUPRA
Higher alcohols						
n-Propyl alcohol	ST	8134.00 ± 162.68 a	18,214.70 ± 910.70 b	17,998.00 ± 413.95 b	19,415.70 ± 100.96 b	29,103.90 ± 203.72 c
Isobutanol	ST	11,320.00 ± 396.20 a	31,725.00 ± 1078.65 b	29,327.10 ± 1055.77 b	31,000.10 ± 314.21 b	43,641.30 ± 916.47 c
Amyl alcohol	ST	14,982.50 ± 419.49 a	43,114.80 ± 776.05 b	39,081.50 ± 172.43 c	43,057.80 ± 516.69 b	33,019.80 ± 363.22 d
Isoamyl alcohol	ST	65,148.30 ± 1280.18 a	183,491.00 ± 1853.31 b	172,004.20 ± 1408.06 c	176,007.70 ± 893.09 d	149,989.00 ± 1109.92 e
Total		99,585.00 ± 1394.32	276,545.50 ± 1021.46	258,410.80 ± 1065.09	269,481.30 ± 1481.87	244,760.90 ± 1223.77
% Higher alcohols		77.34	87.11	87.24	88.79	85.73
Methanol	ST	25,827.00 ± 371.50 a	46,562.50 ± 744.99 b	51,314.00 ± 451.56 c	58,670.90 ± 557.37 d	46,998.00 ± 831.98 b
Total		25,827.00 ± 371.50	46,562.50 ± 744.99	51,314.00 ± 451.56	58,670.90 ± 557.37	47,998.00 ± 831.98
% Methanol		20.1	14.7	17.3	19.3	16.5
Acids						
Hexanoic acid	ST	392.03 ± 4.37 a	431.49 ± 10.49 a	381.09 ± 2.42 a	423.36 ± 14.36 a	320.34 ± 10.14 b
Heptanoic acid	ST	3.48 ± 1.14 a	5.55 ± 0.39 b	4.88 ± 0.27 b	4.90 ± 0.17 b	5.36 ± 0.26 b
Octanoic acid	ST	971.99 ± 30.35 a	993.69 ± 25.31 a	967.12 ± 3.75 a	984.04 ± 35.21 a	526.09 ± 63.05 b
n-Decanoic acid	ST	27.99 ± 10.67 a	128.59 ± 7.21 b	46.12 ± 4.01 a	153.78 ± 11.76 b	26.93 ± 0.15 a
9-Decenoic acid	LB	52.84 ± 15.49 a	156.62 ± 12.49 b	124.73 ± 10.37 b	287.84 ± 25.14 c	2.00 ± 0.19 d
Benzoic acid	ST	241.76 ± 30.88 a	54.45 ± 7.52 b	42.99 ± 3.73 b	53.62 ± 3.83 b	nd
Total		1690.08	1770.38	1566.92	1907.56	880.73
% Acids		1.32	0.56	0.53	0.63	0.30
C6 alcohols						
1-Hexanol	ST	110.10 ± 6.26 a	92.66 ± 0.25 a	100.42 ± 0.52 a	103.75 ± 0.09 a	27.83 ± 2.08 b
(E)-3-Hexen-1-ol	ST	0.96 ± 0.11 a	0.50 ± 0.02 b	0.51 ± 0.21 b	0.52 ± 0.01 b	0.25 ± 0.10 c
(Z)-3-Hexen-1-ol	ST	6.95 ± 0.71 a	5.11 ± 0.26 b	4.49 ± 0.13 b	4.81 ± 0.35 b	4.83 ± 0.30 b
Total		118.02	98.27	105.42 ± 7.09	109.07 ± 15.46	32.91 ± 5.69
% C6 alcohols		0.09	0.03	0.04	0.04	0.01
Alcohols		-	-	-	-	-
4-Methyl-1-pentanol	ST	1.78 ± 0.24	1.31 ± 0.19	1.47 ± 0.12	1.35 ± 0.14	1.64 ± 0.22
3-Methyl-1-pentanol	LB	0.71 ± 0.16 a	4.42 ± 0.17 b	4.82 ± 0.06 b	4.71 ± 0.11 b	3.43 ± 0.38 c
1-Octanol	ST	3.18 ± 0.22 a	2.73 ± 0.14 b	2.65 ± 0.12 b	3.03 ± 0.18 a	2.86 ± 0.57 ab
Phenylethyl alcohol	ST	1457.09 ± 35.53 a	1286.68 ± 15.32 b	938.30 ± 43.57 c	1218.67 ± 58.06 d	519.80 ± 83.55 e
Total		1462.77	1295.14	947.24	1227.75	527.73
% Alcohols		1.14	0.41	0.32	0.41	0.18

Table 1. *Cont.*

Volatile Compounds	ID	Control	PM4	PM8	PM12	SUPRA
Terpenes						
Linalool	ST	nd	nd	nd	nd	47.20 ± 9.44
Linalool oxide	LB	nd	nd	nd	nd	5.67 ± 0.92
α-Terpineol	ST	nd	nd	nd	nd	26.09 ± 5.22
β-Citronellol	ST	4.12 ± 0.31 a	4.31 ± 0.29 a	2.90 ± 0.58 b	4.38 ± 0.64 a	25.38 ± 3.01 c
Nerol	ST	nd	nd	nd	nd	10.10 ± 2.02
2,6-Dimethyl-3,7-octadien-2,6-diol	LB	24.35 ± 0.73 a	23.97 ± 1.05 a	19.71 ± 0.25 b	22.93 ± 0.10 c	119.76 ± 13.49 d
Total		28.48	28.29	22.61	27.31	234.20
% Terpenes		0.02	0.01	0.01	0.01	0.08
Esters						
Ethyl acetate	ST	8023.04 ± 180.71 a	19,107.14 ± 114.64 b	18,988.94 ± 170.90 b	18,179.64 ± 363.59 c	20,899.73 ± 285.98 d
Ethyl butyrate	ST	0.53 ± 0.07 a	0.12 ± 0.05 b	0.51 ± 0.03 a	0.56 ± 0.12 a	2.53 ± 0.28 c
Isoamyl acetate	ST	23.49 ± 2.97 a	11.74 ± 0.02 b	27.21 ± 2.34 a	20.04 ± 1.53 c	73.80 ± 7.11 d
Hexyl acetate	ST	2.61 ± 0.38 a	2.93 ± 0.56 a	4.38 ± 0.36 b	2.89 ± 0.34 a	0.98 ± 0.23 c
Ethyl lactate	ST	1.47 ± 0.55 a	0.82 ± 0.36 a	0.91 ± 0.23 a	1.15 ± 0.35 a	4.25 ± 0.39 b
Ethyl 2-hydroxy-4-methyl butyrate	LB	5.56 ± 0.20 a	5.73 ± 0.23 a	5.32 ± 0.16 a	6.63 ± 0.56 a	3.94 ± 0.23 b
Ethyl 2-hydroxy-4-methylpentanoate	LB	6.83 ± 0.52 a	5.67 ± 0.50 a	5.50 ± 0.47 a	6.03 ± 0.29 a	nd
Ethyl pentanoate	ST	168.91 ± 9.81 a	156.03 ± 4.45 a	118.64 ± 19.92 b	147.11 ± 3.47 a	99.34 ± 9.84 b
Ethyl hexanoate	ST	19.76 ± 2.19 a	19.46 ± 0.58 a	36.33 ± 2.79 b	31.25 ± 2.56 b	41.78 ± 3.11 c
Isoamyl hexanoate	LB	nd	0.16 ± 0.07	0.19 ± 0.10	0.24 ± 0.03	nd
Ethyl octanoate	ST	117.89 ± 7.73 a	157.57 ± 4.45 b	238.61 ± 23.05 c	257.98 ± 26.93 c	140.58 ± 3.21 a
Diethyl malonate	LB	0.16 ± 0.03	0.19 ± 0.01	0.19 ± 0.02	0.18 ± 0.01	0.20 ± 0.05
Ethyl decanoate	ST	21.21 ± 4.90 a	27.11 ± 5.34 a	40.20 ± 6.71 b	36.82 ± 7.43 b	22.14 ± 1.13 a
Diethyl succinate	ST	50.81 ± 3.00 a	51.92 ± 3.04 a	44.35 ± 4.55 a	64.81 ± 4.51 b	61.68 ± 1.54 b
Ethyl 9-decenoate	LB	82.77 ± 4.51 a	100.84 ± 4.55 b	108.30 ± 5.63 b	106.09 ± 4.52 b	31.56 ± 7.24 c
2-Phenethyl acetate	ST	83.66 ± 6.09 a	83.81 ± 7.26 a	80.55 ± 2.00 a	69.25 ± 6.58 b	50.31 ± 4.72 c
Ethyl cinnamate	ST	4.80 ± 0.98 a	11.31 ± 1.79 b	nd	4.72 ± 0.99 a	nd
Total		8613.52 ± 167.40	19,742.53 ± 137.83	19,700.13 ± 111.62	18,935.38 ± 139.41	21,432.81 ± 181.00
% Esters		6.72	6.20	6.67	6.19	7.29
Aldehydes						
Acetaldehyde	ST	17,235.10 ± 172.32 a	17,956.90 ± 538.70 b	15,434.50 ± 134.28 c	11,784.00 ± 400.66 d	17,629.50 ± 458.37 ab
Total		17,235.10 ± 172.32	17,956.90 ± 538.70	15,434,50 ± 134.28	11,784.00 ± 400.66	17,629.50 ± 458.37
% Aldehydes		13.29	5.68	5.08	3.96	5.76

Table 1. *Cont.*

Volatile Compounds	ID	Control	PM4	PM8	PM12	SUPRA
Thiols						
3-Methylthio-1-propanol	LB	9.13 ± 1.64 a	6.98 ± 0.82 b	4.90 ± 0.39 c	5.57 ± 0.94 b	3.42 ± 0.60 c
Total		9.13	6.98	4.90	5.57	3.42
% Thiols		0.01	0.01	0.01	0.01	0.01
Phenols						
2-Methoxy-4-vinilphenol	ST	5.35 ± 0.46 a	9.01 ± 1.86 b	9.54 ± 2.02 b	13.93 ± 2.93 c	nd
2,6-Di-tert-butyl-4-ethylphenol	LB	9.63 ± 1.02 a	8.39 ± 0.21 a	7.64 ± 0.66 a	8.74 ± 0.92 a	5.77 ± 0.55 b
Total		14.98	17.40	17.18	22.66	5.77
% Phenols		0.01	0.01	0.01	0.01	0.01

nd: not detected. PM4, PM8 and PM12: pellicular maceration for 4, 8 and 12 h, respectively; SUPRA: supra-extraction wine; ID: Identification method; ST: compounds detected using pure standards and with Xcalibur v.1.1. Library Browser; LB: compounds detected using with Xcalibur v.1.1. Library Browser. Different letters indicate significant differences concentration volatile compounds analyzed between for Palomino fino wines studied ($p < 0.05$). Data are expressed as mean ± standard deviation ($n = 3$).

Table 2. Volatile compound concentrations (μg/L) of Palomino fino wines made using aroma-release techniques.

Volatile Compounds	ID	F	F-12G	F-AR	PC	PC-12G	PC-AR	CK	CK-12G	CK-AR	ENSIS	ENSIS-12G	ENSIS-AR
Higher alcohols		-	-	-	-	-	-	-	-	-	-	-	-
n-Propyl alcohol	ST	52,340.00 ± 1046.80 a	55,084.00 ± 550.84 b	36,210.00 ± 784.25 c	54,214.00 ± 231.04 b	52,416.00 ± 314.53 a	51,580.00 ± 191.32 a	45,304.00 ± 214.50 c	44,103.00 ± 119.060 d	45,080.00 ± 165.70 d	51,320.00 ± 231.45 a	51,875.00 ± 182.04 a	40,824.00 ± 189.60 e
Isobutanol	ST	58,108.00 ± 552.03 a	60,110.0 ± 947.20 b	45,408.00 ± 791.05 c	61,320.10 ± 320.01 b	54,220.00 ± 208.64 d	61,000.00 ± 401.08 b	53,986.70 ± 289.56 d	52,216.00 ± 487.33 e	54,254.00 ± 614.90 d	56,512.00 ± 713.25 f	63,024.50 ± 682.78 g	47,600.00 ± 503.44 h
Amyl alcohol	ST	24,024.00 ± 288.09 a	25,050.00 ± 151.50 a	18,860.00 ± 334.12 b	24,977.00 ± 146.51 a	23,740.00 ± 203.60 a	24,070.00 ± 114.58 a	31,278.00 ± 407.82 c	29,998.60 ± 216.94 c	31,065.00 ± 387.23 c	27,048.00 ± 145.66 d	30,300.00 ± 278.19 c	22,090.00 ± 106.07 e
Isoamyl alcohol	ST	85,100.0 ± 1021.20 a	87,960.0 ± 985.14 b	64,150.0 ± 879.03 c	89,103.0 ± 915.74 b	81,500.0 ± 1190.06 d	85,260.0 ± 827.54 a	104,005.0 ± 1198.70 e	101,600.0 ± 998.67 f	101,520.0 ± 1010.50 f	112,107.3 ± 778.38 g	118,910.0 ± 963.21 h	89,610.0 ± 532.17 b
Total		219,00.00	228,000.00	164,410.00	229,00.00	210,00.00	221,91.00	234,000.00	227,000.00	231,520.00	246,000.00	264,000.00	200,000.00
% Higher alcohols		54.68	61.24	45.85	46.22	62.21	41.23	65.58	68.28	65.34	68.03	65.11	63.66
Methanol	ST	49,321.00 ± 138.52 a	55,010.00 ± 247.25 b	27,763.00 ± 98.74 c	52,024.00 ± 316.07 d	53,117.00 ± 278.90 bd	54,210.00 ± 179.85 b	49,206.50 ± 204.13 a	54,009.00 ± 405.11 b	51,180.50 ± 180.26 d	53,612.00 ± 713.71 b	50,178.00 ± 227.29 a	42,090.00 ± 462.38 e
Total		49,321.00 ± 138.52	55,010.00 ± 247.25	27,763.00 ± 98.74	52,024.00 ± 316.07	53,117.00 ± 278.90	54,210.00 ± 179.85	49,206.50 ± 204.13	54,009.00 ± 405.11	51,180.50 ± 180.26	53,612.00 ± 713.71	50,178.00 ± 227.29	42,090.00 ± 462.38
% Methanol		12.3	14.8	7.7	10.5	15.6	10.1	13.8	16.2	14.4	14.8	12.4	13.4
Acids		-	-	-	-	-	-	-	-	-	-	-	-
Hexanoic acid	ST	134.04 ± 15.85 a	144.67 ± 12.45 a	120.19 ± 14.32 a	85.58 ± 31.20 b	119.98 ± 39.87 a	78.93 ± 21.24 b	155.04 ± 26.41 c	140.79 ± 19.87 a	125.17 ± 23.44 a	135.63 ± 15.67 a	126.79 ± 17.6 a	149.28 ± 21.92 c
Octanoic acid	ST	504.44 ± 50.48 a	569.55 ± 46.78 a	370.83 ± 49.60 b	358.74 ± 80.03 b	441.28 ± 85.61 b	284.07 ± 50.11 c	582.00 ± 65.12 a	507.05 ± 58.70 a	300.36 ± 38.69 c	478.23 ± 60.48 a	410.32 ± 62.11 b	421.82 ± 70.90 b
n-Decanoic acid	ST	67.09 ± 28.03 a	62.76 ± 25.10 a	nd	nd	43.98 ± 19.20 a	nd	69.46 ± 18.94 a	21.24 ± 2.09 b	nd	58.87 ± 15.78 a	10.72 ± 0.84 c	53.47 ± 16.74 a
Total		705.57	776.99	491.01	444.32	605.24	363.00	806.50	669.07	425.53	672.72	547.83	624.57
% Acids		0.18	0.21	0.14	0.09	0.18	0.07	0.23	0.20	0.12	0.19	0.14	0.20
C6 alcohols		-	-	-	-	-	-	-	-	-	-	-	-
1-Hexanol	ST	132.35 ± 11.05 a	93.96 ± 6.59 b	125.60 ± 8.79 a	96.28 ± 3.20 b	80.81 ± 4.57 c	104.17 ± 6.19 d	121.97 ± 4.98 a	88.62 ± 8.91 b	122.27 ± 8.99 a	94.75 ± 2.83 b	71.50 ± 6.48 c	95.70 ± 5.50 b
(E)-3-Hexen-1-ol	ST	0.43 ± 0.04 a	0.38 ± 0.13 a	0.45 ± 0.08 a	0.13 ± 0.01 b	0.42 ± 0.09 a	0.15 ± 0.03 b	0.52 ± 0.12 a	0.45 ± 0.05 a	0.65 ± 0.17 a	0.40 ± 0.01 a	0.40 ± 0.09 a	0.44 ± 0.07 a
(Z)-3-Hexen-1-ol	ST	6.20 ± 0.10 a	5.24 ± 0.36 b	6.53 ± 0.32 a	3.68 ± 0.06 c	4.91 ± 0.13 b	4.26 ± 0.41 b	5.57 ± 0.18 a	4.86 ± 0.23 b	5.70 ± 0.67 a	4.37 ± 0.50 b	4.38 ± 0.95 b	4.88 ± 0.86 b
Total		138.97	99.57	132.58	100.09	86.14	108.57	128.06	93.93	128.62	99.53	76.28	101.03
% C6 alcohols		0.03	0.03	0.04	0.02	0.03	0.02	0.04	0.03	0.04	0.03	0.02	0.03
Alcohols		-	-	-	-	-	-	-	-	-	-	-	-
4-Methyl-1-pentanol	ST	4.62 ± 0.35 a	1.11 ± 0.06 b	4.02 ± 0.12 a	0.44 ± 0.09 c	nd	0.49 ± 0.15 c	nd	nd	nd	1.23 ± 0.11 b	nd	1.29 ± 0.24 b
3-Methyl-1-pentanol	LB	nd	5.18 ± 0.18 a	0.00 ± 0.00	nd	nd	nd	2.43 ± 0.12 b	2.31 ± 0.17 b	2.60 ± 0.22 b	3.35 ± 0.27 c	1.62 ± 0.11 d	3.28 ± 0.37 c
3-Ethoxy-1-propanol	LB	7.88 ± 0.25 a	7.64 ± 0.26 a	nd	4.82 ± 0.41 b	6.86 ± 0.36 c	5.15 ± 0.43 b	3.78 ± 0.20 d	3.31 ± 0.46 d	4.30 ± 0.19 b	6.10 ± 0.18 c	4.99 ± 0.25 b	6.59 ± 0.48 c
3-Ethyl-2-pentanol	ST	nd	7.71 ± 0.31 a	nd	1.56 ± 0.11 b	3.65 ± 0.11 c	nd	4.79 ± 0.18 d	nd	nd	nd	Nd	nd
Benzyl alcohol	ST	19.44 ± 0.19 a	9.63 ± 0.9 b	nd	14.25 ± 0.34 c	15.11 ± 0.40 c	nd	7.56 ± 0.23 d	6.54 ± 0.15 d	nd	9.68 ± 0.30 b	10.66 ± 0.74 b	nd
Phenylethyl alcohol	ST	1249.95 ± 81.23 a	1804.51 ± 41.54 b	1925.52 ± 87.41 b	955.23 ± 34.76 c	1117.23 ± 76.76 a	993.57 ± 35.90 c	1573.88 ± 57.98 d	1448.35 ± 43.68 d	1356.26 ± 59.30 d	1192.92 ± 48.60 a	1204.32 ± 47.69 a	1407.93 ± 45.90 d
Total		1281.90	1835.78	1929.54	976.30	1142.85	999.21	1592.44	1460.51	1363.16	1213.28	1221.59	1419.10
% Alcohols		0.32	0.49	0.54	0.20	0.34	0.19	0.45	0.44	0.38	0.33	0.30	0.45
Terpenes		-	-	-	-	-	-	-	-	-	-	-	-
β-Citronellol	ST	12.43 ± 0.69 a	9.41 ± 0.32 b	15.29 ± 1.43 c	6.57 ± 0.36 d	6.03 ± 0.27 d	3.94 ± 0.36	13.24 ± 0.80 a	8.34 ± 0.29 b	18.78 ± 1.06 c	10.14 ± 0.64 b	6.35 ± 0.27 d	17.02 ± 0.84 c
2,6-Dimethyl-3,7-Octadien-2,6-diol	LB	13.04 ± 1.24 a	11.52 ± 3.18 a	11.42 ± 2.20 a	9.05 ± 0.51 b	8.37 ± 0.32 b	8.77 ± 0.22 b	10.49 ± 0.50 ab	10.23 ± 0.45 ab	7.62 ± 0.57 b	8.59 ± 0.30 b	14.54 ± 2.04 a	10.43 ± 0.83 ab
Total		25.47	20.93	26.72	15.62	14.40	12.71	23.73	18.57	26.40	18.73	20.89	27.45
% Terpenes		0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01

Table 2. *Cont.*

Volatile Compounds	ID	F	F-12G	F-AR	PC	PC-12G	PC-AR	CK	CK-12G	CK-AR	ENSIS	ENSIS-12G	ENSIS-AR
Esters		-	-	-	-	-	-	-	-	-	-	-	-
Ethyl acetate	ST	49,958.0 ± 134.91 a	38,081.0 ± 284.23 b	47,140.4 ± 304.78	63,125.0 ± 150.03	33,080.0 ± 97.95	55,730.0 ± 173.49	40,070.0 ± 171.00	24,328.0 ± 121.10	33,780.5 ± 254.71	26,007.0 ± 86.05	38,040.0 ± 58.24 b	27,635.0 ± 91.79
Ethyl butyrate	ST	1.21 ± 0.14 a	nd	nd	nd	nd	nd	1.03 ± 0.11 a	nd	nd	0.63 ± 0.05 b	nd	nd
Ethyl isovaleriate	LB	2.09 ± 0.23 a	nd	2.09 ± 0.17 a	1.14 ± 0.28 b	nd	1.14 ± 0.16 b	1.89 ± 0.36 a	nd	1.89 ± 0.34 a	0.98 ± 0.17 b	nd	0.98 ± 0.16 b
Isoamyl acetate	ST	nd	nd	nd	0.35 ± 0.02 a	nd	nd	nd	nd	nd	0.09 ± 0.01 b	nd	nd
Ethyl lactate	ST	15.77 ± 0.68 a	17.24 ± 1.84 a	23.19 ± 1.04 b	8.67 ± 0.11 c	20.53 ± 0.87 d	13.16 ± 0.94 e	13.87 ± 0.65 e	19.50 ± 0.58 d	21.12 ± 1.73 d	7.81 ± 0.22 c	16.59 ± 0.75 a	12.31 ± 0.32 e
Ethyl pentanoate	ST	98.32 ± 6.20 a	68.47 ± 4.80 b	85.01 ± 3.10 c	39.23 ± 1.05 d	87.99 ± 2.05 c	35.10 ± 1.45 e	53.44 ± 1.07 f	41.46 ± 1.17 d	44.78 ± 2.99 d	59.99 ± 4.43 f	53.77 ± 1.12 f	66.48 ± 1.19 b
Ethyl hexanoate	ST	14.89 ± 0.75 a	nd	8.69 ± 0.95 b	6.67 ± 0.33 c	nd	5.36 ± 0.11 d	14.42 ± 0.55 a	19.50 ± 0.43 e	3.47 ± 0.17 f	9.98 ± 0.31 b	0.99 ± 0.07 g	1.17 ± 0.19 g
Ethyl octanoate	ST	51.64 ± 1.98 a	15.73 ± 0.84 b	43.70 ± 1.75 c	31.30 ± 0.97 d	20.31 ± 0.76 e	24.34 ± 0.76 f	49.94 ± 0.97 a	14.02 ± 0.39 b	55.47 ± 3.08 a	51.26 ± 0.87 a	13.72 ± 0.82 b	46.82 ± 1.44 c
Ethyl decanoate	ST	83.59 ± 2.09 a	32.84 ± 1.15 b	11.43 ± 0.69 c	52.77 ± 1.15 d	30.98 ± 1.09 b	nd	72.10 ± 1.13 e	30.28 ± 0.92 b	27.06 ± 1.86 f	87.42 ± 1.39 a	26.06 ± 0.66 f	25.11 ± 1.38 f
Diethyl succinate	ST	25.66 ± 1.04 a	51.06 ± 1.99 b	41.89 ± 1.12 c	11.77 ± 0.99 d	31.53 ± 0.54 e	19.40 ± 0.32 f	35.57 ± 0.86 g	58.17 ± 1.24 h	60.25 ± 2.55 h	32.63 ± 0.55 e	40.84 ± 0.50 c	53.83 ± 1.67 b
Ethyl 9-decenoate	LB	15.61 ± 0.97 a	10.40 ± 0.32 b	10.72 ± 0.64 b	9.95 ± 0.20 b	9.56 ± 0.58 b	6.11 ± 1.11 c	41.74 ± 0.49 d	17.14 ± 0.78 e	29.62 ± 0.42 f	21.41 ± 0.29 g	7.94 ± 0.69 c	14.57 ± 0.23 a
Phenethyl acetate	ST	57.64 ± 2.04 a	72.58 ± 1.78 b	54.81 ± 3.67 a	38.25 ± 0.67 c	37.73 ± 1.32 c	32.54 ± 1.03 d	97.22 ± 1.00 e	79.47 ± 2.96 f	75.03 ± 0.81 b	76.71 ± 2.50 bf	39.81 ± 1.17 c	71.86 ± 1.36 b
Ethyl palmitate	LB	51.05 ± 0.87 a	nd	nd	nd	90.91 ± 4.98 b	nd	139.40 ± 3.03 c	nd	nd	45.65 ± 0.84 d	nd	nd
Ethyl laurate	ST	nd	nd	nd	nd	nd	nd	23.22 ± 0.77 a	nd	nd	49.40 ± 1.25 b	nd	nd
Ethyl nonadecanoate	ST	25.00 ± 0.27 a	8.97 ± 0.25 b	nd	4.55 ± 0.09 c	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl linoleate	LB	nd	182.53 ± 4.56 a	nd	nd	103.70 ± 2.09 b	nd	49.45 ± 1.03 c	nd	nd	70.57 ± 2.03 d	nd	nd
Ethyl hexadecanoate	ST	nd	36.51 ± 0.75 a	nd	nd	48.25 ± 0.41 b	nd	nd	nd	nd	28.41 ± 0.66 c	nd	nd
Ethyl oleate	LB	nd	90.67 ± 2.05 a	nd	nd	29.29 ± 0.17 b	nd	nd	nd	nd	nd	nd	nd
Diethyl 2-hydroxy-3-methylsuccionate	LB	nd	10.23 ± 0.76 a	nd	nd	10.21 ± 0.14 a	nd	nd	11.77 ± 1.22 a	nd	nd	8.66 ± 0.09 b	nd
Total		50,400.48	38,678.23	47,421.94	63,629.63	33,600.98	55,867.16	40,663.32	24,619.28	34,099.19	26,549.93	38,248.38	27,928.13
% Esters		12.55	10.38	13.21	12.75	9.87	10.38	11.37	7.38	9.61	7.31	9.43	8.88
Aldehydes		-	-	-	-	-	-	-	-	-	-	-	-
Acetaldehyde	ST	80,094.0 ± 920.10 a	48,002.00 ± 720.03 b	116,670.0 ± 1,944.5 c	150,315.0 ± 1100.00 d	40,108.50 ± 661.79 e	204,760.00 ± 819.04 f	30,721.0 ± 536.60 g	25,011.0 ± 525.23 h	35,810.50 ± 438.52 i	33,895.5 ± 152.05 j	51,214.0 ± 286.74 k	42,040.0 ± 371.10 l
Total		80,094.0	48,002.00	116,670.00	150,315.0	40,108.5	204,760.00	30,721.0	25,011.0	35,810.5	33,895.5	51,214.0	42,040.0
% Aldehydes		19.95	12.88	32.49	30.20	11.78	38.04	8.59	7.49	10.09	9.34	12.63	13.37
Thiols		-	-	-	-	-	-	-	-	-	-	-	-
3-Methylthio-1-Propanol	LB	5.13 ± 0.16 a	7.12 ± 0.29 b	8.75 ± 0.98 c	2.13 ± 0.30 d	5.90 ± 0.99 a	2.06 ± 0.11 d	8.63 ± 0.87 c	8.33 ± 0.56 c	8.87 ± 0.69 c	6.13 ± 0.48 b	6.01 ± 0.76 b	7.73 ± 0.95 b
Total		-	-	-	-	-	-	-	-	-	-	-	-
% Thiols		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

nd: not detected. PC, F, CK and ENSIS: wine elaborated by different yeast strains; 12G and AR: correspond to enzymatic extracts used; ID: Identification method; ST: compounds detected using pure standards and with Xcalibur v.1.1. Library Browser; LB: compounds detected using with Xcalibur v.1.1. Library Browser. Different letters indicate significant differences concentration volatile compounds analyzed between for Palomino fino wines studied ($p < 0.05$). Data are expressed as mean ± standard deviation ($n = 3$).

On the other hand, it has been reported that the production of fatty acids is dependent on the yeast strains and their abilities to synthesize/convert precursors or break carbon chains [45]. These varying abilities could explain the differences in acids observed when different yeast strains were used (Table 2). The CK and F wines showed the highest content in fatty acids, highlighting octanoic acid (>500 μg/L), while PC wines showed the lowest content. Likewise, although no references have been found, treatment with glycosidase enzymes also affected the acid content, but the behaviour of each enzyme differed according to the strain. So, the F and PC wines treated with 12G showed higher concentrations of fatty acids than the same untreated wines, while the opposite effect was observed in CK and ENSIS wines. However, the AR enzyme treatment led to lower content of fatty acids than their respective untreated wines.

3.1.3. C6 Alcohols

C6 alcohols are known to contribute to the aroma of many fruits and vegetables [46] and have also been proposed as potential markers of varietal authenticity [47]. Some authors [48,49] have even described them as major contributors to the varietal aroma of neutral grape varieties. Besides, according to Denis et al. [50], hexan-1-ol, hexenal, (E)-2-hexen-1-ol and (E)-2-hexenal are all precursors to hexyl acetate, although their metabolization by yeast depends on the concentration and type of C6 compounds. On the one hand, the C6 alcohol levels of Palomino fino wines varied according to the treatment used, with 1-hexanol as a highlight (Tables 1 and 2). The SUPRA wines (Table 1) showed the lowest C6 compounds' content because of the low levels of 1-hexanol and also (E)-3-hexen-1-ol (27.83 and 0.25 μg/L, respectively). On the other hand, the wines elaborated by the PC strain (Table 2) showed lower contents of these compounds, principally (E)-3-hexen-1-ol, while no significant effect of enzyme treatment was observed. Bakker et al. [51] pointed out that C6 compounds are originally present in crushed grape must, resulting from the enzymatic oxidation of grape polyunsaturated fatty acids through the lipoxygenase pathway. In this sense, extractive treatments did not favour the yield of C6 alcohols in our study, contrary to what was observed in other grape varieties [14,37,41].

3.1.4. Alcohols

As shown in Tables 1 and 2, the type of technological treatment applied also had an impact on the alcohol content of wines, ranging between 0.5 and 2 mg/L. Phenylethyl alcohol was the principal contributor to this content, and the SUPRA wines showed the lowest level.

Only 1-octanol was detected in the control, PM and SUPRA wines, while 3-ethoxy-1-propanol, 3-ethyl-2-pentanol and benzyl alcohol were present in the other wines. Likewise, each alcohol's formation and its content in wine depended on the yeast strain and enzyme used. The highest levels were obtained with the CK strain and the lowest with the PC. Glycosidase enzymes showed different behaviour depending on the wine characteristics. The AR enzyme increased the alcohol content in the F, PC and ENSIS wines, and 12G had a stronger effect on the F and PC wines. Therefore, alcohol contents were dependent on the raw material and the biochemical and technological changes during winemaking, as indicated by some authors [52].

3.1.5. Terpenes

Terpenes are considered as the main part of the varietal compounds derived from grapes, where they are often found to be glycosidically bound [42], depending on the variety and the relative proportions of free and bound forms [52]. The presence of terpenes in wine is typically due to the direct extraction of these compounds and their skin glycoconjugates [14,42,53]. However, during fermentation, terpene glycosides are hydrolysed to free volatile terpenes by yeast glycosidase and by the acidic fermentation conditions.

According to Genovés et al. [54], 2,6-dimethyl-3,7-octadiene-2,6-diol is, together with geraniol, one of the major terpene compounds in Palomino musts. Other terpene com-

pounds, such as linalool, nerol and a-terpineol, have been found for the same variety by some authors [5,8]. However, as can be observed in Tables 1 and 2, the content of terpenes in Palomino fino wines is represented by 2,6-dimethyl-3,7-octadien-2,6-diol and a small fraction of β-citronellol, except in the SUPRA wines (Table 1). In fact, the latter showed a terpene content higher than 200 μg/L, where 2,6-dimethyl-3,7-octadiene-2,6-diol was the majority terpene compound. On the one hand, this technique favoured the extraction of other terpenic compounds, such as linalool, linalool oxide, α-terpineol, β-citronellol and nerol, or their precursors. On the other hand, no increase in terpenes due to pellicular maceration and the use of yeast strains or glycosidase enzymes was observed, contrary to other studies [5,8,55]. In general, the terpene content was similar, except when compared to the PC wines (12–15 μg/L), which showed the lowest contents. Therefore, this autochthonous yeast strain generally used for sherry wine elaboration seems to have a lower capacity to release glycosidic compounds. The content of aromatic precursors extracted from the grape, their presence in the must and the glycosidase activity of the yeasts during fermentation conditioned the release of glycosylated terpenes [56].

The disruption of membranes by freezing [57] produced during the supra-extraction favours the selective extraction of precursors and aromatic compounds and their subsequent diffusion into the must, even in varieties such as Palomino fino, which is considered neutral by some authors [54].

3.1.6. Esters

Esters were found in a range between 8.0 and 63.0 mg/L (Tables 1 and 2), showing the higher contents (>24.0 mg/L) in wines made with different yeast strains and glycosidase enzymes, most likely due to the must characteristics used in winemaking. The biosynthesis of these compounds is directly dependent on the availability of fatty acids, and both depend on fermentation conditions such as yeast strain, nutrient status of the must (e.g., sugar, assimilable nitrogen) and temperature [58]. On the one hand, the SUPRA and PM techniques increased the total esters, but no influence of maceration time was observed. Regarding the yeast used, a greater influence of the must composition was observed compared to the yeast strain. On the other hand, the β-glycosidase enzymes used led to a lower content of esters, contrary to what has been shown in other studies for the same variety [2,5]. The observed decrease was greater with the use of 12G, except for the ENSIS wines. This fact shows that both preparations possess some type of secondary hydrolytic activity that mainly affects these esters [59].

The compound responsible for the total ester content and the differences found between Palomino fino wines was ethyl acetate, with the highest concentration. The wines elaborated using the PC strain stood out due to their levels of ethyl acetate, but the use of enzymes caused a decrease of this compound. Significantly high levels (>50.0 μg/L) of ethyl pentanoate, ethyl octanoate, ethyl decanoate, diethyl succinate, phenethyl acetate and ethyl 9-decenoate were found in these wines, although this last did not reach higher values than 16.0 and 10 μg/L in the F and PC wines, respectively. The behaviour of these esters depended on both the technique used and the compound itself. The pellicular maceration favoured the formation of ethyl octanoate, whose content increased with time. The SUPRA wines were characterized by their isoamyl acetate, ethyl pentanoate and ethyl octanoate content. The differences observed in the content of these esters in wines made with different yeast strains were due to the formation and concentration of the yeast used in the present study, but similar levels were reached with all of them except the PC yeast strain. Similar findings have been reported by several other authors [60] about the influence of *S. cerevisiae* on ester formation.

According to Amores-Arrocha et al. [22], the ester levels observed could be explained by the presence of fatty acids in wines, because their synthesis is conditioned by the greater or lesser content of fatty acids and alcohols, both substrates of the esterification reactions.

3.1.7. Acetalehydes

Acetaldehyde is formed mainly by the metabolism of yeasts [6], but also by acetic acid bacteria, andcoupled auto-oxidation of ethanol and phenolic compounds [61]. This aroma compound is one of the most important sensory carbonyl compounds formed during vinification [61] and is associated with fruity aromas and notes of nuts or dried fruits [6]. As can be observed from the results in Tables 1 and 2, significant differences were observed between the studied wines. Acetaldehyde production differed significantly between the two types of must, ranging from 11 to 18 mg/L and 30 to 150 mg/L in wines obtained by extractive and aroma-release techniques, respectively. The control and the PM4 and SUPRA wines showed similar levels of acetaldehyde, but longer maceration times led to a decrease. Various factors influence the formation of acetaldehyde, including the medium composition [62]. These results show that the ability to produce acetaldehyde is a property of yeasts and that the *S. cerevisiae* strains used produce relatively higher levels of acetaldehyde than those described by other authors [62,63]. Therefore, the yeast strain represents a prominent factor in determining the content of acetaldehyde in wine distinguishing between two different phenotypes: high and low acetaldehyde producers [63]. In this sense, the PC strain's ability to produce acetaldehyde is worth highlighting, since it could be considered a high acetaldehyde producer; hence the interest in its use for sherry winemaking, where acetaldehyde is a well-known and desirable constituent [64].

The glycosidase enzymes had a different effect on acetaldehyde content. The 12G enzyme gave rise to lower acetaldehyde content, except in the ENSIS wines, while AR increased this content. No studies were found on the effect of the use of enzymes on the acetaldehyde content in wines. However, the accumulation of acetaldehyde during fermentation has been reported to be dependent on the equilibrium between the alcohol, dehydrogenase and aldehyde dehydrogenase enzymes [65]. The residual activity of these enzymes still present in the wine or of the enzyme extracts used could be responsible for the results obtained.

3.1.8. Other Compounds

Other compounds, such as thiols and phenols, were found in exceptionally low concentrations in some of the Palomino fino wines. These compounds were 3-methylthio-1-propanol, 2-methoxy-4-vinilphenol and 2,6-di-*tert*-butyl-4-ethylphenol, representing under 0.1% of the total amount of volatile compounds. The first was found in all the elaborated wines, ranging from 2 to 9 µg/L, while 2-methoxy-4-vinilphenol was found in the control and PM wines and its content increased with maceration time. Only the control, PM and SUPRA wines showed 2,6-di-tert-butyl-4-ethylphenol, with remarkably similar concentrations, except in the latter with the lowest content (5.8 µg/L).

3.2. Odour Activity Values (OAVs) and Aromatic Series

Odour activity values (OAVs) were used to estimate the sensory contribution of the aromatic compounds to the overall flavour of the wines. The contribution of one specific volatile compound to the perception of the aroma depends not only on the concentration of the volatile compound itself, but also on its odour threshold value. In this regard, the odour perception threshold (OPT) value, odour descriptors and odorant series (by the main odour descriptor) of the volatile compounds found in Palomino fino wines were revised and listed in Table 3. According to Sánchez-Palomo et al. [6], compounds present in both higher and lower concentrations compared to their odour threshold were included; the latter due to synergistic effects with other odorant compounds.

Table 3. Odour perception threshold (OPT), odour descriptors and odorant series of the volatile compounds found in Palomino fino.

Volatile Compounds	OPT [a]	Odour Descriptor	Odorant Series
Higher alcohols			
n-Propyl alcohol	50	Fresh, fruity	Grassy
Isobutanol	30	Fruity, wine-like	Fruity
Amyl alcohol	30	Fruity	Fruity
Isoamyl alcohol	30	Ripe fruit, sweet	Fruity
Acids			
Hexanoic acid	8	Cheese, rancid	Fatty
Heptanoic acid	1	Fatty, dry	Fatty
Octanoic acid	0.55	Vegetable oil, rancid, harsh	Fatty
n-Decanoic acid	1	Fatty, unpleasant	Fatty
9-Decenoic acid	0.04	Waxy, fatty, soapy	Fatty
Benzoic acid	1	Chemical	Chemical
C6 alcohols			
1-Hexanol	1.62	Herbaceous, grass, woody	Grassy
(E)-3-Hexen-1-ol	0.4	Fresh	Grassy
(Z)-3-Hexen-1-ol	0.4	Freshly cut grass	Grassy
Alcohols			
4-Methyl-1-pentanol	50	Nutty	Dried fruit
3-Methyl-1-pentanol	1	Soil, mushroom	Earthy, mushroom
3-Ethoxy-1-propanol	50	Fruity	Fruity
1-Octanol	0.12	Intense citrus, roses	Fruity
3-Ethyl-2-pentanol	n.f.	-	-
Benzyl alcohol	0.9	Fruity, blackberry	Fruity
Phenylethyl alcohol	10	Roses, honey, lilac, floral, pollen	Floral
Terpenes			
Linalool	0.006	Floral, rose	Floral
Linalool oxide	0.006	Fresh, sweet, floral	Floral
α-Terpineol	0.25	Floral	Floral
β-Citronellol	0.018	Floral	Floral
Nerol	0.015	Floral	Floral
2,6-Dimethyl-3,7-octadien-2,6-diol	n.f.	-	-
Esters			
Ethyl acetate	12	Pineapple	Fruity
Ethyl butyrate	0.020	Sour fruit, apple	Fruity
Ethyl isovaleriate	0.003	Fresh fruit, orange, berry, blackberry	Fruity
Isoamyl acetate	0.03	Banana	Fruity
Hexyl acetate	0.02	Pear	Fruity
Ethyl lactate	150	Fruity, buttery	Fatty
Ethyl 2-hydroxy-4-methylbutanoate	0.126	Pineapple, strawberry, tea, honey	Fruity
Ethyl 2-hydroxy-4-methylpentanoate	0.051	Blueberry, valerian oil aroma	Fruity
Ethyl pentanoate	1	Apple	Fruity
Ethyl hexanoate	0.014	Green apple, fruity	Fruity
Isoamyl hexanoate	n.f		
Ethyl octanoate	0.005	Pineapple, pear, sweet	Fruity
Diethyl malonate	n.f.		
Ethyl decanoate	0.2	Sweet fruity, dry fruit	Fruity
Diethyl succinate	1.2	Fruity, melon	Fruity
Ethyl 9-decenoate	0.1	Rose	Floral
Phenethyl acetate	0.25	Floral, roses, honey	Floral
Ethyl cinnamate	0.0011	Balsamic, fruity, honey	Fruity
Aldehydes			
Acetaldehyde	100	Bitter almond	Dried fruit
Thiols			
3-Methylthio-1-propanol	1	Earthy, onion, garlic	Earthy
Phenols			
2-Methoxy-4-vinilphenol	0.04	Cloves, spice	Spicy

n.f.: not found. [a]: Odour Perception Threshold (OPT) (mg/L) and odour descriptors reported by other authors [22,25–30].

Considering the ΣOAV by odorant series (Figure 2) and the total OAV, two clearly differentiated groupings in the wines are observed: on the one hand, the control and wines treated with extractive techniques whose musts were obtained by horizontal membrane pressing; and on the other hand, the wines elaborated using different yeast strains and glycosidase enzymes to release aroma, whose musts were obtained by horizontal plate pressing. Considering the control and F wines, both fermented by the same yeast strain, although the total volatile content was higher in the F wines, their ΣOAV was lower and the opposite occurred in the control wines. Therefore, the total volatile content was not directly related to the aromatic profile given by the compounds contributing to aroma.

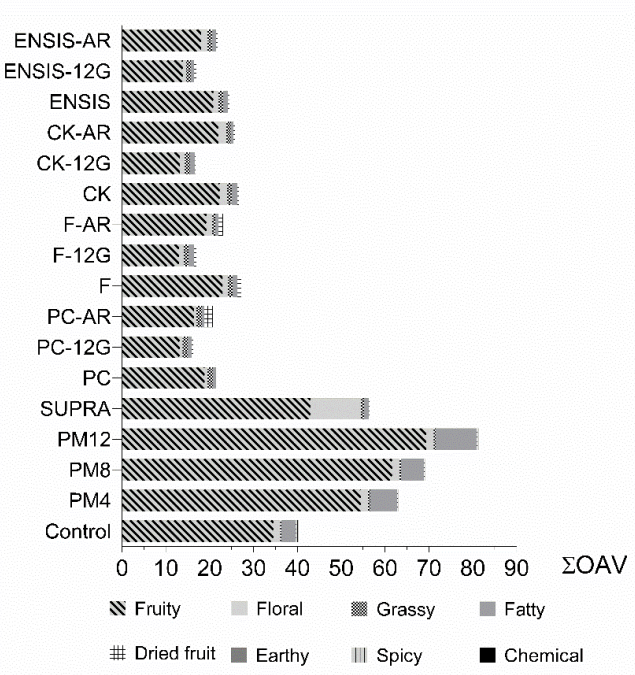


Figure 2. Summary of odour activity values (ΣOAV) by odorant series. PM4, PM8 and PM12: pellicular maceration for 4, 8 and 12 h, respectively; SUPRA: supra-extraction wine; PC, F, CK and ENSIS: wine elaborated by different yeast strains; 12G and AR: correspond to enzymatic extracts used.

Fruity was the predominant odorant series contributed by esters, principally isoamyl alcohol and ethyl octanoate. These compounds have a low perception threshold, so contributed more significantly to the OAV. Their values increased with the pellicular maceration time, reaching maximum levels at 12 h. The time of contact with the skins favoured the grassy, fatty and spicy series, increasing them over time. However, the greatest effect was observed in the fatty series through greater extraction of octanoic acid.

The supra-extraction technique led to an increase of fruity and floral series compared to the control, with the floral series reaching the highest levels in SUPRA wines. Terpene compounds are the main contributors to this series, highlighting linalool and β-citronellol besides subthreshold compounds, such as linalool oxide, nerol and α-terpineol. Other studies on Palomino fino have highlighted the neutral character of this variety and its low potential to produce varietal aromas [2,5,8,55]. These results show supra-extraction as a technique capable of selectively extracting terpenes and aromatic precursors from the Palomino fino grape, highlighting its varietal character.

Regarding the yeast strains used, the results were similar, but slight differences were observed in the fruity, floral and fatty series. The CK strain enhanced the floral series, followed by F and ENSIS, while PC was characterized by its higher levels of the dried fruit series.

The β-glycosidase enzymes showed a different effect on the principal aromatic series. Both enzymes decreased the ΣOAV of the fruity series, with a greater effect of 12G observed. Likewise, the floral series decreased with the use of this enzyme but increased when the AR enzyme was used, except in the PC wines. This enzyme was characterized by producing higher levels of citronellol.

Therefore, the ΣOAV results indicate that the vinification technique used can improve the fruity and floral characteristic in Palomino fino wines and even highlight varietal aromas.

3.3. Principal Component Analysis of Odour Activity Values

The OAVs, related to the aromatic profile of the wine, were used as variables to carry out the principal component analysis (PCA) (Table 4). Three principal components that accounted for 75.1% of the total variance of the data were extracted by PCA. PC1 (44.7% of the total variability) was mainly affected, with positive values, by acids, amyl alcohol, isoamyl alcohol, 1-octanol and 2-methoxy-4-vinilphenol. This factor represents the effects of winemaking techniques on the fruity and fatty series. PC2 was positively affected by terpenes, heptanoic acid, ethyl butyrate and isoamyl acetate, which explained 29.9% of the total variability between the samples. This component shows the main effects that techniques had on the floral series, directly related to varietal character.

Table 4. Loadings of principal components of volatile compounds' OAVs in Palomino fino wines.

Volatile Compounds	PC1	PC2
n-Propyl alcohol	−0.820	−0.214
Isobutanol	−0.768	−0.539
Amyl alcohol	0.671	0.146
Isoamyl alcohol	0.781	0.272
Hexanoic acid	0.919	0.303
Heptanoic acid	0.365	0.657
Octanoic acid	0.924	−0.035
n-Decanoic acid	0.692	−0.125
9-Decenoic acid	0.912	−0.127
Benzoic acid	0.515	−0.074
1-Hexanol	−0.117	−0.710
(E)-3-Hexen-1-ol	−0.695	−0.214
(Z)-3-Hexen-1-ol	-0.247	−0.174
1-Octanol	0.881	0.292
Benzyl alcohol	−0.426	−0.269
Phenylethyl alcohol	−0.272	−0.556
Linalool	0.006	0.990
Linalool oxide	0.006	0.990
α-Terpineol	0.006	0.990
β-Citronellol	-0.507	0.662
Nerol	0.006	0.990
Ethyl acetate	−0.631	−0.283
Ethyl butyrate	0.169	0.797
Ethyl isovaleriate	−0.560	−0.227
Isoamyl acetate	0.090	0.951
Hexyl acetate	0.943	0.058
Ethyl lactate	−0.814	−0.324
Ethyl 2-hydroxy-4-methylbutyrate	0.951	0.227
Ethyl 2-hydroxy-4-methylpentanoate	0.934	−0.124
Ethyl pentanoate	0.809	0.094
Ethyl hexanoate	0.972	0.570
Ethyl octanoate	0.908	0.219
Ethyl decanoate	−0.065	−0.188
Diethyl succinate	0.309	0.388
Ethyl 9-decenoate	0.950	−0.12
Phenylethyl acetate	0.357	−0.131
Ethyl cinnamate	0.739	−0.131
Acetaldehyde	−0.442	−0.256
3-Methylthio-1-propanol	0.064	−0.448
2-Methoxy-4-vinilphenol	0.963	−0.130

Loadings of rotated component matrix. Quartimax with Kaiser normalization of principal component analysis (PCA) of volatile compounds in Palomino fino wines.

It is clearly seen from the score plot of the samples on the plane defined by PC1 and PC2 (Figure 3) that three types of wines are identified according to their aromatic profile: firstly, there are the closely grouped wines resulting from the use of yeast strains and glycosidase enzymes (negative values of PC1 and PC2); secondly, slightly separated, there are the control and PM wines (positive values of PC1 and negative values of PC2); thirdly, there are the SUPRA wines (negative values of PC1 and positive values of PC2).

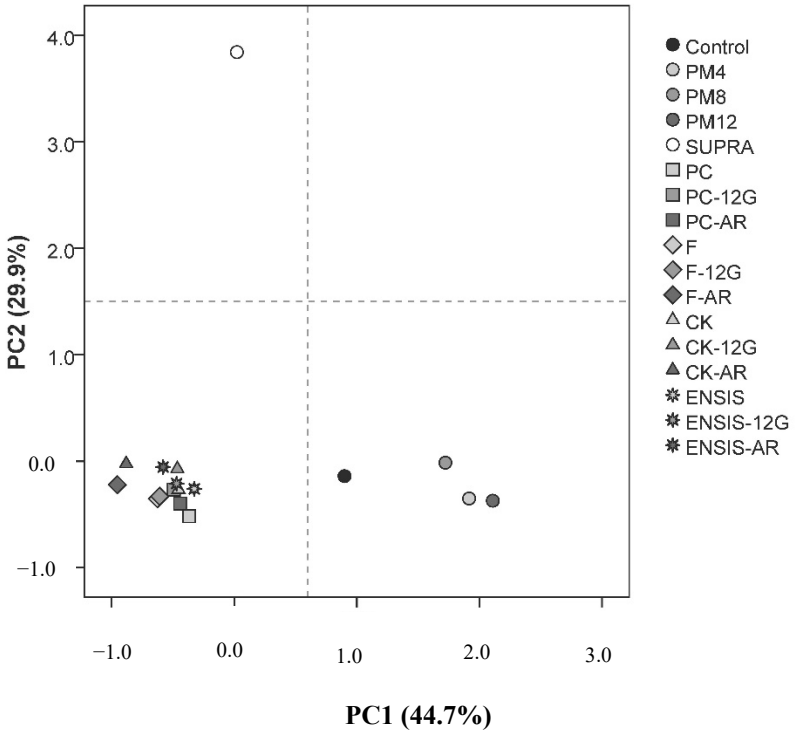


Figure 3. PCA scores of Palomino fino wines elaborated using different vinification techniques. PM4, PM8 and PM12: pellicular maceration for 4, 8 and 12 h, respectively; SUPRA: supra-extraction wine; PC, F, CK and ENSIS: wines elaborated by different yeast strains; 12G and AR: correspond to enzymatic extracts used.

The loadings of each compound on the principal components clearly show that terpenic compounds are mainly responsible for the grouping of the floral wines. Fatty acids and their esters are responsible for the differentiation of other wines due to their fruity character, separating those made using strains and enzymes from the control and those from the pellicular maceration.

3.4. Sensory Analysis

The spider plots (Figure 4) show the mean values for the attributes of the control and treated wines with different winemaking techniques. In general, the wines vinified by extractive techniques (Figure 4a) showed the highest scores for most attributes, especially intensity and aroma quality, indicating an improvement over the control and other wines. As shown in the sensorial analysis, SUPRA Palomino fino wines obtained higher values in the global judgment and were evaluated as superior in terms of aromatic and taste quality. In addition, the tasters valued them as more floral and, above all, fruity, with notes of pineapple, pear, plum and other aromas, such as caramel and raisins, which is consistent with the results obtained in the aromatic profile (OAVs) of these wines. The results described in this study show that the supra-extraction-treated wines had a higher free aromatic profile in comparison to the control wines, mainly due to extraction and hydrolysis of the glycosidically bound terpenes.

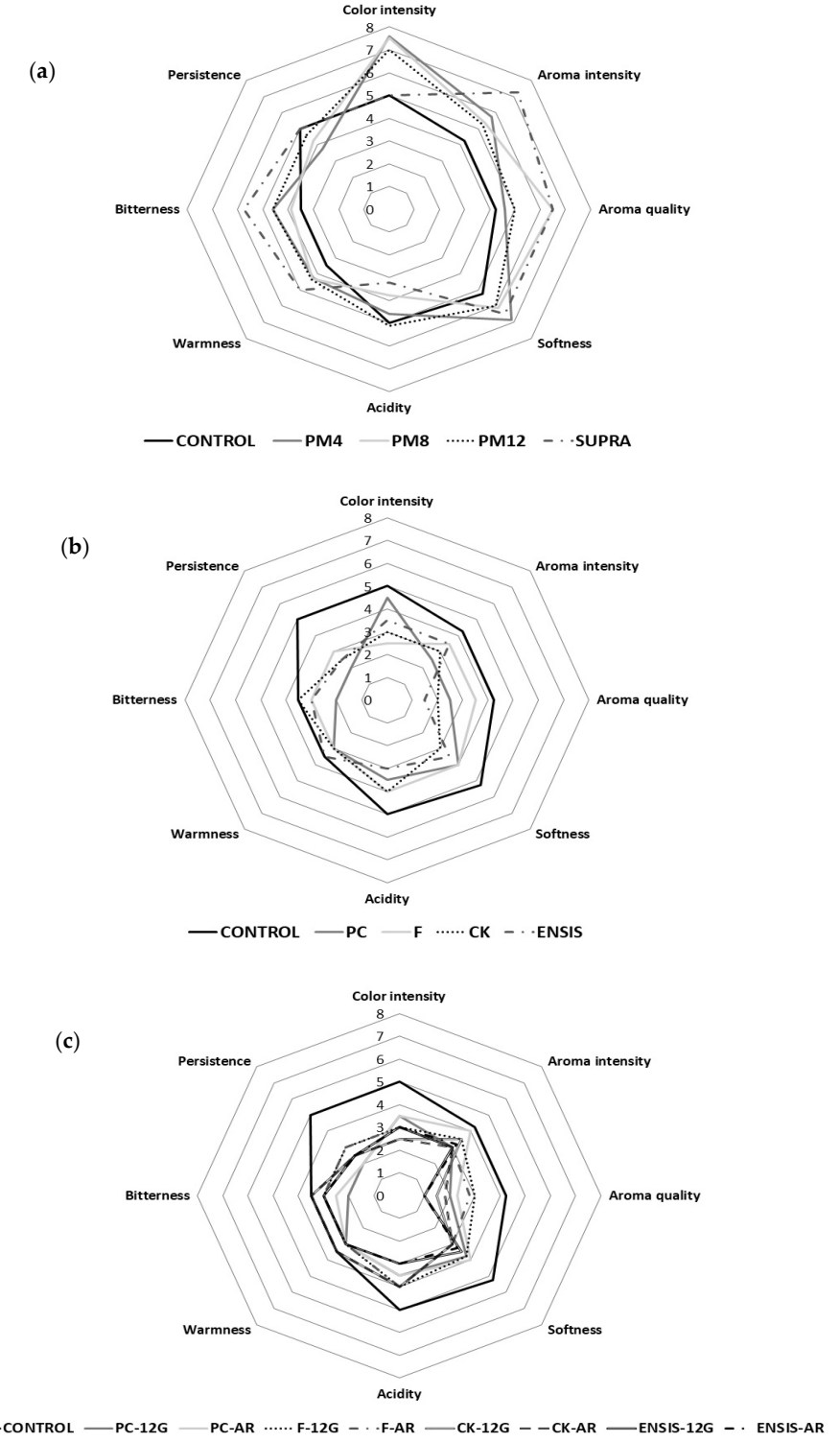


Figure 4. Effect of winemaking technique on sensorial analysis: results of Palomino fino wines compared to the control wine. (**a**) Extractive techniques of pellicular maceration (PM) and supra-extraction (SUPRA); (**b**) aroma-release technique using yeast strains (PC, F, CK and ENSIS); (**c**) aroma release-technique using the β-glycosidase enzymes AR and 12G.

On the other hand, the grape origin and the way of obtaining the must have a greater influence on the sensorial profile than the use of yeast strains (Figure 4b) and glycosidase enzymes (Figure 4c), with clear differences in the analysed attributes compared to the control. F wines were the best valued for their aromatic quality with fruity and spicy notes,

while PC wines were valued as sherried wines mainly due to their aroma of green apple, nuts and almond, characteristic of the biological aging of sherry wines. This result confirms that obtained in the OAV analysis and explains why the PC yeast strain is selected for the fermentation of Palomino fino musts and its subsequent aging under biofilm of "flor" velum.

The β-glycosidase enzymes led to a significant loss of aromatic quality, resulting in wines classified as "lacking typicality" by the tasters.

4. Conclusions

The current study was the first to investigate the influence of different pre-fermentation (pellicular maceration and supra-extraction), fermentation (use of yeasts) and post-fermentation (use of β-glycosidase enzymes) treatments on the volatile compounds, aromatic profile and sensorial evaluation of Palomino fino wines. The magnitude of analytical and sensorial differences observed depended on the origin of the grape variety and the type of press and technique used.

Until now, the Palomino variety has been considered as neutral, with few remarkable aromas, in which the fruity character slightly increases after certain treatments, such as cold skins, fermentation by co-inoculation or the use of glycosidase enzymes. However, the supra-extraction technique showed an increase of volatile compounds in Palomino fino wines and ΣOAVs, improving their aromatic intensity and quality with a greater floral character.

According to the results, supra-extraction is a viable alternative to Palomino fino white wine elaboration, favouring their differentiation from the sherry wines and other white wines made with the same variety.

Author Contributions: Conceptualization, A.M.R., V.M.P. and L.P.-R.; methodology, A.M.R. and V.M.P.; software, A.M.R. and F.S.-G.; validation, A.M.R., V.M.P., L.P.-R. and F.S.-G.; formal analysis, A.M.R. and F.S.-G.; investigation, A.M.R., V.M.P. and L.P.-R.; resources, V.M.P. and L.P.-R.; data curation, A.M.R. and F.S.-G.; writing—original draft preparation, A.M.R.; writing—review and editing, A.M.R.; visualization, A.M.R., V.M.P., L.P.-R. and F.S.-G.; supervision, V.M.P. and L.P.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Vinos de Jerez. Sherry Wines. Available online: https://www.sherry.wine/es/marco-de-jerez/el-consejo-regulador (accessed on 15 November 2020).
2. Puertas, B.; Jiménez, M.J.; Cantos-Villar, E.; Cantoral, J.M.; Rodríguez, M.E. Use of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in semi-industrial sequential inoculation to improve quality of Palomino and Chardonnay wines in warm climates. *J. Appl. Microbiol.* **2016**, *122*, 733–746. [CrossRef] [PubMed]
3. Sancho-Galán, P.; Amores-Arrocha., A.; Palacios, V.; Jiménez-Cantizano, A. Preliminay study of somatic variants of Palomino fino (*Vitis vinífera* L.) grown in a warm climate region (Andalusia, Spain). *Agronomy* **2020**, *10*, 654.
4. Pozo-Bayón, M.A.; Moreno-Arribas, M.V. Sherry Wines. In *Advances in Food and Nutrition Research*, 1st ed.; Jackson, R.S., Ed.; Academic Press, Elsevier Ltd.: London, UK, 2011; Volume 63, pp. 17–40.
5. Valcárcel, M.C.; Palacios, V. Influence of 'Novarom G' Pectinase b-glycosidase Enzyme on the Wine Aroma of Four White Varieties. *Food Sci. Technol. Int.* **2008**, *14* (Suppl. 5), 95–102.
6. Sánchez-Palomo, E.; Trujillo, M.; García, A.; González, M.A. Aroma profile of malbec red wines from La Mancha region: Chemical and sensory characterization. *Food Res. Int.* **2017**, *100*, 201–208. [CrossRef]
7. King, E.S.; Kievit, R.L.; Curtin, C.; Swiegers, J.H.; Pretorius, I.S.; Bastian, S.E.P.; Leigh Francis, I. The effect of multiple yeasts co-inoculations on Sauvignon Blanc wine aroma composition, sensory properties, and consumer preference. *Food Chem.* **2010**, *122*, 618–626. [CrossRef]
8. Piñeiro, Z.; Natera, R.; Castro, R.; Palma, M.; Puertas, B.; Barroso, C.G. Characterisation of volatile fraction of monovarietal wines: Influence of winemaking practices. *Anal. Chim. Acta* **2006**, *563*, 165–172. [CrossRef]

9. Cabrita, M.J.; Costa Freitas, A.M.; Laureano, O.; Borsa, D.; Di Stefano, R. Aroma compounds in varietal wines from Alentejo, Portugal. *J. Food Compos. Anal.* **2007**, *20*, 375–390. [CrossRef]
10. González-Barreiro, C.; Rial-Otero, R.; Cancho-Grande, B.; Simal-Gándara, J. Wine aroma compounds in grapes: A critical review. *Crit. Rev. Food Sci. Nutr.* **2015**, *55*, 202–218. [CrossRef]
11. Slegers, A.; Angers, P.; Oullet, P.; Trucho, T.; Pedneault, K. Volatile compounds from grape skin, juice and wine from five interspecific hybrid grape cultivars grown in Québec (Canada) for wine production. *Molecules* **2015**, *20*, 10980–11016. [CrossRef]
12. Chang, E.; Jung, S.; Hur, Y. Changes in the aromatic composition of grape cv. Cheongsoo wine depending on the degree of grape ripening. *Food Sci. Biotechnol.* **2014**, *23*, 1761–1771. [CrossRef]
13. Wang, J.; Hue, S.; Zhang, Y.; Liu, Y.; Fan, W. Effect of different pre-fermentation treatments on polyphenols, color, and volatile compounds of three wine varieties. *Food Sci. Biotechnol.* **2016**, *25*, 735–743. [CrossRef]
14. Selli, S.; Canbas, A.; Cabaroglu, T.; Erten, H.; Gunata, Z. Aroma components of cv. Muscat of Bornova wines and influence of skin contact treatment. *Food Chem.* **2006**, *94*, 319–326. [CrossRef]
15. Zhang, B.; Luan, Y.; Duan, C.Q.; Yan, G.L. Use of *Torulaspora delbrueckii* co-fermentation with two *Saccharomyces cerevisiae* strains with different aromatic characteristic to improve the diversity of red wine aroma profile. *Front. Microbiol.* **2018**, *9*, 606. [CrossRef]
16. Losada, M.M.; López, J.F.; Añón, A.; Andrés, J.; Revilla, E. Influence of some oenological practices on the aromatic and sensorial characteristics of white Verdejo wines. *Int. J. Food Sci. Technol.* **2012**, *47*, 1826–1834. [CrossRef]
17. Garde, T.; Ancín-Azpilicueta, C. Effect of oak barrel type on the volatile composition of wine: Storage time optimization. *LWT Food Sci. Technol.* **2006**, *39*, 199–205.
18. Fernández de Simón, B.; Martínez, J.; Sanz, M.; Cadahía, E.; Esteruelas, E.; Muño, A.M. Volatile compounds and sensorial characterisation of red wine aged in cherry, chestnut, false acacia, ash and oak wood barrels. *Food Chem.* **2014**, *147*, 346–356. [CrossRef]
19. Wang, J.; Huo, S.; Zhang, Y.; Liu, Y.; Fan, W. Impact of various maceration techniques on the phenolic and volatile composition of Chenin Blanc wines. *Int. J. Food Sci. Technol.* **2016**, *51*, 2360–2366. [CrossRef]
20. Cabaroglu, T.; Selli, S.; Canbas, A.; Leproutre, J.P.; Gunata, Z. Wine flavour enhancement through the use of exogenus fungal glycosidases. *Enzyme Microb. Technol.* **2003**, *33*, 581. [CrossRef]
21. Ugliano, M.; Bartowsky, E.J.; McCarthy, J.; Moio, L.; Henschke, P.A. Hydrolysis and transformation of grape glycosidically bound volatile compounds during fermentation with three *Saccharomyces yeast* strains. *J. Agric. Food Chem.* **2006**, *54*, 6322–6331. [CrossRef]
22. Amores-Arrocha, A.; Roldán, A.; Jiménez-Cantizano, A.; Palacios, V. Evaluation of the use of multiflora bee pollen on the volatile compounds and sensorial profile of Palomino fino and Riesling young wines. *Food Res. Int.* **2018**, *105*, 197–209. [CrossRef] [PubMed]
23. Di Stefano, R. Proposition d'une méthode de préparation de l'echantillon pour la détermination des terpènes libres et glycosides des raisins et des vins. *Bull. OIV* **1991**, *721–722*, 219–223.
24. Francis, I.L.; Newton, J.L. Determining wine aroma from compositional data. *Aust. J. Grape Wine Res.* **2005**, *11*, 114–126. [CrossRef]
25. Ferreira, V.; López, R.; Cacho, J.F. Quantitative determination of the odorants of young red wines from different grape varieties. *J. Sci. Food Agric.* **2000**, *80*, 1659–1667. [CrossRef]
26. Peinado, R.A.; Moreno, J.A.; Muñoz, D.; Medina, M.; Moreno, J. Gas chromatographic quantification of major volatile comp ounds and polyols in wine by direct injection. *J. Agric. Food Chem.* **2004**, *2*, 6389–6393. [CrossRef] [PubMed]
27. Moreno, J.A.; Zea, L.; Moyano, L.; Medina, M. Aroma compounds as markers of the changes in sherry wines subjected to biological ageing. *Food Control.* **2005**, *16*, 333–338. [CrossRef]
28. Moyano, L.; Zea, L.; Moreno, J.; Medina, M. Analytical study of aromatic series in sherry wines subjected to biological aging. *J. Agric. Food Chem.* **2002**, *50*, 7356–7361. [CrossRef]
29. Welke, J.E.; Zanus, M.; Lazzarotto, M.; Zini, C.A. Quantitative analysis of headspace volatile compounds using comprehensive two-dimensional gas chromatography and their contribution to the aroma of Chardonnay wine. *Food Res. Int.* **2014**, *59*, 85–99. [CrossRef]
30. Zea, L.; Moyano, L.; Moreno, J.; Cortes, B.; Medina, M. Discrimination of the aroma fraction of Sherry wines obtained by oxidative and biological ageing. *Food Chem.* **2001**, *75*, 79–84. [CrossRef]
31. Peinado, R.A.; Moreno, J.A.J.; Bueno, J.E.; Moreno, J.A.J.; Mauricio, J.C. Comparative study of aromatic compounds in two young white wines subjected to pre-fermentative cryomaceration. *Food Chem.* **2004**, *84*, 585–590. [CrossRef]
32. ISO NORM. 3591(1977). *Sensory Analysis: Apparatus Wine Tasting Glass*; ISO: Geneva, Switzerland, 1977.
33. Jackson, R.S. *Wine Tasting: A Professional Handbook*; Academic Press: London, UK, 2009; p. 350.
34. Vilanova, M.; Zamuz, S.; Vilariño, F.; Sieiro, C. Effect of terroir on the volatiles of *Vitis vinifera* cv. *Albariño. J. Sci. Food Agric.* **2007**, *87*, 1252–1256. [CrossRef]
35. Darias-Martíin, J.; Díaz-González, D.; Díaz-Romero, D. Influence of two pressing processes on the quality of must in white wine production. *J. Food Eng.* **2004**, *63*, 35–340. [CrossRef]
36. Aleixandre, J.L.; García, M.J. Influencia del prensado en la calidad del vino. *Viticultura y Enología Prof.* **1993**, *24*, 31–37.
37. Moreno-Pérez, A.; Vila-López, R.; Fernández-Fernández, J.I.; Martínez-Cutillas, A.; Gil-Muñoz, R. Influence of cold pre-fermentation treatments on the major volatile compounds of three wine varieties. *Food Chem.* **2013**, *139*, 770–776. [CrossRef] [PubMed]

38. Ribéreau-Gayón, P.; Dubourdieu, D.; Donèche, B.; Lonvau, A. *Handbook of Enology: The Microbiology of Wine and Vinifications*; John Wiley & Sons Ltd.: Hoboken, NJ, USA, 2003; p. 417.
39. Lukic´, I.; Radeka, S.; Grozaj, S.; Staver, M.; Peršuric´, D. Changes in physico-chemical and volatile aroma compound composition of Gewürztraminer wine as a result of late and ice harvest. *Food Chem.* **2016**, *196*, 1048–1057. [CrossRef]
40. Klingshirn, L.M.; Liu, J.R.; Gallanger, J.F. Higher alcohol formation in wines as related to the particle size profiles of juice insoluble solids. *Am. J. Enol. Vitic.* **1987**, *38*, 207–210.
41. Guitart, A.; Hernandez-Orte, P.; Cacho, J. Effects of maceration on the amino acid content of Chardonnay musts and wines. *Vitis* **1997**, *36*, 43–47.
42. Zhang, S.; Petersen, M.A.; Liu, J.; Toldam-Andersen, T.B. Influence of pre-fermentation treatments on wine volatile and sensory profile of the new disease tolerant cultivar Solaris. *Molecules* **2015**, *20*, 21609–21625. [CrossRef]
43. Gnekow, B.; Ough, C.S. Methanol in Wines and Musts: Source and Amounts. *Am. J. Enol. Vitic.* **1976**, *27*, 1–6.
44. RESOLUTION OENO 19/2004. Maximum Content Limits of Methanol of Wines. Available online: https://www.oiv.int/public/medias/644/oeno-19--2004-en.pdf (accessed on 28 January 2020).
45. Lin, X.; Hu, X.; Wu, W.; Liu, S.; Li, C. Evaluation of the volatile profile of wax apple (*Syzygium samarangense*) wines fermented with different commercial *Saccharomyces cerevisiae* strains. *Food Sci. Biotechnol.* **2019**, *28*, 657–667. [CrossRef]
46. Schwab, W.; Davidovich-Rikanati, R.; Lewinsohn, E. Biosynthesis of plant-derived flavor compounds. *Plant. J.* **2008**, *54*, 712–732. [CrossRef]
47. Oliveira, J.M.; Faria, M.; Sá, F.; Barros, F.; Araújo, I.M. C6-alcohols as varietal markers for assessment of wine origin. *Anal. Chim. Acta* **2006**, *563*, 300–309. [CrossRef]
48. Gómez, E.; Martínez, A.; Laencina, J. Changes in volatile compounds during maturation of some grape varieties. *J. Sci. Food Agric.* **1995**, *67*, 229–233. [CrossRef]
49. Watkins, P.; Wijesundera, C. Application of zNose™ for the analysis of selected grape aroma compounds. *Talanta* **2006**, *70*, 595–601. [CrossRef]
50. Denis, E.G.; Keyzers, R.A.; Kaula, C.M.; Maffei, S.M.; Nicholson, E.L.; Boss, P.K. Grape Contribution to Wine Aroma: Production of Hexyl Acetate, Octyl Acetate, and Benzyl Acetate during Yeast Fermentation Is Dependent upon Precursors in the Must. *J. Agric. Food Chem.* **2012**, *60*, 2638–2646. [CrossRef]
51. Bakker, J.; Clarke, R.J. Volatile Components. In *Wine Flavour Chemistry*; Bakker, J., Clarke, R.J., Eds.; Wiley-Blackwell: Chichester, UK, 2011; pp. 155–238.
52. Ribereau-Gayon, P.; Glories, Y.; Maujeau, A.; Dubordieu, P. *Handbook of Enology. The Chemistry of Wine Stabilization and Treatments*, 2nd ed.; John Wiley and Sons, Ltd.: Chichester, UK, 2006; Volume 2.
53. Lopez-Tamames, E.; CarroMarino, N.; Gunata, Y.Z.; Sapis, C.; Baumes, R.; Bayonove, C. Potential aroma in several varieties of Spanish grapes. *J. Agric. Food Chem.* **1997**, *45*, 1729–1735. [CrossRef]
54. Genovés, S.; Gil, J.V.; Vallés, S.; Casas, J.A.; Manzanares, P. Assesment of the aromatic potential of Palomino fino grape must using glicosidasas. *Am. J. Enol. Vitic.* **2005**, *56*, 188–191.
55. Fernández-González, M.; Di Stefano, R. Fractionation of glycoside aroma precursors in neutral grapes. Hydrolysis and conversion by *Saccharomyces cerevisiae*. *Lebensm. Wiss. Technol.* **2004**, *37*, 467–473. [CrossRef]
56. Claus, H.; Mojsov, K. Enzymes for Wine Fermentation: Current and Perspective Applications. *Fermentation* **2018**, *4*, 52. [CrossRef]
57. Álvarez, I.; García, M.A.; González, R.; Martin, P. Avances en Ciencias y Técnicas Enológicas. *ITACyL y GIENOL* **2000**, *121*.
58. Ugliano, M.; Henschke, P.A. Yeasts and wine flavour. In *Wine Chemistry and Biochemistry*; Moreno-Arribas, M.V., Polo, M.C., Eds.; Springer: New York, NY, USA, 2009; pp. 313–392.
59. Tamborra, P.; Martino, N.; Esti, M. Laboratory tests on glycosidase preparations in wine. *Anal. Chim. Acta* **2004**, *513*, 299–303. [CrossRef]
60. Patel, S.; Shibamoto, T. Effect of 20 different yeast strains on the production of volatile components in Symphony wine. *J. Food Compost. Anal.* **2003**, *16*, 469–476. [CrossRef]
61. Liu, S.Q.; Pilone, G.O. An overview of formation and roles of acetaldehyde in winemaking with emphasis on microbiological implications. *Int. J. Food Sci. Technol.* **2000**, *35*, 49–61. [CrossRef]
62. Delfini, C.; Costa, A. Effects of the grape must lees and insoluble materials on the alcoholic fermentation rate and the production of acetic acid, pyruvic acid, and acetaldehyde. *Am. J. Enol. Vitic.* **1993**, *44*, 86–92.
63. Romano, P.; Suzzi, G.; Turbanti, L.; Polsinelli, M. Acetaldehyde production in *Saccharomyces cerevisiae* wine yeasts. *FEMS Microbiol. Lett.* **1994**, *118*, 213–218. [CrossRef]
64. Amerine, M.A. Composition of wines. I. Organic constituents. *Ad. Food Res.* **1954**, *5*, 353–510.
65. Millan, C.; Ortega, J.M. Production of ethanol, acetaldehyde, and acetic acid in wine by various yeast races: Role of alcohol and aldehyde dehydrogenase. *Am. J. Enol. Vitic.* **1988**, *39*, 107–112.

foods

MDPI

Article

Comparative Evaluation of Brandy de Jerez Aged in American Oak Barrels with Different Times of Use

Manuel J. Valcárcel-Muñoz [1], María Guerrero-Chanivet [1,2], M. Valme García-Moreno [2,*], M. Carmen Rodríguez-Dodero [2] and Dominico A. Guillén-Sánchez [2]

[1] Bodegas Fundador S.L.U. Departamento de Investigación y Desarrollo, C/San Ildefonso, n 3, 11403 Jerez de la Frontera, Cádiz, Spain; m.valcarcel@bodegasfundador.com (M.J.V.-M.); maria.guerreroch@uca.es (M.G.-C.)

[2] Departamento de Química Analítica, Facultad de Ciencias, Instituto Investigación Vitivinícola y Agroalimentaria (IVAGRO), Campus Universitario de Puerto Real, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain; maricarmen.dodero@uca.es (M.C.R.-D.); dominico.guillen@uca.es (D.A.G.-S.)

* Correspondence: valme.garcia@uca.es; Tel.: +34-956-016-456

Abstract: Brandy de Jerez is a European Geographical Indication for grape-derived spirits aged in oak casks that have previously contained any kind of Sherry wine and, therefore, are known as *Sherry Casks*®. Wood compounds have a substantial influence in the quality of the brandies that are aged in the barrels. In the cellar, the barrels that have been used for many years to keep Sherry wine or other wine spirits are often used for this purpose. When wooden barrels are used for the first time, they release a large amount of compounds into the liquid contained in them. Such amount decreases over time but casks life cycle has remained unexplored until now. The present work has the aim to study the brandies obtained from the same wine spirit after two years ageing in three differently oak casks: namely new, 7 years of use (4 years containing Oloroso wine and 3 years containing wine spirits) and 32 years of use (8 years containing Oloroso wine and 24 years containing wine spirits). According to the results from our experiments, even after 32 years of use, the wood barrels still contribute to modify the organoleptic characteristics of brandy. Moreover, the brandies aged in used barrels were judged more balanced than those aged in new barrels.

Keywords: Brandy de Jerez; *Sherry Cask*®; oak wood; aroma; ageing

Citation: Valcárcel-Muñoz, M.J.; Guerrero-Chanivet, M.; García-Moreno, M.V.; Rodríguez-Dodero, M.C.; Guillén-Sánchez, D.A. Comparative Evaluation of Brandy de Jerez Aged in American Oak Barrels with Different Times of Use. *Foods* **2021**, *10*, 288. https://doi.org/10.3390/foods10020288

Academic Editors: Fernando M. Nunes and Susana Río Segade

Received: 10 December 2020
Accepted: 27 January 2021
Published: 31 January 2021

1. Introduction

Brandy de Jerez is a spirit produced in the Southern Spanish area known as *Marco de Jerez*, which includes the cities of Jerez de la Frontera, El Puerto de Santa María and Sanlúcar de Barrameda. It is elaborated following the specifications provided by the Technical File of the Geographical Indication under such name [1,2]. This document defines as a grape-derived spirit with a minimum alcoholic strength of 36% vol. (normally between 36–45% vol.), obtained from wine spirits and distillates aged in under 1000 L oak barrels which have been previously seasoned with Sherry wine. The production process follows a traditional dynamic system employed in the Sherry area known as *Criaderas and Solera*.

Brandy de Jerez exhibits a number of specific organoleptic characteristics that make it different from other spirits. Such characteristics are conferred to brandy by the casks where it is aged. This is so because the barrels used to produce Brandy de Jerez must have contained for a certain length of time some type of Sherry wine, i.e., Fino, Amontillado, Oloroso, or Pedro Ximénez. This conditioning process is known as seasoning and every barrel that has undergone such seasoning process according to the rules established by the Technical File that regulates its elaboration [3], is then referred to as *Sherry Cask*®.

Naturally, the characteristics of the final product will be influenced by a number of characteristics related to the wooden cask that have an effect on the ageing process [4], namely the geographical origin and botanical species [5–10], the container volume [11] and

its wood toasting grade [4,7,12]), the temperature and humidity in the cellar, the ageing time [5,13–15] and, only in the case of Brandy de Jerez, the seasoning of the barrel [16–20]. The organoleptic characteristics of a particular brandy will be different if it has been aged in a cask with one type of Sherry wine or another. For these reasons, the characteristics of each barrel represent a crucial element that will determine the outcome of the brandy ageing process. Traditionally, American oak (*Quercus alba*) is the wood type that is most often used by cooperage companies in the Sherry area for the manufacturing of barrels.

As previously said, casks are active contributors to the organoleptic properties of brandy. Wood composition, atmospheric conditions as well as the type of distillate and its alcoholic strength have an influence on the physical-chemical phenomena in which certain compounds from the wood and other components in the distillate are involved. Extraction processes are the most common among such phenomena, but oxidation, esterification, hydrolysis, ethanolysis, Maillard reactions, polymerization, and polycondensation reactions also take place during the ageing process [4,21,22]. Also, some water evaporates and trickles out of the barrel through its pores.

Most of the compounds that are transferred from the wood into the liquid are responsible for the organoleptic profile of the resulting brandy. Wood is mostly composed of polysaccharides (cellulose and hemicellulose) and lignin. This represents around 90% of the total wood matter. The remaining 10% is composed of extractive compounds such as phenolic compounds (polyphenols or simple phenols), fatty acids, alcohols and inorganic substances [22]. The thermal degradation of lignin during the manufacturing of the barrels or its degradation by ethanolysis and hydrolysis during spirit and wine ageing, together with is acid character [23], make it release certain compounds such as vanillin, coniferylaldehyde, syringaldehyde, sinapaldehyde, and cinnamic and benzoic acids into the distillate [4,22]. The degradation of hemicellulose gives place to compounds such as furfural and its derivatives [24,25]. Hydrolysable tannins, such as gallotannins and ellagitannins, are highly soluble in ethanol-water solutions and their transformation into gallic acid or ellagic acid by hydrolysis is very common [22]. Brandy de Jerez also contains other compounds that come from Sherry wine, such as tartaric, lactic, or succinic acid [20]. In those cases, the barrels act as transfer vectors between the Sherry wine that had been previously contained in the cask and the newly ageing distillate [26].

American oak (*Quercus alba*) toasted wood contains between 460 and 3620 μg/g wood of low molecular weight phenols [7]. 745.24 ± 51.28 μg/g wood of phenolic acids, such as ellagic acid, gallic acid, vanillic acid, or syringic acid, 1608.18 ± 346.20 μg/g wood of phenolic aldehydes and a certain amount of volatile compounds that range between 1919.13 and 2660.91 μg/g wood [27]. With repeated use, the amount of these components extracted from the wood and transferred into the ageing spirit becomes gradually smaller compared to the *Sherry Cask*®'s first use [28]. Nevertheless, since new *Sherry Casks*® contain a huge amount of extractable compounds, a study on how the repeated use of the same barrels over the years may affect their capacity to enrich the distillates and, therefore, to have an impact on the final product's organoleptic profile should be of the utmost interest.

Wine makers use the same barrels again and again for many years and only when they are seriously deteriorated or damaged they are finally discarded. This study intends to confirm that even after many years of use, wooden casks can be used to produce brandy. For that purpose, the same wine spirit has been aged for two years in different oak casks: new, 7 years of use (4 years containing Oloroso wine and 3 years containing wine spirits), and 32 years of use (8 years containing Oloroso wine and 24 years containing wine spirits).

2. Materials and Methods

2.1. Samples

The study was carried out in 500 L wooden barrels (Tonelerías Domecq, Jerez de la Frontera, Spain) made out of American oak (AO) (*Quercus alba*) staves of a medium toasting grade. The toasting procedure was carried out according to the traditional practices in the Sherry area [29].

The Oloroso wine employed for the seasoning the barrels is a white wine fortified at 18% vol. and aged following the traditional oxidation process in the Sherry area, *Criaderas and Solera* system.

The wine spirit (grapes of the Airén variety) that was aged in all the experiments was supplied by Bodegas Fundador, S.L.U. It had been obtained by column distillation at 77 % vol. and it was hydrated with demineralized water at 65% vol.

The conditions of the three experiments in this study are specified in Table 1: *New barrels*, *4 + 3 Used barrels* with 7 years of use (4 years containing Oloroso wine and 3 years containing wine spirits) and *8 + 24 Used barrels* with 32 years of use (8 years containing Oloroso wine and 24 years containing wine spirits). For the experiments *4 + 3 Used barrels* and *8 + 24 Used barrels* were emptied and refilled with fresh wine spirit.

Table 1. Experimental conditions under study.

Experience	Previous Use of the Barrels		
	Sherry Seasoning		Brandy Ageing
	Type	No. of Years	No. of Years
New barrels	None	No seasoning	No previous use
4 + 3 Used barrels	Oloroso	4	3
8 + 24 Used barrels	Oloroso	8	24

Each experimental group comprised ten barrels divided in two set of five barrels each. Individual samples were taken from each barrel and were combined into a pooled sample of each set of five barrels in order to reduce the variability of the barrels (Figure 1). Two pooled samples were obtained (n = 2) in each sampling time. This sampling procedure were carried out during two years, at 1, 2, 4, 6, 8, 10, 12, 15, 18, 21, and 24 months, for the three proposed experiences (Table 1). The initial wine spirit was also analyzed. A total of 67 samples were studied. Each sample was analyzed in duplicate.

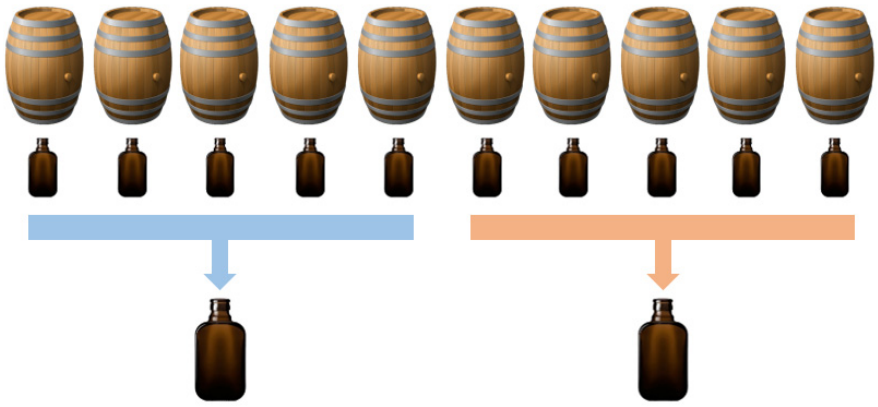


Figure 1. Experimental group of barrels (two set of five barrels) and definition of the sampling plan for the physical-chemical analysis.

Since the parameters studied had not been shown a great evolution, only the most relevant results after the first and the second year of ageing have been included in the tables. Ethyl acetate, volatile acids, and Total Polyphenol Index had experimented a significant increase during the ageing, so the complete evolution have been graphically represented.

For the tasting sessions, the two pooled samples of the same experiment were combined into an individual sample of each experiment (n = 1). The initial wine spirit and the samples after 1 year and after 2 years of ageing were tasted. A total of 7 samples were tasted in duplicate in two different sessions.

2.2. Reagents

To determine the Total Polyphenol Index (TPI), Folin–Ciocalteu reagent, anhydrous sodium carbonate and gallic acid were purchased from Merck (Darmstadt, Germany).

UHPLC grade acetonitrile from Panreac (Barcelona, Spain), acetic acid from Merck (Darmstadt Germany) were used to prepare the UHPLC phases. The standards for calibration were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Ultrapure water from EMD Millipore (Bedford, MA, USA) was used to prepare the chromatography phases, the reagents and the calibration standards.

2.3. Enological Control Parameters

A pH-Meter Basic 20 (Crison Instruments SA, Barcelona, Spain) was used to measure the pH. The alcoholic strength (% Alcohol by Volume, ABV) was obtained by density measurement of the distillate in a DMA-5000 densimeter (Anton Paar, Ashland, OR, USA). The volatile acids expressed as mg acetic acid/100 mL of 100% vol. alcohol, were measured by means of a segmented flow analyzer AA3 HR Autoanalyzer (Seal Analytical, Norderstedt Stadt, Germany) following the iodide/iodate procedure [30–32] according to the method established by the International Organization of Vine and Wine (OIV). The potassium in brandy was determined in mg/L by means of a PinAAcle 900F Atomic Absorption Spectrometer (Perkin Elmer, Boston, MA, USA) and WinLab32 AA (Perkin Elmer, Boston, MA, USA) was the software application used for data acquisition and to perform the data analyses. Each sample was measured in duplicate.

2.4. Phenolic Compounds and Furfurals

The phenolic compounds and furfurals were quantified by UHPLC following the method previously established by our research group [33,34]. A Waters Acquity UPLC equipped with a PDA detector and an Acquity UPLC C18 BEH, 100 × 2.1 mm (i.d.) with 1.7 µm particle size (Waters Corporation, Milford, MA, USA) column was employed for the analysis. Nine phenolic compounds (gallic acid, ellagic acid, p-hydroxybenzaldehyde, vanillic acid, vanillin, syringic acid, syringaldehyde, sinapaldehyde, and coniferylaldehyde) and three furanic aldehydes (furfural, 5-methylfurfural, and 5-hydroxymethylfurfural) were identified.

The samples and standards were filtered through 0.22 µm nylon membranes, and they were injected in duplicate. The absorption was determined by UV scanning at between 250 and 400 nm, with a resolution of 1.2 nm. The linear standard curve ranges from 0.1 mg/L to 10 mg/L. The compounds were identified by comparing the retention times and UV-Vis spectra of the sample peaks against those previously obtained from the standards. The results were expressed in mg of compound per 100 mL of 100% vol. alcohol.

2.5. Total Polyphenol Index

A Lambda 25 spectrophotometer (Perkin Elmer, Boston, MA, USA) was used to determine the TPI. This instrument was calibrated based on gallic acid aliquots in the range 0–750 mg/L. The total polyphenolic index was measured following the Folin–Ciocalteau method according to the official method established by the International Organization of Vine and Wine (OIV). 0.5 mL of sample, 25 mL of ultrapure water, 2.5 mL of Folin–Ciocalteau reagent and 10 mL of 20% sodium carbonate in strict order were introduced in a 50 mL volumetric flask, and made up to the mark by adding ultrapure water. The dilutions were carried out in duplicate, and the absorbance was measured at 750 nm [35]. Glass cells with a 1 cm optical path were used. The samples were measured in duplicate. The results were expressed in mg gallic acid equivalent (GAE) per 100 mL of 100% vol. alcohol.

2.6. Color Measurements

The samples' color was measured by means of a Lambda 25 spectrophotometer (Perkin Elmer, Boston, MA, USA) at 420 nm, 520 nm and 620 nm absorbance, according to the

official method established by the International Organization of Vine and Wine (OIV). Glass cells with a 1 cm optical path were used. Each sample was directly measured in duplicate.

2.7. Aldehydes, Acetal, Methanol, Esters, and Higher Alcohols

Aldehydes, acetal, methanol, esters and higher alcohols (also known as fusel alcohols) were quantified by GC-FID. Twenty-one compounds were determined following two different procedures. In both cases, the equipment used was an Agilent 7890B Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with Flame Ionization Detector.

For the analysis of acetaldehyde, acetaldehyde—diethyl acetal, methanol, ethyl acetate, n-propyl alcohol, 2-butyl alcohol, isobutyl alcohol, n-butyl alcohol, 2-methyl-1-butanol and 3-methyl-1-butanol, the samples were injected in a split mode (split 1:46, 250 °C) into a DB-624 (30 m × 250 µm × 1.4 µm, Agilent Technologies, Santa Clara, CA, USA) column. The oven temperature for the analysis was programmed as follows: 30 °C (30 min), then 6 °C/min to 100 °C (0 min). Temperatures of the injector and the detector were 250 °C and 300 °C, respectively. Nitrogen was used as a carrier at flow of 1.0 mL/min. Data acquisition and analyses were performed using OpenLAB CDS Chemstation (Agilent Technologies, Santa Clara, CA, USA) software.

For the analysis of n-hexanol, 2-phenylethyl alcohol, ethyl lactate, ethyl succinate, ethyl caproate, ethyl caprylate, ethyl caprate, ethyl laureate, ethyl myristate and ethyl palmitate, samples were injected in a splitless mode (1 min, 250 °C) onto CP-WAX 57 CB (25 m × 250 µm × 0.2 µm, Agilent Technologies, Santa Clara, CA, USA) column. The oven temperature program during analysis was as follows: 45 °C (20 min), then 3 °C/min to 170 °C (20 min). Temperatures of the injector and the detector were 250 °C and 300 °C respectively. Nitrogen was used as the carrier gas at a flow of 1.3 mL/min. The data acquisition and analyses were performed using OpenLAB CDS Chemstation (Agilent Technologies, Santa Clara, CA, USA) software.

Standards were made in an ethanol/ultrapure water solution at 40%vol. The linear standard curve of 3-methyl-1-butanol ranges from 1 to 250 mg/100 mL of 100% vol. alcohol. The linear standard curve of methanol, ethyl acetate, n-propyl alcohol, isobutyl alcohol and 2-methyl-1-butanol ranges from 1 to 100 mg/100 mL of 100% vol. alcohol. The linear standard curve of acetaldehyde and acetaldehyde—diethyl acetal ranges from 1 to 50 mg/100 mL of 100% vol. alcohol. The linear standard curve of ethyl lactate ranges from 0.5 to 25 mg/100 mL of 100% vol. alcohol. The linear standard curve of 2-butyl alcohol, n-butyl alcohol, n-hexanol, 2-phenylethyl alcohol, ethyl succinate, ethyl caproate, ethyl caprylate, ethyl caprate, ethyl laureate, ethyl myristate, and ethyl palmitate ranges from 0.1 to 5 mg/100 mL of 100% vol. alcohol. The samples were diluted at 40%vol. with ultrapure water and injected in duplicate. The results were expressed in mg of compound per 100 mL of 100% vol. alcohol.

2.8. Tasting Sessions

The tasting sessions took place in a room adequately furnished with individual workspaces to facilitate the concentration and isolation of the tasters [36] at a controlled temperature (20 °C). The 4 tasters were all experts belonging to the staff of Bodegas Fundador, S.L.U. with over 20 years experience in the field and members of the official tasting panel for the Denomination of Origin *Jerez-Xérès-Sherry* and the Geographical Indication *Brandy de Jerez*.

72 h before the tasting sessions the samples were hydrated with demineralized water up to 36% vol. of alcoholic strength, which is the standard alcohol content for the commercial product. 50 mL of each sample was served in black standardized glasses [37], which remained covered by a glass for 10 min in order to stabilize the headspace before the tasting. In each session the set of 7 samples was presented in a random order to the tasters.

The selected descriptors of odor and flavor (Table 2) were chosen following the indications of the Technical File of the Geographical Indication of *Brandy de Jerez* [1]. Table 2

includes a description of the descriptors and also the odor and flavor patterns employed for the training of the tasters. For the evaluation of the brandies, a numerical scale was used, as defined in ISO 4121:2003 [38]. The pattern (Table 2) would represent a 5 score in the 5-point scale used for the evaluation (5 = very high). Neutral wine alcohol hydrated at 36% vol. would represent a 1 score (1 = absence). The samples were tasted in duplicate in two different sessions.

Table 2. Odor and flavor descriptors and patterns used for the training of the tasters.

Descriptor	Definition	Pattern
	Odor	
Aromatic complexity	Diversity of positive aromatic sensations.	5 years old brandy, produced with pot stills, hydrated at 36% vol.
Fruity	Fruit aromas characteristic of the grape varieties used to produce the wine and its distillate (apple, pear, banana, pineapple, tropical fruits, etc.).	Wine spirit from pot stills hydrated at 36% vol., with a level of fatty acids ethyl esters and higher alcohols acetates of 35 mg/L.
Vinous	'Memories' of the original wine used to produce the distillate and compounds acquired during ageing in *Sherry Casks*®.	5 years old brandy, produced with pot stills, hydrated at 36% vol.
Vanilla	Compounds released during lignin degradation. 'Noble' wood sensations.	Wine spirit hydrated at 36% vol. with lignin vanilla addition.
Toasted/caramel	Sensations of toasted wood.	Wine spirit hydrated at 36% vol. after adding hydroalcoholic medium toasted American oak extract.
Spicy/aniseed	Sensations of non-toasted wood and the characteristic smell of medium chain wine ethyl esters (C6).	Wine spirit hydrated at 36% vol. after adding hydroalcoholic raw American oak extract and wine lees.
	Flavor	
Alcoholic	Burning sensation in the oral cavity.	Neutral wine alcohol hydrated at 36% vol., with addition of distillation heads and tails, with a level of volatile compounds of 500 mg/100 mL of 100% vol. alcohol.
Smoothness	Pleasant as it goes down the throat.	5 years old brandy hydrated at 36% vol.
Oxidative sweetness	Velvety sensation in the oral cavity.	5 years old brandy hydrated at 36% vol., after adding 3 g/L of rectified concentrate grape must.
Equilibrium	General evaluation of all the sensations, from the first contact with the liquid until it is swallowed: structured, absence of astringency or bitterness, etc.	8 years old brandy Solera Gran Reserva hydrated at 36% vol.
Oak/woody	Wood sensation; seasoned, unseasoned.	5 years old brandy hydrated at 36% vol., after adding hydroalcoholic medium toasted American oak extract.

2.9. Statistical Analysis

Statgraphics 18 software package (Statgraphics Technologies, Inc., The Plains, VA, USA) was employed for ANOVA and Fisher's Least Significant Difference test. Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA) was employed for other statistical parameters.

3. Results and Discussion

3.1. Enological Control Parameters

The data corresponding to pH, ABV, volatile acids, and potassium of brandies ageing in new barrels, barrels with 7 years of use and barrels with 32 years of use during the first and second year of ageing are shown in Table 3.

Table 3. pH, alcoholic strength (% Alcohol by Volume, ABV), volatile acids (mg acetic acid/100 mL of 100% vol. alcohol) and potassium (mg/L) of brandies aged for one or two years in *New barrels*, *4 + 3 Used barrels*, and *8 + 24 Used barrels*.

	Initial	*New Barrels*		*4 + 3 Used Barrels*		*8 + 24 Used Barrels*	
		1 Year Ageing	2 Years Ageing	1 Year Ageing	2 Years Ageing	1 Year Ageing	2 Years Ageing
pH	4.52	4.33 ± 0.01 ^{a}	4.20 ± 0.03 ^{a}	4.12 ± 0.03 ^{b}	3.99 ± 0.01 ^{b}	4.10 ± 0.02 ^{b}	4.00 ± 0.03 ^{b}
Volatile acids	10.3	58.6 ± 0.4 ^{a}	76.9 ± 0.4 ^{a}	15.9 ± 0.5 ^{b}	22.3 ± 0.7 ^{b}	14.0 ± 0.8 ^{b}	20.0 ± 0.4 ^{c}
Alcoholic strength	65.27	65.36 ± 0.04 ^{a}	65.57 ± 0.02 ^{a}	65.13 ± 0.03 ^{b}	65.15 ± 0.04 ^{b}	65.14 ± 0.03 ^{b}	65.16 ± 0.00 ^{b}
Potassium	n.d.	5.90 ± 0.30 ^{a}	8.00 ± 0.15 ^{a}	15.50 ± 0.71 ^{b}	17.00 ± 1.41 ^{b}	7.00 ± 0.00 ^{a}	8.50 ± 0.71 ^{a}

Mean values ± standard deviation (n = 2); significant differences ($p < 0.05$) of a particular parameter in the same ageing year are indicated by different letters; n.d.: Not detected.

pH value is around 4, which is characteristics of young brandies [39]. In all the cases studied, their pH decreased over time. In the case of *Used barrels*—*Sherry Casks*®—the distillate is enriched with acid compounds from the wood [4,17] or the wine during the ageing process [20]. Some oxidation reactions of the ethanol molecules produce acetic acid during this process too, which also explains why aged brandy contains a greater amount of volatile acids than younger ones (Figure 2). Significant differences associated to used or unused barrels have been observed, but no differences between the brandy aged in *4 + 3 Used barrels* or *8 + 24 Used barrels* were registered.

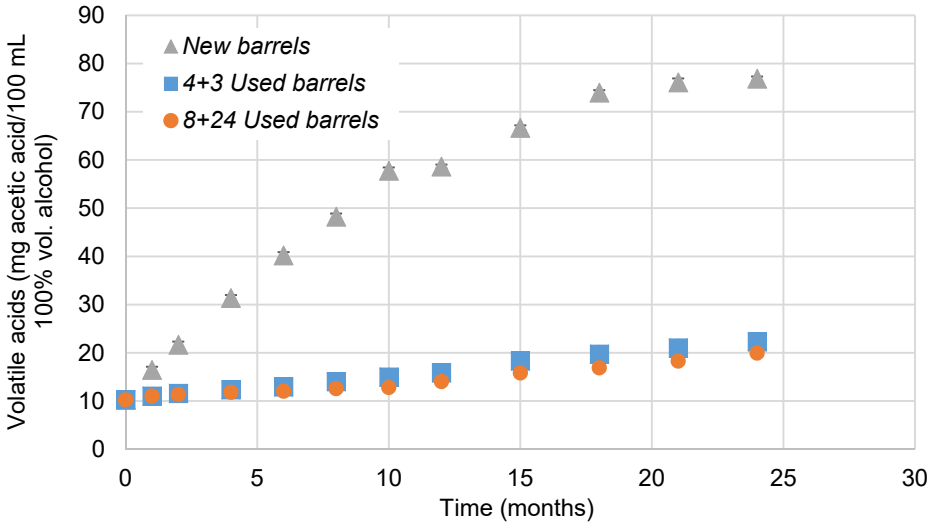


Figure 2. Evolution of volatile acids in brandies aged in *New barrels*, *4 + 3 Used barrels*, and *8 + 24 Used barrels* for two years. When the standard deviation was lower than 0.8 cannot be noticed in the graph.

The alcoholic strength of all the studied brandies was approximately 65% vol. This percentage decreased when aged in the *Used barrels*, while in the *New barrels* it increased. Since the barrels are not airtight, the evaporation processes that take place inside the barrel are compensated by the perspiration of water molecules to the outside through the wood [40,41]. The volume losses during brandy ageing are shown in Table 4. A greater volume loss was detected in *New barrels* than in *3 + 4* and *8 + 24 Used barrels*. In the *New barrels* experiments, part of the brandy in them was absorbed by the barrel dry wood. This fact explains the higher concentration of compounds such as higher alcohols in brandies kept in *New barrels* compared to that of the brandies in the *Used barrels*.

Table 4. Brandy volume loss after two years of ageing.

Experiment	*New Barrels*	*4 + 3 Used Barrels*	*8 + 24 Used Barrels*
Average volume loss after the first year (L/500 L barrel)	28.1 ± 3.4 [a]	14.1 ± 3.3 [b]	14 ± 3.4 [b]
Average volume loss after the second year (L/500 L barrel)	13.7 ± 3.3	13 ± 3.2	14.6 ± 3.4
Average percentage of volume loss per year (%)	4.2 ± 0.7 [a]	2.7 ± 0.7 [b]	2.9 ± 0.7 [a,b]

Data are mean values ± standard deviation (n = 2); values in the same row with different letters are significantly different ($p < 0.05$).

With regard to potassium, significant differences were found depending on the type of barrel where the brandy was aged. The smallest amount of potassium was found in the brandies aged in *New barrels*. Although new wood would release inorganic compounds into the distillate, such as potassium [42], in this case, seasoning had a greater effect on the final product, since Sherry wines contain inorganic salts such as potassium bitartrate [43]. During the seasoning process, these compounds precipitate and dissolve in the distillate and, as a consequence, the amount of potassium that can be found in brandies aged in *Sherry Casks*® is greater than the one detected in *New barrels* without any previous contact with wine.

3.2. Phenolic Composition and Total Polyphenol Index of the Aged Brandies

The data corresponding to low molecular weight phenolic compounds content as determined by means of UHPLC in the two-year aged brandies are indicated in Table 5 as mg per 100 mL of 100% vol. alcohol. All the compounds in the study exhibited the same trend: significant differences were observed depending on the barrel type, but no differences were noted between the brandy aged in *4 + 3 Used barrels* and *8 + 24 Used barrels*, excluding the content of syringic acid, vanillin and syringaldehyde. Except for furfural, all of the above mentioned compounds could not be detected in the initial wine spirit, since they are provided by the wood or the wine in the case of seasoned barrels, and they are easily and generally found in brandies aged in wood [4,17,19–21,44]. When wood is used for the first time, a large amount of these compounds are transferred into the liquid [45]. Therefore, the brandy held in *New barrels* contains a larger concentration of them than those held in *Used barrels*, because these wood compounds had already been extracted in large quantities during the previous use. From the tasters' point of view this high concentration of wood compounds might be perceived as a sort of 'aggressive' taste, as described in Section 3.5. On the other hand, *Used barrels* release into the distillate Sherry wine compounds that provide the brandy with rather pleasant organoleptic characteristics. Although the phenolic compounds concentration levels detected in the brandies aged in *8 + 24 Used barrels* was slightly lower than in those brandies aged in *4 + 3 Used barrels*, such difference, that could be explained by a lower availability of such compounds in older barrels, could not be regarded as relevant.

With regard to the concentration of the phenolic acids (gallic acid, vanillic acid, syringic acid, and ellagic acid) their values in the different brandies follow the expected pattern (Table 5). Thus, their concentration increased over the ageing period and greater concentrations were observed in the brandies aged in *New barrels* than in those aged in *3 + 4 Used barrels* or *8 + 24 Used barrels*, because of the lower availability of these compounds. Phenolic aldehydes as p-hydroxybenzaldehyde, vanillin, syringaldehyde, coniferylaldehyde, and sinapaldehyde resulting from the thermal degradation of lignin [23,25,46], were found in all the brandies studied (Table 5). Significant amounts of 5-hydroxymethylfurfural, furfural and 5-methylfurfural were also detected with similar content trends in all the experiments (Table 5).

Table 5. Phenolic compounds content (mg/100 mL of 100% vol. alcohol) in brandies aged in *New barrels*, *4 + 3 Used barrels*, and *8 + 24 Used barrels* after the first and the second year of ageing.

	Initial	*New Barrels*		*4 + 3 Used Barrels*		*8 + 24 Used Barrels*	
		1 Year Ageing	2 Years Ageing	1 Year Ageing	2 Years Ageing	1 Year Ageing	2 Years Ageing
Gallic acid	n.d.	0.83 ± 0.05 ^{a}	1.02 ± 0.05 ^{a}	0.29 ± 0.02 ^{b}	0.47 ± 0.03 ^{b}	0.22 ± 0.02 ^{b}	0.36 ± 0.02 ^{b}
Vanillic acid	n.d.	0.52 ± 0.02 ^{a}	0.60 ± 0.06 ^{a}	0.06 ± 0.00 ^{b}	0.09 ± 0.01 ^{b}	0.05 ± 0.01 ^{b}	0.08 ± 0.01 ^{b}
Syringic acid	n.d.	0.47 ± 0.02 ^{a}	0.58 ± 0.02 ^{a}	0.14 ± 0.01 ^{b}	0.24 ± 0.02 ^{b}	0.08 ± 0.01 ^{c}	0.12 ± 0.01 ^{c}
Ellagic acid	n.d.	2.48 ± 0.05^{a}	2.90 ± 0.05 ^{a}	1.52 ± 0.03 ^{b}	1.97 ± 0.03 ^{b}	1.57 ± 0.07 ^{b}	1.90 ± 0.08 ^{b}
p-Hydroxybenzaldehyde	n.d.	0.06 ± 0.01	0.05 ± 0.01	n.d.	n.d.	n.d.	n.d.
Vanillin	n.d.	0.64 ± 0.02 ^{a}	0.78 ± 0.03 ^{a}	0.09 ± 0.01 ^{b}	0.15 ± 0.01 ^{b}	0.06 ± 0.01 ^{b}	0.09 ± 0.01 ^{c}
Syringaldehyde	n.d.	1.39 ± 0.07 ^{a}	1.59 ± 0.07 ^{a}	0.24 ± 0.01 ^{b}	0.39 ± 0.02 ^{b}	0.12 ± 0.01 ^{b}	0.21 ± 0.01 ^{c}
Coniferylaldehyde	n.d.	1.60 ± 0.05 ^{a}	1.57 ± 0.04 ^{a}	0.05 ± 0.00 ^{b}	0.07 ± 0.01 ^{b}	0.03 ± 0.00 ^{b}	0.05 ± 0.01 ^{b}
Sinapaldehyde	n.d.	2.59 ± 0.09 ^{a}	2.99 ± 0.07 ^{a}	0.03 ± 0.00 ^{b}	0.06 ± 0.01 ^{b}	0.03 ± 0.00 ^{b}	0.05 ± 0.01 ^{b}
5-Hydroxymethylfurfural	n.d.	1.63 ± 0.06 ^{a}	1.60 ± 0.06 ^{a}	0.04 ± 0.01 ^{b}	0.06 ± 0.01 ^{b}	0.02 ± 0.00 ^{b}	0.03 ± 0.01 ^{b}
Furfural	0.083	3.72 ± 0.17 ^{a}	4.04 ± 0.12 ^{a}	0.18 ± 0.01 ^{b}	0.23 ± 0.01 ^{b}	0.16 ± 0.01 ^{b}	0.20 ± 0.01 ^{b}
5-Methylfurfural	n.d.	0.54 ± 0.02	0.52 ± 0.03	n.d.	n.d.	n.d.	n.d.

Data are mean value ± standard deviation (n = 2); significant differences ($p < 0.05$) of a particular parameter in the same ageing year are indicated by different letters; n.d.: Not detected.

The data corresponding to the TPIs of the studied brandies, expressed in mg GAE/100 mL of 100% vol. alcohol, can be seen in Figure 3. As expected, TPI increased with ageing in all the cases. However, depending on the wood previous usage, it did so in a lesser or greater extent. Thus, the TPI closely followed a similar evolution pattern as the one for low molecular weight phenolic compounds that has been previously discussed [47], since the amount of phenolic compounds available in the wood and, therefore, their concentration in the aged brandy also depend on whether the wood has been previously used or not.

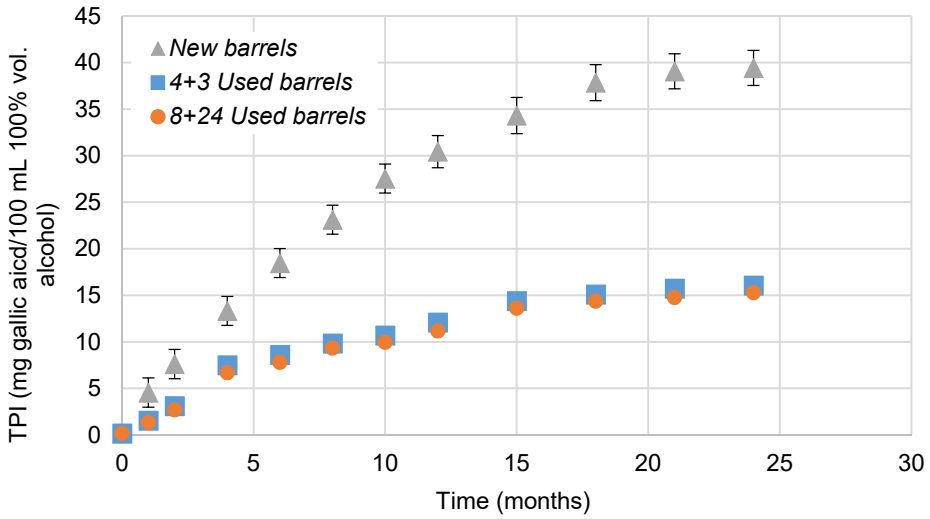


Figure 3. Evolution of Total Polyphenol Index (TPI) in brandies aged in *New barrels*, *4 + 3 Used barrels* and *8 + 24 Used barrels* for two years, expressed in mg gallic acid/100 mL of 100% vol. alcohol. The standard deviation between *4 + 3 Used barrels*-aged brandies and *8 + 24 Used barrels*-aged brandies was lower than 0.7 and cannot be noticed in the graph.

In this way, when wood is used for the first time (*New barrels*) it releases a much larger quantity of compounds into the distillate than when the wood has been previously seasoned. Nevertheless, a close look at Figure 3 and Table 5 let us see that no significant differences between *4 + 3 Used barrels* and *8 + 24 Used barrels* were noted. All in all, it can be confirmed that, regardless of the length of time that a particular barrel may have been used, typically seasoned barrels will release similar levels of phenolic compounds into the brandy. In fact, even after 32 years of use, barrels will continue to provide brandies

with the compounds that confer this spirit with its characteristic and highly appreciated organoleptic profile.

3.3. Chromatic Characteristics

The samples were measured for the following color absorbances: A420, A520 and A620. As expected, the color intensity of the brandies increased with ageing (Table 6).

Table 6. Chromatic characteristics of the brandies studied after 1 and 2 years of ageing.

	Initial	*New Barrels*		*4 + 3 Used Barrels*		*8 + 24 Used Barrels*	
		1 Year Ageing	**2 Years Ageing**	**1 Year Ageing**	**2 Years Ageing**	**1 Year Ageing**	**2 Years Ageing**
A420	n.d.	0.327 ± 0.020^{a}	0.410 ± 0.018^{a}	0.189 ± 0.008^{b}	0.268 ± 0.008^{b}	0.122 ± 0.007^{c}	0.198 ± 0.006^{c}
A520	n.d.	0.053 ± 0.004^{a}	0.086 ± 0.004^{a}	0.041 ± 0.001^{b}	0.065 ± 0.001^{b}	0.028 ± 0.003^{c}	0.042 ± 0.003^{c}
A620	n.d.	0.012 ± 0.001^{a}	0.015 ± 0.001^{a}	0.011 ± 0.001^{a}	0.016 ± 0.000^{b}	0.008 ± 0.000^{b}	0.011 ± 0.000^{c}

Data are mean value ± standard deviation ($n = 2$); significant differences ($p < 0.05$) of a particular parameter in the same ageing year are indicated by different letters; n.d.: Not detected.

The distillates aged in *New barrels* presented the greatest intensity growth. The absorbance at 420 nm, which corresponds to the yellow zone, was higher than the absorbance at 520 nm and 620 nm in all the cases studied. Since the color of the brandy is closely related to the presence of phenolic compounds in the distillate, the increment in A420, A520 and A650 intensity would be related to the ageing process in the wood barrels and to the subsequent extraction and oxidation reactions that take place between the compounds that are being extracted from the wood and those already present in the distillate [44,46,48].

Some studies associate the increment in the yellow shade intensities (A420) with the oxidation of the ellagitannins from the wood [49] and with the condensation reactions among tannins in the presence of acetaldehyde and phenolic aldehydes [44]. The intensity of the A420 shade as measured in the experimental brandies in the study presented significant differences that could be associated to the type of barrel used for the ageing of the spirit. Thus, the brandies aged in *New barrels* registered the highest values for this parameter, while those brandies that had been aged in *8 + 24 Used barrels* exhibited the lowest values, i.e., the least intense A420 shade. Hence, it can be concluded that even though this shade intensity increased in all the cases, the brandies that had been aged in *4 + 3 Used barrels* or *8 + 24 Used barrels* presented a paler yellow tone than those brandies aged in *New barrels*.

3.4. Aldehydes, Acetal, Methanol, Esters, and Higher Alcohols

Regarding acetaldehyde and acetaldehyde—diethyl acetal, their content did not experiment any marked evolution during the ageing period (Table 7). This is due to the equilibrium that affects these two compounds, influenced by alcoholic strength and pH. Acetaldehyde stabilized its presence during the second year of brandy ageing [50], when its losses due to evaporation and its oxidation into acetic acid are compensated by the concentration of the compound caused by the ethanol evaporation and the perspiration of the water through the wood [40,41] (Table 4). It can be seen in Table 7 that both compounds do not follow a clear evolution.

Table 7. Aldehydes, acetal, methanol, esters and higher alcohols contents (mg/100 mL of 100% vol. alcohol) in the brandies studied.

	Initial	*New Barrels*		*4 + 3 Used Barrels*		*8 + 24 Used Barrels*	
		1 Year Ageing	2 Years Ageing	1 Year Ageing	2 Years Ageing	1 Year Ageing	2 Years Ageing
Acetaldehyde	21.8	23.8 ± 0.5 ^{a}	24.1 ± 0.6 ^{a}	21.9 ± 0.6 ^{b}	22.0 ± 0.3 ^{b}	21.9 ± 0.3 ^{b}	21.9 ± 0.3 ^{b}
Diethyl acetal	28.8	31.3 ± 0.3 ^{a}	32.1 ± 0.3 ^{a}	29.4 ± 0.3 ^{b}	29.5 ± 0.7 ^{b}	29.3 ± 0.4 ^{b}	29.4 ± 0.5 ^{b}
Methanol	68.0	66.2 ± 0.3 ^{a}	66.9 ± 0.3	67.4 ± 0.4 ^{a,b}	68.1 ± 0.2	67.9 ± 0.7 ^{b}	68.0 ± 0.7
Ethyl acetate	31.1	47.7 ± 0.7 ^{a}	65.7 ± 0.4 ^{a}	37.8 ± 0.5 ^{b}	44.9 ± 0.6 ^{b}	37.3 ± 0.5 ^{b}	43.1 ± 0.9 ^{b}
Higher alcohols							
N-propyl alcohol	33.2	37.3 ± 0.2 ^{a}	37.8 ± 0.1 ^{a}	34.2 ± 0.6 ^{b}	34.2 ± 0.6 ^{b}	34.0 ± 0.5 ^{b}	34.1 ± 0.4 ^{b}
2-butyl alcohol	0.5	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Isobutyl alcohol	33.2	33.3 ± 0.1	37.2 ± 0.2 ^{a}	34.0 ± 0.6	34.5 ± 0.1 ^{b}	34.4 ± 0.7	34.4 ± 0.9 ^{b}
n-butyl alcohol	2.1	2.3 ± 0.1 ^{a}	2.6 ± 0.0 ^{a}	2.2 ± 0.0 ^{b}	2.2 ± 0.0 ^{b}	2.2 ± 0.0 ^{b}	2.2 ± 0.0 ^{b}
2-methyl-1-butanol	40.3	46.5 ± 0.5 ^{a}	50.5 ± 0.2 ^{a}	41.4 ± 0.6 ^{b}	41.8 ± 0.1 ^{b}	40.9 ± 1.0 ^{b}	41.4 ± 0.4 ^{b}
3-methyl-1-butanol	182.6	184.5 ± 0.0	185.9 ± 0.0	186.3 ± 0.8	188.1 ± 1.1	186.7 ± 1.8	188.3 ± 1.5
N-hexanol	2.8	2.8 ± 0.0	2.9 ± 0.0	2.8 ± 0.0	2.8 ± 0.0	2.8 ± 0.0	2.8 ± 0.0
2-phenylethyl alcohol	0.6	0.6 ± 0.0	0.7 ± 0.0 ^{a}	0.6 ± 0.0	0.6 ± 0.0 ^{b}	0.6 ± 0.0	0.6 ± 0.0 ^{b}
Total	295.3	308.1 ± 0.6	318.0 ± 0.1 ^{a}	302.0 ± 2.5	304.7 ± 1.8 ^{b}	302.2 ± 3.0	304.4 ± 2.3 ^{b}
Ethyl esters from organic acids							
Ethyl lactate	13.2	13.4 ± 0.0 ^{a}	13.5 ± 0.0 ^{a}	14.1 ± 0.1 ^{b}	14.3 ± 0.1 ^{b}	13.9 ± 0.1 ^{b}	14.0 ± 0.1 ^{c}
Ethyl succinate	1.1	1.1 ± 0.0 ^{a}	1.1 ± 0.0 ^{a}	1.8 ± 0.1 ^{b}	2.0 ± 0.1 ^{b}	1.5 ± 0.1 ^{c}	1.5 ± 0.1 ^{c}
Total	14.3	14.5 ± 0.1 ^{a}	14.6 ± 0.0 ^{a}	15.9 ± 0.3 ^{b}	16.5 ± 0.1 ^{b}	15.3 ± 0.1 ^{c}	15.6 ± 0.1 ^{c}
Ethyl esters from fatty acids							
Ethyl caproate	0.2	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Ethyl caprylate	1.4	1.4 ± 0.0 ^{a}	1.5 ± 0.0	1.5 ± 0.0 ^{b}	1.5 ± 0.0	1.5 ± 0.0 ^{b}	1.5 ± 0.0
Ethyl caprate	1.0	1.0 ± 0.0	1.0 ± 0.0 ^{a}	1.0 ± 0.0	1.1 ± 0.0 ^{b}	1.0 ± 0.0	1.1 ± 0.0 ^{b}
Ethyl laureate	0.2	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
Ethyl myristate	0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Ethyl palmitate	0.4	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Total	3.2	3.3 ± 0.2	3.4 ± 0.1	3.6 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.7 ± 0.1

Mean value ± standard deviation (n = 2); significant differences ($p < 0.05$) of a particular parameter in the same ageing year are indicated by different letters.

A similar slight evolution was also observed in the methanol concentration. This is a volatile compound that may evaporate during ageing, but its concentration is also influenced by ethanol evaporation and water perspiration [40,41], thus, its concentration could increase as a consequence of a total volume decrease (Table 4).

Ethyl acetate is the compound in these families of volatile substances that most increased its concentration with the ageing process (Figure 4). Its initial content in the wine spirit is determined by the quality of the distilled wine and the type of still or distillation column used. Ethyl acetate is involved in the esterification reactions between the acetic acid (formed during the ageing) and the ethanol. The content levels of this compound in *New barrels* are higher than in *Used barrels*. In recent studies published by our research group [34], it was demonstrated that wood has the capacity to release acetic acid into the distillate, thus increasing its content in the liquid with ageing. After the esterification of the acetic acid has been completed, the amount of ethyl acetate in brandy increases. Significant differences were observed attending to the usage conditions of the barrel, but there were no differences between the brandy aged in *4 + 3 Used barrels* or *8 + 24 Used barrels*.

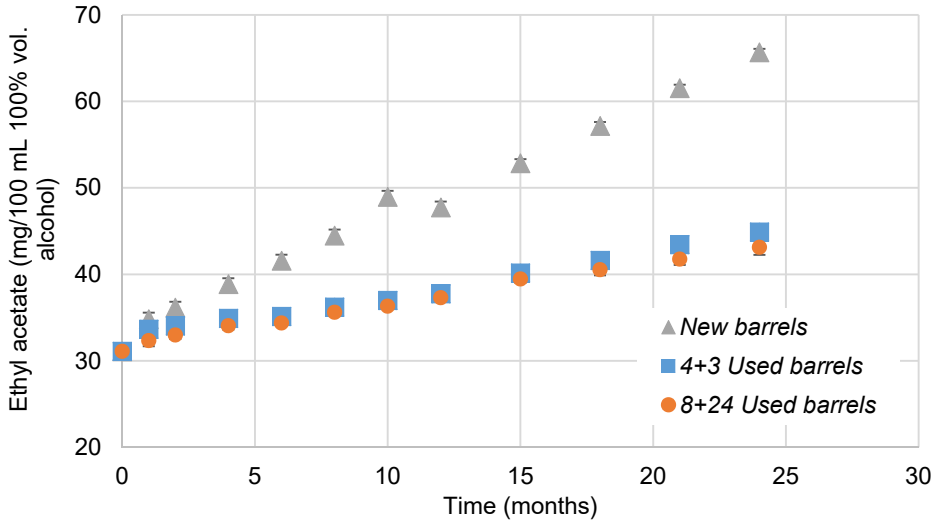


Figure 4. Evolution of ethyl acetate in brandies aged in *New barrels*, *4 + 3 Used barrels* and *8 + 24 Used barrels* for two years, expressed in mg/100 mL of 100% vol. alcohol. When the standard deviation was lower than 0.9 cannot be noticed in the graph.

In relation to the higher alcohols (n-propyl alcohol, 2-butyl alcohol, isobutyl alcohol, n-butyl alcohol, 2-methyl-1-butanol, 3-methyl-1-butanol, n-hexanol and 2-phenylethyl alcohol), slight increments were observed in most of the brandies. Although in some cases, their content level remained invariable. These compounds are not influenced by the wood, since they come from the distillate. They hardly evaporate through the wood pores, because their molecular volume is larger than water or ethanol's. Thus, their concentration increment could be attributed to the ethanol evaporation and water perspiration during the process [40,41]. After the two years of ageing, a high total amount of higher alcohols was determined in those brandies aged in *New barrels*. This could be explained by the considerable loss of volume that took place over the experiment (Table 4).

The esters (ethyl lactate, ethyl succinate, ethyl caproate, ethyl caprylate, ethyl caprate, ethyl laureate, ethyl myristate, and ethyl palmitate) either remained stable or increased slightly. In general, their concentration is affected by hydrolysis, but this reaction rarely takes place during the ageing process, since pH and alcoholic strength values remain almost stable. Similarly to the higher alcohols, esters' concentration increment was a result of the ethanol evaporation and water perspiration during the process (Table 4) [40,41].

The amount of ethyl esters that come from organic acids, such as lactic acid and succinic acid, is larger in *4 + 3* and *8 + 24 Used barrels* than it is in *New barrels* due to the seasoning of the casks, since the presence of these compounds in brandies is explained by the organic acids content in wine (lactic acid and succinic acid), which are involved in the esterification reactions that take place in the liquid over the brandy ageing process. Naturally, their concentration levels were higher in those brandies that had been aged in *3 + 4 Used barrels* than it was in *8 + 24 Used barrels* because of the lower availability of these compounds after the repeated use of each barrel. Thus, significant differences were registered between the three experiments. Furthermore, it should be noted that the concentration of esters derived from fatty acids also increased during the ageing process as a consequence of volume losses. Nevertheless, these compounds were found in very low concentrations in all the Brandies studied here and hardly any differences could be observed between them.

3.5. Tasting Results

According to the tasting sessions carried out, the best perceived descriptors were fruity and vinous notes with medium-high intensity values (between 3 and 5 in a 5-point scale). The ageing notes (vanilla, toasted/caramel, spicy/aniseed, oxidative sweetness, and oak) were evaluated with scores between 1 and 3 points, with the highest values awarded to the brandies that had already been aged for two years in new oak barrels. All of them were characterized by their complex aromatic profile, soft and balanced sensations in the oral cavity, with a remarkable presence of the alcoholic component.

The highest intensity of vinous note was found in the two-year aged brandies, while the lowest intensity was attributed to the young wine spirits. Similar scores were awarded to aromatic complexity, vanilla, toasted/caramel, spicy/aniseed, softness, oxidative sweetness and oak notes. On the other hand, in terms of flavor balance, 2-year brandies were scored higher by the tasters than the other two younger samples. As expected, fruity character and alcoholic note decreased significantly with longer ageing times. The differences between the aromatic notes of wine, vanilla, toasted/caramel and spicy/aniseed, as well as for the woody note and the balance in the flavor evaluation, were confirmed to be associated to wood age and usage. Thus, when the brandy was aged in new oak barrels, the samples were less vinous and balanced and presented higher notes of vanilla, toasted/caramel and oak than the others brandies. When wood is used for the first time, a large amount of phenolic compounds are transferred into the liquid [45], as can be seen in Section 3.2. It should be highlighted that the 4 tasters associated the highest flavor notes of oak, that had been attributed to the brandies aged in *New Barrels*, with astringent sensations and 'aggressiveness'. The tasters related the 'aggressive' term with the gustatory evaluation: high alcoholic character, high bitter oak notes, low smoothness and low oxidative sweetness, which is the opposite of a balanced product. However, they did not establish such association in the case of the brandies aged in used casks. Regarding these descriptors, no big differences were perceived between aged spirits in *4 + 3 Used barrels* and *8 + 24 Used barrels* according to the tasters' opinion. The spicy/aniseed note is the only one that was perceived at different intensity between the three types of casks. Thus, the tasters associated the highest values to the samples aged in *New barrels* and the lowest for the samples aged in *8 + 24 Used barrels*. The softness sensation perceived in the oral cavity was higher in brandies aged in *4 + 3 Used barrels* than in brandies aged in *8 + 24 Used barrels*, followed by the spirits aged in *New barrels*.

When focusing on mean values (Figure 5), the differences in the taste profile of the brandies aged in *Used barrels* with respect to the Brandies aged in *New barrels* grows wider as wood contact time increases.

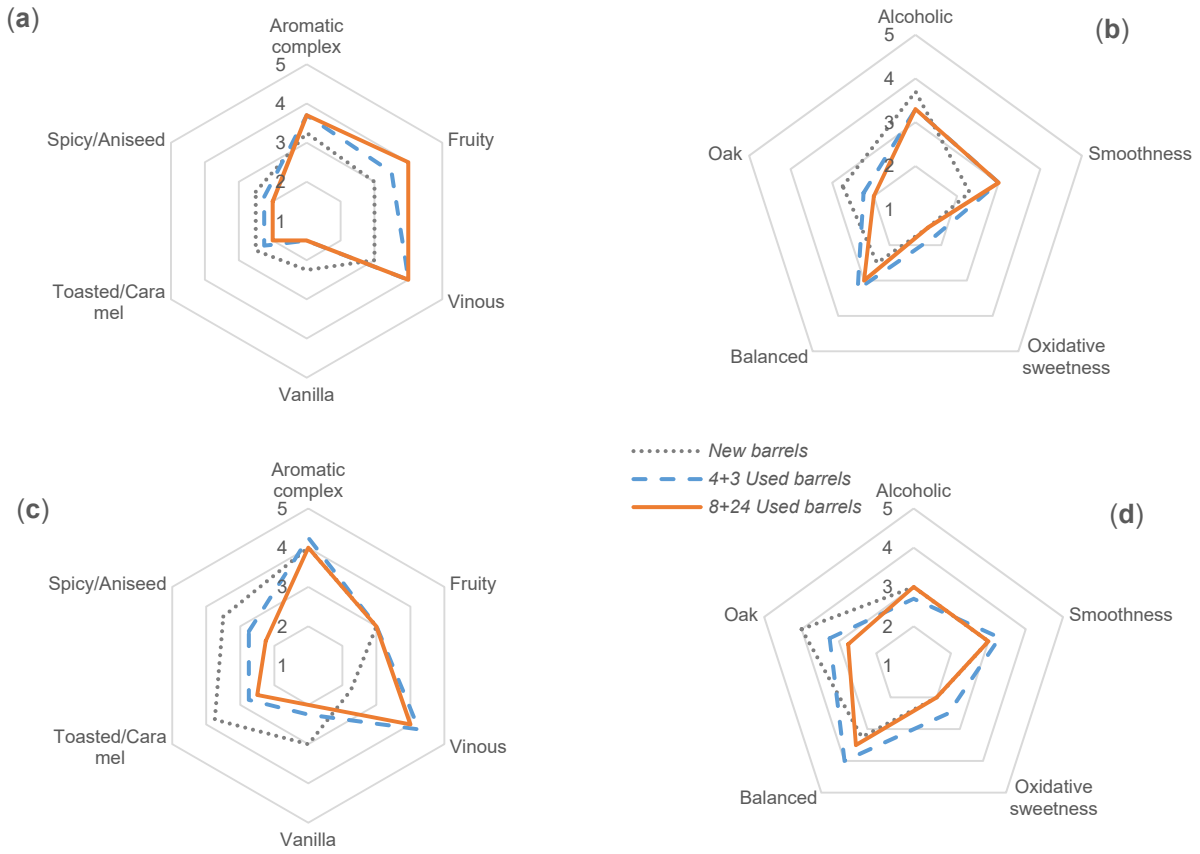


Figure 5. Radar charts of the tasting results of brandies aged for 1 and 2 years in barrels with different previous usage: *New barrels*, *4 + 3 Used barrels*, *8 + 24 Used barrels*. (**a**) Odor evaluation after 1 year of ageing; (**b**) Flavor evaluation after 1 year of ageing; (**c**) Odor evaluation after 2 years of ageing; (**d**) Flavor evaluation after 2 years of ageing (Legend in Figure 5d).

4. Conclusions

There is a clear difference between brandies aged in *New barrels* and those aged in used or seasoned barrels. When barrels are used for the first time, they release much larger amounts of wood compounds into the distillate than in later uses of the same barrel. However, once the barrel has been used, no significant differences can be observed in relation to the number of years of use. In fact, although slightly higher levels of volatile acids, phenolic compounds, A420, A520, and A620, as well as aldehydes, acetal, methanol, esters, and higher alcohols have been detected in the brandies aged in *4 + 3 Used barrels* compared to *8 + 24 Used barrels*, such differences were irrelevant in most cases.

The tasters described as 'aggressive' the brandies aged in the *New barrels*, that released a high number of compounds. Moreover, the final product was not considered as well balanced as those obtained from *Used barrels*. On the other hand, in most cases the taster did not perceived differences between brandies aged in *4 + 3* or *8 + 24 Used barrels*.

It has been confirmed that even after 32 years of use the barrels' wood would still contribute to the organoleptic properties of brandy. Obviously, *Used barrels* do not yield the same amount of wood compounds into the distillates, but, attending to the organoleptic properties of the final product, this could be considered a positive factor. According to the main results of this study, it can be concluded that wooden barrels that have been used for a high number of years can still be used for ageing distillates, providing them with different organoleptic characteristics than *New barrels*.

Author Contributions: Conceptualization, methodology, formal analysis, D.A.G.-S., M.C.R.-D.; M.G.-C., M.J.V.-M. and M.V.G.-M.; investigation, M.G.-C. and M.J.V.-M.; writing—original draft preparation, M.G.-C.; writing—review and editing, D.A.G.-S., M.J.V.-M. and M.V.G.-M.; supervision, D.A.G.-S., M.J.V.-M. and M.V.G.-M.; project administration, D.A.G.-S. and M.V.G.-M.; funding acquisition, D.A.G.-S. and M.V.G.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been supported by the University of Cadiz and Bodegas Fundador, S.L.U. (ref.: OT2019/108). The authors wish to thank the University of Cadiz and Bodegas Fundador, S.L.U. for the industrial pre-doctoral contract TDI-8-18, granted to the author María Guerrero Chanivet.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Consejería de Agricultura Pesca y Desarrollo rural. Orden de 28 de junio de 2018, por la que se aprueba el expediente técnico de Indicación Geográfica "Brandy de Jerez". *Boletín Oficial la Junta Andalucía* **2018**, *127*, 19–20.
2. European Parliament and of the Council. Regulation (EC) No 2019/787 of 17 April 2019, on the definition, description, presentation and labelling of spirit drinks, the use of the names of spirit drinks in the presentation and labelling of other foodstuffs, the protection of geographical indications for spirit drinks, the use of ethyl alcohol and distillates of agricultural origin in alcoholic beverages, and repealing Regulation (EC) No 110/2008. *Off. J. Eur. Union* **2019**, *L130*, 17–27.
3. Consejo Regulador de Vinos y Vinagres de Jerez. Especificación Técnica de Envinado de Vasijas; Consejo Regulador de Vinos y Vinagres de Jerez: 2017. MicrosoftWord-Documento3. Available online: www.sherry.wine (accessed on 29 January 2020).
4. Canas, S. Phenolic Composition and Related Properties of Aged Wine Spirits: Influence of Barrel Characteristics. A Review. *Beverages* **2017**, *3*, 55. [CrossRef]
5. Caldeira, I.; Mateus, A.M.; Belchior, A.P. Flavour and odour profile modifications during the first five years of Lourinhã brandy maturation on different wooden barrels. *Anal. Chim. Acta* **2006**, *563*, 264–273. [CrossRef]
6. Jordao, A.M.; Ricardo-da-Silva, J.M.; Laureano, O. Comparison of volatile composition of cooperage oak wood of different origins (Quercus pyrenaica vs. Quercus alba and Quercus petraea). *Mitt. Klosterneubg.* **2005**, *55*, 22–31.
7. Martínez-Gil, A.; del Alamo-Sanza, M.; Sánchez-Gómez, R.; Nevares, I. Different Woods in Cooperage for Oenology: A Review. *Beverages* **2018**, *4*, 94. [CrossRef]
8. Canas, S.; Silva, V.; Belchior, A.P. Wood related chemical markers of aged wine brandies. *Ciência e Técnica Vitivinícola* **2008**, *23*, 45–52.
9. De Rosso, M.; Cancian, D.; Panighel, A.; Dalla Vedova, A.; Flamini, R. Chemical compounds released from five different woods used to make barrels for aging wines and spirits: Volatile compounds and polyphenols. *Wood Sci. Technol.* **2009**, *43*, 375–385. [CrossRef]
10. Prida, A.; Puech, J.L. Influence of geographical origin and botanical species on the content of extractives in American, French, and East European oak woods. *J. Agric. Food Chem.* **2006**, *54*, 8115–8126. [CrossRef]
11. Pérez-Prieto, L.J.; López-Roca, J.M.; Martínez-Cutillas, A.; Pardo Mínguez, F.; Gómez-Plaza, E. Maturing wines in oak barrels. Effects of origin, volume, and age of the barrel on the wine volatile composition. *J. Agric. Food Chem.* **2002**, *50*, 3272–3276. [CrossRef]
12. Soares, B.; Garcia, R.; Freitas, A.M.C.; Cabrita, M.J. Phenolic compounds released from oak, cherry, chestnut and robinia chips into a syntethic wine: Influence of toasting level. *Ciência e Técnica Vitivinícola* **2012**, *27*, 17–26.
13. Rodríguez Madrera, R.; Blanco Gomis, D.; Mangas Alonso, J.J. Influence of distillation system, oak wood type, and aging time on volatile compounds of cider brandy. *J. Agric. Food Chem.* **2003**, *51*, 5709–5714. [CrossRef] [PubMed]
14. Cruz, S.; Canas, S.; Belchior, A.P. Effect of ageing system and time on the quality of wine brandy aged at industrial-scale. *Ciência e Técnica Vitivinícola* **2013**, *27*, 83–93.
15. Canas, S.; Caldeira, I.; Belchior, A.P. Extraction/oxidation kinetics of low molecular weight compounds in wine brandy resulting from different ageing technologies. *Food Chem.* **2013**, *138*, 2460–2467. [CrossRef]
16. Martínez Montero, C.; Rodríguez Dodero, M.D.C.; Guillén Sánchez, D.A.; García Barroso, C. Sugar contents of Brandy de Jerez during its aging. *J. Agric. Food Chem.* **2005**, *53*, 1058–1064. [CrossRef]
17. Rodríguez Dodero, M.C.; Guillén Sánchez, D.A.; Rodríguez, M.S.; Barroso, C.G. Phenolic Compounds and Furanic Derivatives in the Characterization and Quality Control of Brandy de Jerez. *J. Agric. Food Chem.* **2010**, *58*, 990–997. [CrossRef]
18. Schwarz, M.; Rodríguez, M.C.; Guillén, D.A.; Barroso, C.G. Analytical characterisation of a Brandy de Jerez during its ageing. *Eur. Food Res. Technol.* **2011**, *232*, 813–819. [CrossRef]
19. Schwarz, M.; Rodríguez, M.C.; Martínez, C.; Bosquet, V.; Guillén, D.; Barroso, C.G. Antioxidant activity of Brandy de Jerez and other aged distillates, and correlation with their polyphenolic content. *Food Chem.* **2009**, *116*, 29–33. [CrossRef]
20. Sánchez-Guillén, M.M.; Schwarz-Rodríguez, M.; Rodríguez-Dodero, M.C.; García-Moreno, M.V.; Guillén-Sánchez, D.A.; García-Barroso, C. Discriminant ability of phenolic compounds and short chain organic acids profiles in the determination of quality parameters of Brandy de Jerez. *Food Chem.* **2019**, *286*, 275–281. [CrossRef]

21. Nishimura, K.; Matsuyama, R.R. Maturation and Maturation Chemistry. In *The Science and Technology of Whiskies*; Piggott, J., Sharp, R., Duncan, R.E.B., Eds.; Longman Scientific and Technical: London, UK, 1989; pp. 253–263.
22. Mosedale, J.; Puech, J.-L. Wood maturation of distilled beverages. *Trends Food Sci. Technol.* **1998**, *9*, 95–101. [CrossRef]
23. Conner, J.M.; Paterson, A.; Piggott, J.R. Analysis of lignin from oak casks used for the maturation of Scotch whisky. *J. Sci. Food Agric.* **1992**, *60*, 349–353. [CrossRef]
24. Sarni, F.; Moutounet, M.; Puech, J.L.; Rabier, P. Effect of heat treatment of oak wood extractable compounds. *Holzforschung* **1990**, *44*, 461–466. [CrossRef]
25. Le Floch, A.; Jourdes, M.; Teissedre, P.-L. Polysaccharides and lignin from oak wood used in cooperage: Composition, interest, assays: A review. *Carbohydr. Res.* **2015**, *417*, 94–102. [CrossRef] [PubMed]
26. Coelho, E.; Teixeira, J.A.; Domingues, L.; Tavares, T.; Oliveira, J.M. Factors affecting extraction of adsorbed wine volatile compounds and wood extractives from used oak wood. *Food Chem.* **2019**, *295*, 156–164. [CrossRef] [PubMed]
27. Martínez-Gil, A.; Cadahía, E.; Fernández de Simón, B.; Gutiérrez-Gamboa, G.; Nevares, I.; del Álamo-Sanza, M. Phenolic and volatile compounds in Quercus humboldtii Bonpl. wood: Effect of toasting with respect to oaks traditionally used in cooperage. *J. Sci. Food Agric.* **2019**, *99*, 315–324. [CrossRef]
28. Reazin, G.H. Chemical analysis of whisky maturation. In *Flavour of Distilled Beverages: Origin and Development*; Piggott, J.R., Ed.; Ellis Horwood: Chichester, UK, 1983; pp. 225–240.
29. The Cooperage. The Making of A Sherry Cask. Available online: https://www.sherry.wine/sherry-cask/sherrycask-the-cooperage (accessed on 8 December 2020).
30. Saris, J.; Morfaux, J.N.; Dervin, L. Détermination automatique de l'acidité volatile du vin. *Ind. Alim. Agric.* **1970**, *87*, 115–121.
31. Pilone, G.J. Effect of lactic acid on volatile acid determination of wine. *Am. J. Enol. Vitic.* **1967**, *18*, 149–156.
32. Dubernet, M. Dosage automatique de l'acidité volatile dans les vins. *Connaiss. Vigne Vin* **1976**, *10*, 297–309. [CrossRef]
33. Schwarz, M.; Rodríguez, M.C.; Guillén, D.A.; Barroso, C.G. Development and validation of UPLC for the determination of phenolic compounds and furanic derivatives in Brandy de Jerez. *J. Sep. Sci.* **2009**, *32*, 1782–1790. [CrossRef]
34. Guerrero-Chanivet, M.; Valcárcel-Muñoz, M.J.; García-Moreno, M.V.; Guillén-Sánchez, D.A. Characterization of the Aromatic and Phenolic Profile of Five Different Wood Chips Used for Ageing Spirits and Wines. *Foods* **2020**, *9*, 1613. [CrossRef]
35. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158. [CrossRef]
36. International Organization for Standardization (ISO) Sensory analysis. General Guidance for the Design of test rooms (ISO 8589:2007). 2007. Available online: https://www.iso.org/standard/36385.html (accessed on 29 January 2020).
37. International Organization for Standardization (ISO) Sensory Analysis. Apparatus. Wine-tasting glass (ISO 3591:1977). 1977. Available online: https://www.iso.org/standard/52497.html (accessed on 29 January 2020).
38. International Organization for Standardization (ISO) Sensory Analysis. Guidelines for the Use of Quantitative Response Scales (ISO 4121:2003). 2003. Available online: https://www.iso.org/standard/33817.html (accessed on 29 January 2020).
39. Tsakiris, A.; Kallithraka, S.; Kourkoutas, Y. Grape brandy production, composition and sensory evaluation. *J. Sci. Food Agric.* **2014**, *94*, 404–414. [CrossRef] [PubMed]
40. Singleton, V.L. Maturation of Wines and Spirits—Comparisons, Facts, and Hypotheses. *Am. J. Enol. Vitic.* **1995**, *46*, 98–115.
41. Guymon, J.F.; Crowell, E.A. Brandy aging. Some comparisons of American and French oak cooperage. *Wines Vines* **1970**, 23–25.
42. Maga, J.A. The contribution of wood to the flavor of alcoholic beverages. *Food Rev. Int.* **1989**, *5*, 39–99. [CrossRef]
43. García-Ruiz, J.M.; Alcántara, R.; Martín, J. Effects of Reutilized Potassium Bitartrate Seeds on the Stabilization of Dry Sherry Wine. *Am. J. Enol. Vitic.* **1995**, *46*, 525–528.
44. Canas, S.; Caldeira, I.; Anjos, O.; Belchior, A.P. Phenolic profile and colour acquired by the wine spirit in the beginning of ageing: Alternative technology using micro-oxygenation vs traditional technology. *Lwt* **2019**, *111*, 260–269. [CrossRef]
45. Baldwin, S.; Andreasen, A.A. Congener Development in Bourbon Whisky Matured at Various Proofs for Twelve Years. *J. AOAC Int.* **1974**, *57*, 940–950. [CrossRef]
46. Cernîşev, S. Analysis of lignin-derived phenolic compounds and their transformations in aged wine distillates. *Food Control* **2017**, *73*, 281–290. [CrossRef]
47. Belchior, A.; Caldeira, I.; Costa, S.; Lopes, C.; Tralhão, G.; Ferrão, A.; Mateus, A.; Carvalho, E. Evolução das Características Fisico-Químicas e Organolépticas de Aguardentes Lourinhã ao Longo de Cinco Anos de Envelhecimento em Madeiras de Carvalho e de Castanheiro. *Ciência e Técnica Vitivinícola* **2001**, *16*, 81–94.
48. Espejo, F.; Armada, S. Colour Changes in Brandy Spirits Induced by Light-Emitting Diode Irradiation and Different Temperature Levels. *Food Bioprocess Technol.* **2014**, *7*, 2595–2609. [CrossRef]
49. Fujieda, M.; Tanaka, T.; Suwa, Y.; Koshimizu, S.; Kouno, I. Isolation and structure of whiskey polyphenols produced by oxidation of oak wood ellagitannins. *J. Agric. Food Chem.* **2008**, *56*, 7305–7310. [CrossRef] [PubMed]
50. Álvarez Batista, M.Á. *Influencia del "Envinado" de las Botas de Roble Americano en la Evolución de los Aguardientes de vino Durante su Envejecimiento*; Universidad de Sevilla: Sevilla, Spain, 1997.

foods

MDPI

Article

Analysis of Intact Glycosidic Aroma Precursors in Grapes by High-Performance Liquid Chromatography with a Diode Array Detector

Cristina Cebrián-Tarancón [1], José Oliva [2], Miguel Ángel Cámara [2], Gonzalo L. Alonso [1] and M. Rosario Salinas [1,*]

[1] Cátedra de Química Agrícola, E.T.S.I. Agrónomos y Montes, Departamento de Ciencia y Tecnología Agroforestal y Genética, Universidad de Castilla-La Mancha, Avda. de España s/n, 02071 Albacete, Spain; cristina.ctarancon@uclm.es (C.C.-T.); gonzalo.alonso@uclm.es (G.L.A.)

[2] Departamento de Química Agrícola, Geología y Edafología, Facultad de Química, Universidad de Murcia, Campus de Espinardo s/n, 30100 Murcia, Spain; josoliva@um.es (J.O.); mcamara@um.es (M.Á.C.)

* Correspondence: Rosario.Salinas@uclm.es; Tel.: +34-967-599210; Fax: +34-967-599238

Abstract: Nowadays, the techniques for the analysis of glycosidic precursors in grapes involve changes in the glycoside structure or it is necessary the use of very expensive analytical techniques. In this study, we describe for the first time an approach to analyse intact glycosidic aroma precursors in grapes by high-performance liquid chromatography with a diode array detector (HPLC-DAD), a simple and cheap analytical technique that could be used in wineries. Briefly, the skin of Muscat of Alexandria grapes was extracted using a microwave and purified using solid-phase extraction combining Oasis MCX and LiChrolut EN cartridges. In total, 20 compounds were selected by HPLC-DAD at 195 nm and taking as a reference the spectrum of phenyl β-D-glucopyranoside, whose DAD spectrum showed a first shoulder from 190 to 230 nm and a second around 200–360 nm. After that, these glycosidic compounds were identified by High-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (HPLC-qTOF-MS). Disaccharides hexose pentose were the most abundant group observed with respect to the sugars and monoterpendiols the main aglycones found.

Keywords: aroma precursors; grapes; HPLC-DAD; HPLC-qTOF-MS; intact glycosides

Citation: Cebrián-Tarancón, C.; Oliva, J.; Cámara, M.Á.; Alonso, G.L.; Salinas, M.R. Analysis of Intact Glycosidic Aroma Precursors in Grapes by High-Performance Liquid Chromatography with a Diode Array Detector. *Foods* **2021**, *10*, 191. https://doi.org/10.3390/foods10010191

Received: 28 December 2020
Accepted: 15 January 2021
Published: 19 January 2021

1. Introduction

Aroma glycosides are compounds produced during the secondary metabolism in plants formed by a sugar moiety linked to a volatile aglycone by a β-glycosidic bond. The main sugar is β-D-glucose however, another sugar molecule may be added to form higher glycosides such as disaccharide, which have been indicated as the most abundant in grapes [1].

In glycosylated aroma precursors of grapes, the aglycone moiety is responsible for the potential aromatic character of the molecule since, although these compounds originally have no odour, the volatile aglycones are released during winemaking contributing to the wine aroma profile [2,3]. The aglycone can belong to different chemical families, such as terpenes, phenols or norisoprenoids [1] and even though their nature depends mainly on the grapes' variety, it may also be influenced by other factors, such as soil or climatic conditions or viticultural practices. In fact, several studies have proven that it is possible to modify the glycosidic aroma profile of grapes by treating the leaves with oak or guaiacol extracts [4–6].

Such an important role of glycosylated aroma precursors has stimulated the development of analytical methods for their quantification [7]. Typically, such precursors are analysed by isolating their glycosides, followed by the release of aglycones via enzymatic

hydrolysis [8,9] or acidic hydrolysis [10–12], and finally the analysis of volatile aglycones by gas chromatography-mass spectrometry (GC-MS). However, in both cases, the molecular structure of glycosylated compounds is modified during the analysis process, a partial breaks occur in the glycosides, generating fragments and causing major structural changes in the aglycones, which do not reflect the original glycoside chemistry of grapes [1,2].

In a different line, Serrano de la Hoz (2014) [13] developed a fast method for analysing the potential aroma of grapes (IPAv) by measuring the amount of glycosidic glucose released at equimolecular proportions of volatile aglycones by acid hydrolysis. Although the simplicity of this method makes it useful in viticulture and wine production, it provides an estimate of the total content of glycosidic aroma precursors, but not a concentration.

The firsts identifications of aroma precursors by high-performance liquid chromatography–mass spectrometry (HPLC-MS/MS) was described by Nasi et al. 2008 [14] and Schievano et al. 2013 [15] and later several researchers have been proposed different strategies for the direct quantification of aglycones using ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS/MS) [16–18]. However, due to the complexity of these techniques and the high cost of the equipment needed, it is difficult to employ them in viticulture or wineries. For its part, the high-performance liquid chromatography with a diode array detector (HPLC-DAD) has been used for the quantification of glycosylated compounds in other crops, as for example steviol glycosides, which are glycoconjugated terpenols whose structures are very similar than glycoside terpene aroma precursors [19,20]. In these articles it is reported the maximum absorption wavelengths of these compounds in 210 nm, probably associated to the aglycone. But, to increase the sensitivity, measurements could be done at a lower wavelength (e.g., 195 nm), where sugars have their maximum wavelengths of absorption.

It could be a simple and cheap analytical technique that allows wineries to determine intact glycosidic aroma precursors but, to our knowledge, no previous studies on the use of this technique in grapes have been performed. Therefore, this work addresses the possibility of analyzing intact glycosidic grape aroma precursors by HPLC-DAD.

2. Materials and Methods

2.1. Grape Material

Fifty kilograms of Muscat of Alexandria grapes were collected in a representative way of the plot from a vineyard of O.D. La Mancha (Castilla-La Mancha, Spain) during the 2019 harvest under proper sanitary conditions and at the optimal stage of maturity. All grapes were immediately frozen at -20 ± 2 °C until extraction.

2.2. Extract Preparation

Grapes frozen were skinned, the pulp and seeds were removed, and the skins were freeze-dried (LyoAlfa 6–50; Telstar, Terrassa, Spain). Then, 20 g of homogenised freeze-dried skin from 0.5 kg of frozen grapes was ground to a fine powder and moisturised with 70 mL of water for 2 h at room temperature (20 ± 3 °C). Then, extraction was performed using a NEOS microwave device (Milestone, Sorisole, Italy) at 70 °C (600 W) for 1 min [21]. The mixture was then centrifuged at 4000 rpm ($3000\times g$) for 10 min, and the total supernatant volume was taken and divided into two similar fractions of approximately 25 mL each, which is the adequate volume for each cartridge.

2.3. Isolation of Intact Glycosidic Aroma Precursors

Glycosidic aroma precursors were extracted and purified using solid-phase extraction (SPE) according to the method described by Hernández-Orte et al. (2015) [22], with minor modifications, as seen in Figure 1.

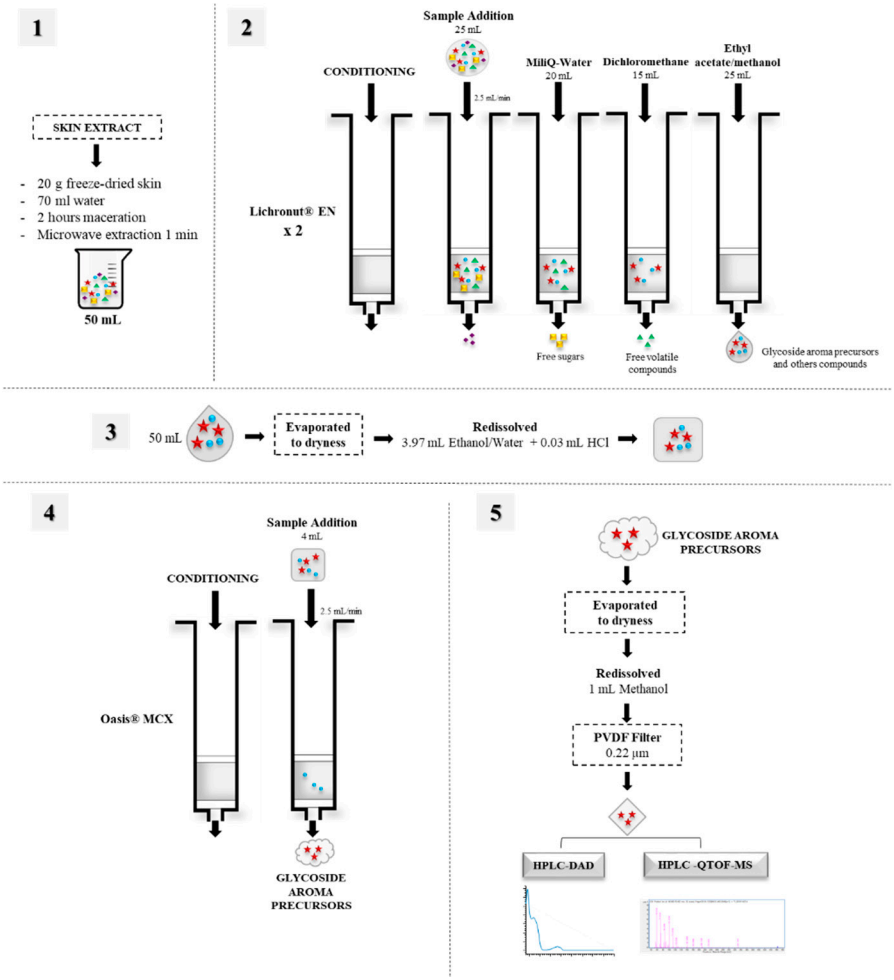


Figure 1. Extraction and isolation procedure of glycosidic aroma precursors.

As a first step, each fraction (25 mL) was passed through a LiChrolut EN cartridge (40–120 µm, 500 mg; Millipore Corp., Bedford, MA, USA), followed by the use of an Oasis MCX SPE cartridge (60 µm, 500 mg; Waters Corp., Milford, MA, USA). The LiChrolut EN cartridge was conditioned with 16 mL of dichloromethane, 16 mL of methanol and 32 mL of Milli-Q water, and approximately 25 mL of skin extract was passed through the cartridge at a flow rate of 2.5 mL/min. Then, the cartridges were washed with 20 mL of water to remove free sugars, followed by 15 mL of dichloromethane to remove the free volatile fraction. The glycosidic aroma precursor fraction was then recovered using 25 mL of ethyl acetate/methanol (90:10, v/v). The two eluates were mixed and the total volume (50 mL) was evaporated to dryness using a rotary vacuum evaporator (LABOROTA 4000eco; Heidolph Instruments, Schwabach, Germany). Then, the dryness mixture was re-dissolved in 3.97 mL of ethanol/water (50:50, v/v) together with 30 µL of HCl (pH 1). To remove polyphenols, all solutions (4 mL) were passed through an Oasis MCX cartridge previously conditioned with 5 mL of methanol, 5 mL of Milli-Q water, 5 mL of HCl (pH 1) and finally 5 mL of Milli-Q water. Later, the eluted fraction was evaporated to dryness, recovered with 1 mL of methanol and filtered through a Durapore polyvinylidene fluoride filter (0.22 µm; Millipore Corp.) for HPLC-DAD and HPLC-qTOF MS analysis. Extraction were made in duplicate.

2.4. HPLC-DAD Analysis

To determine the intact glycosidic aroma precursors of grapes, an analysis was performed using an Agilent 1200 HPLC chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD (Agilent G1315D; Agilent Technologies) coupled to an Agilent

ChemStation (version B.03.01) data processing system. Taking as a reference the phenyl β-D-glucopyranoside spectrum DAD at 195 nm (Figure S1), the possible intact glycoside compounds were selected.

Separation was performed using an ACE C18-PFP column (4.6 mm × 150 mm, 3 µm particle size) connected to an Excel HPLC Pre-Column Filter 1PK (0.5 µm particle size) at 30 °C. Brisa LC2 C18 column (250 mm × 4.6 mm, 5 µm particle size) purchased from Teknokroma (Barcelona, Spain) was previously tested, but a worse results were obtained

The eluents used in both systems were water (A) and acetonitrile (B) with different gradients and different elution times. The flow rate was 0.8 mL/min, and the sample injection volume was 20 µL. The DAD (Agilent Technologies Deutschland GmbH, Wald bronn, Germany) was set at 190, 195, 210, 230, 280 and 324 nm. Each extract was injected in duplicate, so a total of 4 chromatograms were obtained.

2.5. HPLC-qTOF-MS Analysis

Identification of glycosidic aroma precursors previously selected using HPLC-DAD was performed using HPLC-qTOF MS, MassHunter PCDL Manager (Agilent Technologies) personal database and MS/MS fragmentation. For this purpose, the ACE C18-PFP column was used and the same solvents, flow rates and elution gradients to those used for the HPLC-DAD analysis were used to minimise any changes in the compounds' retention time and the possible formation of adducts. Only the times of the peaks observed in HPLC-DAD chromatogram were tested in the HPLC-qTOF-MS and not all the peaks observed in this chromatogram. 20 µL of the sample were injected.

Samples were run in the negative mode using an Agilent 1290 Series II HPLC (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) device coupled to an Agilent 6550 Q-TOF(Agilent Technologies Deutschland GmbH, Waldbronn, Germany) with a Jet Stream dual electrospray ionisation source. Nitrogen was used for both the drying gas and the sheath gas in the source. The capillary voltage was set to 4000 V, the nozzle voltage was set to 500 V and the fragmentor voltage was set to 350 V. The drying gas flow was set to 16 L/min at 150 °C, the sheath gas flow was set to 12 L/min at 300 °C and the nebuliser was set to 30 psig. The scan range was set to m/z 50–1100 for the MS and MS/MS mode. Before running each sequence, the instrument was calibrated according to the manufacturer's specifications.

Reference mass ions were run continuously into the dual electrospray ionisation source at a rate of 50 µL/min throughout the run to ensure accurate mass calibration. The reference masses were 121.985587 and 1033.988109 m/z.

In the first run, MS/MS spectra were acquired using the target MS/MS mode of the instrument. The source conditions were the same as for single MS mode acquisition. The compounds were fragmented with a collision energy of 30 eV, and the precursor ions, established previously according to the literature, were acquired in a narrow isolation width (~1.3 u) to ensure ion selectivity with a minimum threshold of 200 counts. However, better results were obtained when the MS/MS spectra were acquired using the all ions MS/MS analysis mode of the instrument. The source conditions were again the same as for single MS mode acquisition, and three different collision energies (0, 10 and 40 eV) were selected for each run cycle.

3. Results

3.1. Isolation of Intact Glycosidic Aroma Precursor

The content of aroma glycosides in grapes is mainly concentrated in the skin, with a lower concentration in the pulp and juice [1]. Therefore, to isolate intact glycosides, grape skins were extracted using a microwave.

Glycosidic aroma precursors were extracted and purified using SPE with two different cartridges. The interference caused by free glucose and volatiles present in grapes needs to be removed. This was achieved through extraction using a LiChrolut EN cartridge (40–120 µm, 500 mg; Millipore Corp.). This is commonly used to analyse aroma precursors

in grapes [23–25] since it resin was proved to be the most suitable for extracting precursor compounds and aromatic components [26] and it is particularly suitable for the extraction of polar analytes and a wide range of less polar solutions, such as hydroalcoholic solutions or wine [27]. Following the above procedure, an Oasis MCX SPE cartridge (60 µm, 500 mg; Waters Corp.) was used, which contains a mixture of reversed-phase and cationic-exchanger materials that allow the isolation of grape and wine flavonols, anthocyanin and other phenolic compounds [28–30]. Notably, these compounds coexist in grapes with aroma precursors and may lead to interference during chromatographic analyses. When the skin extract was passed through both cartridges, a decrease in phenolic compounds was observed, and hence a better glycosidic aroma precursor peak resolution was obtained.

3.2. HPLC-DAD Conditions

To choose the best HPLC-DAD conditions, two columns with different elution types were tested. First, separation was performed on a reversed-phase C_{18} column (Brisa LC2) because of its similarity to those used by some authors to analyse glycosidic phenolic compounds [31]. However, a better peak resolution was obtained with the ACE Excel 3 C18-PFP column, possibly because of its smaller particle size. In terms of elution, different gradients were tested with water (A) and acetonitrile (B) as eluents. The best results were observed with the following gradient for Solvent B: 0 min, 1%; 3 min, 2.5%; 13 min, 2.5%; 14 min, 5%; 34 min, 5%; 35 min, 9%; 35.5 min, 9%; 36 min, 10%; 56 min, 10%; 57 min, 11%; 57.5 min, 11%; 58 min, 12%; 78 min, 12%; 79 min, 15%; 83 min, 15%; 88 min, 100%; 93 min 100%. The wavelength used was 195 nm because this is the most adequate for our objective.

It should be noted that the literature has established a cut-off UV wavelength of acetonitrile of 190 nm [32], below which absorption may cause fluctuating baselines and higher noise levels. However, the measured UV absorbance may be influenced by several factors, such as the solvent's purity, instrumental parameters or the reference substance [33]. Therefore, a single blank was run with the previously described gradient conditions and no interference on the baseline was observed.

3.3. Identification of Intact Glycosidic Aroma Precursors by HPLC-qTOF-MS

In general, in grapes, the monosaccharides glycosides are less present in grapes than disaccharides. In this line, Hjelmeland et al. 2015 [2] studied the monoterpene glycosides profile over three developmental stages in Muscat of Alexandria grapes, being monoterpenol hexose pentose the most abundant group. At time, Ghaste et al. 2015 [17] identified 15 glycosylated precursors of volatiles, mainly disaccharides glycosides. More reciently, Godshaw et al. 2019 [18] studied the intact profile of monoterpenyl glycosides in six *Vitis vinifera* grapes and they identified the malonylated monoterpenol glucosides, monoterpenol hexose-pentoses and monoterpendiol hexose-pentoses as predominated in all samples. In the same line, Caffrey et al. 2020 [34] described a method to characterize glycosidically bound precursors of monoterpenoids, norisoprenoids, volatile phenols, aliphatic alcohols and sesquiterpenoids in grapes. Therefore, before the analysis, a potential list of compounds was created on the basis of previously identified glycosidic aroma precursors in Muscat of Alexandria grapes [2,17,18,34].

All compounds were grouped according to their structure and molecular formula. A personal compound database was automatically generated by MassHunter PCDL Manager (Agilent Technologies, Deutschland GmbH, Waldbronn, Germany) using the exact mass of each compound according to the molecular formula imported. Final tentative identification was performed by comparing the fragmentation spectra with known glycoconjugate fragmentation patterns of these authors. These are the compounds included:

- *ALCOHOLS: Group C4: pentose-hexose* ($C_{15}H_{28}O_{10}$; m.w. 368.1682); *pentose-hexose-hydroxy* ($C_{15}H_{28}O_{11}$; m.w. 384.1632). *Group C5: pentose-hexose* ($C_{16}H_{30}O_{10}$; m.w. 382.1839); *deoxyhexose-hexose* ($C_{17}H_{32}O_{10}$; m.w. 396.1995); *pentose-hexose-hydroxy* ($C_{16}H_{30}O_{11}$;

m.w. 398.1788); *Group C6: pentose-hexose* ($C_{17}H_{32}O_{10}$; m.w. 396.1995); *deoxyhexose-hexose* ($C_{18}H_{34}O_{10}$; m.w. 410.2152); *pentose-hexose-dehydro-hydroxy* ($C_{17}H_{32}O_{11}$; m.w. 412.1945); *hexose-hexose-dehydro* ($C_{18}H_{32}O_{11}$; m.w. 424.1945); *hexose-hexose-hexose* ($C_{24}H_{42}O_{16}$; m.w. 586.2473); *Group C8: pentose-hexose-dehydro-hydroxy* ($C_{19}H_{36}O_{11}$; m.w. 440.2258).

- MONOTERPENOLS: *glycosides* ($C_{16}H_{28}O_{6;}$ m.w. 316.1886); *hexose-pentose-glycosides* ($C_{21}H_{36}O_{10}$; m.w. 448.2309); *malonylated glycosides* ($C_{21}H_{34}O_{11}$; m.w. 462.2101); *hexose-deoxyhexose-glycoside* ($C_{22}H_{38}O_{10}$; m.w. 462.2465); *dihexose-pentose-glycosides* ($C_{27}H_{46}O_{15}$; m.w. 610.2837); *hexose-dipentose* ($C_{26}H_{44}O_{14}$; m.w. 580.2731); *dipentose-hexose-glycosides* ($C_{26}H_{44}O_{14}$; m.w. 580.2731).
- MONOTERPENEDIOLS: *hexose-pentose-glycoside* ($C_{21}H_{36}O_{11}$; m.w. 464.2258); *dihydro-hexose-pentose-glycoside* ($C_{21}H_{38}O_{11}$; m.w. 466.2414); *dihydro-deoxyhexose-hexose-glycoside* ($C_{22}H_{38}O_{11}$; m.w. 478.2414); *pentose-hexose glycoside* ($C_{21}H_{36}O_{12}$; m.w. 480.2207).
- MONOTERPENETRIOLS: *hexose-dehydro* ($C_{16}H_{26}O_8$; m.w. 346.2414): *dihydro-hexose* ($C_{16}H_{30}O_8$; m.w. 350.1941); *dihydro-hexose-pentose* ($C_{21}H_{38}O_{12}$; m.w. 482.2362); *deoxyhexose-hexose* ($C_{22}H_{38}O_{12}$; m.w. 494.2363).
- NORISOPRENOIDS: In this group of compounds, the aglycone were groups according to the database used by (Caffrey et al., 2020). *Norisoprenoids (Group G): hexose* ($C_{19}H_{34}O_8$; m.w. 390.2254); *hexose-hydroxy* ($C_{19}H_{34}O_9$; m.w. 406.2203); *hexose-hexose-hydroxy* ($C_{25}H_{44}O_{14}$; m.w. 568.2731); *hexose-hexose* ($C_{25}H_{44}O_{13}$; m.w. 552.2782). *Norisoprenoids (Group F): hexose* ($C_{19}H_{39}O_8$; m.w. 388.2097); *hexose-hydroxy* ($C_{19}H_{34}O_9$; m.w. 406.2203); *hexose-hexose* ($C_{25}H_{42}O_{13}$; m.w. 550.2625). *Norisoprenoids (Group A): hexose-hexose* ($C_{25}H_{42}O_{13}$; m.w. 550.2625). *Norisoprenoids (Group E): hexose* ($C_{19}H_{30}O_8$; m.w. 386.1941); *hexose-pentose* ($C_{24}H_{38}O_{12}$; m.w. 518.2363).
- *PHENOLS: pentose-hexose* ($C_{18}H_{26}O_{10}$; m.w. 402.1500); *pentose-pentose-hexose* ($C_{25}H_{40}O_{13}$; m.w. 548.2469).
- *SESQUITERPENOLS: hexose-pentose* ($C_{26}H_{44}O_{10}$; m.w. 516.2934).

As seen in Figure 2, 20 intact glycosidic aroma precursors were previously selected by using the HPLC-DAD method, and it was possible to identify aglycones in 12 of them by HPLC-qTOF-MS. All peaks selected as glycosidic aroma precursor were identified taken as a reference the phenyl β-D-glucopyranoside spectrum at 195 nm (Figure S1), which showed maximum UV absorbance at 195, 210 and 266 nm. This compound ($C_{12}H_{16}O_6$, MW 256.25 g/mol) is commonly used as a standard in the analysis of glycosidic aroma precursors [7]. In all cases, the results indicated that there was a significant amount of absorption in the UV area. Specifically, the maximum absorption ranged from 190 to 230 nm, which could be attributed with sugars of the molecule by due to the similarity with their UV spectrum (Figure S2). Moreover, a shoulder was observed around 200–360 nm, which could be attributed to the aglycone.

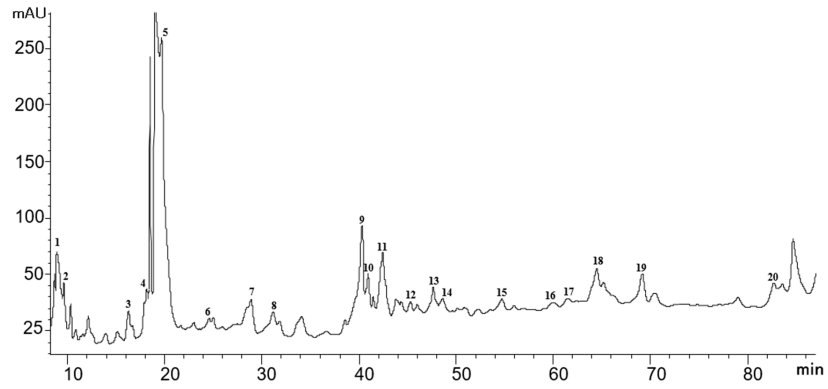


Figure 2. High-performance liquid chromatography with a diode array detector (HPLC-DAD) chromatogram at 195 nm with peaks tentatively selected as intact glycosidic aroma precursors in Muscat of Alexandria grapes.

As seen in Figure 3, at 195 nm, it is possible to obtain a chromatogram in which glycosides are tentatively selected, when compared to the reference standard, but not all responses were similar, depending on the molar absorptivity at each specific wavelength. However, in the absence of a standard to perform correct quantification, the response can be approximated with the standard used.

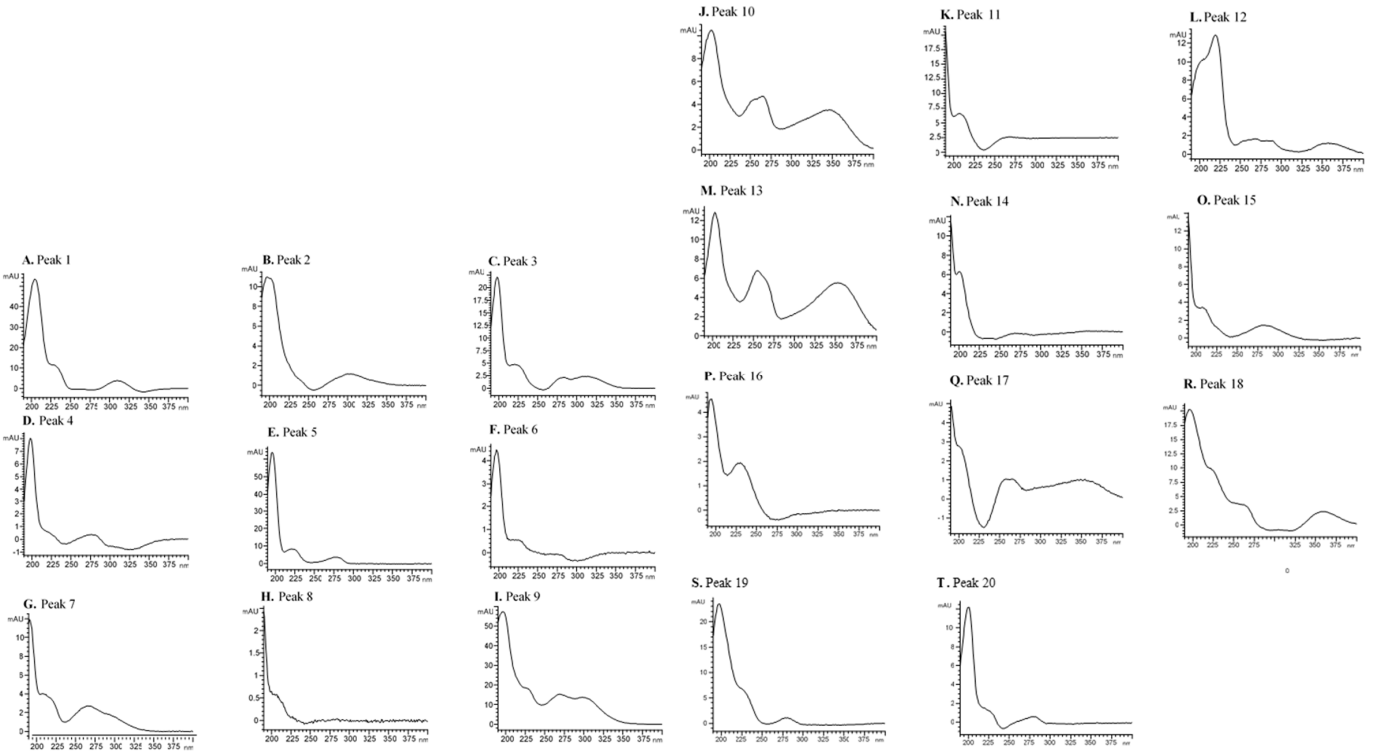


Figure 3. Intact glycosylated aroma precursor spectra (195 nm) of Muscat of Alexandria (peaks A-T). (**A**) Peak 1: C4 alcohol, RT 8.90. (**B**) Peak 2: Undefined glycoside 1, RT 9.74. (**C**) Peak 3: Undefined glycoside 2, RT 16.33. (**D**) Peak 4: C6 alcohol, RT 17.96. (**E**) Peak 5: Undefined glycoside 3, RT 19.68. (**F**) Peak 6: C6 alcohol, RT 24.49. (**G**) Peak 7: Undefined glycoside 4, RT 29.07. (**H**) Peak 8: Undefined glycoside 5, RT 29.07. (**I**) Peak 9: Dihydromonoterpentriol. RT 40.41. (**J**) Peak 10: Norisoprenoid. RT 41.43. (**K**) Peak 11: Undefined glycoside 6, RT 42.51. (**L**) Peak 12: Undefined glycoside 7, RT 45.29. (**M**) Peak 13: Monoterpendiol, RT 47.71. (**N**) Peak 14: Monoterpenetriol, RT 48.64. (**O**) Peak 15: Monoterpendiol, RT 54.89. (**P**) Peak 16: Monoterpendiol, RT 59.97. (**Q**) Peak 17: Monoterpendiol, RT 61.55. (**R**) Peak 18: Monoterpendiol, RT 64.47. (**S**) Peak 19: Monoterpendiol, RT 69.21. (**T**) Peak 20: Undefined glycoside, RT 82.69.

To the identification of these peaks with the glycosylated precursors, MS/MS fragmentation at three different collision energies (10 and 40 eV) obtained by HPLC-qTOF MS/MS was studied (Table 1), taking as a reference the retention time of the HPLC-DAD chromatogram ($\pm$0.5 min). Peaks were confirmed as glycosidic aroma precursors by means of MS/MS fragmentation of sugar rings or sequential loss of sugars or aglycones from glycosides, and when possible aglycones were identified. The existence of sugars was confirmed by the presence of most or all of the following masses (m/z), associated with sugar ring fragments [2,18,34]: 59.0139, 71.0139, 73.0295, 85.0295, 89.0244, 101.0244, 113.0244, 115.0401, 119.0350, 125.0244 and 143.0350. Notably, according to Caffrey et al. (2020) [34], the specific sugar fragment m/z values included 131.0350 and 149.0455 for pentose sugars, 161.0455 and 179.0561 for hexose sugars and 145.0506 and 163.0612 for deoxyhexose sugars.

Table 1. Intact glycosidic aroma precursors tentatively identified by HPLC-qTOF-MS/MS and the correspondent area peaks by HPLC-DAD in Muscat of Alexandria grapes.

Peak Number	*RT*	*Compound Name*				*ESI MS/MS*			*DAD*	
		Sugars	**Aglycone**	**Formula**	**Exact Mass**	***m/z* [M-H]$^-$**	***m/z* Experimental [M-H]$^-$**	**MS/MS Products Ions**	**λ Max (UV)**	**Peak Area (λ195)**
1	8.90	Pentose Hexose Hydroxy	C4 alcohol	$C_{15}H_{28}O_{11}$	384.1632	383.1554	383.1559	59.0135; 71.0131; 73.0288; 89.0234; 101.0236; 113.0236; 119.0492; 149.0082; 161.0439; 179.054; 251.1123; 383.1552	205; 231; 312	753 ± 41
2	9.74	Hexose	Undefined aglycone 1	NF	NF	NF	NF	59.0135; 71.0132; 73.0288; 89.0235; 101.0237; 113.0237; 161.0439; 163.0392; 179.0536	196; 297	240 ± 15
3	16.33	Hexose	Undefined aglycone 2	NF	NF	NF	NF	59.0139; 71.0135; 73.0292; 89.0238; 101.0241; 113.024; 119.0343; 161.0444; 366.1191; 443.193; 444.1957	198; 218; 284; 313	575 ± 49
4	17.96	Hexose Hexose Dehydro	C6 alcohol	$C_{18}H_{32}O_{11}$	424.1945	423.1867	423.1872	59.0139; 71.0136; 73.0292; 89.0239; 101.0241; 113.024; 119.0348; 125.024; 161.0445; 179.0551; 243.1229; 261.1337; 423.1856	198; 221; 276	406 ± 42
5	19.68	Hexose	Undefined aglycone 3	NF	NF	NF	NF	59.014; 71.0137; 73.0294; 89.0239; 101.0242; 119.0347; 161.0446; 179.0552; 287.1496; 403.1604	196;220;276	8415 ± 545
6	24.49	Hexose Hexose Dehydro	C6 alcohol	$C_{18}H_{32}O_{11}$	424.1945	423.1867	423.1872	59.0139; 71.0137; 73.0294; 89.024; 101.0242; 113.0242; 119.0346; 161.0446; 179.0556; 261.1335; 423.1868	197; 224	229 ± 22
7	29.07	Pentose Hexose	Undefined aglycone 4	NF	NF	NF	NF	59.0141; 71.0139; 89.0241; 101.0244; 119.0347; 131.0347; 149.045; 161.0449; 179.0557; 347.1169; 359.1346; 429.2129	191; 265	845 ± 73

Table 1. *Cont.*

Peak Number	*RT*	*Compound Name*			*ESI MS/MS*				*DAD*	
		Sugars	**Aglycone**	**Formula**	**Exact Mass**	***m/z* [M-H]$^-$**	***m/z* Experimental [M-H]$^-$**	**MS/MS Products Ions**	**λ Max (UV)**	**Peak Area (λ195)**
	8	33.58	Pentose Hexose	Undefined aglycone 5	NF	NF	NF	NF	59.0142; 71.0139; 73.0295; 89.0242; 101.0245; 113.0245; 119.035; 131.035; 149.0451; 161.045; 179.0559; 193.0501; 379.1607	190; 207	265 ± 26
	9	40.41	Pentose Hexose	Dihydromonoterpentriol	$C_{21}H_{38}O_{12}$	482.2363	481.2285	481.2291	59.0142; 71.0139; 89.0243; 101.0245; 113.0245; 119.0348; 131.0348; 143.0346; 149.0451; 161.0449; 179.0556; 481.2283;	197; 228; 269; 298	651 ± 45
	10	41.43	Pentose Hexose	Norisoprenoid	$C_{24}H_{38}O_{12}$	518.2363	517.2285	517.2291	59.0143; 71.0140; 73.0296; 89.0243; 101.0245; 113.0245; 119.0348; 125.0244; 143.0345; 149.0453; 161.0449; 179.0554; 205.1228; 223.1332; 385.1870; 517.2283	202; 257; 264; 345	111.93 ± 8
	11	42.51	Pentose Hexose	Undefined aglycone 6	NF	NF	NF	NF	59.0142; 71,014; 73,0295; 89,0243; 101,0245; 113,0245; 119,0349; 143,0345; 149,0448; 161,0449; 287,1495; 449,2027	190; 202	961 ± 56
	12	45.29	Pentose Hexose	Undefined aglycone 7	NF	NF	NF	NF	59,0143; 71,014; 73,0296; 89,0243; 101,0246; 113,0245; 119,035; 149,0452; 161,045; 179,0558; 287,1499; 449,2031	220; 267; 359	195 ± 15
	13	47.71	Pentose Hexose	Monoterpendiol	$C_{21}H_{36}O_{11}$	464.2258	463.218	463.2175	59.0143; 71.0140; 89.0243; 101.0246; 113.0245; 119.0349; 131.0351; 149.0451; 161.0450; 179.0557; 463.2175	203; 254; 352	339 ± 36

Table 1. *Cont.*

Peak Number	*RT*	*Compound Name*			*ESI MS/MS*				*DAD*	
		Sugars	**Aglycone**	**Formula**	**Exact Mass**	***m/z* [M-H]$^-$**	***m/z* Experimental [M-H]$^-$**	**MS/MS Products Ions**	**λ Max (UV)**	**Peak Area (λ195)**
14	48.64	Hexose Dehydro	Monoterpene triol	$C_{16}H_{26}O_8$	346.1628	345.155	345.1555	59.0143; 71.0140; 73.0296; 89.0243; 101.0246; 113.0245; 119.0348; 131.0345; 143.0346; 149.0449; 161.0449; 179.0555; 183.1020; 345.1559	190; 200	267 ± 22
15	54.89	Pentose Hexose	Monoter-pendiol	$C_{21}H_{36}O_{11}$	464.2258	463.2258	463.2185	59.0143; 71.014; 89.0243; 101.0246; 113.0246; 119.0351; 131.0348; 149.0451; 161.045; 179.0558; 293.0875 *; 331.1755 *; 463.2185	190; 205; 282	387 ± 29
16	59.97	Pentose Hexose	Monoter-pendiol	$C_{21}H_{36}O_{11}$	464.2258	463.218	463.2185	59.0143; 71.0141; 89.0244; 101.0247; 113.0247; 119.0351; 125.0246; 131.0353; 149.0453; 161.045; 179.0557; 293.0877 *; 331.1756 *; 463.2185	193; 231	239 ± 19
17	61.55	Pentose Hexose	Monoter-pendiol	$C_{21}H_{36}O_{11}$	464.2258	463.218	463.2186	59.0144; 71.0141; 89.0244; 101.0247; 113.0247; 119.0351; 131.035; 143.0348; 149.0453; 161.045; 163.0613; 179.0558; 293.0875 *; 331.1754 *; 463.2185	190; 204	208 ± 21
18	64.47	Pentose Hexose	Monoter-pendiol	$C_{21}H_{36}O_{11}$	464.2258	463.218	463.2185	59.0143; 71.0141; 73.0297; 89.0244; 101.0246; 113.0249; 119.0352; 131.0347; 143.0347; 149.0451; 161.0450; 179.0556; 331.1754 *; 463.2185	196; 223; 262; 358	763 ± 59
19	69.21	Pentose Hexose	Monoter-pendiol	$C_{21}H_{36}O_{11}$	NF	NF	463.2185	59.0144; 89.0244; 101.0248; 119.0357; 161.0449; 179.0555; 331.1755 *; 463.219	197; 228; 280	848 ± 89

Table 1. *Cont.*

Peak Number	*RT*	*Compound Name*			*ESI MS/MS*				*DAD*	
		Sugars	**Aglycone**	**Formula**	**Exact Mass**	***m/z* [M-H]⁻**	***m/z* Experimental [M-H]⁻**	**MS/MS Products Ions**	**λ Max (UV)**	**Peak Area (λ195)**
20	82.69	Pentose Hexose Deoxyhexose	Undefined aglycone 8	NF	NF	NF	NF	59.0144; 73.0297; 89.0244; 101.0248; 119.0353; 149.0452; 161.0451; 163.0613; 179.0557; 301.1653	200; 226; 279	618 ± 62

* MS/MS sugar of aglycone loss; RT: Retention time (min); NF: nof found; peak area: mean value ($n = 4$) ± standard deviation.

3.3.1. Monoterpenediol Glycosides

Monoterpenediols are the polyhydroxylated form of monoterpenes ($C_{10}H_{18}O$, MW 170.2487 g/mol). In the studied grapes, six monoterpenediol hexose-pentose glycosides (MW 464.2258 g/mol) were selected (peak numbers 13, 15, 16, 18 and 19; Table 1). These compounds were recognised and confirmed using MS/MS analysis in the presence of $[M\text{-}H]^-$ ions with m/z 463.2175. The first identification was performed in the presence of ions m/z 331.1755 and 293.0875 in the MS/MS spectra, corresponding to the neutral loss of a terminal pentose and aglycone unit, according to Caffrey et al. (2020) [34] and Godshaw et al. (2019) [18]. Moreover, the presence of both sugars was confirmed by their representative fragments: m/z 131.0344 and 149.0450 for the pentose sugar and m/z 161.045 and 179.0556 for the hexose sugar.

All compounds of this group appeared between 47 and 69 min on the chromatogram (Figure 2), at which point the elution gradient for acetonitrile ranged from 10% to 12%. The most abundant monoterpenediol glycoside appeared at 69.21 min (peak 19; Figure 2), with a DAD spectrum maximum at 197, 228 and 280 nm (Figure 3S). The second important monoterpenediol appeared at 64.47 min (peak 18; Figure 2), with shoulder wavelength absorbance at 196, 223, 262 and 358 nm (Figure 3R). Peak 15 (54.89 min; Figure 2) was the next abundant monoterpenediol, with a higher absorbance at 190, 205 and 282 nm (Figure 3O). Several researchers have pointed out that the most abundant monoterpenediols in Muscat of Alexandria grapes are 8-hydroxylinalool, 2,6-dimethyl-3,7-octadiene-2,6-diol, 3,7-dimethyl-1,5-octadiene-3,7-diol, 3,7-dimethyl-1,7-octadiene-3,6-diol, 8-hydroxynerol and 8-hydroxygeraniol [16,35–37]. Therefore, it could be associated with aglycone of the more important monoterpenediols identified above. Although these compounds make no direct contribution to aroma, they may break down to yield volatile compounds with a pleasant aroma, such as hotrienol and linalool, whose precursors are 3,7-dimethyl-1,5-octadiene-3,7-diol glycoside and 8-hydroxylinalool, respectively [37]. These compounds, along with other monoterpenes, such as geraniol, have been described as two of the most important compounds in wine, which contribute to the varietal characteristics of wine thanks to their flowery and sweet aroma nature [36,38].

3.3.2. Monoterpenetriol Glycosides

One possible dihydromonoterpenetriol hexose pentose (MW 482.2363 g/mol) was selected in the studied grapes (peak 9; Table 1). Putative identification was performed in the presence of pentose (m/z 131.0344 and 249.0450) and hexose (m/z 161.045 and 179.0556) moieties and $[M\text{-}H]^-$ ions with m/z 463.2175. Although no aglycone fragments were found in the fragmentation spectra, with no discernible fragments that can unequivocally identify this glycosidic compound, this is the only compound in our database with this parent ion. In the HPLC-DAD chromatogram, however, it was identified at 40.41 min (Figure 2), with 10% acetonitrile in the gradient. Notably, the spectrum of this compound showed three maxima at 197, 269 and 298 nm and a shoulder at 228 nm (Figure 3I). According to Caffrey et al. (2020) [34], the dihydromonoterpenetriol aglycone of this compound may be dihydroxy-dihydrolinalool (triol).

One monoterpenetriol hexose dehydro (MW 346.1628 g/mol) peak was also found (peak 14; Table 1). This compound was tentatively identified by the presence of an $[M\text{-}H]^-$ ion parent with m/z 345.1628 and MS/MS product ion 183.1020, in line with the fragmentation proposed by Caffrey et al. (2020) [34]. The retention time in HPLC-DAD was 48.64 min (Figure 2), with an elution gradient similar to what has previously been used (i.e., monoterpenetriol). However, in this case, the DAD spectrum only showed two maximum absorbance values at 190 and 200 nm (Figure 3N).

3.3.3. Norisoprenoid Glycosides

Norisoprenoids are a group of aroma compounds that originate from the oxidative degradation of carotenoids and contribute to the varietal characteristics of many types of wine, especially to aromatic varieties. In this study, peak 10 on the chromatogram (Figure 2)

was correlated to a norisoprenoid (Group E) hexose pentose (MW 518.2363 g/mol) [34]. Similar to previously described glycosidic aroma precursors, identification was performed using a parent ion with m/z 517.2363 and product ions of MS/MS fragmentation m/z 205.1228, m/z 223.1332 and m/z 385.187, consistent with the fragmentation of Caffrey et al. (2020) [34]. Moreover, the presence of both sugars was confirmed by their representative fragments: m/z 149.0450 for the pentose sugar and m/z 161.045 and 179.0556 for the hexose sugar.

The retention time in the HPLC-DAD chromatogram was 41.43 min (Figure 2), with an elution gradient between 9% and 10% for acetonitrile. The DAD spectrum showed higher absorbance at 202, 257, 264 and 345 nm (Figure 3J). The aglycone of the detected norisoprenoid belonged to Group E of the above-mentioned glycosides, with 3,4-dihydroxy-β-ionone and 3-hydroxy-5,6-epoxy-β-ionone being the most abundant in the variety under study, which are associated with a pleasant flowery and fruity aroma.

3.3.4. Alcohol Glycosides

In total, three intact aliphatic alcohol glycosides were tentatively identified in this study. Notably, peak 1 on the chromatogram (Figure 2) was identified as a C_4 alcohol pentose hexose hydroxy (MW 384.1632 g/mol) by the presence of $[M\text{-}H]^-$ ions with m/z 383.1550 and MS/MS product ions m/z 251.1136. This was the first glycosidic aroma precursor observed, which appeared with an elution gradient between 1% and 2.5% for acetonitrile, suggesting that it is the most polar compound among all the identified compounds. The absorbance peak of the spectrum was observed at 205 and 312 nm, as well as a shoulder at 231 nm (Figure 3A). The possible aglycone associated with it must have a molecular mass of 74.12 g/mol based on the number of carbons, it could be a C_4 alcohol such as isobutyl alcohol.

Peaks 4 and 6 were associated with C_6 alcohols hexose hexose dehydro (MW 424.1945 g/mol) by the presence of $[M\text{-}H]^-$ ions with m/z 423.1945 and neutral loss of sugar or aglycone with m/z 261.1335. The first peak showed maximum absorbance at 198 and 276 nm and a shoulder at 221 nm (Figure 3D), whereas the second one showed a higher spectrum at 197 and 224 nm (Figure 3F).

Aliphatic alcohols are commonly associated with negative characteristics in grapes and wine, such as vegetable or herbaceous aroma [39]. However, when these compounds are present in a glycosidic form, they have a lower impact on the aroma of grapes and wine.

3.3.5. Other Unconfirmed Peaks

In this study, DAD spectra corresponding to peaks 2, 3, 5, 7, 8, 11, 12 and 20 (Figure 2) as well as the subsequent association with MS/MS spectra were putatively identified as glycosidic conjugates, peak 5 being the most abundant of all those detected. Nevertheless, their MS/MS fragmentation was not associated with any of the groups of compounds described previously. Peaks 2, 3 and 5 were associated with monoglucosides as only characteristic fragmentation ions m/z 161.045 and 179.0556 for hexose were found. On the other hand, peaks 7, 8, 11 and 12 on the HPLC-DAD chromatogram were associated with disaccharides as, along with glucose ion fragmentation, ions m/z 131.0344 and 149.0450 for pentose were detected. Finally, peak 20 on the chromatogram was associated with a trisaccharide, pentose hexose deoxyhexose glycoside, because the fragmentation pattern was found to share the ion m/z 163.0613 that is associated with deoxyhexose.

The fact that there aren't commercial standard of glycosidic aroma precursors of grapes makes it difficult their quantification. However, the use of an internal standard such as, for example, the phenyl β-D-glucopyranoside used as a reference in this article, it could allow to make an approximate quantification of them.

4. Conclusions

In this study, an innovative method for analysing intact glycosidic aroma precursors in grapes was developed. In total, 20 glycosidic aroma precursors were selected using HPLC-

DAD and tentatively identified by HPLC-qTOF-MS/MS. Moreover, all interferences due to free sugars and polyphenols were resolved using LiChrolut EN and Oasis MCX cartridges, respectively. It was also found that disaccharide glycosides, mainly monoterpenediols and alcohols, are the most abundant form in Muscat of Alexandria grapes.

The results obtained in this study prove that it is possible to determine intact glycosidic aroma precursors in grapes by a widely used analytical technique as HPLC-DAD, making it useful in viticulture or oenology to study the impact of vineyard treatments on the aroma potential of grapes and wines.

Supplementary Materials: The following are available online at https://www.mdpi.com/2304-8158/10/1/191/s1, Figure S1: Diode array detector (DAD) spectra of phenyl β-D-glucopyranoside (195 nm), Figure S2: Diode array detector (DAD) spectra of D-glucose (maximum at 192 nm).

Author Contributions: C.C.-T.: Methodology, Writing and Investigation J.O.: Resources M.Á.C.: Resources G.L.A.: Conceptualization, Supervision, Project administration, Writing-Review M.R.S.: Conceptualization, Supervision, Project administration, Writing-review. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Junta de Comunidades of Castilla-La Mancha and the Ministry of Economy and Competitiveness/FEDER of the Spanish government for Project no. SBPLY/17/180501/000191.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Liu, J.; Zhu, X.-L.; Ullah, N.; Tao, Y.-S. Aroma Glycosides in Grapes and Wine. *J. Food Sci.* **2017**, *82*, 248–259. [CrossRef]
2. Hjelmeland, A.K.; Zweigenbaum, J.; Ebeler, S.E. Profiling monoterpenol glycoconjugation in Vitis vinifera L. cv. Muscat of Alexandria using a novel putative compound database approach, high resolution mass spectrometry and collision induced dissociation fragmentation analysis. *Anal. Chim. Acta* **2015**, *887*, 138–147. [CrossRef] [PubMed]
3. Martínez-Gil, A.M.; Pardo-García, A.I.; Zalacain, A.; Alonso, G.L.; Salinas, M.R. Lavandin hydrolat applications to Petit Verdot vineyards and their impact on their wine aroma compounds. *Food Res. Int.* **2013**, *53*, 391–402. [CrossRef]
4. Martínez-Gil, A.M.; Angenieux, M.; Pardo-García, A.I.; Alonso, G.L.; Ojeda, H.; Rosario Salinas, M. Glycosidic aroma precursors of Syrah and Chardonnay grapes after an oak extract application to the grapevines. *Food Chem.* **2013**, *138*, 956–965. [CrossRef] [PubMed]
5. Pardo-Garcia, A.I.; Wilkinson, K.L.; Culbert, J.A.; Lloyd, N.D.R.; Alonso, G.L.; Salinas, M.R. Accumulation of glycoconjugates of 3-methyl-4-hydroxyoctanoic acid in fruits, leaves, and shoots of Vitis vinifera cv. Monastrell following foliar applications of oak extract or oak lactone. *J. Agric. Food Chem.* **2015**, *63*, 4533–4538. [CrossRef] [PubMed]
6. Pardo-Garcia, A.I.; Wilkinson, K.L.; Culbert, J.A.; Lloyd, N.D.R.; Alonso, G.L.; Salinas, M.R. Accumulation of guaiacol glycoconjugates in fruit, leaves and shoots of Vitis vinifera cv. Monastrell following foliar applications of guaiacol or oak extract to grapevines. *Food Chem.* **2017**, *217*, 782–789. [CrossRef] [PubMed]
7. Salinas, M.R.; De La Hoz, K.S.; Zalacain, A.; Lara, J.F.; Garde-Cerdán, T. Analysis of red grape glycosidic aroma precursors by glycosyl glucose quantification. *Talanta* **2012**, *89*, 396–400. [CrossRef]
8. Dziadas, M.; Jeleń, H.H. Comparison of enzymatic and acid hydrolysis of bound flavor compounds in model system and grapes. *Food Chem.* **2016**, *190*, 412–418. [CrossRef]
9. Loscos, N.; Hernández-Orte, P.; Cacho, J.; Ferreira, V. Evolution of the aroma composition of wines supplemented with grape flavour precursors from different varietals during accelerated wine ageing. *Food Chem.* **2010**, *120*, 205–216. [CrossRef]
10. Alegre, Y.; Arias-Pérez, I.; Hernández-Orte, P.; Ferreira, V. Development of a new strategy for studying the aroma potential of winemaking grapes through the accelerated hydrolysis of phenolic and aromatic fractions (PAFs). *Food Res. Int.* **2020**, *127*, 108728. [CrossRef]
11. Hampel, D.; Robinson, A.L.; Johnson, A.J.; Ebeler, S.E. Direct hydrolysis and analysis of glycosidically bound aroma compounds in grapes and wines: Comparison of hydrolysis conditions and sample preparation methods. *Aust. J. Grape Wine Res.* **2014**, *20*, 361–377. [CrossRef]
12. Loscos, N.; Hernández-Orte, P.; Cacho, J.; Ferreira, V. Comparison of the suitability of different hydrolytic strategies to predict aroma potential of different grape varieties. *J. Agric. Food Chem.* **2009**, *57*, 2468–2480. [CrossRef] [PubMed]

13. Serrano de la Hoz, K. *Determinación de Precursores Aromáticos Glicosídicos de Uvas, Mostos y Vinos, Blancos y Tintos. Aplicación en Bodega*; Universidad de Castilla-La Mancha: Ciudad Real, Spain, 2014.
14. Nasi, A.; Ferranti, P.; Amato, S.; Chianese, L. Identification of free and bound volatile compounds as typicalness and authenticity markers of non-aromatic grapes and wines through a combined use of mass spectrometric techniques. *Food Chem.* **2008**, *110*, 762–768. [CrossRef]
15. Schievano, E.; D'Ambrosio, M.; Mazzaretto, I.; Ferrarini, R.; Magno, F.; Mammi, S.; Favaro, G. Identification of wine aroma precursors in Moscato Giallo grape juice: A nuclear magnetic resonance and liquid chromatography-mass spectrometry tandem study. *Talanta* **2013**, *116*, 841–851. [CrossRef] [PubMed]
16. Flamini, R.; De Rosso, M.; Panighel, A.; Dalla Vedova, A.; De Marchi, F.; Bavaresco, L. Profiling of grape monoterpene glycosides (aroma precursors) by ultra-high performanceliquid chromatography-high resolution mass spectrometry (UHPLC/QTOF). *J. Mass Spectrom.* **2014**, *49*, 1214–1222. [CrossRef]
17. Ghaste, M.; Narduzzi, L.; Carlin, S.; Vrhovsek, U.; Shulaev, V.; Mattivi, F. Chemical composition of volatile aroma metabolites and their glycosylated precursors that can uniquely differentiate individual grape cultivars. *Food Chem.* **2015**, *188*, 309–319. [CrossRef]
18. Godshaw, J.; Hjelmeland, A.K.; Zweigenbaum, J.; Ebeler, S.E. Changes in glycosylation patterns of monoterpenes during grape berry maturation in six cultivars of Vitis vinifera. *Food Chem.* **2019**, *297*. [CrossRef]
19. Lorenzo, C.; Serrano-Díaz, J.; Plaza, M.; Quintanilla, C.; Alonso, G.L. Fast methodology of analysing major steviol glycosides from Stevia rebaudiana leaves. *Food Chem.* **2014**, *157*, 518–523. [CrossRef]
20. Geuns, J.M.C.; Struyf, T.; Bartholomees, U.; Ceunen, S. *Analysis of Steviol Glycosides: Development of an Internal Standard and Validation of the Methods*; Nova Science Publishers, Inc.: Hauppauge, NY, USA, 2014; pp. 73–95. ISBN 9781634630849.
21. Serrano de la Hoz, K.; Carmona, M.; Zalacain, A.; Alonso, G.L.; Salinas, M.R. The varietal aroma potential index (IPAv): A tool to evaluate the quality of grapes and wines, white and red. In Proceedings of the 37th World Congress of Vine and Wine, Mendoza, Argentina, 9–14 November 2014.
22. Hernandez-Orte, P.; Concejero, B.; Astrain, J.; Lacau, B.; Cacho, J.; Ferreira, V. Influence of viticulture practices on grape aroma precursors and their relation with wine aroma. *J. Sci. Food Agric.* **2015**, *95*, 688–701. [CrossRef]
23. Jesús Ibarz, M.; Ferreira, V.; Hernández-Orte, P.; Loscos, N.; Cacho, J. Optimization and evaluation of a procedure for the gas chromatographic-mass spectrometric analysis of the aromas generated by fast acid hydrolysis of flavor precursors extracted from grapes. *J. Chromatogr. A* **2006**, *1116*, 217–229. [CrossRef]
24. López, R.; Aznar, M.; Cacho, J.; Ferreira, V. Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. *J. Chromatogr. A* **2002**, *966*, 167–177. [CrossRef]
25. Zhao, P.; Qian, Y.; He, F.; Li, H.; Qian, M. Comparative Characterization of Aroma Compounds in Merlot Wine by LiChrolut-EN-Based Aroma Extract Dilution Analysis and Odor Activity Value. *Chemosens. Percept.* **2017**, *10*, 149–160. [CrossRef]
26. Loscos, N.; Hernandez-Orte, P.; Cacho, J.; Ferreira, V. Release and formation of varietal aroma compounds during alcoholic fermentation from nonfloral grape odorless flavor precursors fractions. *J. Agric. Food Chem.* **2007**, *55*, 6674–6684. [CrossRef] [PubMed]
27. López, P.; Batlle, R.; Nerín, C.; Cacho, J.; Ferreira, V. Use of new generation poly(styrene-divinylbenzene) resins for gas-phase trapping-thermal desorption. Application to the retention of seven volatile organic compounds. *J. Chromatogr. A* **2007**, *1139*, 36–44. [CrossRef] [PubMed]
28. Castillo-Muñoz, N.; Gómez-Alonso, S.; García-Romero, E.; Hermosín-Gutiérrez, I. Flavonol profiles of Vitis vinifera red grapes and their single-cultivar wines. *J. Agric. Food Chem.* **2007**, *55*, 992–1002. [CrossRef]
29. González-Manzano, S.; Santos-Buelga, C.; Pérez-Alonso, J.J.; Rivas-Gonzalo, J.C.; Escribano-Bailón, M.T. Characterization of the mean degree of polymerization of proanthocyanidins in red wines using Liquid Chromatography-Mass Spectrometry (LC-MS). *J. Agric. Food Chem.* **2006**, *54*, 4326–4332. [CrossRef] [PubMed]
30. Gómez Gallego, M.A.; Sánchez-Palomo, E.; Hermosín-Gutiérrez, I.; González Viñas, M.A. Polyphenolic composition of Spanish red wines made from Spanish Vitis vinifera L. red grape varieties in danger of extinction. *Eur. Food Res. Technol.* **2013**, *236*, 647–658. [CrossRef]
31. Sepúlveda, L.; Romaní, A.; Aguilar, C.N.; Teixeira, J. Valorization of pineapple waste for the extraction of bioactive compounds and glycosides using autohydrolysis. *Innov. Food Sci. Emerg. Technol.* **2018**, *47*, 38–45. [CrossRef]
32. Williams, S. Ghost peaks in reversed-phase gradient HPLC: A review and update. *J. Chromatogr. A* **2004**, *1052*, 1–11. [CrossRef]
33. Grob, R.L.; Cao, K.B. Acetonitrile-Phosphate Buffer System under Gradient Condition. *J. Environ. Sci. Health Part A Environ. Sci. Eng. Toxicol.* **1990**, *25*, 167–183. [CrossRef]
34. Caffrey, A.J.; Lerno, L.A.; Zweigenbaum, J.; Ebeler, S.E. Direct Analysis of Glycosidic Aroma Precursors Containing Multiple Aglycone Classes in Vitis vinifera Berries. *J. Agric. Food Chem.* **2020**, *68*, 3817–3833. [CrossRef] [PubMed]
35. Corona, O.; Planeta, D.; Bambina, P.; Giacosa, S.; Paissoni, M.A.; Squadrito, M.; Torchio, F.; Segade, S.R.; Cinquanta, L.; Gerbi, V.; et al. Influence of different dehydration levels on volatile profiles, phenolic contents and skin hardness of alkaline pre-treated grapes cv Muscat of alexandria (Vitis vinifera L.). *Foods* **2020**, *9*, 666. [CrossRef] [PubMed]
36. Darriet, P.; Thibon, C.; Dubourdieu, D. Aroma and Aroma Precursors in Grape Berry. *Biochem. Grape Berry* **2012**, 111–136. [CrossRef]

37. Rocha, S.M.; Coelho, E.; Zrostlíková, J.; Delgadillo, I.; Coimbra, M.A. Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry of monoterpenoids as a powerful tool for grape origin traceability. *J. Chromatogr. A* **2007**, *1161*, 292–299. [CrossRef] [PubMed]
38. Salinas, M.R.; Alonso, G.L.; Pardo, F.; Bayonove, C. Free and bound volatiles of Monastrell wines. *Sci. Aliment.* **1998**, *18*, 223–231.
39. Canosa, P.; Oliveira, J.M.; Masa, A.; Vilanova, M. Study of the volatile and glycosidically bound compounds of minority Vitis vinifera red cultivars from NW Spain. *J. Inst. Brew.* **2011**, *117*, 462–471. [CrossRef]

foods

MDPI

Article

HPLC-DAD-MS and Antioxidant Profile of Fractions from Amontillado Sherry Wine Obtained Using High-Speed Counter-Current Chromatography

Mónica Schwarz [1,2], Fabian Weber [3], Enrique Durán-Guerrero [4,*], Remedios Castro [4], María del Carmen Rodríguez-Dodero [4], Maria Valme García-Moreno [4], Peter Winterhalter [5] and Dominico Guillén-Sánchez [4]

1 "Salus Infirmorum" Faculty of Nursing, University of Cadiz, 11001 Cadiz, Spain; monica.schwarz@uca.es
2 Nutrition and Bromatology Area, Faculty of Medicine, University of Cadiz, Plaza Falla, 9, 11003 Cadiz, Spain
3 Institute of Nutritional and Food Sciences, Molecular Food Technology, University of Bonn, Endenicher Allee 19b, D-53115 Bonn, Germany; fabian.weber@uni-bonn.de
4 Analytical Chemistry Department, Faculty of Sciences-IVAGRO, University of Cadiz, Agrifood Campus of International Excellence (ceiA3), Pol. Río San Pedro, s/n, Puerto Real, 11510 Cadiz, Spain; remedios.castro@uca.es (R.C.); maricarmen.dodero@uca.es (M.d.C.R.-D.); valme.garcia@uca.es (M.V.G.-M.); dominico.guillen@uca.es (D.G.-S.)
5 Institute of Food Chemistry, Technische Universität Braunschweig, Schleinitzstrasse 20, 38106 Braunschweig, Germany; p.winterhalter@tu-braunschweig.de
* Correspondence: enrique.duranguerrero@uca.es; Tel.: +34-956-016-456

Abstract: In the present work, the polyphenolic profile of a complex matrix such as Amontillado sherry has been processed by means of high-speed counter-current chromatography (HSCCC) and characterized by HPLC-DAD-MS. An Amberlite XAD-7 column was used to obtain the wine extract, and three different biphasic solvent systems were applied for HSCCC separation: MTBE (methyl *tert*-butyl ether)/n-butanol/acetonitrile/water (1.1/3/1.1/5+0.1% trifluoroacetic acid), MTBE/n-butanol/acetonitrile/water (2/2/1/5), and hexane/ethyl acetate/ethanol/water (1/5/1/5). As a result, 42 phenolic compounds and furanic derivatives have been identified by means of HPLC-DAD-MS, with 11 of them being identified for the first time in Sherry wines: 3-feruloylquinic acid, isovanillin, ethyl vanillate, furoic acid, dihydro-*p*-coumaric acid, 6-O-feruloylglucose, ethyl gallate, hydroxytyrosol, methyl protocatechuate, homoveratric acid and veratraldehyde. In addition, the antioxidant capacity (ABTS) of the obtained fractions was determined, revealing higher values in those fractions in which compounds such as gallic acid, protocatechuic acid, protocatechualdehyde, *trans*-caftaric acid, syringic acid, isovanillin or tyrosol, among others, were present. This is the first time that HSCCC has been used to characterize the phenolic composition of Sherry wines.

Keywords: HSCCC; sherry wine; Amontillado; phenolic compounds; antioxidant activity

Citation: Schwarz, M.; Weber, F.; Durán-Guerrero, E.; Castro, R.; Rodríguez-Dodero, M.d.C.; García-Moreno, M.V.; Winterhalter, P.; Guillén-Sánchez, D. HPLC-DAD-MS and Antioxidant Profile of Fractions from Amontillado Sherry Wine Obtained Using High-Speed Counter-Current Chromatography. *Foods* **2021**, *10*, 131. https://doi.org/10.3390/foods10010131

Received: 15 December 2020
Accepted: 6 January 2021
Published: 9 January 2021

1. Introduction

Sherry wines, i.e., Finos, Amontillados and Olorosos, are famous throughout the world. From a legal point of view, the denomination of Sherry wines should only be used for wines produced under the Denomination of Origin Jerez-Xérès-Sherry, in the Southwest of Spain. These are wines that come from the same grape variety Palomino Fino, and that, after being pressed to different degrees, undergo different aging conditions [1]. The different degree of pressing during winemaking already determines the composition of the initial must with regard to many of its constituents, especially polyphenolic compounds. It is known that polyphenols possess various biological activities that may vary according to their structure [2]. The high structural diversity and complexity of the polyphenolic fraction, however, make it difficult to separate individual compounds and to study them in more detail. As the structural diversity is even increasing during the subsequent

wine ageing process, novel analytical approaches such as, e.g., the preparative all-liquid chromatographic technique of countercurrent chromatography are required to fractionate the complex polyphenolic mixture to ensure a subsequent structure elucidation by HPLC-MS analysis.

Finos are wines that age under reducing conditions caused by the layer of yeast, known as veil of *flor*, which protects them from environmental oxygen and gives rise to a lower alcohol content compared to Olorosos and Amontillados wines. Olorosos are aged in American oak barrels under oxidizing conditions and with an alcohol content of around 18% alcohol, while Amontillados go through a period of biological ageing and are then fortified to 17–18% alcohol and subjected to oxidative ageing. All of them spend a fixed period of time in the cask according to a dynamic system known as *criaderas y solera*.

As can be seen from the above description, Amontillado is a very peculiar wine, with a phase of biological ageing and another later phase of oxidative ageing. Its phenolic composition must therefore be intermediate between Fino and Oloroso wines. In 1986, Estrella et al. [3] identified different low molecular weight phenols and other compounds in Fino, Oloroso and Amontillado Sherry wines. Benzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic and gentisic), cinnamic acids (caffeic, *p*-hydroxycinnamic and ferulic), phenolic aldehydes (*p*-hydroxybenzaldehyde, *p*-vanillinic, syringaldehyde and protocatechualdehyde), and the coumarins esculetin and scopoletin were among the compounds identified by them. Later, in 1996, Guillén et al. [4], after developing the method of automatic extraction by SPE of polyphenols from sherry wines, proceeded to determine them by HPLC. Their results confirmed this intermediate composition of the Amontillado wines compared to Fino and Oloroso ones in terms of polyphenolic content. Thirteen benzoic and cinnamic acids together with some aldehydes (protocatechualdehyde, vanillin, syringaldehyde, *p*-hydroxybenzaldehyde) and flavanols (catechin) were found in Amontillado wines by Guillén et al., highlighting the content of gallic acid, *trans*-*p*-coutaric acid and protocatechuic acid [4]. Furthermore, in 2002, García-Moreno and García-Barroso studied the evolution of the phenolic compounds in the three most important types of Sherry wines. In addition to those already found in previous works, they identified hydroxymethylfurfural (HMF) and tyrosol in samples of Amontillado wines [5]. More recently, Ortega et al. [6] studied the evolution of Olorosos' polyphenolic content during their oxidative aging at different temperatures and observed that fluctuating temperatures caused the wines to age more rapidly with a greater increment in polyphenolic content. Flavanols were the polyphenolic fractions that appeared in the Olorosos studied in the highest concentration, and the (+)-catechin and procyanidin B1 contents were the most important. Other compounds, such as syringaldehyde and vanillin, showed clear gains as ageing time was increased.

Regarding Finos, it seems that the most abundant polyphenols are benzoic acid derivatives derived from the barrels' lignin and from the deamination of the nitrogen compounds generated during the autolysis of the *flor* yeast [7]. It has been observed that with increasing ageing time the polyphenolic profile of Amontillado becomes closer to that of Oloroso than to Fino's, with some aldehydes such as vanillin and *p*-hydroxybenzaldehyde increasing along with ageing time [5]. In this sense, López de Lerma et al. [8] failed to use an electronic nose to differentiate Amontillados from Olorosos, although the differentiation was complete with respect to Finos, both for the young and the sweet varieties.

As it can be seen from the scarce background presented above, the polyphenolic profile of this particular Sherry wine, Amontillado, has been barely studied until now. Therefore, in this work, we intend to develop a simple and effective method to isolate and identify simultaneously, and at a preparative scale, the different polyphenolic compounds present in Amontillado wine while considering the requirements for quantitative analysis of the bioactive compounds. For this purpose, we are proposing the use of high-speed counter-current chromatography (HSCCC) together with off-line analysis by HPLC-ESI-MS. Counter-current chromatography is a chromatographic technique that separates solutes by their different distribution coefficients between two immiscible solvents. This type

of chromatography presents multiple advantages over other techniques. Among these, we would like to mention the following: high efficiency to separate and isolate bioactive natural products in short elution periods [9]; high recovery of injected samples, as no chromatographic column is required and, hence, irreversible adsorption does not occur [10]; high versatility of the application range; and high repeatability. In addition to these advantages, the simpler polyphenolic fractions that can be obtained allow us to evaluate their antioxidant activity and relate them with the identified polyphenols. This is a frequent application of HSCCC [11].

HSCCC has been widely used for separating bioactive compounds in numerous food matrices [12–14]. In the oenological field, there are also many studies that have used HSCCC. Thus, it has been successfully applied to the fractioning and separation of polyphenols in red wine [15]. Seventeen polyphenolic compounds including phenolic acids, catechins, proanthocyanidins and anthocyanins have been separated with high purity levels and large yields. In addition, Weber and Winterhalter [16], isolated and identified different anthocyanin oligomers by HSCCC and NMR, while Noguer et al. [17] investigated the antioxidant properties of different fractions obtained by HSCCC. This technique has also been successfully applied to identify and isolate polyphenolic compounds in grape skins and seeds [18], white wine [19,20] and rum [21], among other matrices.

The main objective of the present work is to characterize the polyphenolic compounds and furanic derivatives in a complex matrix such as Amontillado sherry by obtaining simpler fractions through HSCCC, and also by evaluating their antioxidant activity. To date, this is the first time that this technique has been applied to Sherry wine.

2. Materials and Methods

2.1. Reagents

For the preparation of the extracts for HSCCC separation, the following analytical grade solvents were used: methanol, ethyl acetate, ethyl methyl ketone, MTBE (methyl *tert*-butyl ether), n-butanol, acetonitrile, and ethanol, which were acidified with either acetic acid, formic acid, or trifluoroacetic acid, all of which were purchased from Merck (Darmstadt, Germany).

For the HPLC–DAD–MS analyses, HPLC gradient grade methanol, acetic acid and water (Merck) were used. De-ionized water was purified by means of a Milli-Q system provided by Millipore (Bedford, MA, USA). The reference standards were purchased from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), and Sigma (St. Louis, MO, USA).

To determine the extracts' antioxidant activity, a saturated solution of $Zn(CH_3COO)_2$ (Panreac, Barcelona, Spain) and a solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma–Aldrich, Madrid, Spain) were mixed in phosphate buffer medium (pH 6). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) supplied by Sigma–Aldrich was used for calibration.

2.2. Wine Samples

Samples of Amontillado wine (21.5% alcohol by volume), VORS (Vinum Optimum Rare Signatum) category with 30 years of mean ageing, supplied by González Byass S.A. winery (Jerez de la Frontera, Spain) were used for this research.

2.3. Preparation of the Extracts

Nine liters of Amontillado wine diluted in water (1:4) to minimize the effect of its high alcoholic degree were loaded onto an Amberlite XAD-7 column (100 cm × 7 cm) to eliminate proteins, organic acids, residual sugars, and minerals. The flow rate was approx. 10 mL/min. The Amberlite column was pre-conditioned with 2 L of water. The diluted sample was washed using 3 L of water and subsequently eluted with 2 L methanol acidified with acetic acid (19:1 *v/v*). The extract was concentrated with a rotary evaporator under vacuum and lyophilized to obtain a total of 11.2 g of XAD-7 extract.

In order to obtain less polar fractions, the XAD-7 extract was extracted with ethyl acetate by a liquid–liquid extraction. For that purpose, the lyophilized XAD-7 extract (2 g) was dissolved in 500 mL water and was extracted with ethyl acetate (1:1 *v/v*) in a separation funnel. The organic phase was removed and the extraction was repeated three times using 500 mL of ethyl acetate for each extraction. The process was carried out in duplicate and the pooled organic phases were concentrated by means of a rotary evaporator and lyophilized to obtain 900 mg of dry ethyl acetate extract. We were interested in less polar compounds, so we focussed on the ethyl acetate extract, and the water fraction was removed.

2.4. Selection of Two-Phases HSCCC Solvents Systems

Based on the literature data and preliminary experiments, different biphasic solvents systems were tested to perform the fractionation of the samples by HSCCC [22–24]. Preliminary experiments consisted on the visual estimation of the distribution of the extract within both phases (organic and aqueous). The intensity of color in both phases gave us an initial estimation of the suitability of the solvent systems. The systems tested were: hexane/ethyl acetate/methanol (1/1.2/1), MTBE (methyl *tert*-butyl ether)/n-butanol/acetonitrile/water (2/2/1/5), hexane/ethyl acetate/n-butanol/water (1/10/1/10), MTBE/n-butanol/acetonitrile/water (1/3/1/5), n-butanol/acetonitrile/water (4/1/5), MTBE/n-butanol/acetonitrile/water [1.1/3/1.1/5+0.1% trifluoroacetic acid (TFA)], MTBE/n-butanol/acetonitrile/water (1/3/1/5+0.1% TFA), MTBE/n-butanol/acetonitrile/water (0.5/3.5/1/5), MTBE/n-butanol/acetonitrile/water (1/4/1/5). For the ethyl acetate extract, the following solvent systems were tested: hexane/ethyl acetate/ethanol/water (1/5/1/5), ethyl acetate/ethanol/water (5/1/5), ethyl acetate/n-butanol/water (1/5/6), ethyl acetate/n-butanol/ethanol/water (30/6/10/50).

For this purpose, 5 mg from each extract were dissolved in 10 mL of the different systems tested at a 1:1 ratio of aqueous phase to organic phase [25]. Later, an aliquot from each phase was analyzed by liquid chromatography with diode array detection, and the signal was recorded at 280 nm in order to determine the one with the best partition coefficient.

2.5. Separation by Means of High-Speed Countercurrent Chromatography (HSCCC)

In the case of the XAD-7 extract, the solvent applied to HSCCC consisted on solvent system I: MTBE/n-butanol/acetonitrile/water (1.1/3/1.1/5+0.1% trifluoroacetic acid) and solvent system II: MTBE/n-butanol/acetonitrile/water (2/2/1/5). On the other hand, for the ethyl acetate extract, the following solvent system III was used: hexane/ethyl acetate/ethanol/water (1/5/1/5).

The HSCCC equipment used was a CCC-1000 by Pharma-Tech Research (Baltimore, MD, USA), together with a Biotronik HPLC BT3020 pump from Jasco (Pfungstadt, Germany). The separation was conducted at ambient temperature, at 850 rpm and at 3 mL min^{-1} flow rate, classical operation conditions for this type of CCC instrument. Based on the previous experience of the research group, the aqueous phase was used as mobile phase in the *head to tail* elution mode. The employment of *head to tail* mode with aqueous phase as mobile phase in HSCCC would be the equivalent of employing reversed phase elution mode in regular liquid chromatography. Previous studies have demonstrated this elution mode to be effective in the separation of polyphenols in wine matrixes [17]. A quantity of 800 mg of the extract was dissolved in 20 mL of a 1:1 mixture of the organic and aqueous phases and this was injected via a sample loop.

The separation was monitored at 280 nm, using a K-2501 detector (Knauer, Berlin, Germany). The fractions were collected using test tube racks and an LKB SuperFrac collector (Pharmacia, Bromma, Sweden). The thin layer chromatography (TLC) analyses of all the recovered HSCCC fractions were performed on 60 F254 Merck silica gel plates (Darmstadt, Germany), with an ethyl acetate/ethyl methyl ketone/formic acid/water elution system (4/3.5/1/1). After being developed, the detection was performed by UV detection and derivatization with *p*-anisaldehyde-sulfuric acid–glacial acetic acid reagent followed by heating up to 105 °C. The homogeneity of the final fractions was checked

with the help of the chromatograms and the TLC of the different fractions, which were subsequently characterized.

2.6. Characterisation of the Fractions Separated by HSCCC

The resulting fractions were analyzed by HPLC-DAD-MS, using a Waters 2695 equipment coupled to a Waters 2996 photodiode detector and to a Waters micromass ZQ mass spectrometer fitted with electrospray interface (ESI). The column was a Phenomenex Gemini C18 (250 × 2.0 mm, 5 µm particle size). Elution system: A solvent (3% methanol, 2% acetic acid, and 95% water) and B solvent (93% methanol, 2% acetic acid, and 5% water) under gradient conditions: 0 min 100% A, 5 min 95% A, 30 min 50% A, 40 min 50% A, 50 min 100% B. The column was re-equilibrated before the following injection. Prior to their injection into the HPLC equipment, the samples were filtered through a 0.22 µm pore-size membrane (Millipore, Burlington, MA, USA). The injection volume was 20 µL and the flow rate 0.2 mL min^{-1}. The following parameters were used for ESI-MS identification: positive and negative ionization modes with N_2 as drying gas at a flow of 11 mL min^{-1}, 250 °C drying temperature, 3500 V capillary voltage, and 15 V capillary exit. The m/z scanning range covered the 100–1000 uma interval. The injection volume was 20 µL.

2.7. Antioxidant Activity Determination

In order to determine the antioxidant activity of the fractions, 1 mg of each fraction was dissolved into 500 µL of methanol. The method used was a previously developed electrochemical method [26] consisting on electrochemical oxidation in a solution of ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid)) (50 M), to which the sample to be tested was added.

3. Results and Discussion

3.1. HSCCC Fractionation Using Different Solvent Systems

XAD-7 Amontillado extract (800 mg) was fractionated by HSCCC using the biphasic solvent system I, which consists on: MTBE/n-butanol/acetonitrile/water (1.1/3/1.1/5 *v/v/v/v*) acidified with 0.1% trifluoroacetic acid. Nine fractions were obtained, where fractions 6–9 were coil-fractions. In order to obtain simpler fractions, another solvent system was used. The second fractionation was carried out using the following solvent system II: MTBE/n-butanol/acetonitrile/water (2/2/1/5 *v/v/v/v*). After 12 h, the separation of 800 mg of XAD-7 Amontillado extract produced ten fractions in the normal elution mode and eight additional fractions in the extrusion mode. Upon injection of the solutes, the first stage in elution/extrusion CCC is a classical elution. In order to recover the compounds with a high affinity to the stationary phase, the extrusion mode is initiated by switching the solvent reservoir of the liquid pump system from mobile phase to stationary phase, i.e., making the formerly stationary phase the mobile liquid. As a consequence, the solutes located inside the column are subsequently pushed out of the column. The advantage of the elution/extrusion technique is that solutes cannot stay trapped in the column, i.e., a full sample recovery is achieved during an acceptable separation time [27]. The resulting fractions were simpler, but the coil-fractions still contained too many compounds. In order to simplify these fractions, the XAD-7 extract was re-extracted using ethyl acetate. A quantity of 700 mg of extract was employed and fractionated using solvent system III: hexane/ethyl acetate/methanol/water (1/5/1/5 *v/v/v/v*), and 12 fractions were obtained. Figure 1 shows the HSCCC fractionations obtained using the different solvent systems.

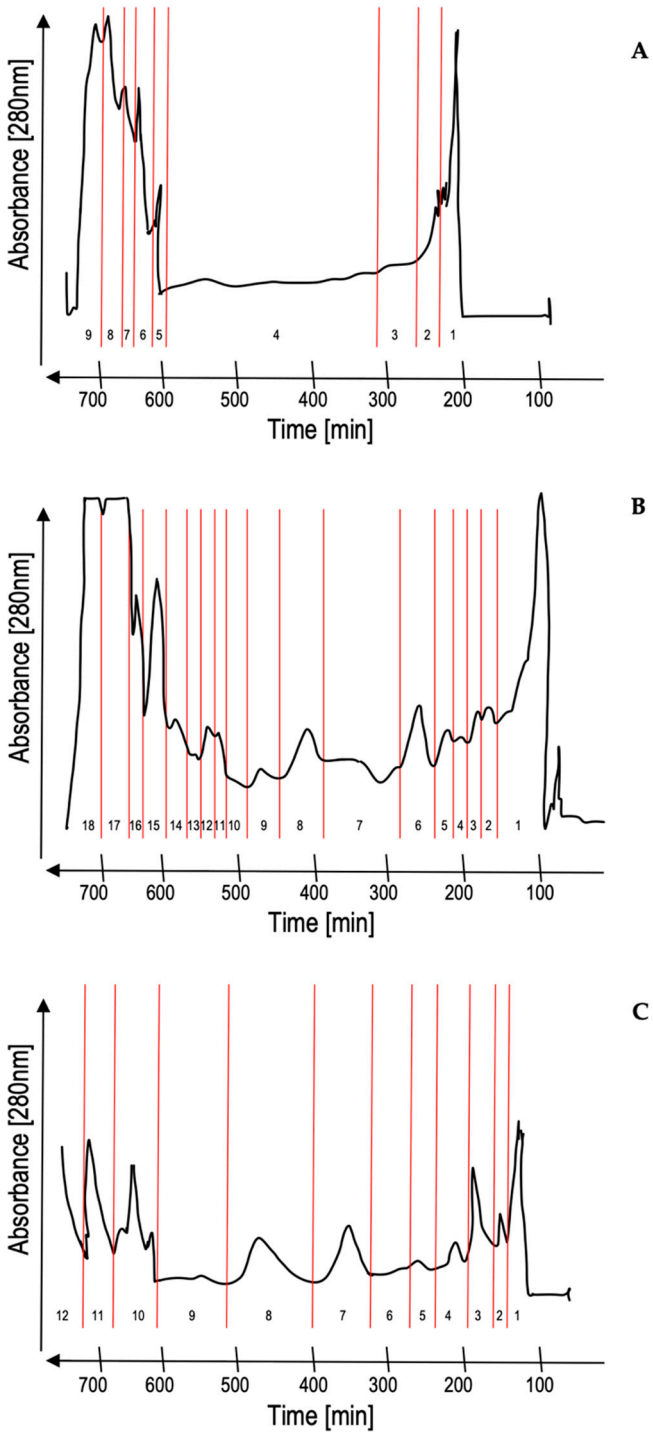


Figure 1. High-speed counter-current chromatography (HSCCC) fractionation obtained using the different solvent systems. (**A**)—solvent system I: MTBE (methyl *tert*-butyl ether)/n-butanol/acetonitrile/water (1.1/3/1.1/5+0.1% trifluoroacetic acid); (**B**)—solvent system II: MTBE/n-butanol/acetonitrile/water (2/2/1/5); (**C**)—solvent system III: hexane/ethyl acetate/methanol/water (1/5/1/5).

The quantities obtained in the three different fractionations after being concentrated and freeze-dried are shown in Table 1. The recovery of the sample from the three different fractionations was 50, 57 and 53%, respectively. The retained material was discharged and only the recovered sample was analyzed.

Table 1. Weight measured for the different fractions obtained from the different HSCCC solvent systems.

System I		System II		System III	
Fractions	**Weight (mg)**	**Fractions**	**Weight (mg)**	**Fractions**	**Weight (mg)**
F1	56	F1	75.3	F1	30
F2	32	F2	20.5	F2	13
F3	30	F3	8	F3	33
F4	22	F4	7.1	F4	14
F5	56.6	F5	8.4	F5	20
F6	20.9	F6	8	F6	30
F7	34.2	F7	7.2	F7	12.3
F8	52	F8	9.2	F8	50
F9	101.6	F9	5.9	F9	80
Total	405.3	F10	12.2	F10	24
		F11	8.5	F11	12.9
		F12	19.2	F12	58.5
		F13	12.5	Total	377.7
		F14	27		
		F15	17.3		
		F16	8.6		
		F17	112.5		
		F18	36.9		
		Total	404.3		

3.2. Identification of the Compounds in the Different Fractions Obtained by HSCCC

By analyzing the different fractions obtained by the HSCCC technique through HPLC-PDA-MS, a total of 42 phenolic compounds and furan derivatives were identified. The guidelines that were followed to carry out the identification of the phenolic compounds in the different fractions consisted of the acquisition of the UV-Vis and ESI-MS spectra, both in positive and negative mode, in order to ensure an identification of the molecular peak. Wherever reference compounds were available, the identification was confirmed by injecting the standard compound. In these cases, in addition to the corresponding MS and UV spectra, the retention time was also employed for the identification. For the identification of the remaining compounds, identification was carried out based on the mass spectrum, the UV-Vis spectrum, and the bibliographic data collected from the databases that are accessible on the Internet, such as Phenol-Explorer (Rothwell JA, 2015), PubChem, (PubChem, 2020), SpectraBaseTM (John Wiley & Sons, Inc., 2020), and ChemSpider (Royal Society of Chemistry, 2020). Table 2 shows the identifications that have been completed.

All the compounds identified, obtained with the different solvent systems, are reported in Figure 2. Only the chromatograms of the fractions in which any compound was identified have been presented. In the rest of the fractions, a very scarce number of compounds was present and no identification was carried out.

By comparing the MS and UV spectra and retention time of reference compounds, where available, 21 phenolic compounds and furan derivatives were successfully identified (Table 2). Thus, different hydroxybenzoic acids were identified, such as gallic acid (N.28), protocatechuic acid (N.5) and *p*-hydroxybenzoic acid (N.10); some methoxybenzoic acids as syringic acid (N.9), vanillic acid (N.12) and veratric acid (N.33); hydroxycinamic acids, such as caffeic acid (N.14), *trans*-*p*-coumaric acid (N.18) and *trans*-ferulic acid (N.19); esters of caffeic acid with tartaric acid (*cis*-caftaric acid (N.1) and *trans*-caftaric acid (N.7)); some hydroxybenzaldehydes, such as protocatechuic aldehyde (N.6) and *p*-hydroxybenzaldehyde (N.11); methoxybenzaldehydes, such as syringaldehyde (N.3), vanillin (N.40) and veratraldehyde (N.41); some hydroxycoumarins like esculetin (N.37) and scopoletin (N.39); and finally tyrosol (2) and 5-hydroxymethylfurfural (N.26).

Table 2. Identifications of the compounds detected in the different fractions obtained using the three solvent systems (S_I, S_II, S_III).

N.	Compounds	Fraction	RetentionTime (min)	UV Bands (nm)	Mass Weight	Molecular Formula	$[M + H]^+$	$[M - H]^-$
1	*cis*-Caftaric acid [a]	F5 S_I	20.1	327	312.23	C13H12O9	231	179/229/311
2	Tyrosol [a]	F6 S_I	17.7	274	138.16	C8H10O2	121	137
3	Syringaldehyde [a]	F6 S_I	26.1	309	182.17	C9H10O4	183	181
4	3-Feruloylquinic acid [b]	F6 S_I	43.9	332	368.30	C17H20O9	164/370	163/206/368
5	Protocatechuic acid [a]	F7 S_I	14.4	260, 290	154.12	C7H6O4	153	155
6	Protocatechualdehyde [a]	F7 S_I	17.4	280, 311	138.12	C7H6O3	139	137
7	*trans*-Caftaric acid [a]	F7 S_I	20.2	328	312.23	C13H12O9		179/311
8	Isovanillin [a]	F7 S_I	20.8	277, 310	152.15	C8H8O3		151
9	Syringic acid [a]	F7 S_I	24.8	275	198.17	C9H10O5		197
10	*p*-Hydroxybenzoic acid [a]	F8 S_I	20.2	255	138.12	C7H6O3		137
11	*p*-Hydroxybenzaldehyde [a]	F8 S_I	22.0	284	122.12	C7H6O2		121
12	Vanillic acid [a]	F8 S_I	23.1	260, 290	168.15	C8H8O4		167
13	*Cis-p*-Coutaric acid [b]	F8 S_I	23.9	312	296.23	C13H12O8		150/163/295
14	Caffeic acid [a]	F8 S_I	24.8	323	180.16	C9H8O4		135/179
15	*trans-p*-Coutaric acid [b]	F8 S_I	24.9	313	296.23	C13H12O8		150/163/295
16	Caffeic acid-C-hexoside 1 [b]	F8 S_I	34.3	330	342.30	C15H18O9		179/341
17	*cis-p*-Coumaric acid [b]	F9 S_I	28.3	297	164.16	C9H8O3		119/ 163
18	*trans-p*-Coumaric acid [a]	F9 S_I	29.2	310	164.16	C9H8O3		119/163
19	*trans*-Ferulic acid [a]	F9 S_I	30.3	323	194.18	C10H10O4		194
20	Caffeic acid-C-hexoside 2 [b]	F9 S_I	33.3	332	342.30	C15H18O9		179/341
21	Derivative Caffeic acid 1 [b]	F9 S_I	35.8	313	326.00	C15H18O8		178/324
22	Derivative Caffeic acid 2 [b]	F9 S_I	36.4	315	326.00	C15H18O8		178/324
23	Coumaroyl hexoside [b]	F9 S_I	37.5	315	326.30	C15H18O8		162/178/325
24	*cis*-Ferulic acid [b]	F9 S_I	45.2	310	194.18	C10H10O4		192/193
25	Ethyl vanillate [b]	F7 S_II	17.9	281, 320	196.20	C10H12O4		123/195
26	Hydroxymethylfurfural [a]	F9 S_II	9.9	284	126.11	C6H6O3	127	
27	Fertaric acid [b]	F13 S_II	26.1	326	326.25	C14H14O9		193/325
28	Gallic acid [a]	F14 S_II	8.6	271	170.12	C7H6O5	171	169
29	Furoic acid [b]	F14 S_II	14.2	151	112.08	C5H4O3	113	111
30	Dihydro-p-coumaric acid [b]	F17 S_II	27.8	278	166,17	C9H10O3	167	
31	6-O-Feruloylglucose [b]	F17 S_II	33.5	307	356,32	C16H20O9		193/323/355
32	Ethyl gallate [b]	F18 S_II	26.2	272	198.17	C9H10O5	199	197
33	Veratric acid [a]	F18 S_II	32.9	260, 294	182.17	C9H10O4	183	181
34	4-O-beta-D-Glucosyl-4-coumaric acid [b]	F4 S_III	29.7	290, 329	326.30	C15H18O8		163/246/325
35	Hydroxytyrosol [b]	F6 S_III	18.2	275	154.16	C8H10O3		154

Table 2. *Cont.*

N.	Compounds	Fraction	RetentionTime (min)	UV Bands (nm)	Mass Weight	Molecular Formula	$[M + H]^+$	$[M - H]^-$
36	Methyl protocatechuate [b]	F9 S_III	20.2	307	168.15	C8H8O4		145/167
37	Esculetin [a]	F9 S_III	22.8	300, 346	178.14	C9H6O4	129/179	131/177
38	Homoveratric acid [b]	F10 S_III	26.7	279	196.20	C10H12O4		156/195
39	Scopoletin [a]	F10 S_III	28.3	300, 344	192.17	C10H8O4	194	192
40	Vanillin [a]	F11 S_III	24.8	280, 310	152.15	C8H8O3	153	151
41	Veratraldehyde [a]	F11 S_III	27.3	278, 315	166.17	C9H10O3	168	165
42	Ethyl caffeate [b]	F12 S_III	38.6	326	208.21	C11H12O4		207

[a]: Identified based on bibliography and by injection of standards; [b]: Identified based on specific bibliography and databases.

On the other hand, through the use of the different databases previously mentioned as well as literature references, we found several hydroxycinnamic acids and hydroxycinnamic acid derivatives (Table 2), since they all have the similar and characteristic UV spectra of these compounds, with a maximum wavelength between 310 and 330 nm.

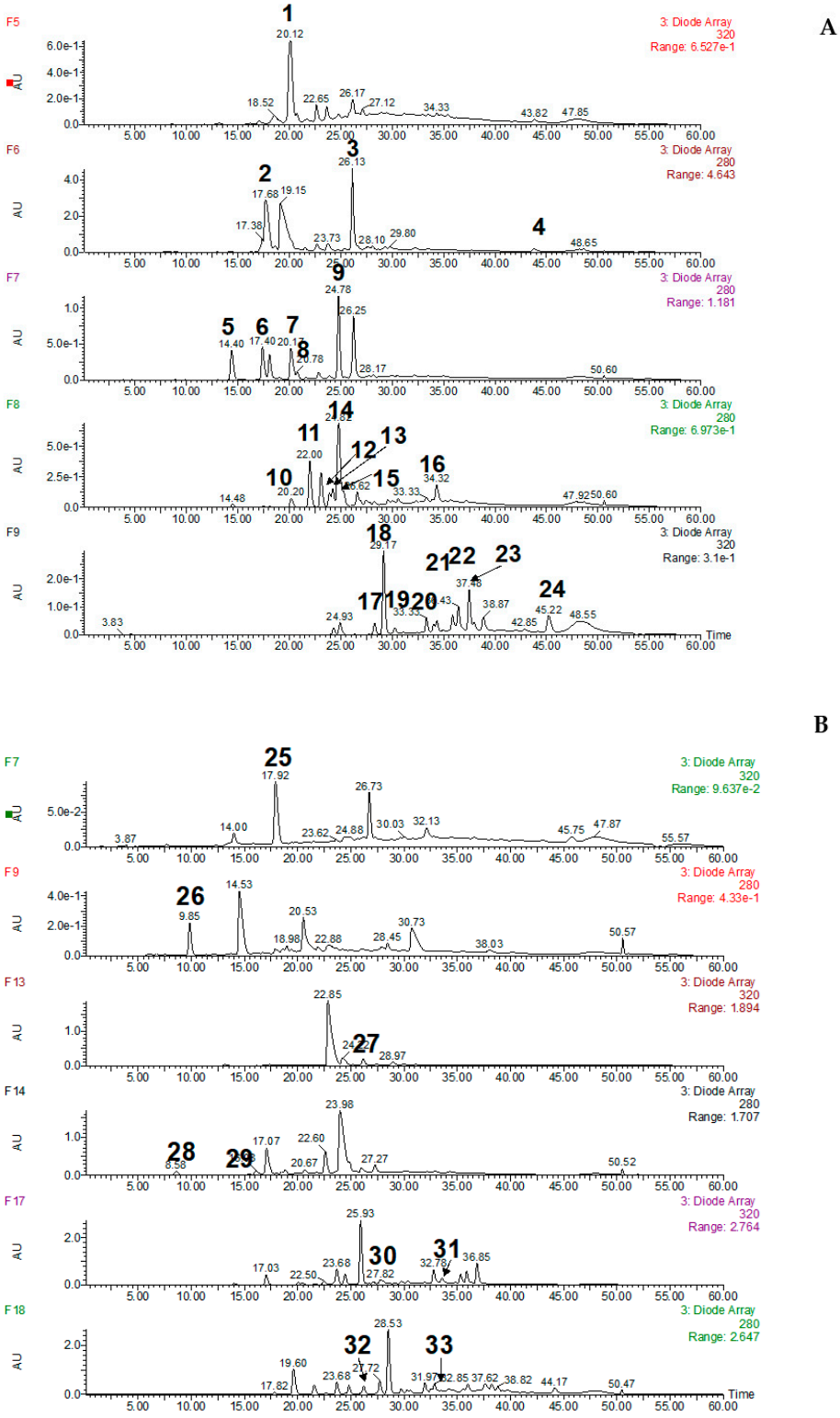


Figure 2. *Cont*.

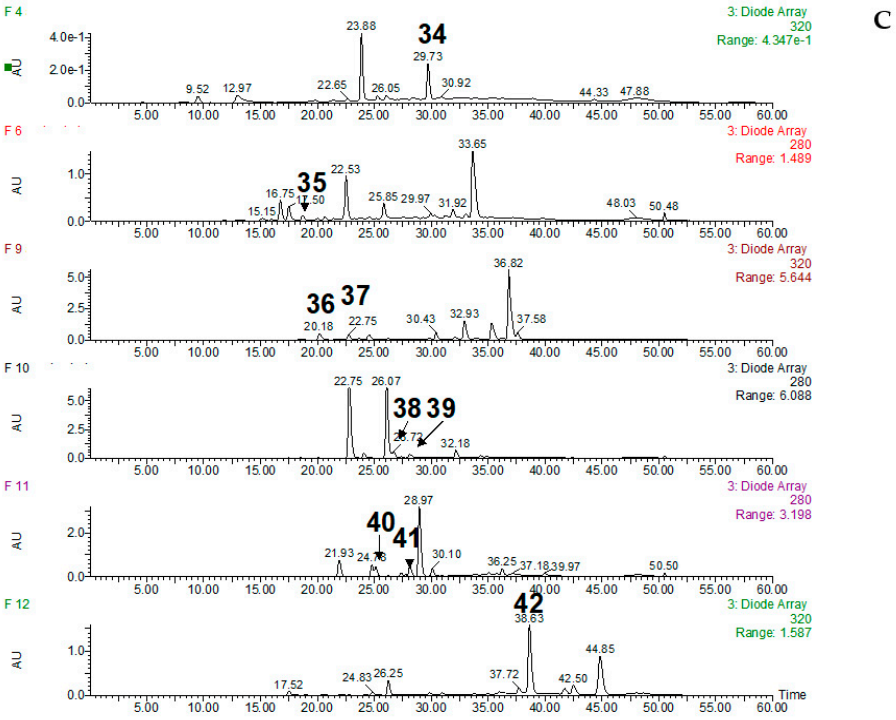


Figure 2. HPLC Chromatograms of the fractions obtained using the different solvent systems in which any compound was identified. The numbering applied corresponds to that used in Table 2. (**A**)—solvent system I: MTBE/n-butanol/acetonitrile/water (1.1/3/1.1/5+0.1% trifluoroacetic acid); (**B**)—solvent system II: MTBE/n-butanol/acetonitrile/water (2/2/1/5); (**C**)—solvent system III: hexane/ethyl acetate/methanol/water (1/5/1/5).

Thus, *cis-p*-coumaric acid (N.17) was identified with a mass spectrum identical to *trans-p*-coumaric acid (N.18) in both positive and negative modes. However, in the UV spectrum a hypsochromic shift of the absorption band of about 15 nm was observed (which was confirmed by querying PubChem) as well as a longer retention time of the *cis* isomer. Similarly, this behavior was observed in the compound identified as *cis*-ferulic acid (N.24) with respect to the *trans* isomer (N.20). We also encountered the corresponding esters of the two isomers of *p*-coumaric acid with tartaric acid (*cis-p*-coutaric acid (N.13) and *trans-p*-coutaric acid (N.15), Table 2), both with a fragment at $[M - H]^-$ 296 Da, which would indicate the molecular mass of the corresponding ester and have UV spectra similar to those of the hydroxycinnamic acids from which they originate. In the same way, we found the compound N.27, with the UV spectrum characteristic of hydroxycinnamic acids and with fragments in the mass spectrum pointing to a molecular mass of 326 Da, that corresponds to that of fertaric acid. In the mass spectrum, fragments $[M - H]^-$ to 193 Da corresponding to ferulic acid are present, which would confirm the loss of the tartaroyl radical.

The compounds labeled as N.21 and N.22 (Table 2) exhibit a fragment $[M - H]^-$ at 324 Da in their mass spectra, which would point to a possible molecular mass of 326 Da, and both present the fragment corresponding to caffeic acid (179 Da). Therefore, they have been identified as derivatives of caffeic acid 1 and 2, since we have no other data that would allow us to clarify their identification.

Compound N.4 was identified as the ester with the quinic acid of ferulic acid, as it presents a UV spectrum similar to that of ferulic acid, within the mass spectrum of fragments corresponding to the same. In addition, a peak that could be assigned to the loss of quinic acid (−194 Da) was observed.

Based on the UV and MS spectra obtained for compounds N.16, N.20, N.23, N.31 and N.34 (Table 2) we can conclude that all of them are derivatives of hydroxycinnamic acid

based on their UV absorption spectrum, with spectra related to caffeic acid, *p*-coumaric acid and ferulic acid. Furthermore, besides the fragments of the corresponding acids, we found in the mass spectra a fragment corresponding to the loss of the remaining hexose moiety (−162 Da). Thus, in the mass spectrum of compound N.34, we found the fragment at m/z 163, which is characteristic of the derivatives of *p*-coumaric acid. After consulting the MassBank of North America (MoNA) mass spectral database (GLP License) by introducing the fragmentation of the mass spectrum, it could be observed that it coincides with the spectrum of coumaric acid O-glucoside. In this group of compounds, we found those labeled as N.16 and N.20 (Table 2). The mass spectra of both point at a molecular mass of 342 Da, both with the fragment corresponding to caffeic acid. They also showed the characteristic fragmentation that indicates the excision of the remaining hexose, suggesting that they are hexosides of caffeic acid. After consulting the mass spectra in MoNA spectra database, we identified compound N.16 as caffeic acid 4-O-glucoside and compound N.20 as caffeic acid C-hexoside. Compound N.23 was identified as coumaric acid O-hexoside based on its UV spectrum, which is similar to that of *p*-coumaric acid and its mass spectrum, which displays the characteristic fragments of *p*-coumaric acid (*m/z* 163) and the loss of the hexosyl radical (−162) that was confirmed in the consulted databases. The mass spectrum of compound N.31 reveals a molecular mass of 356 Da, with the characteristic fragments of ferulic acid and the loss of a glycosyl group. On the other hand, the UV spectrum also confirms the presence of a ferulic acid, which has been identified as 6-O-Feruloyl glucose (Table 2).

The compound N.30 (Table 2) presents UV absorption and mass spectra coinciding with those of dihydro-*p*-coumaric acid (phloretic acid), which is a hydroxyphenyl propanoic acid, according to the databases that have been consulted, and presents a molecular mass of 166 Da and a UV absorption band at 277 nm.

The compounds labeled as N.25, N.32 and N.36 have been identified as alkyl esters of 3 benzoic acids (Table 2). The compound N.25 is assigned to ethyl vanillate, having a $[M - H]^-$ of 181 Da and a UV spectrum with two bands at 262 and 295 nm, and thus being the identification confirmed by the literature [21] and the consulted bases. In the same way, compound N.32 (ethyl gallate) is identified by $[M - H]^-$ at 197 Da and a UV absorption band at 272 nm; these data have been confirmed in the literature [21] and in the consulted databases. Following the same guidelines, the compound N.36 was identified as methyl protocatechuate, presenting a $[M - H]^-$ at 167 Da and a UV absorption band at 304 nm.

The compound N.42 presents in the mass spectrum a fragment $[M - H]^-$ at 207 Da and a UV spectrum similar to that of caffeic acid with a maximum lambda at 326 nm, which indicates that it is a derivative of this acid, and after consulting the bibliography and the databases consulted in the present work, we concluded that it is ethyl caffeate.

A fragment $[M - H]^-$ at 195 Da and a UV spectrum with a band at 279 nm are the characteristics of the compound N.38, which, after consulting the bibliography and the databases, was tentatively identified as homoveratric acid.

Finally, furoic acid, with a molecular mass of 112 Da and UV absorption band at 255 nm, was identified as compound N.29. Compound N.35 was identified as hydroxytyrosol, with a UV spectrum similar to tyrosol, with a maximum wavelength at 275 and a fragment $[M - H]^-$ at 153 Da. Both were also confirmed via consultation of the databases used.

3.3. Prevalence of the Identified Phenolic and Furanic Compounds in Wines

According to the information available, some of the compounds mentioned in Table 2, have been identified for the first time in Amontillados or Sherry wines: 3-feruloylquinic acid (N.4), isovanillin (N.8), ethyl vanillate (N.25), furoic acid (N.29), dihydro-*p*-coumaric acid (phloretic acid) (N.30), 6-O-feruloylglucose (N.31), ethyl gallate (N.32), hydroxytyrosol (N.35), methyl protocatechuate (N.36), homoveratric acid (N.38) and veratraldehyde (N.41).

The rest of the compounds identified in the samples have been widely reported in the literature regarding the phenolic content of Amontillado Sherry wines. As it has already been indicated in our introduction, previous studies [3–5] demonstrated the presence

of gallic, caffeic, *p*-coumaric (*cis* and *trans*), *p*-hydroxybenzoic, protocatechuic, syringic, vanillic, and ferulic acids; the aldehydes *p*-hydroxybenzaldehyde, syringaldehyde, protocatechualdehyde and vanillin; the phenolic acid esters caftaric acid, chlorogenic acid and *p*-coutaric acid (*cis* and *trans*); and flavan-3-ol (+)-catechin [4], tyrosol and HMF in Sherry wines. Other publications on the phenolic compounds content in Sherry wines, which are not necessarily Amontillado wines, indicate the presence of other compounds such as ethyl caffeate (N.42), fertaric acid (N.27), *cis*-ferulic acid (N.24), *trans*-ferulic acid (N.19) and furoic acid (N.29) [6,28–32], all of which have also been identified in our wine (Table 2).

The origins of these compounds, in Amontillado wine in particular and in fortified wines in general, are quite different. Some of them are derived from the raw material itself, others are formed during the fermentation processes (alcoholic and malolactic fermentation), and/or they may also come from the extraction phenomena that take place during the final ageing process in wooden barrels.

Gallic acid (N.28, Table 2) can be generated both from the starting grapes and from the hydrolysis of gallotannins in the barrels' wood, especially during the first years of ageing [33]. The esters from phenolic acids such as fertaric acid (N.27), the *cis* and *trans* isomers from caftaric acid (N.1 and N.7), and the *cis* and *trans* isomers from *p*-coutaric acid (N.13 and N.15) (Table 2), come from the grapes themselves and their initial concentration decreases in the first stages of wine ageing (biological ageing stage) while their concentration levels remain the same once the wine enters the oxidative stage [5]. Other esters that have been identified in this work are ethyl caffeate (N.42), ethyl gallate (N.32) and ethyl vanillate (N.25) (Table 2). These compounds have not been detected in fortified wines, although they have been identified in some samples of Riesling grapes and in single-varietal wines [20].

Some aldehydes such as vanillin, syringaldehyde, sinapaldehyde and coniferaldehyde are derived from the decomposition of lignin during the wood toasting process [34]. The oxidation of these aldehydes will eventually give rise to their corresponding acids: vanillic, syringic, ferulic and coniferylic. Of all these compounds, we have identified six in our wine (Table 2): vanillic acid (N.12), syringic acid (N.9), *cis* and *trans* ferulic acid (N.24 and N.19), vanillin (N.40) and syringaldehyde (N.3). The limited presence of aldehyde-type compounds in the samples of Amontillado wine is due to the inhibition of these compounds by the *flor* yeast during its initial biological ageing stage [5]. However, the presence of *p*-hydroxybenzaldehyde (N.11) is characteristic of Sherry wines and vinegars because of their long ageing periods in oak barrels.

The presence of furans is associated with the toasting of the wood, and more specifically with the presence of hydroxymethylfurfural (N.26), which is related to the thermal degradation of the glucose in wood cellulose. We have not found in the literature any author reporting the presence of furoic acid (N.29) in samples of Amontillado or other Sherry wines, but we have found some references indicating the presence of this compound in aged Madeira wines [35] and in barrel-aged spirits such as whisky, brandy or rum [36].

The presence of tyrosol (N.2) in samples of Amontillado wines, and other fortified wines, has also been described by other authors [5,6,29,32], but not that of hydroxytyrosol (N.35) (derived from tyrosol via the hydroxylation of its aromatic ring), even though small amounts (between 1 and 5 mg/l) have been detected in red and white wines [37,38]. These two compounds can be considered as secondary metabolites of the tyrosine formed by the yeast during the alcoholic fermentation [39].

The identified coumarins (Table 2), scopoletin (N.39) and esculetin (N.37), as well as other coumarins identified in wines and distillates, are secondary metabolites that result from an intramolecular esterification of orthohydroxycinnamic acid forming lactones. These two compounds had already been detected in Sherry wine samples (Finos, Olorosos and Amontillados) by Estrella et al. in 1986 [3].

Dihydro-*p*-coumaric acid (N.30) is derived from the reduction of *p*-coumaric acid by lactic acid bacteria during the malolactic fermentation [40]. Isovanillin (N.8) [41] and veratraldehyde (N.41) [42] have been identified in oak-aged vinegar samples.

We have not found any information in the literature on the presence of homoveratric acids (N.38) in grapes or derived products. Nevertheless, veratric acid and veratraldehyde have been found in Sherry wines [43]. Nor have any references been found to indicate the presence of 3-feruloylquinic acid (N.4), which is quite frequently found in coffee beans and in coffee itself [44].

3.4. Antioxidant Activity of the Fractions Obtained

In order to try and determine the relationship between antioxidant activity and phenolic composition, the antioxidant activity of the fractions obtained from XAD-7 extract, and also those from ethyl acetate extract, was determined. Table 3 shows the antioxidant activity as measured in the different fractions, as well as their standard deviation (n = 3).

Table 3. Antioxidant activity (mM Trolox) and standard deviation (n = 3) of the fractions obtained from XAD-7 extract and ethyl acetate extract.

XAD-7 Extract			Ethyl Acetate Extract		
Fractions	**Antioxidant Activity * (mM Trolox)**	**Standard Deviation**	**Fractions**	**Antioxidant Activity * (mM Trolox)**	**Standard Deviation**
F1	0.000 ^{a}	0.000	F1	2.712 ^{bc}	0.740
F2	0.704 ^{abc}	0.823	F2	2.768 ^{bc}	1.041
F3	1.963 ^{cd}	0.183	F3	6.680 ^{d}	0.250
F4	2.250 ^{d}	0.535	F4	2.214 ^{b}	0.256
F5	0.000 ^{a}	0.000	F5	2.720 ^{bc}	0.336
F6	1.451 ^{bcd}	0.469	F6	3.631 ^{bc}	0.952
F7	4.320 ^{e}	0.864	F7	2.749 ^{bc}	0.183
F8	2.180 ^{d}	0.333	F8	4.142 ^{c}	0.536
F9	0.220 ^{ab}	0.249	F9	2.790 ^{bc}	0.205
			F10	2.918 ^{bc}	0.048
			F11	3.846 ^{c}	0.521
			F12	0.236 ^{a}	0.239

*: Different letters in one column mean significant differences between the antioxidant activity of the fractions for each extract, according to Tukey's test ($p < 0.05$).

It can be seen (Table 3), in reference to XAD-7 extract, that the F7 fraction was the one with the significant highest antioxidant activity ($p < 0.05$). Some of the polyphenols identified in this fraction were protocatechuic acid, protocatechualdehyde, *trans*-caftaric acid, syringic acid and isovanillin. Several of these compounds have been previously related to the antioxidant activity of different oenological products [45–49], and therefore the high antioxidant capacity of this fraction is justified. It is also worth mentioning that when comparing the antioxidant activity of the raw extract (2238 mM Trolox), surprisingly, it presents a similar value to that of some of the fractions obtained and listed in Table 3. This would confirm the hypothesis of the antagonistic effect that some polyphenols may present in terms of antioxidant activity, as argued by some authors [50,51].

In relation to the fractions obtained from the ethyl acetate extract, some of them, such as F3, F8 and F11, were found to have high values for antioxidant activity (Table 3). On the one hand, gallic acid and different esters of cinnamic acids can be found in the F3 fraction. The F8 fraction, on the other hand, contains two major compounds: protocatechuic acid and tyrosol. According to some studies, the presence of all these compounds in wines and derivatives is related to their antioxidant capacity [47,52,53]. Besides, some of the polyphenols that, according to these results, seem to contribute to a greater extent to the antioxidant activity, such as protocatechuic acid or tyrosol, have also been reported in this sense by previous works conducted on Sherry wines [54]. Furthermore, hydroxytyrosol was identified in the F6 fraction (Table 3), which although available in wine, is the main antioxidant in virgin and extra virgin olive oils [55], and has been attributed biological activity, and therefore may contribute to an increase in antioxidant activity.

All the above described results lead us to conclude that the prevalence of certain phenolic compounds in Amontillado wine represent a substantial contribution to the in vitro antioxidant activity of the different fractions obtained.

4. Conclusions

The use of the HSCCC technique has been successfully applied to a complex matrix such as Amontillado sherry. This technique facilitates the fractionation of samples and, therefore, the characterization and identification of compounds. In this way, the identification of 42 compounds has been achieved, 11 of which were identified for the first time in sherry wines. HSCCC has been used for the first time to investigate the composition of Sherry wines, and has proven to be useful for the characterization of this type of fortified wine. Therefore, it would be interesting to apply this technique to other types of Sherry wines in the future, in order to broaden the existing knowledge on this type of highly complex wine.

Author Contributions: Conceptualization, M.S. and F.W.; methodology, F.W.; software, M.V.G.-M.; validation, M.d.C.R.-D.; formal analysis, R.C.; investigation, M.S.; resources, P.W.; data curation, F.W.; writing—original draft preparation, M.S., E.D.-G., R.C., M.d.C.R.-D., M.V.G.-M. and D.G.-S.; writing—review and editing, E.D.-G.; visualization, E.D.-G.; supervision, P.W. and D.G.-S.; project administration, D.G.-S.; funding acquisition, D.G.-S. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by the project FEDER INNTERCONECTA "INNTER-VINANDAL ITC-20131018", from the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (ERDF) through the plan I+D+I.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

Acknowledgments: We would like to thank "Bodegas González Byass S.A." for providing us with the wine required for this study. In loving memory of Carmelo García Barroso.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Pereira, V.; Pereira, A.C.; Marques, J.C. *Emerging Trends in Fortified Wines: A Scientific Perspective*; Elsevier Inc.: Amsterdam, The Netherlands, 2019; ISBN 9780128152690.
2. Zhen, J.; Villani, T.S.; Guo, Y.; Qi, Y.; Chin, K.; Pan, M.H.; Ho, C.T.; Simon, J.E.; Wu, Q. Phytochemistry, antioxidant capacity, total phenolic content and anti-inflammatory activity of Hibiscus sabdariffa leaves. *Food Chem.* **2016**, *190*, 673–680. [CrossRef] [PubMed]
3. Estrella, M.I.; Hernández, M.T.; Olano, A. Changes in polyalcohol and phenol compound contents in the ageing of sherry wines. *Food Chem.* **1986**, *20*, 137–152. [CrossRef]
4. Guillén, D.A.; Barroso, C.G.; Pérez-Bustamante, J.A. Automation of sample preparation as a preliminary stage in the high-performance liquid chromatographic determination of polyphenolic compounds in sherry wines. *J. Chromatogr. A* **1996**, *730*, 39–46. [CrossRef]
5. Valme García Moreno, M.; García Barroso, C. Comparison of the evolution of low molecular weight phenolic compounds in typical sherry wines: Fino, amontillado, and oloroso. *J. Agric. Food Chem.* **2002**, *50*, 7556–7563. [CrossRef]
6. Ortega, A.F.; Lopez-Toledano, A.; Mayen, M.; Merida, J.; Medina, M. Changes in color and phenolic compounds during oxidative aging of sherry white wine. *J. Food Sci.* **2003**, *68*, 2461–2468. [CrossRef]
7. Pozo-Bayón, M.Á.; Moreno-Arribas, M.V. Sherry wines: Manufacture, composition and analysis. In *Encyclopedia of Food and Health*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 779–784.
8. López de Lerma, M.N.; Bellincontro, A.; García-Martínez, T.; Mencarelli, F.; Moreno, J.J. Feasibility of an electronic nose to differentiate commercial Spanish wines elaborated from the same grape variety. *Food Res. Int.* **2013**, *51*, 790–796. [CrossRef]
9. Cao, X.; Yang, C.; Pei, H.; Li, X.; Xu, X.; Ito, Y. Application of counter-current chromatography as a new pretreatment method for the determination of polycyclic aromatic hydrocarbons in environmental water. *J. Sep. Sci.* **2012**, *35*, 596–601. [CrossRef]

10. Xie, J.; Sun, B.; Wang, S.; Ito, Y. Isolation and purification of nootkatone from the essential oil of fruits of Alpinia oxyphylla Miquel by high-speed counter-current chromatography. *Food Chem.* **2009**, *117*, 375–380. [CrossRef]
11. Gong, Y.; Huang, X.Y.; Pei, D.; Duan, W.D.a.; Zhang, X.; Sun, X.; Di, D.L. The applicability of high-speed counter current chromatography to the separation of natural antioxidants. *J. Chromatogr. A* **2020**, *1623*, 461150. [CrossRef]
12. Surup, F.; Minh Thi Tran, T.; Pfütze, S.; Budde, J.; Moussa-Ayoub, T.E.; Rohn, S.; Jerz, G. Opuntisines, 14-membered cyclopeptide alkaloids from fruits of Opuntia stricta var. dillenii isolated by high-performance countercurrent chromatography. *Food Chem.* **2021**, *334*, 127552. [CrossRef]
13. Esatbeyoglu, T.; Wray, V.; Winterhalter, P. Isolation of dimeric, trimeric, tetrameric and pentameric procyanidins from unroasted cocoa beans (*Theobroma cacao* L.) using countercurrent chromatography. *Food Chem.* **2015**, *179*, 278–289. [CrossRef]
14. Rodríguez-Rivera, M.P.; Lugo-Cervantes, E.; Winterhalter, P.; Jerz, G. Metabolite profiling of polyphenols in peels of citrus limetta risso by combination of preparative high-speed countercurrent chromatography and LC-ESI-MS/MS. *Food Chem.* **2014**, *158*, 139–152. [CrossRef] [PubMed]
15. Li, Y.; Li, L.; Cui, Y.; Zhang, S.; Sun, B. Separation and purification of polyphenols from red wine extracts using high speed counter current chromatography. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2017**, *1054*, 105–113. [CrossRef] [PubMed]
16. Weber, F.; Winterhalter, P. Synthesis and structure elucidation of ethyliden-linked anthocyanin—Flavan-3-ol oligomers. *Food Res. Int.* **2014**, *65*, 69–76. [CrossRef]
17. Noguer, M.; Cerezo, A.B.; Rentzsch, M.; Winterhalter, P.; Troncoso, A.M.; García-Parrilla, M.C. Simulated digestion and antioxidant activity of red wine fractions separated by high speed countercurrent chromatography. *J. Agric. Food Chem.* **2008**, *56*, 8879–8884. [CrossRef] [PubMed]
18. Luo, L.; Cui, Y.; Zhang, S.; Li, L.; Li, Y.; Zhou, P.; Sun, B. Preparative separation of grape skin polyphenols by high-speed counter-current chromatography. *Food Chem.* **2016**, *212*, 712–721. [CrossRef] [PubMed]
19. Gawel, R.; Schulkin, A.; Smith, P.A.; Waters, E.J. Taste and textural characters of mixtures of caftaric acid and grape reaction product in model wine. *Aust. J. Grape Wine Res.* **2014**, *20*, 25–30. [CrossRef]
20. Baderschneider, B.; Winterhalter, P. Isolation and characterization of novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 2788–2798. [CrossRef]
21. Regalado, E.L.; Tolle, S.; Pino, J.A.; Winterhalter, P.; Menendez, R.; Morales, A.R.; Rodríguez, J.L. Isolation and identification of phenolic compounds from rum aged in oak barrels by high-speed countercurrent chromatography/high-performance liquid chromatography-diode array detection-electrospray ionization mass spectrometry and screening for antioxidan. *J. Chromatogr. A* **2011**, *1218*, 7358–7364. [CrossRef]
22. Köhler, N.; Wray, V.; Winterhalter, P. Preparative isolation of procyanidins from grape seed extracts by high-speed counter-current chromatography. *J. Chromatogr. A* **2008**, *1177*, 114–125. [CrossRef]
23. Salas, E.; Dueñas, M.; Schwarz, M.; Winterhalter, P.; Cheynier, V.; Fulcrand, H. Characterization of pigments from differents high speed countercurrent chromatography wine fractions. *J. Agric. Food Chem.* **2005**, *53*, 4536–4546. [CrossRef] [PubMed]
24. Köhler, N.; Winterhalter, P. Large-scale isolation of flavan-3-ol phloroglucinol adducts by high-speed counter-current chromatography. *J. Chromatogr. A* **2005**, *1172*, 217–222. [CrossRef] [PubMed]
25. Ito, Y. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J. Chromatogr. A* **2005**, *1065*, 145–168. [CrossRef] [PubMed]
26. Alonso, Á.M.; Guillén, D.A.; Barroso, C.G. Development of an electrochemical method for the determination of antioxidant activity. Application to grape-derived products. *Eur. Food Res. Technol.* **2003**, *216*, 445–448. [CrossRef]
27. Berthod, A.; Ruiz-Angel, M.J.; Carda-Broch, S. Elution-extrusion countercurrent chromatography. Use of the liquid nature of the stationary phase to extend the hydrophobicity window. *Anal. Chem.* **2003**, *75*, 5886–5894. [CrossRef]
28. Pereira, V.; Câmara, J.S.; Cacho, J.; Marques, J.C. HPLC-DAD methodology for the quantification of organic acids, furans and polyphenols by direct injection of wine samples. *J. Sep. Sci.* **2010**, *33*, 1204–1215. [CrossRef]
29. Ortega, A.F.; Mayen, M.; Medina, M. Study of colour and phenolic compounds in two models of oxidative ageing for sherry type white wines. *Food Control.* **2008**, *19*, 949–956. [CrossRef]
30. Arcari, S.G.; Chaves, E.S.; Vanderlinde, R.; Rosier, J.P.; Bordignon-Luiz, M.T. Brazilian fortified wines: Chemical composition, chromatic properties and antioxidant activity. *Food Res. Int.* **2013**, *53*, 164–173. [CrossRef]
31. Ho, P.; Hogg, T.A.; Silva, M.C.M. Application of a liquid chromatographic method for the determination of phenolic compounds and furans in fortified wines. *Food Chem.* **1999**, *64*, 115–122. [CrossRef]
32. Fernández-Pachón, M.S.; Villaño, D.; Troncoso, A.M.; García-Parrilla, M.C. Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with antioxidant activity. *Anal. Chim. Acta* **2006**, *563*, 101–108. [CrossRef]
33. Canas, S. Phenolic composition and related properties of aged wine spirits: Influence of barrel characteristics. A review. *Beverages* **2017**, *3*, 55. [CrossRef]
34. Sarni, F.; Moutounet, M.; Puech, J.L.; Rabier, P. Effect of heat treatment of oak wood extractable compounds. *Holzforschung* **1990**, *44*, 461–466. [CrossRef]
35. Câmara, J.S.; Alves, M.A.; Marques, J.C. Changes in volatile composition of Madeira wines during their oxidative ageing. *Anal. Chim. Acta* **2006**, *563*, 188–197. [CrossRef]

36. Goldberg, D.M.; Hoffman, B.; Yang, J.; Soleas, G.J. Phenolic constituents, furans, and total antioxidant status of distilled spirits. *J. Agric. Food Chem.* **1999**, *47*, 3978–3985. [CrossRef] [PubMed]
37. Fernández-Mar, M.I.; Mateos, R.; García-Parrilla, M.C.; Puertas, B.; Cantos-Villar, E. Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. *Food Chem.* **2012**, *130*, 797–813. [CrossRef]
38. Barcenilla, J.; Estrella, I.; Gómez-Cordovés, C.; Hernández, T.; Hernández, L. The influence of yeasts on certain non-volatile components of wine. *Food Chem.* **1989**, *31*, 177–187. [CrossRef]
39. Bordiga, M.; Lorenzo, C.; Pardo, F.; Salinas, M.R.; Travaglia, F.; Arlorio, M.; Coïsson, J.D.; Garde-Cerdán, T. Factors influencing the formation of histaminol, hydroxytyrosol, tyrosol, and tryptophol in wine: Temperature, alcoholic degree, and amino acids concentration. *Food Chem.* **2016**, *197*, 1038–1045. [CrossRef]
40. Rodríguez, H.; Curiel, J.A.; Landete, J.M.; de las Rivas, B.; de Felipe, F.L.; Gómez-Cordovés, C.; Mancheño, J.M.; Muñoz, R. Food phenolics and lactic acid bacteria. *Int. J. Food Microbiol.* **2009**, *132*, 79–90. [CrossRef]
41. Cerezo, A.B.; Álvarez-Fernández, M.A.; Hornedo-Ortega, R.; Troncoso, A.M.; García-Parrilla, M.C. Phenolic composition of vinegars over an accelerated aging process using different wood species (acacia, cherry, chestnut, and oak): Effect of wood toasting. *J. Agric. Food Chem.* **2014**, *62*, 4369–4376. [CrossRef]
42. Gálvez, M.C.; Barroso, C.G.; Pérez-Bustamante, J.A. Analysis of polyphenolic compounds of different vinegar samples. *Z. Lebensm. Unters.* **1994**, *199*, 29–31. [CrossRef]
43. Palma, M.; Piñeiro, Z.; Barroso, C.G. Stability of phenolic compounds during extraction with superheated solvents. *J. Chromatogr. A* **2001**, *921*, 169–174. [CrossRef]
44. Bhagat, A.R.; Delgado, A.M.; Issaoui, M.; Chammem, N.; Fiorino, M.; Pellerito, A.; Natalello, S. Review of the role of fluid dairy in delivery of polyphenolic compounds in the diet: Chocolate milk, coffee beverages, matcha green tea, and beyond. *J. AOAC Int.* **2019**, *102*, 1365–1372. [CrossRef] [PubMed]
45. Monteiro, G.C.; Minatel, I.O.; Junior, A.P.; Gomez-Gomez, H.A.; de Camargo, J.P.C.; Diamante, M.S.; Pereira Basílio, L.S.; Tecchio, M.A.; Pereira Lima, G.P. Bioactive compounds and antioxidant capacity of grape pomace flours. *LWT* **2021**, 135. [CrossRef]
46. Cotoras, M.; Vivanco, H.; Melo, R.; Aguirre, M.; Silva, E.; Mendoza, L. In vitro and in vivo evaluation of the antioxidant and prooxidant activity of phenolic compounds obtained from grape (Vitis vinifera) pomace. *Molecules* **2014**, *19*, 21154–21167. [CrossRef]
47. Alonso, Á.M.; Castro, R.; Rodríguez, M.C.; Guillén, D.A.; Barroso, C.G. Study of the antioxidant power of brandies and vinegars derived from Sherry wines and correlation with their content in polyphenols. *Food Res. Int.* **2004**, *37*, 715–721. [CrossRef]
48. Feliciano, R.P.; Bravo, M.N.; Pires, M.M.; Serra, A.T.; Duarte, C.M.; Boas, L.V.; Bronze, M.R. Phenolic content and antioxidant activity of moscatel dessert wines from the setúbal region in portugal. *Food Anal. Methods* **2009**, *2*, 149–161. [CrossRef]
49. Liu, Q.; Tang, G.Y.; Zhao, C.N.; Gan, R.Y.; Li, H. Bin Antioxidant activities, phenolic profiles, and organic acid contents of fruit vinegars. *Antioxidants* **2019**, *8*, 78. [CrossRef]
50. Odabasoglu, F.; Aslan, A.; Cakir, A.; Suleyman, H.; Karagoz, Y.; Bayir, Y.; Halici, M. Antioxidant activity, reducing power and total phenolic content of some lichen species. *Fitoterapia* **2005**, *76*, 216–219. [CrossRef]
51. Parker, T.L.; Miller, S.A.; Myers, L.E.; Miguez, F.E.; Engeseth, N.J. Evaluation of synergistic antioxidant potential of complex mixtures using oxygen radical absorbance capacity (ORAC) and electron paramagnetic resonance (EPR). *J. Agric. Food Chem.* **2010**, *58*, 209–217. [CrossRef]
52. Mudnic, I.; Modun, D.; Rastija, V.; Vukovic, J.; Brizic, I.; Katalinic, V.; Kozina, B.; Medic-Saric, M.; Boban, M. Antioxidative and vasodilatory effects of phenolic acids in wine. *Food Chem.* **2010**, *119*, 1205–1210. [CrossRef]
53. Stefenon, C.A.; Bonesi, C.D.M.; Marzarotto, V.; Barnabé, D.; Spinelli, F.R.; Webber, V.; Vanderlinde, R. Phenolic composition and antioxidant activity in sparkling wines: Modulation by the ageing on lees. *Food Chem.* **2014**, *145*, 292–299. [CrossRef] [PubMed]
54. Schwarz, M.; Rodríguez, M.C.; Guillén, D.A.; Barroso, C.G. Evolution of the colour, antioxidant activity and polyphenols in unusually aged sherry wines. *Food Chem.* **2012**, *133*, 271–276. [CrossRef] [PubMed]
55. Wang, S.T.; Le, J.; Peng, R.; Li, Y. Efficient extraction and sensitive LC-MS quantification of hydroxytyrosol in wine, oil and plasma. *Food Chem.* **2020**, 323. [CrossRef] [PubMed]

foods

MDPI

Article

Rapid PCR Method for the Selection of 1,3-Pentadiene Non-Producing *Debaryomyces hansenii* Yeast Strains

Eva-María Rivas [1,2,†], Petra Wrent [1,2,†] and María-Isabel de Silóniz [1,2,*]

[1] Department of Genetics, Physiology and Microbiology. Biological Sciences Faculty. Complutense University of Madrid. José Antonio Nováis, 12. 28040 Madrid, Spain; evarifer@gmail.com (E.-M.R.); pwrent@ucm.es (P.W.)
[2] CEI Campus Moncloa, UCM-UPM, 28040 Madrid, Spain
* Correspondence: siloniz@bio.ucm.es; Tel.: +34-91-3944962
† These authors contributed equally to this work.

Received: 17 December 2019; Accepted: 28 January 2020; Published: 7 February 2020

Abstract: To prevent microbial growth and its consequences, preservatives such as sorbic acid or its salts, commonly known as sorbates, are added to foods. However, some moulds and yeasts are capable of decarboxylating sorbates and producing 1,3-pentadiene. This is a volatile compound with an unpleasant "petroleum-like "odour, which causes consumer rejection of the contaminated products. In this work, we studied the production of 1,3-pentadiene in 91 strains of the yeast *Debaryomyces hansenii*, and we found that nearly 96% were able to produce this compound. The sequence of the *FDC1Dh* gene was analysed showing differences between 1,3-pentadiene producer (P) and non-producer (NP) strains. A specific PCR assay with degenerated primers based on the gene sequence was developed to discern NP and P strains. It was tested on *D. hansenii* strains and on some physiologically related species frequently isolated from foods, such as *D. fabrii*, *D. subglobosus* and *Meyerozyma guillermondii*. This method could be applied for the selection of NP *D. hansenii* strains, useful in biotechnological food production and as a biocontrol agent.

Keywords: strains-selection; 1,3-pentadiene; sorbate; spoilage-yeast; food-preservation

1. Introduction

Yeasts are beneficial organisms that contribute to the production of certain foods and beverages [1–4] but can cause spoilage [5,6]. There is an increased concern about the spoilage produced by yeasts [7]. They are able to grow in products with low water activity, pH, and low temperatures [7]. Moreover, few species are able to grow in the presence of preservatives such as low molecular weight weak acids [5,8].

Sorbic acids and their salts are weak acid preservatives whose fungistatic activities are favoured at low pH, where they are found in their undissociated forms. The FDA (U.S. Food and Drug Administration), JEDFA (Joint FAO/WHO Expert Committee on Food Additives) and SCF (Scientific Committee in Food) evaluations consider these preservatives to be among the safest and, according to the EU EFSA Panel, the most effective. However, the microbial decarboxylation of sorbates in a single step produces volatile 1,3-pentadiene that has a petroleum-hydrocarbon-like unpleasant off-odour. Fungal sorbate degradation was first demonstrated on *Penicillium* strains isolated from cheddar cheese, all the strains isolated were able to eliminate the sorbic acid [9]. Later in the 1990s, more yeast strains were described as 1,3-pentadiene producers, including *D. hansenii* strains, which were isolated from cheese, margarine, butter or marzipan [10–13]. *D. hansenii* appears in the inventory of microorganisms with technological benefits for its use in food fermentation [1–3,14]. It is also used in cured meat, where it has been proposed as a starter [15,16] and as a biocontrol agent [17–20]. *D. hansenii's* effectiveness as

a biocontrol agent is well studied but its ability to degrade sorbates if strains survey and remain in the final product has not been analysed. Therefore, a method that distinguishes between 1,3-pentadiene producer (P) or non-producer strains (NP) could be of great interest to the industry.

Detection of 1,3-pentadiene is feasible by sensorial, gas chromatography coupled to mass spectrometry (GC-MS) or MWIR (Mid-Wave IR) devices [11,21–24]. These techniques and their implementation are time-consuming and expensive for 1,3-pentadiene detection.

The decarboxylation of sorbic acid in 1,3-pentadiene requires the removal of the carboxyl group of the molecule. The molecular basis of 1,3-pentadiene production has been studied mainly in strains of *Aspegillus niger* and *Saccharomyces cerevisiae* [25,26] and it was shown that it requires the activity of a Pad1 enzyme (named in this work as Phenylacrylic Acid Decarboxylase). Goodney y Tubb [27] described that the *PAD1* gene (named in this work as *POF1*, Fenolic Off Flavour) encoded for a ferulic acid decarboxylase. Sorbic acid is not considered a phenylacrylic acid as ferulic, cumaric or cinnamic acids but shares some structural characteristics with them, such as a carboxylic group and an aliphatic chain with two double bonds. Further studies in *Aspergillus* reported that a second gene was involved [28]. It is an oxidative decarboxylation produced by two enzyme systems: PAD1 and FDC1 (Ferulic Acid Decarboxylase) [29]. More recently, a positive relation has been reported between the number of single nucleotide polymorphisms of *PAD1* and *FDC1* and ferulic acid decarboxylation in several industrial yeast strains [30]. The aim of this work was to develop a simple method for *D. hansenii* NP strains selection using a new PCR protocol based on the *FDC1Dh* gene.

2. Materials and Methods

2.1. Yeast Strains and Culture Conditions

A total of 129 strains, some of them from 1,3-pentadiene spoiled foods, were used in this work from different Culture Collections or isolated in our laboratory (see Supplementary Material, Table S1). Strains were cultured at 28 °C in Yeast Morphology Broth (YMB) and routinely maintained on the same culture medium plus Agar (YMA): 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, MI, USA), 0.3% (w/v) proteose-peptone No.3 (Difco), 0.3% (w/v) malt extract (Difco), 1% (w/v) glucose (Panreac Quimica S.A., Barcelona, Spain), and 2% (w/v) agar.

For 1,3-pentadiene detection, bottles (20 mL chromatographic magnetic screw-capped, LLG Labware, Meckenheim, Germany) containing 9 mL of YMB pH 7 supplemented with potassium sorbate 0.75g/L (Scharlau, Barcelona, Spain) [24] were inoculated with 1 mL of a saline solution suspension of the yeasts (ca 6 McFarland). The bottles were incubated at 28 °C for 4 days.

2.2. 1,3-Pentadiene Detection

Two methods were used for 1,3-pentadiene detection. (1) GC-MS (GC:Varian CP-3800) coupled with Mass Spectrometry (MS:Saturno 2200 GC/MS/MS in automatic mode and with an automatic CombiPal Splitless injector: Two hundred microliters of headspace volatile compounds were analysed. Pure 1,3-pentadiene was used as an internal standard (50% mixture cis-trans isomers, Aldrich-Chemical, Wisconsin, USA). (2) A sensory method: Three independent experts introduced a needle into the headspace of each culture and sniffed the sample to detect the "petroleum smell" as previously described [24]. Once the accuracy of the sensory method was verified, it was applied to the rest of the strains listed in Table 1.

Table 1. 1,3-pentadiene detection in strains of selected species using the sensory method [24] and chromatography (GC/MS) assays.

Species	Strains	Sensorial Detection	GC/MS
D. hansenii	CECT 11369^T	+	+
	Es 4	+	+
	J-12	-	-
	PR 5	-	-
D. fabryi	CECT 11370^T	+	+
	PR 66	+	+
Z. rouxii	T2R	+	+
	Bch	+	+
	TYN 1.3	-	-
	CYC 1484	-	-
S. cerevisiae	BY 4747	+	+
	Y05833 (ΔPAD1)	-	-
	ATCC 7754	+	+
	EPO 1.1.2	-	-

ATCC: American Type Culture Collection; CECT: Colección Española de Cultivos; T: Type strain.

2.3. Primer Design and Sequencing

For the primer design, we used the putative homologous *FDC1 Saccharomyces cerevisiae* region (1500 bp) [29,30] present in *D. hansenii* as a target, whose sequence was obtained from NCBI GenBank accession No. XM_461563.1 [31]. Based on the nucleotide sequence found in both species, the primers FDC1_Dh_Full_Fw 5′ CTATTTATATCCGTACGCAGACC 3′ and FDC1_Dh_Full_Rv 5′ TAATATGAGCAATTTAAGACCAGAG 3′ were designed. With the objective of analysing differences in sequence between the 1,3-pentadiene *D. hansenii* producing (P) or non-producing strains (NP), a DNA template was obtained as described by Lõoke et al. [32]. PCR amplifications were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) following the protocol described below. After purification (Ultraclean™ PCR clean-up Kit (MO-BIO, Larsband, USA), 80 µL of all positive amplicons were sequenced (ABI PRISM 3730XL DNA Analyzer (Applied Biosystem, Foster, CA, USA). All sequences were aligned with ApE (A plasmid Editor, M.W. Davis) which is freely available [33]. After detecting the differences in the sequence between (P) and (NP), a degenerate primer FDC1_Dh_Pentadien 5′CGTAGACCYTTCTCATAATAGCA 3′, where Y = C or T was designed to amplify a 130 bp intermediate region which was used together with the reverse primer FDC1_Dh_Full_Rv in the PCR reaction described below. The primers used were prepared by Conda Labs-Spain Portal at Integrated DNA Technologies. For validation purposes, each strain was tested at least twice.

2.4. PCR Conditions

DNA amplifications were carried out in 25 µL reactions containing 50–100 ng genomic DNA, 1.25 µL of each primer (20 µM), 12.5 µL NZYtaq2x colourless Mastermix (NzyTech, Lisbon, Portugal) and nuclease-free water to a final volume of 25 µL. Different annealing temperatures were tested, ranging from 52 °C to 68 °C. PCR conditions were as follows: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 45 sec at the T_m selected, 72 °C for 45 sec; and then 1 cycle of 72 °C for 8 min. PCR-amplified DNA fragments were separated in 1% (w/v) agarose gels (Bio-Rad) and visualised under UV light. The GeneRuler 100bp plus DNA Ladder (MBI Fermentas) was used as a molecular size marker.

2.5. Analysis of Protein Sequences

The sequences of the *FDC1Dh* of *D. hansenii* were converted into their corresponding amino acid sequence with the ApE programme, taking into account that the CUG codon of *D. hansenii* codes for

serine instead of leucine. Subsequently, these proteins were aligned using MegAlign—CLUSTAL method, (Lasergene, Madison, WI, USA) and web Clustal Omega [34] and Esprit 3.x [35] web applications.

3. Results

The ability to produce 1,3-pentadiene, indicating sorbate degradation, was studied in selected species (Table 1). The results obtained by gas chromatography coupled with mass spectrometry (GC-MS) were compared to a sensorial method [24] based on Casas et al. [36]. In the chromatographic analysis, the same peak was obtained both in the gas collected in the free headspace of the cultures and in the control samples containing 1,3-pentadiene. The fragmentation of the mass spectrum of that peak presents characteristic ions of 39, 53 and 67 m/z (see Supplementary Material, Figure S1). As can be seen, both GC-MS and olfactory sensorial methods provided the same results (Table 1). In the remaining strains, 1,3-pentadiene was detected using the olfactory sensorial method as described in the Material and Methods section (Table 2). We found only four out of 91 *D. hansenii* strains that did not produce 1,3-pentadiene (NP, non-producers), and therefore nearly 96% of the strains of this yeast were able to produce this volatile compound.

Table 2. Sensorial results for 1,3-pentadiene producing and non-producing yeast species and strains. PCR amplification with specific primers for the differentiation between 1,3-pentadiene producers and non- producers.

Species/ Strains	1,3-Pentadiene Production	Amplification with Primers FDC1_Dh_Pentadien and FDC1_Dh_Full_Rv
Debaryomyces hansenii CECT11369^{T}, CECT10026, CECT10352, CECT10378, CBS1102, CYC1265, CYC1307, Es 4, J-01, J-09, J-11, J-15, J-16, J-17, CH2, Pr11, Pr13, EPEC1.3, EPEC4, E.2, 29C1.2, 29Inf1, V1.1, V1.2, V1.3, V1.4, V1.6, V1.7, V1.8, V1.9, V1.10, V2.2, V2.4, V.2.5, V2.6, V2.7, V2.8, V2.10, V3.1, V3.3, V3.4, V3.5, V3.6, V3.7, V3.8, V3.9, V3.10, A4.1, A4.2, A5.1, A5.2, A8.1, A8.2, ent 1, ent 2, ent 9, ent 50.3,ent 56, ent 64.1, ent 64.5, ent 64.6, ent 81.1, ent 81.2, ent 15, ent 19, ent 24, ent 55, ent 63, ent 65, Rec1.1, Rec1.3, Rec2.3, Rec2.4, Rec9.1, Rec9.2, Rec11.5, Rec13.1, Rec13.3, ent 23, ent 25, ent 28, ent 95.1, ent 96.1, ent 102.1, ent 102.2, ent 102.4, ent 102.5	+	+
Debaryomyces hansenii CECT10517, CBS1792, J-12, Pr5	–	–
Other yeast species		
Debaryomyces fabryi CECT11370^{T}, CECT11365, CBS 6066. ***Debaryomyces subglobosus*** CBS1796^{T}, CBS792 ***Saccharomyces cerevisiae*** ATCC7754, YAA1, ***Wickerhamomyces anomalus*** CECT1114^{T}, CECT10320 ***Zygosaccharomyces rouxii*** CECT1232^{T}, Bch, T2R	+	–
Hanseniaspora uvarum CECT10389, YAb. ***Issachenkia orientalis***, Pim A, PR 3. ***Kregervanrija delftensis*** CECT10238$^{T.}$ ***Lachancea cidri*** CECT10657^{T},. ***Lachancea fermentati*** CECT10382^{T} CECT10678. ***Meyerozyma guilliermondii*** CECT1456^{T}. ***Millerozyma farinosa*** CECT1456^{T}. ***Ogatea angusta*** CECT10220. ***Priceomyces carsonii*** CECT10227^{T}, CECT10230. ***Pichia fermentans*** CECT1455^{T}. ***Pichia membranifaciens*** CECT1115^{T}. ***Saccharomyces cerevisiae*** CYC1172, CYC1220. ***Schwanniomyces etchelsii*** CECT11412. ***Torulaspora delbrueckii*** CYC1391^{T}, CYC1176. ***Wickerhamomyces anomalus*** CECT1112. ***Yarrowia lipolytica*** PR 7, PR 12. ***Zygosaccharomyces bailii*** CECT1898^{T}, CECT11042. ***Zygosaccharomyces mellis*** CECT10066.	–	–

ATCC: American Type Culture Collection; CBS: Centraalbureau voor Schimmelcultures; CECT: Colección Española de Cultivos. ^{T}: Type strain. +, 1.3-pentadiene production or amplification with primers pair. -, non produces 1,3 pentadiene or non amplify with primers pair.

Next, to achieve our goal of obtaining specific primers for the detection of *D. hansenii* strains producing 1,3-pentadiene, we designed a primer pair based on a *S. cerevisiae FCD1* gene sequence to amplify putative homologous gene from *D. hansenii* gDNA [29,30]. The amplified region presents a sequence identity of 66% with the *FDC1* gene of *S. cerevisiae*. The best result for the amplification of the *FDCDh* region was obtained after 30 cycles and with an annealing temperature of 59 °C. A single fragment of about 1542 bp was amplified from all of the *D. hansenii* strains. By analysing these sequences we observed nucleotide polymorphism of the *FDC1Dh* gene between 1,3-pentadiene producer (P) and non-producer (NP) strains (see Supplementary Material, Figure S2). Many nucleotide differences were related to amino acid changes (Table 3). Additionally, and most importantly, all NP strains contain at least one deletion in the nucleotide sequence of the *FDC1Dh* gene (Table 3). Specifically, the deletion of adenine or guanine in position 383 alters the reading frame and consequently, it would be responsible for a premature STOP codon. Only one NP strain, PR5, had two more deletions in the positions 281 and 1234, the first of them being responsible for an alteration of the reading frame and a premature STOP (see asterisks in Figure S2).

Table 3. Nucleotide polymorphisms in gene *FDC1Dh* that produce amino acid changes in the putative protein sequence. The numbers indicate the nucleotides positions in the gene.

Nucleotide	127 G-A	145 C-T	156 A-T	281 *	328 A-G	362 T-C	367 C-A	383 G-A	 *	458 A-C
***D. hansenii* 1,3-pentadiene producer strains**										
CECT 11369T										
CECT 10352		+						+		
CECT 10386			+		+	+	+			+
CH2			+		+	+	+			+
EPEC 1.3		+						+		
***D. hansenii* 1,3-pentadiene no producer strains**										
CECT 10517	+		+		+	+	+		+	+
CBS 1792	+		+		+	+	+		+	+
J-12	+		+		+	+	+		+	+
PR 5	+		+	+	+	+	+		+	+

Nucleotide	733 G-A	775 A-G	798 T-A	1127 A-G	1183 C-A	1234 *	1251 T-A	1329 G-A	1389 A-C	1434 A-T
D. hansenii 1,3-pentadiene producer strains										
CECT 11369T					+					
CECT 10352	+			+			+	+	+	
CECT 10386		+	+	+			+	+	+	
CH2		+	+		+					
EPEC 1.3	+			+			+	+	+	+
D. hansenii 1,3-pentadiene no producer strains										
CECT 10517		+	+	+			+	+	+	+
CBS 1792		+	+	+		+	+	+	+	+
J-12		+	+	+			+	+	+	+
PR 5		+	+	+		+	+	+	+	+

+, Substitution in amino acid; *,Nucleotide deletion.

As mentioned, one of the objectives of this work was to develop a simple method for differentiating P and NP strains of *D. hansenii* by PCR. For this, an FDC1_Dh_Pentadien degenerated primer was designed, based on the sequences of the *FDC1Dh* gene of the strains, as described in the Material and Methods section. It comprises the position 127 where a base change was detected in P and NP strains. The primer also contains a Y in position 125 that hybridises with C or T present in the sequence of P or NP strains, respectively (see Supplementary Material, Figure S2).

The specificity of the primers and the PCR protocol developed was tested on DNA templates obtained from yeast strains listed in Supplementary Material, Table S1. All P strains of *D. hansenii* gave a positive result with clear amplicons of 130 bp, whereas no amplification was found in NP strains (Figure 1). The rest of the yeast species included in the study showed no amplification, although they were 1,3-pentadiene producing strains (Table 1), supporting the specificity of developed PCR assay for *D. hansenii* strains.

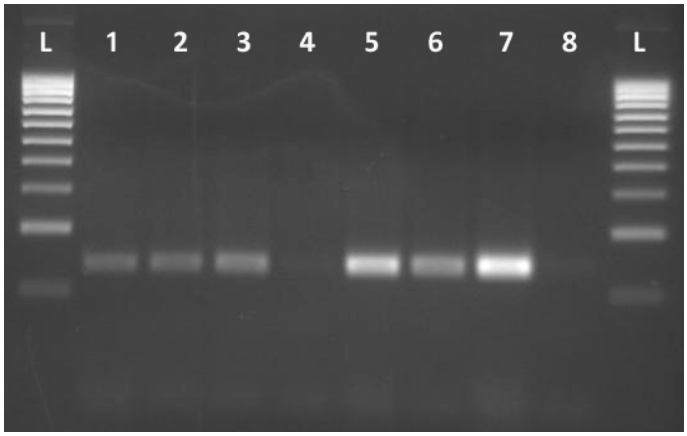


Figure 1. PCR amplification result obtained with primer pair Figure 1. Dh_Full_Rv. L: 100bp ladder. Lines 1-3, 5-7: *Debaryomyces hansenii* 1,3-pentadiene producer strains: CECT 11369T, Es4, EPEC 1.3, CECT 10352, CECT 10378, CH2, respectively. Line 4: *D.hansenii* 1,3-pentadiene no producer strain: J-12; Lane 8: Negative control.

4. Discussion

Debaryomyces hansenii shows a dual role in the food industry. It has different biotechnological applications, but it is also capable of spoiling certain products. Among the positive aspects, the yeast is considered a promising alternative to chemical fungicides used in agriculture and several strains have been proposed as biological control agents [17–20]. However, if potentially spoiling strains are used, such as those that degrade sorbates, the yeasts present on the fruits or vegetables could remain in the final products obtained [37–39]. Taking into account that the decarboxylation of sorbic acid is not a property of the yeast species but of the strain [40], the selection of strains that do not produce unpleasant "petroleum-like"off-odours would be of importance for quality and safety reasons. According to the International Chemical Safety Card (CAS No. 504-60-9), a low exposure (concentrations) of 1,3-pentadiene does not have an adverse effect on humans. Nevertheless, the problem is not only the production of a compound with an offensive odour but the fact that the antimicrobial action of the sorbates disappears and other undesirable fungi or bacteria can grow on the food.

Table 2 shows that the production of volatile 1,3-pentadiene is a common feature in the *D. hansenii* strains studied. Under the study conditions, a conversion of 45% of the sorbate into 1,3-pentadiene was measured by chromatography in *D. hansenii* (data not shown). The ability to produce 1,3-pentadiene is a strain characteristic, surprisingly nearly 96% of 91 *D. hansenii* strains analysed were able to produce this compound. Thus, many strains of *D. hansenii* can cause spoilage. Given that both methods, chromatographic and sensorial, need isolation and cultivation as well as another subsequent cultivation for four days with sorbates, a search for the differences between strains was conducted to develop a fast and accurate molecular method. Based on the *PAD*1 sequence, we previously developed a molecular method for the rapid detection of *D. hansenii* species [41]. However, when beginning this work we did not find differences in the *PAD*1 sequence between (P) and (NP) strains (data not shown). We thus focused our study on the *FDC1* gene. In this work, we describe for the first time a *D. hansenii* putative homologue sequence of the *S. cerevisiae FCD1/YDR539W* gene [42] related to the decarboxylation of sorbates. We developed a PCR protocol based on the differences in the *FDC*1 sequence between (P) and (NP) strains. The primers FDC1_Dh_Pentadien and FDC1_Dh_Full_Rv developed in this assay produce a clear single fragment of 130 bp in all (P) *D. hansenii* strains tested (Table 2), and no false negatives were detected. Additionally, no false positives were found in the other 21 species included in the study. For the industry and control laboratories, this method is easier, quicker and less tedious than the sensorial method, as well as less expensive than the chromatographic method. A 24 h culture,

instead of four days, is the time required by the PCR method to differentiate between *D. hansenii* 1,3-pentadiene producer strains and non-producer strains.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/2/162/s1, Figure. S1: Gas chromatogram (above) and mass spectra (below) from the headspace gas of a suspension in YMB of pure 1,3-pentadiene (A) and from the head gas of D. hansenii CECT 11369T cultured on YMB with 0.75 g/l potassium sorbate (B), Figure. S2: Part of the FDC1Dh nucleotide alignment in selected strains of D. hansenii including the most significant base changes. In blue, the producing strains of 1,3-pentadiene and in orange, the non-producing strains. In red, the different bases are highlighted and framed with a black rectangle. The stars show where there is nucleotide deletion. The black numbers indicate the position of each nucleotide within the gene, Table S1: Yeast species and strains used in this study and origin.

Author Contributions: P.W. and E.-M.R. performed the experiments. P.W. and M.-I.d.S. wrote the paper. M.-I.d.S. conceived and designed the experiments All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported in part by the projects from the Ministry of Economy and Competitiveness (RT2018-0975593-B-C2). Research by Eva Rivas has been supported by a PICATA predoctoral fellowship (CEI Campus Moncloa, UCM-UPM, Madrid, Spain).

Acknowledgments: We would like to thank Nour Kayali and Cristina Gutiérrez for the chromatographic analysis carried out at the Mass Spectrometry Centre of the Complutense University (UCM) and Bill Newton from linguistic department of UCM.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Dolci, P.; Barmaz, A.; Zenato, S.; Pramotton, R.; Alessandria, V.; Cocolin, L.; Rantsiou, K.; Ambrosoli, R. Maturing dynamics of surface microflora in Fontina PDO cheese studied by culture-dependent and -independent methods. *J. Appl. Microbiol.* **2009**, *106*, 278–287. [CrossRef] [PubMed]
2. Padilla, B.; Manzanares, P.; Belloch, C. Yeast species and genetic heterogeneity within *Debaryomyces hansenii* along the ripening process of traditional ewes' and goats' cheeses. *Food Microbiol.* **2014**, *38*, 160–166. [CrossRef]
3. Petersen, K.M.; Westall, S.; Jespersen, L. Microbial succession of *Debaryomyces hansenii* strains during the production of Danish surfaced-ripened cheeses. *J. Dairy Sci.* **2002**, *85*, 478–486. [CrossRef]
4. Rai, A.K.; Pandey, A.; Sahoo, D. Biotechnological potential of yeasts in functional food industry. *Trends Food Sci. Technol.* **2019**, *83*, 129–137. [CrossRef]
5. Deák, T. *Handbook of Food Spoilage Yeasts*, 2nd ed.; CRC Press: Boca Ratón, FL, USA, 2008.
6. Hernández, A.; Pérez-Nevado, F.; Ruiz-Moyano, S.; Serradilla, M.J.; Villalobos, M.C.; Martín, A.; Córdoba, M.G. Spoilage yeasts: What are the sources of contamination of foods and beverages? *Int. J. Food Microbiol.* **2018**, *286*, 98–110. [CrossRef] [PubMed]
7. Rivas, E.M. *Interacción Sorbatos-Levaduras en Alimentos: Modelización de su Efecto en Medio Sólido, Biología Molecular y Detección de su Transformación en 1, 3-Pentadieno por MWIR*; Universidad Complutense de Madrid: Madrid, Spain, 2017.
8. Loureiro, V.; Querol, A. The prevalence and control of spoilage yeasts in foods and beverages. *Trends Food Sci. Technol.* **1999**, *10*, 356–365. [CrossRef]
9. Marth, E.H.; Capp, C.M.; Hasenzahl, L.; Jackson, H.W.; Hussong, R.V. Degradation of Potassium Sorbate by *Penicillium* Species. *J. Dairy Sci.* **1966**, *49*, 1197–1205. [CrossRef]
10. Sensidoni, A.; Rondinini, G.; Peressini, D.; Maifreni, M.; Bortolomeazzi, R. Presence of an off-flavour associated with the use of sorbates in cheese and margarine. *Ital. J. Food Sci.* **1994**, *6*, 237–242.
11. Casas, E.; de Ancos, B.; Valderrama, M.J.; Canob, P.; Peinado, J.M. Pentadiene production from potassium sorbate by osmotolerant yeasts. *Int. J. Food Microbiol.* **2004**, *94*, 93–96. [CrossRef]
12. Casas, E.; Valderrama, M.J.; Peinado, J.M. Spoilage of marzipan products by an osmotolerant yeast. *Adv. Food Sci.* **1996**, *18*, 56–60.
13. Quirós, M.; Martorell, P.; Querol, A.; Barrio, E.; Peinado, J.M.; De Silóniz, M.I. Four new *Candida cretensis* strains isolated from Spanish fermented sausages (chorizo): Taxonomic and phylogenetic implications. *FEMS Yeast Res.* **2008**, *8*, 485–491. [CrossRef] [PubMed]

14. Wrent, P.; Rivas, E.M.; Gil de Prado, E.; Peinado, J.M.; de Silóniz, M.I. Debaryomyces. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Elsevier Ltd., Academic Press: Oxford, UK, 2014; Volume 1, pp. 563–570.
15. Andrade, M.J.; Cordoba, J.J.; Casado, E.M.; Cordoba, M.G.; Rodriguez, M. Effect of selected strains of *Debaryomyces hansenii* on the volatile compound production of dry fermented sausage "salchichón". *Meat Sci.* **2010**, *85*, 256–264. [CrossRef] [PubMed]
16. Ramos-Moreno, L.; Ruiz-Castilla, F.J.; Bravo, C.; Martínez, E.; Menéndez, M.; Dios-Palomares, R.; Ramos, J. Inoculation with a terroir selected Debaryomyces hansenii strain changes physico-chemical characteristics of Iberian cured pork loin. *Meat Sci.* **2019**, *157*, 107875. [CrossRef] [PubMed]
17. Gil-Serna, J.; Patiño, B.; Cortés, L.; González-Jaén, M.T.; Vázquez, C. Mechanisms involved in reduction of ochratoxin A produced by *Aspergillus westerdijkiae* using *Debaryomyces hansenii* CYC 1244. *Int. J. Food Microbiol.* **2011**, *151*, 113–118. [CrossRef]
18. Chalutz, E.; Wilson, C.L. Postharvest biocontrol of green and blue mold and sour rot of citrus fruit by *Debaryomyces hansenii*. *Plant Dis.* **1990**, *74*, 134–137. [CrossRef]
19. Liu, S.-Q.; Tsao, M. Biocontrol of dairy moulds by antagonistic dairy yeast *Debaryomyces hansenii* in yoghurt and cheese at elevated temperatures. *Food Control* **2009**, *20*, 852–855. [CrossRef]
20. Czarnecka, M.; Żarowska, B.; Połomska, X.; Restuccia, C.; Cirvilleri, G. Role of biocontrol yeasts Debaryomyces hansenii and Wickerhamomyces anomalus in plants' defence mechanisms against Monilinia fructicola in apple fruits. *Food Microbiol.* **2019**, *83*, 1–8. [CrossRef]
21. Liewen, M.B.; Marth, E.H. Growth and inhibition of microorganisms in the presence of sorbic acid: A review. *J. Food Prot.* **1985**, *48*, 364–375. [CrossRef]
22. Pinches, S.; Apps, P. Production in food of 1,3-pentadiene and styrene by Trichoderma species. *Int. J. Food Microbiol.* **2007**, *116*, 182–185. [CrossRef]
23. Nieminen, T.; Neubauer, P.; Sivela, S.; Vatamo, S.; Silfverberg, P.; Salkinojasalonen, M. Volatile compounds produced by fungi grown in strawberry jam. *LWT Food Sci. Technol.* **2008**, *41*, 2051–2056. [CrossRef]
24. Rivas, E.M.; Maldonado, M.; Diezma, B.; Wrent, P.; Peinado, J.; de Silóniz, M.I.; Vergara, G.; García Hierro, J.; Robla, J.I.; Barreiro, P. Detection of Biological CO2 and 1,3-Pentadiene Using Non-refrigerated Low-Cost MWIR Detectors. *Food Anal. Methods* **2016**, *9*, 1451–1460. [CrossRef]
25. Plumridge, A.; Stratford, M.; Lowe, K.C.; Archer, D.B. The weak-acid preservative sorbic acid is decarboxylated and detoxified by a phenylacrylic acid decarboxylase, PadA1, in the spoilage mold Aspergillus niger. *Appl. Environ. Microbiol* **2008**, *74*, 550–552. [CrossRef] [PubMed]
26. Stratford, M.; Plumridge, A.; Archer, D.B. Decarboxylation of Sorbic Acid by Spoilage Yeasts Is Associated with the PAD1 Gene. *Appl. Environ. Microbiol.* **2007**, *73*, 6534–6542. [CrossRef] [PubMed]
27. Goodey, A.R.; Tubb, R.S. Genetic and Biochemical Analysis of the Ability of *Saccharomyces cerevisiae* to Decarboxylate Cinnamic Acids. *Microbiology* **1982**, *128*, 2615–2620. [CrossRef]
28. Plumridge, A.; Melin, P.; Stratford, M.; Novodvorska, M.; Shunburne, L.; Dyer, P.S.; Roubos, J.A.; Menke, H.; Stark, J.; Stam, H. The decarboxylation of the weak-acid preservative, sorbic acid, is encoded by linked genes in *Aspergillus* spp. *Fungal Genet. Biol.* **2010**, *47*, 683–692. [CrossRef]
29. Mukai, N.; Masaki, K.; Fujii, T.; Kawamukai, M.; Iefuji, H. *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* **2010**, *109*, 564–569. [CrossRef]
30. Mukai, N.; Masaki, K.; Fujii, T.; Iefuji, H. Single nucleotide polymorphisms of *PAD1* and *FDC1* show a positive relationship with ferulic acid decarboxylation ability among industrial yeasts used in alcoholic beverage production. *J. Biosci. Bioeng.* **2014**, *118*, 50–55. [CrossRef]
31. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinform.* **2009**, *10*, 421. [CrossRef]
32. Lõoke, M.; Kristjuhan, K.; Kristjuhan, A. Extraction of genomic DNA from yeasts for PCR-based applications. *BioTechniques* **2011**, *50*, 325–328. [CrossRef]
33. ApE-A plasmid Editor. Available online: http://www.biology.utah.edu/jorgensen/wayned/ape/ (accessed on 1 June 2017).
34. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T.J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Soding, J.; et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **2011**, *7*, 539. [CrossRef]

35. Robert, X.; Gouet, P. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res.* **2014**, *42*, W320–W324. [CrossRef]
36. Casas, E.; Valderrama, M.J.; Peinado, J.M. Simplified Test for the Rapid Detection of Dangerous Yeasts and Filamentous Fungi in Marzipan Products. In *Improved Tradicional Foods for the Next Century*; Toldrá, F., Ramón, D., Navarro, J.L., Eds.; Gráficas Barrastil: Valencia, Spain, 1999; pp. 304–307.
37. Núñez, F.; Lara, M.S.; Peromingo, B.; Delgado, J.; Sánchez-Montero, L.; Andrade, M.J. Selection and evaluation of Debaryomyces hansenii isolates as potential bioprotective agents against toxigenic penicillia in dry-fermented sausages. *Food Microbiol.* **2015**, *46*, 114–120. [CrossRef]
38. Kinay, P.; Yildiz, M. The shelf life and effectiveness of granular formulations of *Metschnikowia pulcherrima* and *Pichia guilliermondii* yeast isolates that control postharvest decay of citrus fruit. *Biol. Control* **2008**, *45*, 433–440. [CrossRef]
39. Wrent, P.; Rivas, E.M.; Gil de Prado, E.; Peinado, J.M.; de Silóniz, M.I. Assessment of the factors contributing to the growth or spoilage of *Meyerozyma guilliermondii* in organic yogurt: Comparison of methods for strain differentiation. *Microorganisms* **2015**, *3*, 428–440. [CrossRef]
40. Wrent, P.; Rivas, E.M.; Peinado, J.M.; de Silóniz, M.I. Strain typing of *Zygosaccharomyces* yeast species using a single molecular method based on polymorphism of the intergenic spacer region (IGS). *Int. J. Food Microbiol.* **2010**, *142*, 89–96. [CrossRef]
41. Wrent, P.; Rivas, E.M.; Gil de Prado, E.; Peinado, J.M.; de Silóniz, M.I. Development of species-specific primers for rapid identification of *Debaryomyces hansenii*. *Int. J. Food Microbiol.* **2015**, *193*, 109–113. [CrossRef]
42. Saccharomyces genome database. Available online: http://www.yeastgenome.org (accessed on 1 June 2017).

foods

MDPI

Article

Optimization of a Novel Method Based on Ultrasound-Assisted Extraction for the Quantification of Anthocyanins and Total Phenolic Compounds in Blueberry Samples (*Vaccinium corymbosum* L.)

María José Aliaño-González [1], José Antonio Jarillo [1], Ceferino Carrera [1], Marta Ferreiro-González [1], José Ángel Álvarez [2], Miguel Palma [1], Jesús Ayuso [2], Gerardo F. Barbero [1,*] and Estrella Espada-Bellido [1]

[1] Department of Analytical Chemistry, Faculty of Sciences, Agrifood Campus of International Excellence (ceiA3), IVAGRO, University of Cadiz, 11510 Puerto Real, Cadiz, Spain; mariajose.alianogonzalez@alum.uca.es (M.J.A.-G.); joseantoniojarillo@gmail.com (J.A.J.); ceferino.carrera@uca.es (C.C.); marta.ferreiro@uca.es (M.F.-G.); miguel.palma@uca.es (M.P.); estrella.espada@uca.es (E.E.-B.)

[2] Department of Physical Chemistry, Faculty of Sciences, Institute of Biomolecules (INBIO), University of Cadiz, 11510 Puerto Real, Cadiz, Spain; joseangel.alvarez@uca.es (J.Á.Á.); jesus.ayuso@uca.es (J.A.)

* Correspondence: gerardo.fernandez@uca.es; Tel.: +34-956-016355; Fax: +34-956-016460

Received: 3 November 2020; Accepted: 26 November 2020; Published: 28 November 2020

Abstract: In recent years, consumers' preference for fruits such as blueberry has increased noticeably. This fact is probably related to their bioactive components such as anthocyanins, phenolic compounds, vitamins, minerals, and tannins that have been found in blueberries by the latest research studies. Both total anthocyanins (TA) and total phenolic compounds (TPC) are known for their multiple beneficial effects on our health, due to their anti-inflammatory, anti-oxidant, and anti-cancer properties. This is the reason why the development of new methodologies for the quality control analysis of raw materials or derived products from blueberry has a great relevance. Two ultrasound-assisted extraction methods (UAE) have been optimized for the quantification of TA and TPC in blueberry samples. The six variables to be optimized were: solvent composition, temperature, amplitude, cycle, extraction solvent pH, and sample/solvent ratio using response surface methodology. The optimized methods have proven to be suitable for the extraction of the TPC and TA with good precision (repeatability and intermediate precision) (coefficient of variation (CV) < 5%) and potentially for application in commercial samples. This fact, together with the multiple advantages of UAE, makes these methods a good alternative to be used in quality control analysis by both industries and laboratories.

Keywords: anthocyanins; antioxidants; blueberry; Box–Behnken design; phenolic compounds; response surface methodology; ultra-high-performance liquid chromatography; UHPLC; UV–Vis; *Vaccinium corymbosum* L.

1. Introduction

Blueberry is a small reddish or bluish-black fruit with a size of around 1.5 cm in diameter that grows in deciduous shrubs from the Ericaceae family [1,2]. Blueberry has been extensively cultivated worldwide with an estimated production of over 600,000 tons per year, being Canada, Chile, China, Spain, USA, and Morocco, among other 30 countries, the major blueberry distributors [3].

Although blueberry is not so often consumed as fresh fruit, it is increasingly common to find it as the main ingredient in jams and pastry preparations or as a secondary ingredient in different candies or desserts (yogurts, cookies, etc.) [4–6]. Moreover, recent investigations have proven its high content in anthocyanins, phenolic compounds, vitamins, and minerals. A fact that has caused blueberry to be included in different extracts and numerous food supplements [7].

The Ericaceae family includes nearly 4000 different plant species, where the *Vaccinium* genus is one of the best known and more frequently consumed [8]. The *Vaccinium* genus can be categorized into four main varieties: bilberry (*V. myrtillus*), blueberry (*V. angustifolium, V. ashei,* and *V. corymbosum*), cranberry (*V. macrocarpon* and *V. oxycoccos*), and lingonberry (*V. Vitis-idaea*), with *V. corymbosum* being one of the most cultivated varieties. Its fruit, with a bittersweet and rather pleasant flavor, exhibits very interesting nutritional properties because of its high content in compounds of biological interest such as phenolic compounds, like catechol, coumaric acid, chlorogenic acid, ellagic acid, epicathechin, gallic acid, anthocyanins (cyanidin 3-*O*-galactoside, delphinidin 3-*O*-arabinoside, petunidin 3-*O*-galactoside, malvidin 3-*O*-galactoside, etc.) and flavonols (mainly quercetin derivatives) [9–13]. Culture conditions of *V. corymbosum* have shown a clear influence on its composition, including phenolic compounds. In relation to temperature, different authors have shown that temperatures between 20 and 30 °C ensure the highest phenolic concentration in blueberries, while higher temperatures have adverse impacts on their growth and composition [14–16]. In addition, the use of ammonium compounds (RNH_4^+) improved the plant performance and the phenolic compounds composition. Similar effects were detected when phytochemicals with acid pH were applied, indicating that acidification is an important mechanism in the composition of blueberry [17,18].

Different research works have proven that blueberry, similarly to other dark-colored berries, contains a high polyphenols concentration, such as total anthocyanins (TA) and phenolic compounds (TPC) [19]. Consequently, blueberry extracts have exhibited beneficial properties for human health that have been associated with the presence of these compounds. They have been confirmed as a considerable advancement for the treatment of cardiovascular diseases [20,21], diabetes [22] or even for the prevention of atherosclerosis [23,24]. In addition, decisive anti-inflammatory activity has been appreciated [25], since they reduce cell death and diminish the morphological criteria that are associated with inflammation in microglia cell cultures. This discovery represents a novel way for the treatment of some neurological diseases [26]. They have also shown a significant activity to decrease oxidative stress (anti-oxidant) with a significant effect against pulmonary arterial hypertension [27], skin damage by UV radiation [28,29] and in the microbial diversity with health benefits [30,31]. Finally, blueberry extracts have exhibited a meaningful activity that favors the treatment of different types of cancer including leukemia [32–34].

Therefore, it has been proven that extracts rich in TA and TPC obtained from blueberry are suitable for a considerable number of applications in the nutrition, cosmetics or medicine industries. For this reason, the development of new methodologies that allow the extraction of these compounds from the blueberries in a fast, easy to use and reliable way is of great importance. Several analytical extraction techniques such as maceration [35–37], Soxhlet [38,39], pressurized liquid extraction [40,41], microwave-assisted extraction [42–44], have been employed to obtain rich anthocyanins and phenolic compound extracts from berries. Among them, ultrasound-assisted extraction (UAE) [45] and pressurized liquid extraction (PLE) [46,47] are the most commonly used for the extraction of both bioactive compounds in blueberries.

UAE has been successfully used for the recovery of TA and TPC. This technique produces the extraction of the organic compounds that can be found in different matrices making use of the energy derived from ultrasounds. Its efficiency is attributed to acoustic cavitation, a phenomenon which consists on the formation, development and collapse of microbubbles on the surface of the solid, which allows the penetration of the solvent into the solid and favors mass transfer processes [48]. In addition, ultrasounds exert a mechanical effect that can contribute to both the release of intracellular material and the desorption of compounds from the solid surface, resulting in higher extraction rates. Finally,

the use of a wider temperature range results in an increased production of cavitation bubbles and consequently improved extraction efficiency [49].

In addition, UAE presents some critical advantages, such as its simplicity, low acquisition cost, no specific maintenance requirements and availability in most laboratories. For all these reasons, this technique has been largely applied to the extraction of bioactive compounds from similar matrices such as myrtle [50], açai [51], black chokeberry [52], maqui [53], sesame [54–56]. In fact, different researchers has previously employed UAE to study the blueberry composition [57–62]. However, as far as the authors are concerned, an exhaustive study (evaluating seven extraction variables) using UAE for the extraction of these bioactive compounds from blueberries has not yet been performed. Different solvents have been applied on UAE of several fruits, with methanol being one of the most employed due to its high effectivity in TA and TPC extraction. It is true that methanol has been classified as class 2 according to FDA (Food and Drug Administration) [63] due to the toxicity and the health consequences when it is consumed. Nevertheless, it is extensively recommended for analytical purposes due to its polarity, viscosity and small size, which makes it very easy to penetrate into cell membranes.

This research is focused on the optimization of two methods based on UAE. Both methods have been combined with spectrometric techniques to confirm that the developed optimized methods produce a successful recovery of TA and TPC from blueberry and, therefore, could be used by industries and laboratories. Following such purposes, repeatability and intermediate precision analyses have been performed and the methods that have been developed have been tested on real samples to demonstrate their applicability.

2. Materials and Methods

2.1. Blueberry Samples

To optimize the extraction methods, 5 kg of blueberries at their optimal state of maturity (*Vaccinium corymbosum* L. var. Legacy) was collected from a 1.5 ha plantation at the village of Ballota (Asturias-Spain; 43.552571, −6.329105) during the month of August. The sampling was carried out randomly throughout the entire crop plot, picking blueberries from all parts of the plant (upper, middle and lower zone). Afterward, the fresh berries were freeze-dried and crushed by means of a conventional electric grinder and the final matrix was homogenized and kept at −20 °C in a freezer until use.

Once the two methods were optimized, they were tested on commercial samples to ensure their actual suitability for industrial and research analysis. In order to guarantee a diversity of samples, five blueberry (*Vaccinium corymbosum* L.) jams were acquired from three different sources and with different content percentages of blueberry according to the respective product's label. Their descriptions can be seen in Table 1. The commercial samples were also kept at −20 °C until analysis.

Table 1. Commercial samples acquired to corroborate the validity of the optimized extraction methods.

Commercial Mark	Origin of Production	% Blueberry
Jam 1	Spain	45
Jam 2	Spain	50
Jam 3	Spain	60
Jam 4	Brazil	50
Jam 5	France	65

2.2. Solvents and Chemical Agents

Methanol (Fisher Scientific, Loughborough, UK), and formic acid (Panreac, Barcelona, Spain), both high-performance liquid chromatography (HPLC) grade, were used for extraction and chromatographic analysis. Ultra-pure water was obtained from a Milli-Q water purifier system

from Millipore (Bedford, MA, USA) and hydrochloric acid (Panreac, Barcelona, Spain; "for analysis" grade) was employed to adjust the pH of the solvents for the extraction.

For the anthocyanin quantification, the standard cyanidin chloride was acquired with a purity higher than 95% from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) whereas for total phenolic compounds analysis, Folin–Ciocalteu reactive (EMD Millipore, Darmstadt, Germany), sodium carbonate anhydrous (Panreac, Barcelona, Spain), and gallic acid standard ≥ 99% (Sigma–Aldrich Chemical Co., St. Louis, MO, USA) were used.

2.3. Ultrasound-Assisted Extraction

A UP200S sonifier (200 W, 24 kHz) (Dr. Hielscher. GmbH, Teltow, Germany) equipment was selected to carry out this study. The sonifier included a water bath coupled to a temperature controller (FRIGITERM-10, J.P. Selecta, S.A., Barcelona, Spain) to allow the optimization of this variable during the development of the method.

Six variables were selected for the optimization and three different levels were established for the range of study (low, medium and high): extraction solvent (25–50–75% MeOH in water), temperature of extraction (10–40–70 °C), amplitude (30–50–70% of the maximum amplitude—200 W), cycle (0.2–0.45–0.7 s), pH of the extraction solvent (2–4.5–7) and samples' mass (g):solvent volume (mL) ratio (0.5:10–0.5:15–0.5:20). Both the selected variables to be optimized and their range of study were chosen according to the existing bibliography and the previous experience of the research group with similar matrices and compounds [51,53,64]. The amount of sample and the time of the experiment were set at 0.5 g and 10 min, respectively, based on similar studies previously completed by our research group [43,65].

In this way, 0.5 g of the sample was weighted and the specific amount of solvents to reach the ratio corresponding to each experiment was added. These solvents had been previously prepared to ensure the right percentage of methanol in water corresponding to each experiment.

The required volume of solution was added to the amount of sample, the extraction probe was put in the mixture and the extraction was carried out under the specific conditions of each experiment. After 10 min, the centrifugation process was realized twice for 5 min at 7500 rpm (9.5 cm orbital radius). The supernatant was moved to a 25 mL volumetric flask and a 0.20 µm nylon syringe filter (Membrane Solutions, Dallas, TX, USA) was employed before the analysis.

2.4. Box–Behnken Design

This research was aimed at developing two extraction methods that would achieve the maximum possible recoveries of TA and TPC from freeze-dried blueberry samples. For this purpose, a Box–Behnken design with response surface methodology (BBD-RSM) was used to optimize the six extraction variables. The amount of TA (mg of anthocyanins per g of sample) determined by ultra-high-performance liquid chromatography (UHPLC) was used as the response variable and the TPC (mg of gallic acid equivalents (GAE) per g of sample) determined by the method of Folin–Ciocalteu was considered as the other response variable. The resulting design included a total of 54 extractions including 6 at the central point (Table S1).

The second-order polynomial equation below, which includes all the six variables, was applied to the responses obtained from all the extractions.

$$\begin{gathered} Y = \beta_0 + \beta_{1\times 1} + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \\ \beta_{16} X_1 X_6 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{26} X_2 X_6 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{36} X_3 X_6 + \beta_{45} X_4 X_5 + \\ \beta_{46} X_4 X_6 + \beta_{56} X_5 X_6 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{44} {X_4}^2 + \beta_{55} {X_5}^2 + \beta_{66} {X_6}^2 \end{gathered} \quad (1)$$

In our equation, Y is the corresponding response, β_0 is the ordinate at the origin; X_1 (percentage of MeOH in the extraction solvent), X_2 (the extraction temperature), X_3 (the ultrasound amplitude), X_4 (the cycle), X_5 (the solvent pH), and X_6 (the ratio of solid sample (g): extraction volume (mL)) are the

independent variables; β_i represents the linear coefficients; β_{ij} depicts the cross product coefficients and βii shows the quadratic coefficients.

Statgraphic Centurion (version XVII) (Statgraphics Technologies, Inc., The Plains, VA, USA) was the software used to calculate the effects of the extraction variables on the final response, the second-order mathematical model, the surface plots, the optimal levels of the significant variables and the variance of the analysis.

2.5. Anthocyanins Identification by UHPLC-Q-ToF-MS

The TA present in the blueberry samples were identified by an ultra-high-performance liquid chromatography (UHPLC) system coupled to quadrupole time-of-flight (Q-ToF-MS) mass spectrometry (XEVO G2, Waters Corp., Milford, MA, USA). The chromatography column employed was a C18 with dimensions of 2.1 mm × 100 mm and a particle size of 1.7 µm (Acquity UPLC BEH C18, Waters). The injection volume was set at 3 µL. The chromatographic conditions used as well as the analysis conditions in the mass spectrometer (Q-ToF-MS) are those described by Aliaño et al. [42].

The identification of the TA present in blueberries was determined by the exact *m*/*z* mass/charge ratio obtained for each of the chromatographic peaks corresponding to anthocyanins. The ratio was compared with other works found in the literature that describe the anthocyanins present in blueberries [66], and by the order of elution of the anthocyanins. A total of 14 anthocyanins were identified in the blueberry extracts; the data regarding each compound and the theoretical and measured mass/charge ratios can be found in Table 2.

Table 2. Mass information and regression equation data of the 14 anthocyanins identified in blueberry extracts.

Anthocyanins	Molecular Formula	*m*/*z*	Regression Equation	R^2 *	LOD ** (ppm)	LOQ *** (ppm)
Malvidin 3-*O*-galactoside	$C_{23}H_{25}O_{12}$	493.1346	y = 170,426.24x − 4292.66	0.9999	0.303	1.012
Malvidin 3-*O*-glucoside	$C_{23}H_{25}O_{12}$	493.1346	y = 170,426.24x − 4292.66	0.9999	0.303	1.012
Petunidin 3-*O*-galactoside	$C_{22}H_{23}O_{12}$	479.1190	y = 175,412.35x − 4292.66	0.9999	0.294	0.983
Petunidin 3-*O*-glucoside	$C_{22}H_{23}O_{12}$	479.1190	y = 175,412.35x − 4292.66	0.9999	0.294	0.983
Delphinidin 3-*O*-galactoside	$C_{21}H_{21}O_{12}$	465.1033	y = 180,699.41x − 4292.66	0.9999	0.286	0.955
Delphinidin 3-*O*-glucoside	$C_{21}H_{21}O_{12}$	465.1033	y = 180,699.41x − 4292.66	0.9999	0.286	0.955
Peonidin 3-*O*-galactoside	$C_{22}H_{23}O_{11}$	463.1240	y = 181,468.74x − 4292.66	0.9999	0.284	0.951
Peonidin 3-*O*-glucoside	$C_{22}H_{23}O_{11}$	463.1240	y = 181,468.74x − 4292.66	0.9999	0.284	0.951
Malvidin 3-*O*-arabinoside	$C_{23}H_{23}O_{11}$	463.1240	y = 181,468.74x − 4292.66	0.9999	0.284	0.951
Cyanidin 3-*O*-galactoside	$C_{21}H_{21}O_{11}$	449.1084	y = 187,132.66x − 4292.66	0.9999	0.276	0.922
Cyanidin 3-*O*-glucoside	$C_{21}H_{21}O_{11}$	449.1084	y = 187,132.66x − 4292.66	0.9999	0.276	0.922
Petunidin 3-*O*-arabinoside	$C_{21}H_{21}O_{11}$	449.1084	y = 187,132.66x − 4292.66	0.9999	0.276	0.922
Delphinidin 3-*O*-arabinoside	$C_{20}H_{19}O_{11}$	435.0927	y = 193,161.98x − 4292.66	0.9999	0.267	0.893
Cyanidin 3-*O*-arabinoside	$C_{20}H_{19}O_{10}$	419.0978	y = 200,531.32x − 4292.66	0.9999	0.257	0.860

* Coefficient of determination; ** Limit of detection; *** Limit of quantification.

2.6. Separation and Quantification of Anthocyanins

Once the TA were identified by UHPLC-Q-ToF-MS, its separation and quantification was the next step to be completed. A LaChrom Ultra Elite UHPLC system (VWR Hitachi, Tokyo, Japan) was used for this purpose. This system included an L-2200U autosampler, an L-2160U pump and an L2300 column oven which was set at 50 °C for the analysis. In addition, the UHPLC was equipped with an L-2420U UV–vis Detector that was set at 520 nm for anthocyanin quantification. The anthocyanins were analyzed on a Hitachi LaChrom HaloTM C18 column (100 × 3 mm inside diameter, particle size 2.7 µm).

Acidified water (5% formic acid) was selected as solvent A, while pure methanol was chosen as solvent B, working at a flow rate of 1.0 mL min^{-1}. The chromatographic separation was performed by the following gradient method: 0 min, 15% B; 1.50 min, 20% B; 3.30 min, 30% B; 4.80 min, 40% B; 5.40 min, 55% B; 5.90 min, 60% B; 6.60 min, 95% B; 9.30 min, 95% B; 10 min, 15% B.

Cyanidin chloride was used as anthocyanin standard, obtaining the following calibration curve: y = 300,568.88x − 28,462.43. The regression equation and the determination coefficient (R^2 = 0.9999) were determined by means of Microsoft Office Excel 2010.

The Shapiro–Wilk test and the *t*-test were used to evaluate the normal distribution of the residues, obtaining a W value of 0.8514 (very close to 1) and a *p* value of 0.803 (higher than 0.05), respectively, which demonstrated the normal distribution of the residues.

Finally, the detection limit (0.198 mg L^{-1}) and the quantification limit (0.662 mg L^{-1}) were calculated as was described by Aliaño et al. [42]. The UHPLC chromatogram representing the 14 anthocyanins is shown in Figure 1.

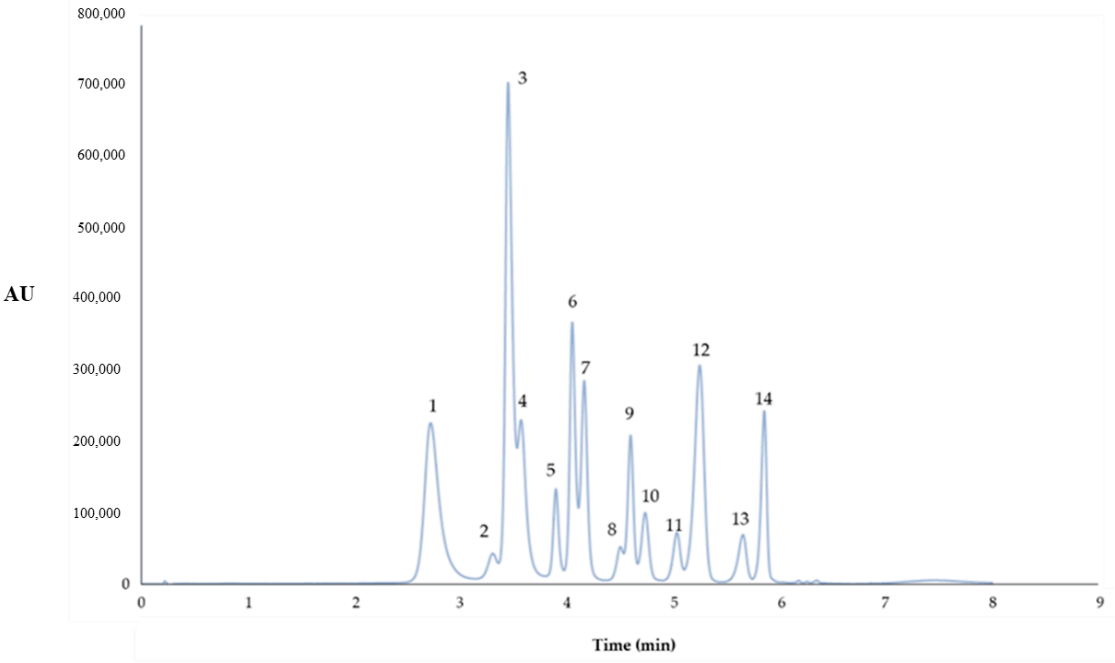


Figure 1. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of the fourteen anthocyanins identified in the blueberry extract (λ = 520 nm). Peak assignment: (1) delphinidin 3-*O*-galactoside; (2) delphinidin 3-*O*-glucoside; (3) cyanidin 3-*O*-galactoside; (4) delphinidin 3-*O*-arabinoside; (5) cyanidin 3-*O*-glucoside; (6) petunidin 3-*O*-galactoside; (7) cyanidin 3-*O*-arabinoside; (8) petunidin 3-*O*-glucoside; (9) peonidin 3-*O*-galactoside; (10) petunidin 3-*O*-arabinoside; (11) peonidin 3-*O*-glucoside; (12) malvidin 3-*O*-galactoside; (13) malvidin 3-*O*-glucoside; (14) malvidin 3-*O*-arabinoside; AU—Absorbance units.

The cyanidin chloride calibration curve was used to quantify the fourteen anthocyanins present in the blueberry extracts, assuming that the different anthocyanins have similar molar attenuation coefficient (ε) and taking into account the molecular weight of each anthocyanin. All the analyses were performed in triplicate and the results were expressed as mg of anthocyanins per g of blueberry. Regression equations of the 14 anthocyanins identified in blueberry extracts are presented in Table 2.

2.7. Total Phenolic Content (TPC)

For the analysis of the TPC of the blueberry extracts, the Folin–Ciocalteu methodology was used, as described by Singleton and Rossi in 1965 [66] but with certain modifications suggested by Singleton et al. in 1999 [67] for extracts of vegetables. A solution of gallic acid in methanol at 1000 mg L^{-1} was used as the standard, and methanol dilutions in the range of 0.1–500 mg L^{-1} were used to draw up the standard curve. This procedure has been described in our previous work [42].

The calibration curve obtained for the gallic acid standard was y = 0.0024x − 0.0031. The determination coefficient was R^2 = 0.9999 while the limit of detection and the limit of quantification

were 1.649 mg L^{-1} and 5.498 mg L^{-1}, respectively; the limits were calculated as explained in Section 2.6 above. The normal distribution of the residues for the gallic acid standard was also studied; the Shapiro–Wilk test showed a W value of 0.9201 and the *p*-value was 0.762, which indicated the normal distribution of the residues.

2.8. Statistical Analysis

As it was previously mentioned, a Box–Behnken design was selected to evaluate the influence of different variables on TA and TPC extraction. For that, the effect of the extraction variables on the final response was studied. The surface plots were evaluated to see how these variables affect the TA and TPC extraction. Once the optimal levels of the significant variables were obtained, a repeatability and intermediate precision study was carried out. Standard deviation and coefficient of variation were the statistical parameters selected to evaluate the precision. Statgraphic Centurion (version XVII) (Statgraphics Technologies, Inc., The Plains, VA, USA) was the software used for the statistical analysis.

3. Results

The aim of this research was the optimization of two different methods both based on UAE to find the maximum recovery of bioactive compounds (TA and TPC) from blueberry samples. Thus, they could be used by industries and laboratories for quality control analysis and to ensure the maximum possible compound concentration in both the raw material and their commercially derived products. For this purpose, a BBD-RSM was selected and a total of 54 experiments with specific conditions for each of them were obtained (Table S1). The extractions were performed in duplicate according to these parameters and after that the TA and TPC were measured. The TA and TPC contents were used to determine the optimal conditions to achieve the maximum yields of these compounds. In all the cases, the analytical parameters of repeatability and intermediate precision have been evaluated to guarantee the suitability of the developed methods. Finally, commercial samples were analyzed under the optimum conditions to prove the suitability of the developed methods.

3.1. Anthocyanins Optimization

3.1.1. Optimization of the Extraction Method

The extracts obtained from the 54 experiments were analyzed by UHPLC–UV–vis to quantify the 14 anthocyanins previously identified. The individual anthocyanin content was aggregated to determine the total anthocyanin content and the average of the two replicates of the same experiment was used as the response variable.

BBD-RSM was applied to determine the influence from each one of the six variables and their possible interaction on the response variable. On the other hand, the correlation between the real values of the TA and the values predicted from Equation (1) was evaluated, and the differences between the actual and the predicted values were computed as relative prediction error (Table S1). The mean prediction error was 4.79%, with values ranging from 0.02% to 15.84%, which implies a clear influence of the extraction variables on the anthocyanin recoveries and, consequently, the possibility of adjusting them to obtain the maximum anthocyanin recovery from the blueberry samples.

The *t*-test was employed to evaluate the influence of the optimized variables, considering a 95% confidence level, which means that the variables with *p*-values lower than 0.05 were considered influential. Table 3 shows the calculated *p*-values. As it can be seen, the most influential variables were: percentage of methanol in the solvent (*p*-value: 0.0005), the quadratic interaction of the percentage of methanol in the water used as extraction solvent (*p*-value: 0.0268), and the interaction percentage of methanol: amplitude (*p*-value: 0.0295). In linear terms, the percentage of methanol in the extraction solvent had a positive influence, which means that the anthocyanin extractions were more favorable when the percentage of methanol was higher.

Table 3. Analysis of variance of the quadratic model adjusted to the extraction of anthocyanins. A: %MeOH; B: temperature; C: amplitude; D: cycle; E: pH; F: ratio.

Variable	Source	Coefficient	Sum of Squares	Degrees of Freedom	Mean Square	*F*-Value	*p*-Value
	β_0	24.0196	325.9582	27			
A	X_1	−0.3187	105.9240	1	105.9240	15.8500	0.0005
B	X_2	0.1353	0.4483	1	0.4483	0.0700	0.7977
C	X_3	−0.2988	23.3445	1	23.3445	3.4900	0.0729
D	X_4	−24.0790	27.6705	1	27.6705	4.1400	0.0522
E	X_5	3.0896	0.4401	1	0.4401	0.0700	0.7995
F	X_6	−0.1373	0.2400	1	0.2400	0.0400	0.8512
A:A	X_1^2	0.0030	36.8281	1	36.8281	5.5100	0.0268
A:B	X_{12}	−0.0006	1.4878	1	1.4878	0.2200	0.6410
A:C	X_{13}	0.0042	35.4482	1	35.4482	5.3000	0.0295
A:D	X_{14}	0.1073	7.1958	1	7.1958	1.0800	0.3090
A:E	X_{15}	−0.0108	3.6585	1	3.6585	0.5500	0.4660
A:F	X_{16}	−0.0058	4.2195	1	4.2195	0.6300	0.4340
B:B	X_2^2	−0.0011	9.2232	1	9.2232	1.3800	0.2507
B:C	X_{23}	−0.0002	0.0861	1	0.0861	0.0100	0.9105
B:D	X_{24}	0.0290	0.3785	1	0.3785	0.0600	0.8138
B:E	X_{25}	−0.0002	0.0053	1	0.0053	0.0000	0.9778
B:F	X_{26}	−0.0014	0.3570	1	0.3570	0.0500	0.8190
C:C	X_3^2	0.0000	0.0041	1	0.0041	0.0000	0.9805
C:D	X_{34}	0.2210	9.7682	1	9.7682	1.4600	0.2375
C:E	X_{35}	−0.0037	0.2665	1	0.2665	0.0400	0.8433
C:F	X_{36}	0.0044	3.1418	1	3.1418	0.4700	0.4990
D:D	X_4^2	−12.4244	6.2022	1	6.2022	0.9300	0.3442
D:E	X_{45}	1.0280	3.3025	1	3.3025	0.4900	0.4883
D:F	X_{46}	1.1570	16.7331	1	16.7331	2.5000	0.1256
E:E	X_5^2	−0.2649	28.1965	1	28.1965	4.2200	0.0501
E:F	X_{56}	−0.0326	1.3285	1	1.3285	0.2000	0.6594
F:F	X_6^2	−0.0030	0.0596	1	0.0596	0.0100	0.9255
Pure Error			173.7380	26	6.6822		
Total			539.3450	53			

A standardized Pareto chart (Figure 2) was used to graphically represent and examine the influence of the variables and their order of importance. As previously mentioned, the content of methanol in water was the most influential variable for anthocyanin extraction. The solvent composition was expected to be one of the most influential variables according to the previous results by this research team, and based on these, a polarity of the solvent similar to that of anthocyanins would be required to ensure its extraction. The composition of the solvent and the quadratic interaction of the extraction solvent have been previously detected as influential variables also for the extraction of anthocyanins from other similar matrices such as sloe [64], black chokeberry [52] or açai [51]. Furthermore, the relationship between the extraction solvent and the amplitude also turned out to be an influential variable; all of them had a positive coefficient but with a significantly lower influence than that exerted by the extraction solvent.

3.1.2. Optimal Conditions

The use of BBD-RSM allows us to determine the optimal extraction variables to ensure the maximum recovery of TA from the blueberry samples. The optimal conditions were determined at 0.5 g of sample extracted at 34 °C with 20 mL of a solvent containing 74.6% MeOH in water at pH 4. It was detected that the optimal amplitude (70%) and cycle (0.7) values corresponded to the maximum values within the studied range. However, the cycle was not detected as an influential variable and the higher amplitude values caused splashes that resulted in some extract losses and, therefore, an optional increment of both ranges of variables was discarded. Finally, it was expected

that relatively low temperature values within the studied range would yield the optimal extraction values, since anthocyanins have shown to be easily degraded under extreme thermal conditions [68]. Other authors have found for bilberry that the extracts after 60 min applying ultrasound had a lower concentration of anthocyanins than the extracts obtained only by conventional maceration [69]. This may be because the sonication process can lead to the formation of free radicals that can enhance polymerization/depolymerization reactions. To see how ultrasound influences our particular extraction conditions, a comparison was made between the total concentration of anthocyanins obtained through the optimal extraction conditions obtained for ultrasound (5 min), with the same optimal extraction conditions, without the application of ultrasound, applying only maceration with magnetic stirring (5 min and 60 min; 300 rpm). The extractions have been carried out in triplicate. Under our extraction conditions, a greater amount of total anthocyanins has been obtained by applying ultrasound, compared to using maceration with magnetic stirring (32.16% for 5 min and 9.42% for 60 min). Although it is true that ultrasound can degrade anthocyanins, by applying ultrasound for a very short period of time, and through cycles, this degradation can be minimized and the positive effects of ultrasound on the extraction of compounds can be optimized. On the other hand, longer ultrasound times present a considerable reduction in the total amount of anthocyanins extracted (Figure 3), so it is recommended to use ultrasound for short periods of time and under optimal extraction conditions.

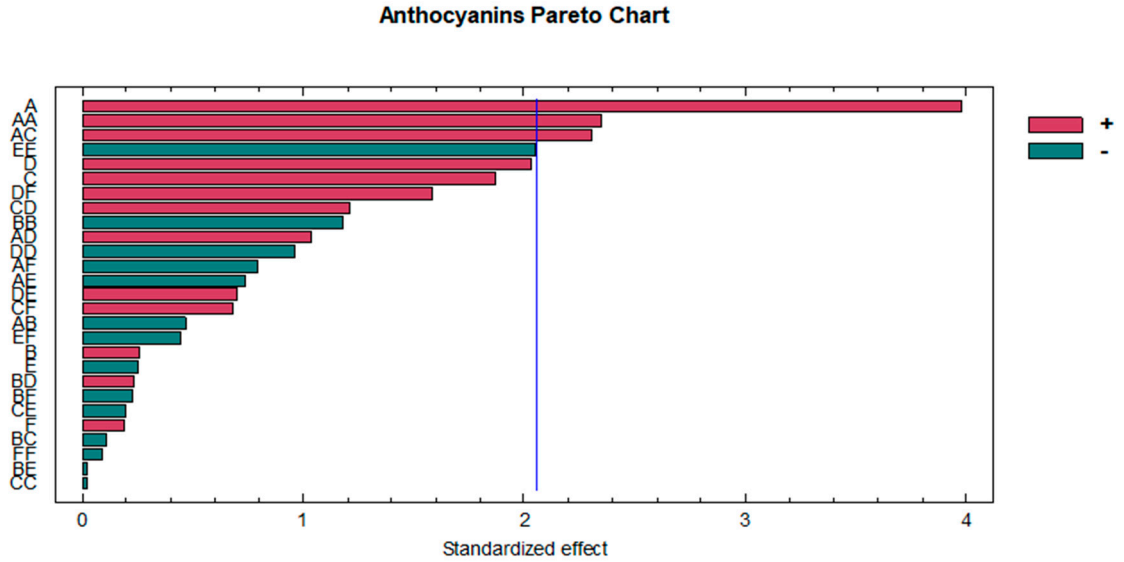


Figure 2. Standardized Pareto chart representing the extraction optimization of total anthocyanins. A: solvent (% MeOH); B: temperature (°C); C: amplitude (%); D: cycle (s); E: pH; F: ratio (mL).

The optimal conditions were compared with recent research studies on the extraction of anthocyanins from berry matrices using UAE. Bonat Celli et al. [68] investigated the extraction of anthocyanin from haskap berries (*Lonicera caerulea* L.) using UAE. The optimal conditions established were similar to those found for this research, such as the ratio (25:1 (mL g^{-1})), temperature (35 °C), or percentage of methanol (80%). However, the extraction time required was 20 min, which is twice the length of the period measured in this research.

On the other hand, Quiang Cheng et al. [70] and R. Albuquerque et al. [71] studied the extraction of anthocyanins from the fruits of *Rubia sylvatica* Nakai and from Jabuticaba's epicarp, respectively. Both authors included pH as an influential variable and demonstrated that acid-level pH (3–4) facilitated the extraction of anthocyanins similarly as it was observed in the present research. Finally, Fibigr et al. [72] investigated the optimal amount of açai berry samples for maximum anthocyanin extraction using UAE. Thus, the experiments performed with 0.4–0.7 g samples allowed the maximum recoveries. As it could be observed, the optimal conditions determined for the extraction of anthocyanins from blueberries were in agreement with the results found in the literature.

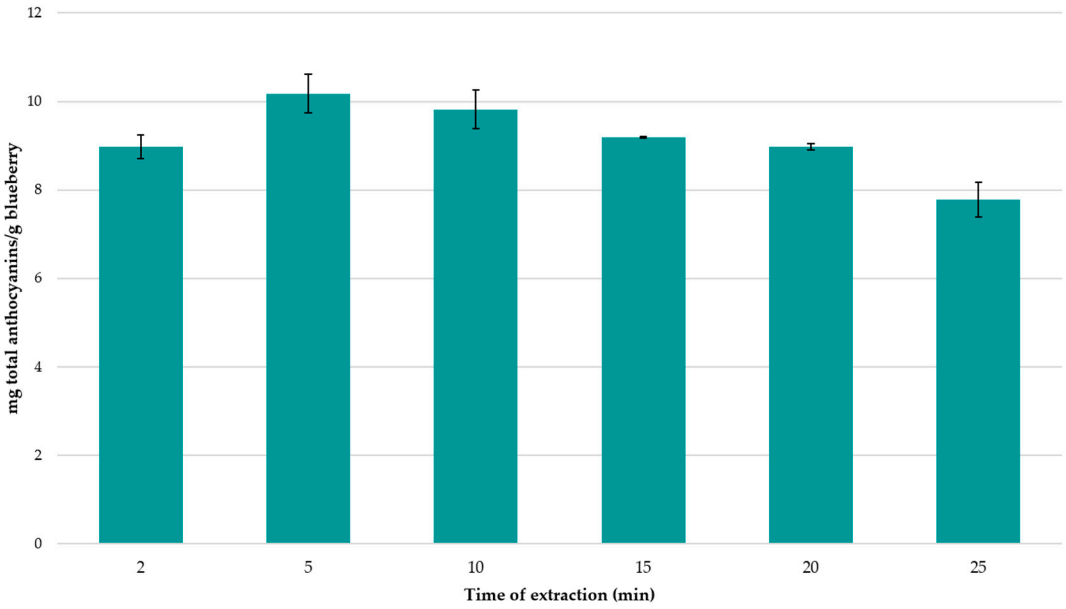


Figure 3. Study of the extraction time to optimize the recovery of anthocyanins from blueberry extracts (n = 3). The error bars represent the uncertainty of the measurement in the three replicates.

3.1.3. Extraction Time

Once the optimal values of the six selected extraction variables had been determined, the next step consisted of studying the extraction time and its influence on the TA extraction. For this purpose, the extractions were carried out under optimal conditions but with six different extraction times (2, 5, 10, 15, 20 and 25 min) each one of them performed in triplicate. Thus, eighteen extracts were obtained which were analyzed by UHPLC–UV–vis and the fourteen anthocyanins were quantified. Total anthocyanins were calculated as the sum of the individual anthocyanins. The average concentration of TA for the different experimental times has been represented in Figure 3. As it can be observed, all the experiments achieved a content of anthocyanins above 7 mg per gram of blueberry sample. However, the maximum concentration of anthocyanins (10.18 mg/g) was reached when 5 min of extraction were used, although no significant differences were noticed when compared to the 10 min-extraction experiments. Therefore, for its lesser energy consumption, 5 min was selected as the optimal extraction time. In fact, a decreasing concentration was detected when 15 min-extraction time was applied and this trend continued up to 25 min-extraction time. This fall in anthocyanin concentrations could be closely related to the high susceptibility of anthocyanins to be degraded that had been observed in similar research studies [73] and for this reason the authors do not recommend any extraction processes that require 10 min or a longer time.

3.1.4. Repeatability and Intermediate Precision

Once optimized, to ensure the suitability of the developed method to be used by different industries and/or laboratories, the evaluation of repeatability and intermediate precision was required.

For this purpose, 10 extractions were carried out under optimal conditions on three different consecutive days and a total of 30 extractions were obtained under the optimal conditions that had been established. These extracts were analyzed by UHPLC–UV-vis and the recovery of TA was calculated as explained before. The coefficient of variation (CV) was the analytical factor selected for this evaluation. The CV of the samples taken on the same day were computed for repeatability while, for intermediate precision, this factor was calculated based on the samples taken on the three consecutive days. The values obtained are shown in Table 4. The coefficient of repeatability was 4.17%, while the coefficient of intermediate precision was 4.32%, both of them below 5%.

Table 4. Repeatability and intermediate precision study for anthocyanins and phenolic compounds recovery from blueberry extracts.

	Repeatability (n = 10)	**Intermediate Precision (n = 30)**
Average mg anthocyanins/g blueberry	10.07	10.13
Standard deviation	0.42	0.44
Coefficient of variation (CV) (%)	4.17	4.32
Average mg total phenolic compounds/g blueberry	31.28	30.94
Standard deviation	1.24	1.19
Coefficient of variation (CV) (%)	3.97	3.85

3.1.5. Re-Extraction Study for Anthocyanins

To check the effectiveness of the extraction method, a re-extraction study of the residues obtained after applying the optimized method was carried out. The residue obtained was subjected to the optimal extraction conditions of the method. This study was carried out in triplicate, obtaining a total amount of anthocyanins less than 5%, so it can be considered a quantitative method.

3.2. Phenolic Compounds Optimization

3.2.1. Optimization of the Extraction Method

The extracts obtained from the 54 extractions were also analyzed by Folin–Ciocalteu methodology and the TPC was determined. The response variable used was the average of the replicates of the same experiment.

The values obtained from the analysis (real values) were correlated with the predicted values from Equation (1), and the differences were considered as relative prediction error (Table S1). As can be seen, the average prediction error was 3.84% and ranged from 0.36% up to 13.08%. These results suggest an influence of the variables that had been considered for the study on the TPC extracted from the blueberry samples. The BBD-RSM method was, therefore, applied in order to determine the influence of those variables as well as their possible interaction on the TPC content. This should allow us to determine the optimal conditions to achieve the maximum recovery of TPC.

The p-values were calculated according to the t-test considering a 95% confidence level, which means that the variables with p-values lower than 0.05 were considered influential. In linear terms, the solvent ratio was an influential variable (p-value: 0.0013) with positive influence, which means that a higher amount of solvent would result in a greater extraction of TPC. Furthermore, the quadratic interaction of the percentage of methanol in the extraction solvent and the interaction of the percentage of methanol and the cycle revealed p-values below 0.05 (p-value: 0.0002 and p-value: 0.0301) which implies that they were influential variables even though they were not so by themselves. On the other hand, the quadratic interaction of the extraction temperature (p-value: 0.0113), the quadratic interaction of the ratio (p-value: 0.003) and the interaction between the extraction temperature and the ratio (p-value: 0.0419) were also influential variables, although the individual variables were not found to be influential by themselves. The calculated p-values can be seen in Table 5.

The standardized Pareto chart (Figure 4) was plotted in order to graphically observe the influence of the variables. It was noted that the quadratic interaction of the methanol percentage was the most influential variable with a negative effect on the extraction of TPC. Recent investigations conducted by our research group on the extraction of TPC from similar matrices such as black chokeberry [52] or myrtle [50] confirmed the influence of methanol percentage on the extraction of these compounds. The solvent ratio was the second most influential variable with a positive effect, as previously mentioned, while the rest of the influential variables (quadratic interaction of the extraction temperature, quadratic interaction of the ratio and the interaction between the extraction temperature and the ratio) showed negative coefficients. Likewise, these quadratic interactions have been observed as influential variables in numerous studies that focused on the extraction of TPC from fruit matrices such as mulberry [65] or açai [40].

Table 5. Analysis of variance of the quadratic model adjusted to the extraction of total phenolic compounds. A: %MeOH; B: temperature; C: amplitude; D: cycle; E: pH; F: ratio.

Variable	Source	Coefficient	Sum of Squares	Degrees of Freedom	Mean Square	*F*-Value	*p*-Value
	β_0	−9.1847		27			
A	X_1	0.2657	0.0241	1	0.0241	0.0100	0.9227
B	X_2	0.2620	1.3348	1	1.3348	0.5300	0.4723
C	X_3	0.0308	7.9465	1	7.9465	3.1700	0.0868
D	X_4	15.8107	7.8318	1	7.8318	3.1200	0.0890
E	X_5	−0.9338	0.1785	1	0.1785	0.0700	0.7918
F	X_6	2.3123	32.7367	1	32.7367	13.0500	0.0013
A:A	$X_1{}^2$	−0.0034	45.9076	1	45.9076	18.3000	0.0002
A:B	X_{12}	0.0004	0.6962	1	0.6962	0.2800	0.6028
A:C	X_{13}	0.0013	3.4061	1	3.4061	1.3600	0.2545
A:D	X_{14}	−0.1454	13.2132	1	13.2132	5.2700	0.0301
A:E	X_{15}	−0.0148	6.8450	1	6.8450	2.7300	0.1106
A:F	X_{16}	0.0081	8.2825	1	8.2825	3.3000	0.0808
B:B	$X_2{}^2$	−0.0015	18.6571	1	18.6571	7.4400	0.0113
B:C	X_{23}	−0.0005	0.7503	1	0.7503	0.3000	0.5891
B:D	X_{24}	−0.0672	2.0301	1	2.0301	0.8100	0.3766
B:E	X_{25}	0.0048	2.0521	1	2.0521	0.8200	0.3741
B:F	X_{26}	−0.0080	11.4960	1	11.4960	4.5800	0.0419
C:C	$X_3{}^2$	−0.0010	1.5125	1	1.5125	0.6000	0.4445
C:D	X_{34}	0.0450	0.4050	1	0.4050	0.1600	0.6911
C:E	X_{35}	0.0080	1.2800	1	1.2800	0.5100	0.4814
C:F	X_{36}	−0.0005	0.0371	1	0.0371	0.0100	0.9042
D:D	$X_4{}^2$	−7.3422	2.1660	1	2.1660	0.8600	0.3614
D:E	X_{45}	1.1820	4.3660	1	4.3660	1.7400	0.1986
D:F	X_{46}	−0.3020	1.1401	1	1.1401	0.4500	0.5062
E:E	$X_5{}^2$	0.0887	3.1619	1	3.1619	1.2600	0.2719
E:F	X_{56}	−0.0142	0.2521	1	0.2521	0.1000	0.7538
F:F	$X_6{}^2$	−0.0647	26.9429	1	26.9429	10.7400	0.0030
Pure Error			65.2306	26	2.5089		
Total			261.2350	53			

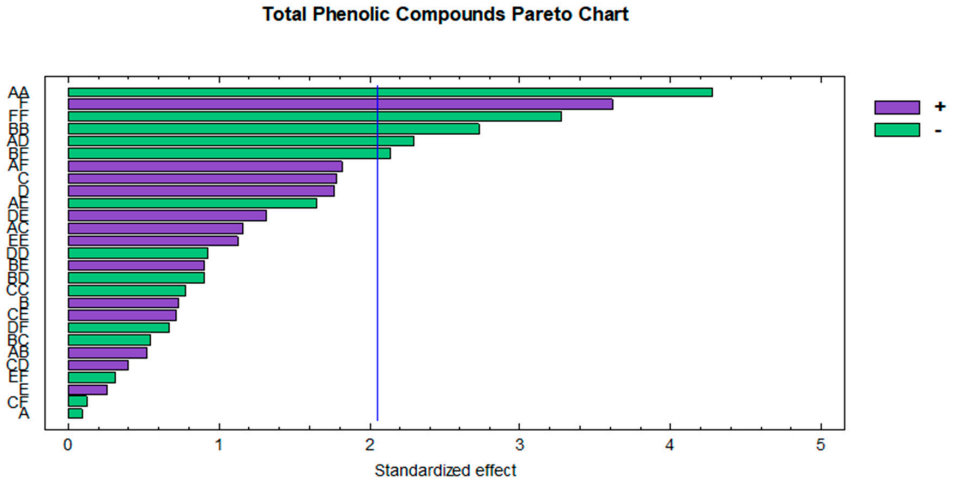


Figure 4. Standardized Pareto chart representing the optimization of total phenolic compounds extraction. A: solvent (% MeOH); B: temperature (°C); C: amplitude (%); D: cycle (s); E: pH; F: ratio (mL).

3.2.2. Optimal Conditions

Once the influential variables for the extraction of total phenolic compounds, had been identified, BBD-RSM was applied to determine the optimal extraction variables that would ensure the maximum

recovery of TPC. The optimal conditions were established at 0.5 g of sample extracted at 33.3 °C with 16 mL of solvent containing 44% MeOH in water at pH 7. Finally, 0.7 cycles at 70% amplitude were selected for the ultrasound system to ensure the maximum extraction of TPC as commented for anthocyanins. Although the optimum pH was set at the maximum value within the range, no higher points were tested since, according to the literature, basic pH could cause the degradation of the phenolic compounds [74,75]. Some similarities were observed with respect to the optimal conditions for the extraction of TA. However, the extraction of TPC was improved when using solvents with a lower percentage of methanol and with a more basic pH than that used for the extraction of TA.

Espada-Bellido et al. [65] carried out an optimization research on the extraction of phenolic compounds from mulberry (*Morus nigra*) pulp using UAE. The optimal conditions were similar to those already determined in this research, namely the solvent pH (7), the ultrasound amplitude (70%), the cycle (0.7) or the ratio (11:1.5 mL g^{-1}). However, the percentage of methanol (61%) and the temperature (64 °C) required were higher than those observed in this study; both factors could be closely interrelated to the nature of the sample.

3.2.3. Extraction Time

Similarly, to the research on TA extraction, a study to determine the optimal extraction time for the recovery of the TPC was required. For this purpose, different extractions were performed under the optimal conditions. Six different extraction times were employed (2, 5, 10, 15, 20 and 25 min) in triplicate. The extracts were analyzed by Folin–Ciocalteu methodology and the total content in phenolic compounds was determined. The average of the three replicates under the same conditions was calculated and represented for each extraction time in Figure 5. The maximum total phenolic content (32.18 mg g^{-1}) was reached when the extraction time was 15 min, without any significant difference when compared to the extraction from the 20 min experiments. For this reason, the optimal extraction time was established at 15 min. Furthermore, it was observed that after that time the TPC began to decrease. This fact could be closely related to the degradation of the compounds, as it has been observed in similar matrices such as açai [51], sloe [64] or blackberries [43].

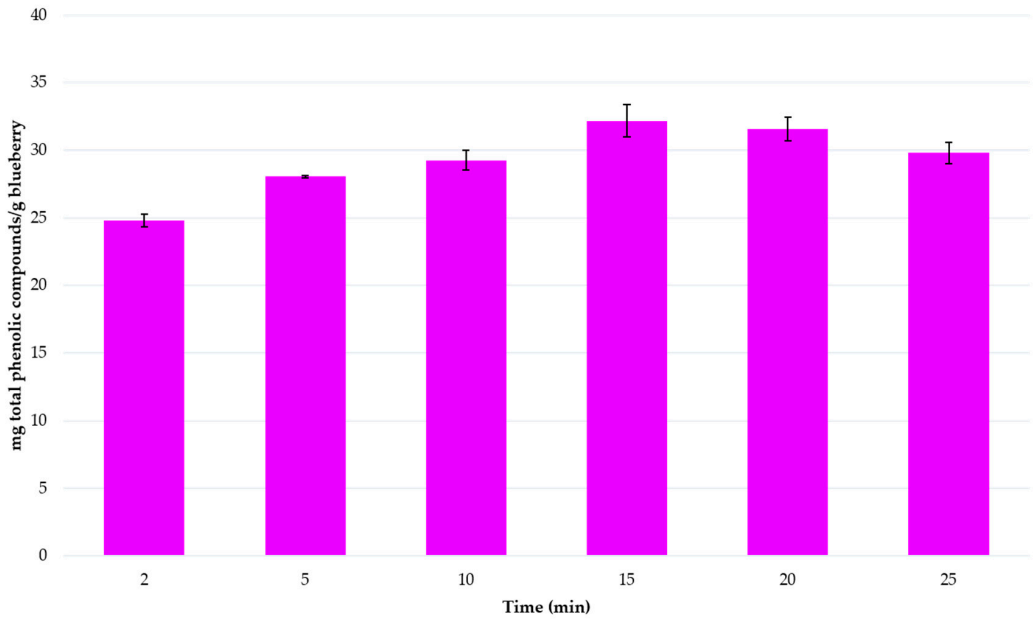


Figure 5. Study of the optimal extraction time study for the recovery of total phenolic compounds from blueberry extracts (n = 3). The error bars represent the uncertainty of the measurement in the three replicates.

3.2.4. Repeatability and Intermediate Precision

The optimized method for the extraction of total phenolic compounds was also evaluated for reliability. For this purpose, in the same way as for the anthocyanins, 10 extractions were carried out on the same day and 10 more extractions on 2 consecutive days. The extracts were analyzed by Folin–Ciocalteu methodology and the total phenolic compounds content was determined. The CV was calculated for the samples analyzed on the same day and on different days. The results can be seen in Table 4.

The method's repeatability exhibited a CV of 3.97% while for its intermediate accuracy the CV obtained was 3.85%; both of them are below 5%, which confirms that the optimized methods are reliable and they could be applied in different industries/laboratories with no relevant differences to be expected between their results.

3.2.5. Re-Extraction Study for Total Phenolic Compounds

As previously carried out for TA, to check the effectiveness of the extraction method, a re-extraction study of the residues obtained after applying the optimized method was carried out. The residue obtained was subjected to the optimal extraction conditions of the method. This study was carried out in triplicate, obtaining a total amount of phenolic compounds less than 5%, so it can be considered a quantitative method for these compounds.

3.3. Application to Real Samples

In the course of this research, two methods based on UAE have been developed. These methods have focused on maximizing the extraction of TA and TPC from blueberry samples. Furthermore, both methods have displayed good repeatability and intermediate accuracy, which means that they could be used both in industrial and research laboratories. Finally, the methods developed were applied to five commercial samples such as jams with blueberry contents (Table 1).

With respect to TA, the samples did not undergo any pre-treatment; 0.5 g of the sample was weighed and extracted under the corresponding optimal conditions for TA before being analyzed by UHPLC–UV–vis. All the samples were extracted in triplicate. The TA were calculated as the sum of 14 individual anthocyanins. The average amount from the replicates of the same sample was calculated and can be seen in Figure 6A. The TA content extracted from the real samples was significantly lower than that obtained from the freeze-dried samples, with the sample from Jam 1 being the one with the largest content. Such lower concentration of anthocyanins in the real samples was expected, since the samples in our experiments were plain fresh berries while the jams contained over 50% sugar, which represents a significant dilution of the blueberry content. In addition, the authors suggest a possible degradation of the anthocyanin because of the temperature reached to obtain the jam matrix, which would greatly depend on the elaboration process applied to each sample. In addition, different research works have shown a considerable reduction in anthocyanins in blueberry jams after several months of storage due to the generation of anthocyanin-procynidin polymers [76], which could be another cause of the decrease in TA observed in jam samples.

Regarding the total phenolic compounds, the jam samples (0.5 g) were extracted under optimal conditions for the extraction of total phenolic compounds. The extracts were filtered through a 0.20 µm nylon syringe filter and Folin–Ciocalteu methodology was applied. All the samples were extracted in triplicate. The average concentrations of the replicates were calculated and the results are shown in Figure 6B. It was detected that, similarly to the anthocyanins, the phenolic content was lower in the real samples than the one obtained from the freeze-dried samples. This could be due to the plain fresh berries used in our experiments, while the blueberries in the jam had been mixed with other elements. It could be observed that Jam 5 had a considerably high content of phenolic compounds, which could be closely related to a lower elaboration temperature and, consequently, to a lesser degradation of the compounds of interest.

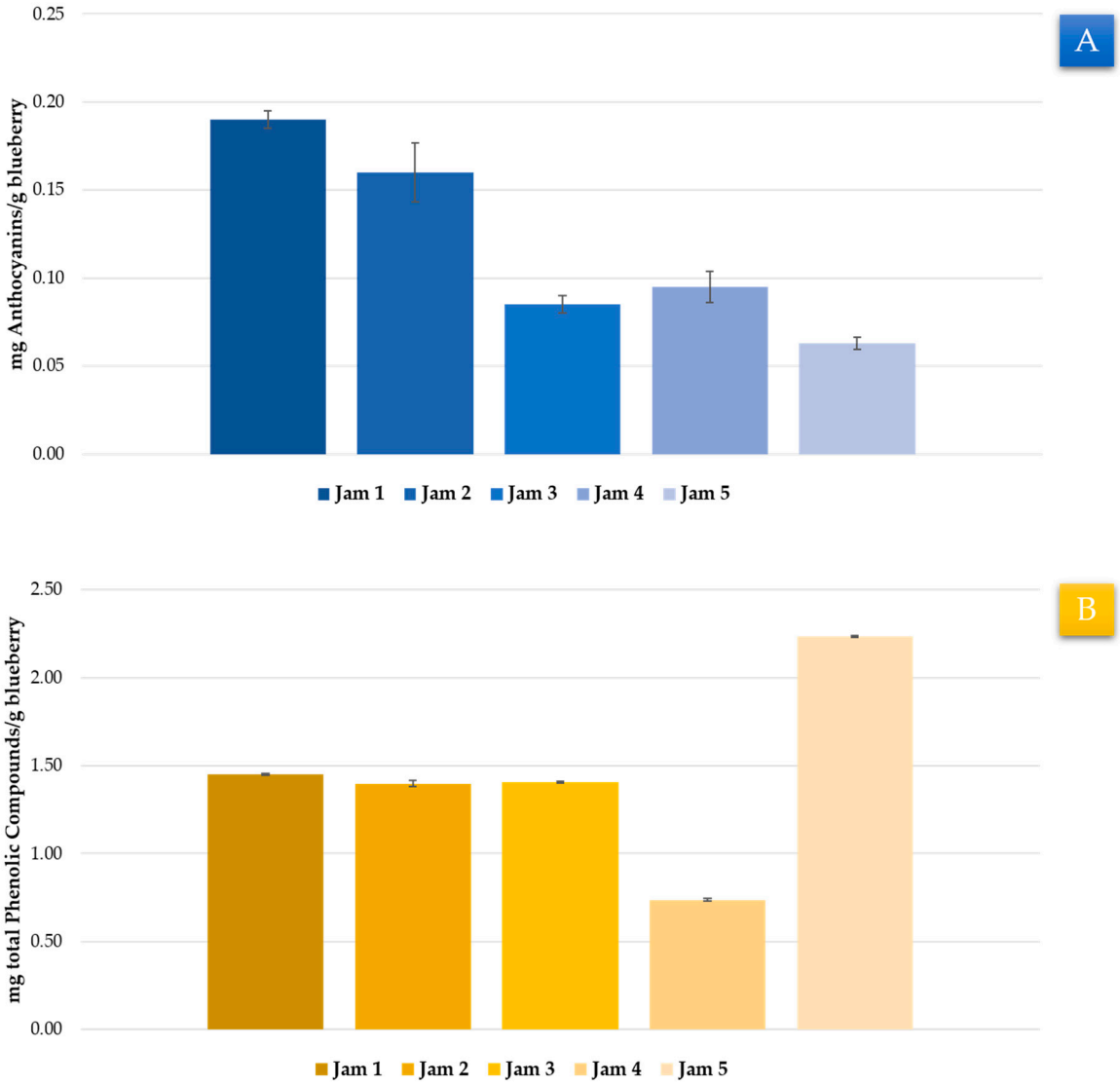


Figure 6. Application of the optimized method to real samples. (**A**) Anthocyanin content in real samples according to the developed method (n = 3). (**B**) Total phenolic compounds in real samples according to the developed method (n = 3). The error bars represent the uncertainty of the measurement in the three replicates.

4. Conclusions

In the present research, two methodologies have been developed for the extraction of TA and TPC from blueberries. These methodologies have been based on UAE because of the many advantages associated with this system, such as ease of use, low power consumption, low cost and the fact that it does not require any maintenance while it is available in most laboratories.

A Box–Behnken design has been carried out to obtain the optimal conditions for the TA and TPC extraction from freeze-dried blueberry samples. In addition, an extraction time study was taken into account and it was observed that only 5 and 15 min were required for the TA and TPC extraction, respectively. In addition, both methods have demonstrated good repeatability and intermediate accuracy with a CV below 5%, which confirms their suitability to be applied by different laboratories and industries. Finally, both optimized methods were tested on real jam samples with a varying blueberry content and proved to be perfectly suitable for the intended purposes.

The results obtained and the important advantages associated with ultrasound-assisted extraction, including among others the fact of being a rapid, repeatable, inexpensive and easy-to-use technique,

suggest that both the methodologies developed could be easily used by laboratories or industries for quality control analysis to assure the content of anthocyanins and total phenolic compounds either in their raw material or in their intermediate or finished blueberry products.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/12/1763/s1, Table S1. Box–Behnken design matrix with the values of the six variables for each experiment and measured and predicted responses ($n = 2$).

Author Contributions: Conceptualization, E.E.-B. and G.F.B.; methodology, E.E.-B. and G.F.B.; software, M.F.-G.; formal analysis, J.A.J., M.J.A.-G. and C.C.; investigation, E.E.-B., C.C. and G.F.B.; resources, J.Á.Á., M.P. and J.A.; data curation, J.A.J., M.J.A.-G., M.F.-G. and G.F.B.; writing—original draft preparation, M.J.A.-G.; writing—review and editing, G.F.B., M.F.-G. and E.E.-B.; supervision, G.F.B., M.F.-G. and E.E.-B.; project administration, G.F.B., J.Á.Á., M.P. and J.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been supported by the project "EQC2018-005135-P" (Equipment for liquid chromatography by means of mass spectrometry and ion chromatography), of the State Subprogram of Research Infrastructures and Technical Scientific Equipment. The authors acknowledge University of Cadiz and V. la Andaluza for the support provided through the project OT2016/046. The authors are grateful to the Instituto de Investigación Vitivinícola y Agroalimentaria (IVAGRO) for providing the necessary facilities to carry out the research and Programa de Fomento e Impulso de la Actividad de Investigación y Transferencia de la Universidad de Cádiz for the payment support of this manuscript.

Acknowledgments: The authors are grateful to IVAGRO (University of Cadiz) for provision of the necessary facilities to carry out the research.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zhao, Y. *Berry Fruit: Value-Added Products for Health Promotion (Food Science and Technology)*, 1st ed.; CRC Press, Ed.; CRC Press: Boca Raton, FL, USA, 2007; pp. 1–444. ISBN 978-0849358029.
2. Retamales, J.B.; Hancock, J.F. *Blueberries*, 1st ed.; CABI, Ed.; CABI: Egham, UK, 2012; pp. 1–352, ISBN 978-1845938260.
3. Ma, L.; Sun, Z.; Zeng, Y.; Luo, M.; Yang, J. Molecular Mechanism and Health Role of Functional Ingredients in Blueberry for Chronic Disease in Human Beings. *Int. J. Mol. Sci.* **2018**, *19*, 2785. [CrossRef] [PubMed]
4. Higuera-Hernández, M.F.; Reyes-Cuapio, E.; Gutiérrez-Mendoza, M.; Budde, H.; Blanco-Centurión, C.; Veras, A.B.; Rocha, N.B.; Yamamoto, T.; Monteiro, D.; Zaldívar-Rae, J.; et al. Blueberry intake included in hypocaloric diet decreases weight, glucose, cholesterol, triglycerides and adenosine levels in obese subjects. *J. Funct. Foods* **2019**, *60*, 103409. [CrossRef]
5. Seo, H.-S.; Adams, S.H.; Howard, L.R.; Brownmiller, C.; Hogan, V.; Chen, J.-R.; Pramudya, R.C. Children's liking and wanting of foods vary over multiple bites/sips of consumption: A case study of foods containing wild blueberry powder in the amounts targeted to deliver bioactive phytonutrients for children. *Food Res. Int.* **2020**, *131*, 108981. [CrossRef]
6. Dimitrellou, D.; Solomakou, N.; Kokkinomagoulos, E.; Kandylis, P. Yogurts Supplemented with Juices from Grapes and Berries. *Foods* **2020**, *9*, 1158. [CrossRef] [PubMed]
7. Krikorian, R.; Kalt, W.; McDonald, J.E.; Shidler, M.D.; Summer, S.S.; Stein, A.L. Cognitive performance in relation to urinary anthocyanins and their flavonoid-based products following blueberry supplementation in older adults at risk for dementia. *J. Funct. Foods* **2020**, *64*, 103667. [CrossRef]
8. Encyclopaedia Britannica List of Plants in the Family Ericaceae|Britannica. Available online: https://www.britannica.com/topic/list-of-plants-in-the-family-Ericaceae-2038184 (accessed on 18 April 2020).
9. Lavefve, L.; Brownmiller, C.; Howard, L.; Reeves, D.; Adams, S.H.; Chen, J.-R.; Diaz, E.C.; Mauromoustakos, A. Changes in Polyphenolics during Storage of Products Prepared with Freeze-Dried Wild Blueberry Powder. *Foods* **2020**, *9*, 466. [CrossRef] [PubMed]
10. Hwang, H.; Kim, Y.-J.; Shin, Y. Assessment of Physicochemical Quality, Antioxidant Content and Activity, and Inhibition of Cholinesterase between Unripe and Ripe Blueberry Fruit. *Foods* **2020**, *9*, 690. [CrossRef]
11. Baenas, N.; Ruales, J.; Moreno, D.A.; Barrio, D.A.; Stinco, C.M.; Martínez-Cifuentes, G.; Meléndez-Martínez, A.J.; García-Ruiz, A. Characterization of Andean Blueberry in Bioactive Compounds, Evaluation of Biological Properties, and In Vitro Bioaccessibility. *Foods* **2020**, *9*, 1483. [CrossRef]

12. Norberto, S.; Silva, S.; Meireles, M.; Faria, A.; Pintado, M.; Calhau, C. Blueberry anthocyanins in health promotion: A metabolic overview. *J. Funct. Foods* **2013**, *5*, 1518–1528. [CrossRef]
13. Lima, M.C.; Paiva de Sousa, C.; Fernandez-Prada, C.; Harel, J.; Dubreuil, J.D.; de Souza, E.L. A review of the current evidence of fruit phenolic compounds as potential antimicrobials against pathogenic bacteria. *Microb. Pathog.* **2019**, *130*, 259–270. [CrossRef]
14. Liu, Y.; Mu, J.; Niklas, K.J.; Li, G.; Sun, S. Global warming reduces plant reproductive output for temperate multi-inflorescence species on the Tibetan plateau. *New Phytol.* **2012**, *195*, 427–436. [CrossRef] [PubMed]
15. Hao, L.; Guo, L.; Li, R.; Cheng, Y.; Huang, L.; Zhou, H.; Xu, M.; Li, F.; Zhang, X.; Zheng, Y. Responses of photosynthesis to high temperature stress associated with changes in leaf structure and biochemistry of blueberry (*Vaccinium corymbosum* L.). *Sci. Hortic. (Amsterdam)* **2019**, *246*, 251–264. [CrossRef]
16. Starast, M.; Paal, T.; Vool, E.; Karp, K.; Albert, T.; Moor, U. The productivity of some blueberry cultivars under Estonian conditions. *Acta Hortic.* **2009**, *810*, 103–108. [CrossRef]
17. Rodgers Dinstel, R.; Cascio, J.; Koukel, S. The antioxidant level of Alaska's wild berries: High, higher and highest. *Int. J. Circumpolar Health* **2013**, *72*, 21188. [CrossRef]
18. Kang, J.; Thakali, K.M.; Jensen, G.S.; Wu, X. Phenolic Acids of the Two Major Blueberry Species in the US Market and Their Antioxidant and Anti-inflammatory Activities. *Plant Foods Hum. Nutr.* **2015**, *70*, 56–62. [CrossRef] [PubMed]
19. Miraghajani, M.; Momenyan, S.; Arab, A.; Hasanpour Dehkordi, A.; Symonds, M.E. Blueberry and cardiovascular disease risk factors: A systematic review and meta-analysis of randomized controlled trials. *Complement. Ther. Med.* **2020**, *53*, 102389. [CrossRef]
20. Zhao, C.-N.; Meng, X.; Li, Y.; Li, S.; Liu, Q.; Tang, G.-Y.; Li, H.-B. Fruits for Prevention and Treatment of Cardiovascular Diseases. *Nutrients* **2017**, *9*, 598. [CrossRef]
21. Basu, A.; Alexander, J.; Ebersole, J.; Planinic, P. Dietary fiber and blueberry intervention lowers gestational weight gain and C-reactive protein in women at high risk for gestational diabetes. *Metabolism* **2020**, *104*, 154117. [CrossRef]
22. Stote, K.; Corkum, A.; Sweeney, M.; Shakerley, N.; Kean, T.; Gottschall-Pass, K. Postprandial Effects of Blueberry (*Vaccinium angustifolium*) Consumption on Glucose Metabolism, Gastrointestinal Hormone Response, and Perceived Appetite in Healthy Adults: A Randomized, Placebo-Controlled Crossover Trial. *Nutrients* **2019**, *11*, 202. [CrossRef]
23. Wu, X.; Wang, T.T.Y.; Prior, R.L.; Pehrsson, P.R. Prevention of Atherosclerosis by Berries: The Case of Blueberries. *J. Agric. Food Chem.* **2018**, *66*, 9172–9188. [CrossRef]
24. Du, C.; Smith, A.; Avalos, M.; South, S.; Crabtree, K.; Wang, W.; Kwon, Y.-H.; Vijayagopal, P.; Juma, S. Blueberries Improve Pain, Gait Performance, and Inflammation in Individuals with Symptomatic Knee Osteoarthritis. *Nutrients* **2019**, *11*, 290. [CrossRef] [PubMed]
25. Su, X.; Zhang, J.; Wang, H.; Xu, J.; He, J.; Liu, L.; Zhang, T.; Chen, R.; Kang, J. Phenolic Acid Profiling, Antioxidant, and Anti-Inflammatory Activities, and miRNA Regulation in the Polyphenols of 16 Blueberry Samples from China. *Molecules* **2017**, *22*, 312. [CrossRef] [PubMed]
26. Debnath-Canning, M.; Unruh, S.; Vyas, P.; Daneshtalab, N.; Igamberdiev, A.U.; Weber, J.T. Fruits and leaves from wild blueberry plants contain diverse polyphenols and decrease neuroinflammatory responses in microglia. *J. Funct. Foods* **2020**, *68*, 103906. [CrossRef]
27. Türck, P.; Fraga, S.; Salvador, I.; Campos-Carraro, C.; Lacerda, D.; Bahr, A.; Ortiz, V.; Hickmann, A.; Koetz, M.; Belló-Klein, A.; et al. Blueberry extract decreases oxidative stress and improves functional parameters in lungs from rats with pulmonary arterial hypertension. *Nutrition* **2020**, *70*, 110579. [CrossRef] [PubMed]
28. Wang, H.; Liu, J.; Pang, D.; Li, T.; Liu, R.H. Mechanisms underlying the protective effects of blueberry extract against ultraviolet radiation in a skin cell co-culture system. *J. Funct. Foods* **2019**, *52*, 603–610. [CrossRef]
29. Zhou, L.; Xie, M.; Yang, F.; Liu, J. Antioxidant activity of high purity blueberry anthocyanins and the effects on human intestinal microbiota. *LWT* **2020**, *117*, 108621. [CrossRef]
30. Sun, X.; Hao, L.; Xie, Q.; Lan, W.; Zhao, Y.; Pan, Y.; Wu, V.C.H. Antimicrobial effects and membrane damage mechanism of blueberry (*Vaccinium corymbosum* L.) extract against *Vibrio parahaemolyticus*. *Food Control* **2020**, *111*, 107020. [CrossRef]
31. Zunino, S.J.; Zhang, Y.; Seeram, N.P.; Storms, D.H. Berry fruit extracts inhibit growth and induce apoptosis of high-risk acute lymphoblastic leukemia cells in vitro. *J. Funct. Foods* **2010**, *2*, 187–195. [CrossRef]

32. Cheng, Z.; Lin, J.; Gao, N.; Sun, X.; Meng, X.; Liu, R.; Liu, Y.; Wang, W.; Li, B.; Wang, Y. Blueberry malvidin-3-galactoside modulated gut microbial dysbiosis and microbial TCA cycle KEGG pathway disrupted in a liver cancer model induced by HepG2 cells. *Food Sci. Hum. Wellness* **2020**, *9*, 245–255. [CrossRef]
33. Poklar Ulrih, N.; Opara, R.; Skrt, M.; Košmerl, T.; Wondra, M.; Abram, V. Part I. Polyphenols composition and antioxidant potential during 'Blaufränkisch' grape maceration and red wine maturation, and the effects of trans-resveratrol addition. *Food Chem. Toxicol.* **2020**, *137*, 111122. [CrossRef]
34. Pace, C.; Giacosa, S.; Torchio, F.; Río Segade, S.; Cagnasso, E.; Rolle, L. Extraction kinetics of anthocyanins from skin to pulp during carbonic maceration of winegrape berries with different ripeness levels. *Food Chem.* **2014**, *165*, 77–84. [CrossRef] [PubMed]
35. Garzón, G.A.; Narváez-Cuenca, C.-E.; Vincken, J.-P.; Gruppen, H. Polyphenolic composition and antioxidant activity of açai (*Euterpe oleracea* Mart.) from Colombia. *Food Chem.* **2017**, *217*, 364–372. [CrossRef] [PubMed]
36. López, V.; Les, F.; Iannarelli, R.; Caprioli, G.; Maggi, F. Methanolic extract from red berry-like fruits of Hypericum androsaemum: Chemical characterization and inhibitory potential of central nervous system enzymes. *Ind. Crops Prod.* **2016**, *94*, 363–367. [CrossRef]
37. Bastías-Montes, J.M.; Monterrosa, K.; Muñoz-Fariña, O.; García, O.; Acuña-Nelson, S.M.; Vidal-San Martín, C.; Quevedo-Leon, R.; Kubo, I.; Avila-Acevedo, J.G.; Domiguez-Lopez, M.; et al. Chemoprotective and antiobesity effects of tocols from seed oil of Maqui-berry: Their antioxidative and digestive enzyme inhibition potential. *Food Chem. Toxicol.* **2020**, *136*, 111036. [CrossRef] [PubMed]
38. Garcia-Mendoza, M.d.P.; Espinosa-Pardo, F.A.; Baseggio, A.M.; Barbero, G.F.; Maróstica Junior, M.R.; Rostagno, M.A.; Martínez, J. Extraction of phenolic compounds and anthocyanins from juçara (Euterpe edulis Mart.) residues using pressurized liquids and supercritical fluids. *J. Supercrit. Fluids* **2017**, *119*, 9–16. [CrossRef]
39. da Silveira, T.F.F.; Cristianini, M.; Kuhnle, G.G.; Ribeiro, A.B.; Filho, J.T.; Godoy, H.T. Anthocyanins, non-anthocyanin phenolics, tocopherols and antioxidant capacity of açaí juice (Euterpe oleracea) as affected by high pressure processing and thermal pasteurization. *Innov. Food Sci. Emerg. Technol.* **2019**, *55*, 88–96. [CrossRef]
40. Aliaño-González, M.J.; Ferreiro-González, M.; Espada-Bellido, E.; Carrera, C.; Palma, M.; Álvarez, J.A.; Ayuso, J.; Barbero, G.F. Extraction of anthocyanins and total phenolic compounds from açai (euterpe oleracea mart.) using an experimental design methodology. part 1: Pressurized liquid extraction. *Agronomy* **2020**, *10*, 183. [CrossRef]
41. de Peredo, A.V.G.; Vázquez-Espinosa, M.; Piñeiro, Z.; Espada-Bellido, E.; Ferreiro-González, M.; Barbero, G.F.; Palma, M. Development of a rapid and accurate UHPLC-PDA-FL method for the quantification of phenolic compounds in grapes. *Food Chem.* **2021**, *334*, 2992. [CrossRef]
42. Aliaño-González, M.J.; Ferreiro-González, M.; Espada-Bellido, E.; Carrera, C.; Palma, M.; Ayuso, J.; Barbero, G.F.; Álvarez, J.Á. Extraction of Anthocyanins and Total Phenolic Compounds from Açai (Euterpe oleracea Mart.) Using an Experimental Design Methodology. Part 3: Microwave-Assisted Extraction. *Agronomy* **2020**, *10*, 179. [CrossRef]
43. Espada-Bellido, E.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Álvarez, J.A.; Barbero, G.F.; Ayuso, J. Extraction of Antioxidants from Blackberry (Rubus ulmifolius L.): Comparison between Ultrasound- and Microwave-Assisted Extraction Techniques. *Agronomy* **2019**, *9*, 745. [CrossRef]
44. Cervantes, L.; Martínez-Ferri, E.; Soria, C.; Ariza, M.T. Bioavailability of phenolic compounds in strawberry, raspberry and blueberry: Insights for breeding programs. *Food Biosci.* **2020**, *37*, 100680. [CrossRef]
45. Paes, J.; Dotta, R.; Barbero, G.F.; Martínez, J. Extraction of phenolic compounds and anthocyanins from blueberry (Vaccinium myrtillus L.) residues using supercritical CO2 and pressurized liquids. *J. Supercrit. Fluids* **2014**, *95*, 8–16. [CrossRef]
46. Chemat, F.; Zill-e-Huma; Khan, M.K. Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrason. Sonochem.* **2011**, *18*, 813–835. [CrossRef] [PubMed]
47. Medina-Torres, N.; Ayora-Talavera, T.; Espinosa-Andrews, H.; Sánchez-Contreras, A.; Pacheco, N. Ultrasound Assisted Extraction for the Recovery of Phenolic Compounds from Vegetable Sources. *Agronomy* **2017**, *7*, 47. [CrossRef]
48. Baiano, A. Recovery of Biomolecules from Food Wastes—A Review. *Molecules* **2014**, *19*, 14821–14842. [CrossRef]

49. Ciulu, M.; Cádiz-Gurrea, M.; Segura-Carretero, A. Extraction and Analysis of Phenolic Compounds in Rice: A Review. *Molecules* **2018**, *23*, 2890. [CrossRef]
50. V. González de Peredo, A.; Vázquez-Espinosa, M.; Espada-Bellido, E.; Ferreiro-González, M.; Amores-Arrocha, A.; Palma, M.; Barbero, G.F.; Jiménez-Cantizano, A. Alternative Ultrasound-Assisted Method for the Extraction of the Bioactive Compounds Present in Myrtle (*Myrtus communis* L.). *Molecules* **2019**, *24*, 882. [CrossRef]
51. Aliaño-González, M.J.; Espada-Bellido, E.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Ayuso, J.; Álvarez, J.Á.; Barbero, G.F. Extraction of Anthocyanins and Total Phenolic Compounds from Açai (Euterpe oleracea Mart.) Using an Experimental Design Methodology. Part 2: Ultrasound-Assisted Extraction. *Agronomy* **2020**, *10*, 326. [CrossRef]
52. Vázquez-Espinosa, M.; V. González-de-Peredo, A.; Espada-Bellido, E.; Ferreiro-González, M.; Toledo-Domínguez, J.J.; Carrera, C.; Palma, M.; Barbero, G.F. Ultrasound-Assisted Extraction of Two Types of Antioxidant Compounds (TPC and TA) from Black Chokeberry (*Aronia melanocarpa* L.): Optimization of the Individual and Simultaneous Extraction Methods. *Agronomy* **2019**, *9*, 456. [CrossRef]
53. Vázquez-Espinosa, M.; V. González de Peredo, A.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Barbero, G.F.; Espada-Bellido, E. Assessment of Ultrasound Assisted Extraction as an Alternative Method for the Extraction of Anthocyanins and Total Phenolic Compounds from Maqui Berries (*Aristotelia chilensis* (Mol.) Stuntz). *Agronomy* **2019**, *9*, 148. [CrossRef]
54. Wang, D.; Zhang, L.; Xu, Y.; Qi, X.; Wang, X.; Wang, X.; Zhang, Q.; Li, P. Optimization of an Ultrasound-Assisted Extraction for Simultaneous Determination of Antioxidants in Sesame with Response Surface Methodology. *Antioxidants* **2019**, *8*, 321. [CrossRef] [PubMed]
55. Wu, Y.; Han, Y.; Tao, Y.; Fan, S.; Chu, D.-T.; Ye, X.; Ye, M.; Xie, G. Ultrasound assisted adsorption and desorption of blueberry anthocyanins using macroporous resins. *Ultrason. Sonochem.* **2018**, *48*, 311–320. [CrossRef] [PubMed]
56. Li, X.; Zhang, L.; Peng, Z.; Zhao, Y.; Wu, K.; Zhou, N.; Yan, Y.; Ramaswamy, H.S.; Sun, J.; Bai, W. The impact of ultrasonic treatment on blueberry wine anthocyanin color and its In-vitro anti-oxidant capacity. *Food Chem.* **2020**, *333*, 127455. [CrossRef] [PubMed]
57. Wang, T.; Guo, N.; Wang, S.-X.; Kou, P.; Zhao, C.-J.; Fu, Y.-J. Ultrasound-negative pressure cavitation extraction of phenolic compounds from blueberry leaves and evaluation of its DPPH radical scavenging activity. *Food Bioprod. Process.* **2018**, *108*, 69–80. [CrossRef]
58. Bamba, B.; Shi, J.; Tranchant, C.; Xue, S.; Forney, C.; Lim, L.-T. Influence of Extraction Conditions on Ultrasound-Assisted Recovery of Bioactive Phenolics from Blueberry Pomace and Their Antioxidant Activity. *Molecules* **2018**, *23*, 1685. [CrossRef]
59. He, B.; Zhang, L.-L.; Yue, X.-Y.; Liang, J.; Jiang, J.; Gao, X.-L.; Yue, P.-X. Optimization of Ultrasound-Assisted Extraction of phenolic compounds and anthocyanins from blueberry (*Vaccinium ashei*) wine pomace. *Food Chem.* **2016**, *204*, 70–76. [CrossRef]
60. Rocha, J.C.G.; Procópio, F.R.; Mendonça, A.C.; Vieira, L.M.; Perrone, Í.T.; de Barros, F.A.R.; Stringheta, P.C. Optimization of ultrasound-assisted extraction of phenolic compounds from jussara (*Euterpe edulis* M.) and blueberry (*Vaccinium myrtillus*) fruits. *Food Sci. Technol.* **2018**, *38*, 45–53. [CrossRef]
61. Jiang, H.-L.; Yang, J.-L.; Shi, Y.-P. Optimization of ultrasonic cell grinder extraction of anthocyanins from blueberry using response surface methodology. *Ultrason. Sonochem.* **2017**, *34*, 325–331. [CrossRef]
62. Yuan, J.; Li, H.; Tao, W.; Han, Q.; Dong, H.; Zhang, J.; Jing, Y.; Wang, Y.; Xiong, Q.; Xu, T. An effective method for extracting anthocyanins from blueberry based on freeze-ultrasonic thawing technology. *Ultrason. Sonochem.* **2020**, *68*, 105192. [CrossRef]
63. FDA. Cder Q3C-Tables and List Guidance for Industry Q3C-Tables and List Guidance for Industry Q3C-Tables and List Guidance for Industry 1. Available online: https://www.fda.gov/media/71737/download (accessed on 16 November 2020).
64. V. González-de-Peredo, A.; Vázquez-Espinosa, M.; Espada-Bellido, E.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Álvarez, J.Á.; Barbero, G.F.; Ayuso, J. Optimization of Analytical Ultrasound-Assisted Methods for the Extraction of Total Phenolic Compounds and Anthocyanins from Sloes (*Prunus spinosa* L.). *Agronomy* **2020**, *10*, 966. [CrossRef]

65. Espada-Bellido, E.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Barroso, C.G.; Barbero, G.F. Optimization of the ultrasound-assisted extraction of anthocyanins and total phenolic compounds in mulberry (*Morus nigra*) pulp. *Food Chem.* **2017**, *219*, 23–32. [CrossRef]
66. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
67. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in Enzymology*; Packer, L., Ed.; Elsevier: Amsterdam, The Netherlands, 1998; pp. 152–178.
68. Celli, G.B.; Ghanem, A.; Brooks, M.S.-L. Optimization of ultrasound-assisted extraction of anthocyanins from haskap berries (*Lonicera caerulea* L.) using Response Surface Methodology. *Ultrason. Sonochem.* **2015**, *27*, 449–455. [CrossRef] [PubMed]
69. Varo, M.A.; Jacotet-Navarro, M.; Serratosa, M.P.; Mérida, J.; Fabiano-Tixier, A.-S.; Bily, A.; Chemat, F. Green Ultrasound-Assisted Extraction of Antioxidant Phenolic Compounds Determined by High Performance Liquid Chromatography from Bilberry (*Vaccinium Myrtillus* L.) Juice By-products. *Waste Biomass Valorization* **2019**, *10*, 1945–1955. [CrossRef]
70. Chen, X.-Q.; Li, Z.-H.; Wang, Z.-J.; Liu, L.-L.; Sun, T.-T.; Ma, J.-Z.; Zhang, Y. Ultrasound-assisted extraction of total anthocyanins from Rubia sylvatica Nakai fruit and radical scavenging activity of the extract. *Ind. Crops Prod.* **2020**, *150*, 112420. [CrossRef]
71. Albuquerque, B.R.; Pinela, J.; Barros, L.; Oliveira, M.B.P.P.; Ferreira, I.C.F.R. Anthocyanin-rich extract of jabuticaba epicarp as a natural colorant: Optimization of heat- and ultrasound-assisted extractions and application in a bakery product. *Food Chem.* **2020**, *316*, 126364. [CrossRef] [PubMed]
72. Fibigr, J.; Šatínský, D.; Solich, P. A UHPLC method for the rapid separation and quantification of anthocyanins in acai berry and dry blueberry extracts. *J. Pharm. Biomed. Anal.* **2017**, *143*, 204–213. [CrossRef] [PubMed]
73. Verbeyst, L.; Van Crombruggen, K.; Van der Plancken, I.; Hendrickx, M.; Van Loey, A. Anthocyanin degradation kinetics during thermal and high pressure treatments of raspberries. *J. Food Eng.* **2011**, *105*, 513–521. [CrossRef]
74. Akbal, F.; Onar, A.N. Photocatalytic degradation of phenol. *Environ. Monit. Assess.* **2003**, *83*, 295–302. [CrossRef]
75. Friedman, M.; Jürgens, H.S. Effect of pH on the Stability of Plant Phenolic Compounds. *J. Agric. Food Chem.* **2000**, *48*, 2101–2110. [CrossRef]
76. Howard, L.R.; Castrodale, C.; Brownmiller, C.; Mauromoustakos, A. Jam Processing and Storage Effects on Blueberry Polyphenolics and Antioxidant Capacity. *J. Agric. Food Chem.* **2010**, *58*, 4022–4029. [CrossRef]

foods

MDPI

Article

Volatile Profiling of Strawberry Fruits Cultivated in a Soilless System to Investigate Cultivar-Dependent Chemical Descriptors

Raúl González-Domínguez [1,2,*], Ana Sayago [1,2], Ikram Akhatou [1,2] and Ángeles Fernández-Recamales [1,2]

1 AgriFood Laboratory, Faculty of Experimental Sciences, University of Huelva, 21007 Huelva, Spain; ana.sayago@dqcm.uhu.es (A.S.); ikram.akhatou@alu.uhu.es (I.A.); recamale@dqcm.uhu.es (Á.F.-R.)
2 International Campus of Excellence CeiA3, University of Huelva, 21007 Huelva, Spain
* Correspondence: raul.gonzalez@dqcm.uhu.es; Tel.: +34-959-219-975

Received: 11 May 2020; Accepted: 9 June 2020; Published: 11 June 2020

Abstract: Volatile compounds are essential for food organoleptic characteristics and of great utility for the food industry as potential markers for authenticity purposes (e.g., variety, geographical origin, adulteration). The aim of this study was to determine the characteristic volatile compounds of strawberry samples grown in a soilless system by using headspace solid phase micro-extraction coupled with gas chromatography and to investigate the influence of cultivar (Festival, Candonga, Camarosa) on this volatile profile. We observed that Festival and, to a lesser extent, Candonga varieties were characterized by the richest aroma-related profiles, including higher levels of esters, furanones and terpenes. In particular, methyl butyrate, hexyl hexanoate, linalool, geraniol and furaneol were the most abundant aromatic compounds detected in the three varieties of strawberries. Complementarily, the application of pattern recognition chemometric approaches, including principal component analysis and linear discriminant analysis, demonstrated that concentrations of specific volatiles can be employed as chemical descriptors to discriminate between strawberry cultivars. Accordingly, geraniol and hexyl hexanoate were found to be the most significant volatiles for the discrimination of strawberry varieties.

Keywords: strawberry; volatile profile; variety; soilless system

1. Introduction

The typical fragrance of strawberry (*Fragaria* × *ananassa* Duch.) results from a balance between different volatile classes, comprising esters, aldehydes, ketones, alcohols, terpenes, furanones, and sulfides [1–5]. Among them, methyl and ethyl esters of butanoic and hexanoic acids, two furanones (furaneol and mesifuran) and methyl anthranilate have been described as the main contributors to the strawberry-like aroma [6–8]. The volatile content in foods is low compared with other nutrients and components, representing less than 0.01% of the fruit fresh weight in strawberry [6], but it is highly influenced by multiple factors, such as the cultivar, climate, production region, sampling time, the degree of ripeness, and post-harvest environment, among others [9–13]. In this vein, several authors have previously reported that some volatiles are highly cultivar-specific (e.g., ethyl butanoate, methyl anthranilate, furaneol, mesifuran), whereas others were found to be general strawberry aroma compounds (e.g., ethyl hexanoate) [4,14]. However, although numerous studies have been performed over the last years to investigate the impact of genotypic and agronomic factors on the chemical composition of strawberry, there is still scarce information about the volatile content of strawberries grown in soilless systems. The soilless culture has experienced strong development in southern

regions of Spain, France and Italy due to the phase out of methyl bromide, a broad-spectrum soil fumigant traditionally employed for strawberry production [15]. In previous studies, the nutritional characteristics of strawberries grown in soilless systems have been compared with those cultivated in soil crops [16], and differences in color and phenolic content among five strawberry varieties cultivated in two soilless substrates have also been evaluated [17,18]. More recently, great efforts have been made to characterize the chemical composition of soilless-grown strawberry and to elucidate the effect of cultivar and agronomic practices by applying complementary analytical approaches, such as metabolomics [19], polyphenolic analysis [20], and the multi-targeted profiling of different nutrients and food components (e.g., sugars, organic acids, minerals) [21–23]. In this context, the study of the volatile fraction from strawberry cultivars grown in soilless systems could be of great interest for the food industry to select optimal breeding and cultivation strategies aimed to produce higher quality fruits with an enhanced aroma. Furthermore, these volatile compounds might serve as markers for detecting adulteration [24] and to predict the shelf life of derived food products and establish the best commercial storage conditions to preserve their quality [25].

Considering this lack of information about the chemical composition of strawberries grown in soilless systems and the effect of cultivar, the aim of this work was to investigate the characteristic volatile profile of strawberries cultivated using this non-conventional crop system. For this purpose, an analytical method based on headspace solid phase micro-extraction (HS-SPME) and gas chromatography (GC) was applied to investigate the effect of cultivar (Festival, Candonga, Camarosa) on these aromatic compounds. Then, two complementary chemometrics approaches were employed, namely principal component analysis and linear discriminant analysis.

2. Materials and Methods

2.1. Samples

The strawberry fruits of three varieties (Camarosa, Candonga and Festival) were grown in Huelva (southwest Spain; latitude 37° 14′ N, longitude 6° 53′ W, altitude 23 m) in a soilless system, as detailed elsewhere [22]. These cultivars were selected with the aim of investigating the impact of soilless growing on the volatile composition of strawberries with a different sensitivity to environmental conditions, from very sensitive varieties (e.g., Festival) to more resistant ones (e.g., Camarosa). Fruits were harvested at commercial ripeness (>75% of the surface showing red color), washed and sepals dissected. Then, the berries (100 g) were gently homogenized with 100 mL of a 20% NaCl solution (*w*/*v*) by using a kitchen mixer, snap-frozen in liquid nitrogen, and stored at −20 °C until analysis. For each cultivar, five independent homogenized samples were prepared by pooling 10 individual fruits from different plants.

2.2. Reagents and Chemicals

Absolute ethanol (>99.5%) and sodium chloride (99.5%) were purchased from Scharlab (Barcelona, Spain). Ultrapure water (18 MΩ cm^{-1}) was obtained from a Milli-Q system (Millipore, Milford, MA, USA). The volatile standards, with purity above 99%, were supplied by Chemservice (West Chester, PA, USA) and Aldrich (Gillingham, UK): methyl butanoate, ethyl butanoate, methyl hexanoate, ethyl hexanoate, hexyl hexanoate, cis-3-hexenyl acetate, trans-2-hexenyl acetate, hexanal, trans-2-hexen-1-al, benzaldehyde, 1-hexanol, trans-2-hexen-1-ol, benzyl alcohol, linalool, geraniol, cis-nerolidol, nerol, mesifurane, furaneol, 2-methylbutanoic acid, 3-methylbutanoic acid, hexanoic acid, γ-nonalactone, and Δ-decalactone. 2-octanol (internal standard, IS) was obtained from Fluka (Madrid, Spain). Individual stock solutions at 1000 mg L^{-1} for each compound and IS were prepared in ethanol and stored at −20 °C.

2.3. Headspace Solid-Phase Micro-Extraction Procedure

A manual fiber holder and 65 µm polydimethylsiloxane-divinylbenzene (PDMS/DVB) fibers (length = 1 cm), supplied by Supelco (Bellefonte, PA, USA), were employed in this study. Fiber was conditioned before use by inserting into the GC injector port at 250 °C during 30 min, following the manufacturer recommendations. A magnetic stirrer equipped with electronic contact thermometer (IKA®-Werke GmbH & Co. KG, Germany) was used to favor the volatilization of aroma-related compounds into the headspace.

The headspace solid phase micro-extraction (HS-SPME) process was carried out by introducing 10 g of fruit puree into a 20 mL glass vial, together with 1 g of sodium chloride and 40 µL of the IS solution at 1000 mg L^{-1}. The vial was crimped with a polytetrafluoroethylene (PTFE)-faced septum, then immersed into a water bath at 65 °C, and magnetically stirred during 30 min for temperature equilibration. Afterwards, headspace sampling was performed for 20 min at 65 °C, stirring the solution at 800 rpm. Finally, the fiber was withdrawn from the vial and the SPME device was transferred to the GC injection port, where thermal desorption of the analytes was carried out at 240 °C for 5 min.

2.4. Chromatographic Analysis

The analyses were carried out on a gas chromatograph equipped with a flame ionization detector (GC-FID) from Agilent Technologies (Palo Alto, CA, USA), following a modification of the method described by Olbricht et al. [9]. The volatile compounds were separated on a CP-Wax 52 CB capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) from Varian (Walnut CreeK, CA, USA), using helium as the carrier gas at a constant flow rate of 1 mL min^{-1}. The injection port was equipped with a 0.75 mm i.d. splitless glass liner (Supelco, Bellefonte, PA, USA) and operated in the splitless mode at 240 °C. The oven temperature was initially maintained at 40 °C for 5 min, raised to 200 °C at 5 °C min^{-1} and held for 5 min, and then raised to 250 °C at 5 °C min^{-1} and held at this temperature for 25 min. The temperature of the FID was set at 250 °C. Under these experimental chromatographic conditions, 24 volatile compounds (+IS) were separated. The identification of volatile compounds was accomplished using an in-house database built by injecting standard compounds, dissolved in solvent and in matrix, under the same chromatographic conditions. Additional experiments were performed on a gas chromatograph (Varian CP 3800) coupled to mass spectrometry (Varian Saturn 2200) to increase the identification reliability using the NIST Mass Spectral Library. Quantification was performed in reference to the IS (mg of 2-octanol equivalents per kg of fresh fruit weight).

2.5. Experimental Design

The working variables for the HS-SPME procedure, namely equilibration time, time and temperature for analyte adsorption into the fiber, time and temperature for analyte desorption, as well as weights of sample and background electrolyte (i.e., sodium chloride), were optimized by using an experimental design methodology. To this end, a fractional factorial design ($2^{(7-3)}$) with three center points was applied to evaluate the main effects of the SPME variables on the recovery of volatile compounds from strawberry samples. Study ranges for these variables were as follows: equilibration time, 5–30 min; extraction time, 20–30 min; extraction temperature, 25–65 °C; desorption time, 1–5 min; desorption temperature, 240–280 °C; weight of sample, 2.5–10 g; weight of background electrolyte, 10–25%. All the extraction tests were performed using a headspace volume in the range 30–50% of the total vial volume, and keeping constant the height of the fiber inside the vial, to ensure reproducibility and extraction efficacy, according to previous literature [26]. In total, 19 trials were conducted in random order using a pooled strawberry sample. To study the effects of the factors on the response variables (i.e., number of peaks and peak areas), a Pareto diagram was used. In this representation, the length of each bar is proportional to the standardized effect (i.e., estimated effect divided by its standard error), and the vertical line can be used to judge which effects are statistically significant at 95% confidence. Subsequently, a response surface methodology (RSM) based on central

composite design (CCD) was developed to optimize those extraction factors that elicited significant effects on dependent variables [27]. CCD consisted of a two-level factorial design, a star design in which experimental points are at a distance α from its center, and the central point. Each factor was studied at five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$). The relationship between dependent and independent variables was fitted by a second-order polynomial model. The validation of the quadratic model obtained by RSM was accomplished by analysis of variance (ANOVA).

2.6. Statistical Analysis

To investigate differences in the volatile profile among strawberry cultivars, the dataset was first submitted to analysis of variance (ANOVA, $p < 0.05$) with the Fisher's Least Significant Difference (LSD) post hoc test. Complementarily, various pattern recognition techniques were applied for the preliminary exploration of data and the characterization of varieties (principal component analysis, PCA), and to discriminate between different samples (linear discriminant analysis, LDA). PCA is one of the most powerful and common techniques for reducing the dimensionality of large datasets without the loss of information. New variables obtained by a linear combination of the original ones are calculated in such a way to keep most of the information present in the original dataset in the lower number of new variables, named principal components (PCs). The eigenvalues generated during PCA give an indication of the amount of information carried by each component. To select the optimal number of components, one of the most used criteria is to retain only factors with eigenvalues higher than 1 (Kaiser criterion). The Bartlett's sphericity test was used as a measure of the sampling adequacy for evaluating the suitability of performing a PCA in our dataset, which is particularly useful for small sample sizes. On the other hand, linear discriminant analysis (LDA) is a supervised classification tool based on the generation of a number of orthogonal linear discriminant functions equal to the number of categories minus one. The discriminant power of each variable is evaluated by measuring the value of the Wilks' lambda parameter for the overall model after removing the selected variable. Then, a forward stepwise algorithm is used to select the variables to be included in the final model. According to this algorithm, the F value is used as a criterion for inclusion or removal of variables in the model. Thus, Wilks' lambda and F values were used in the present work to check the significance of each predictor in the LDA accomplished. Prior to conducting multivariate statistical analyses, data were subjected to logarithmic transformation and Pareto scaling, and data normality was checked by inspecting probability plots. All statistical analyses were conducted on Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. HS-SPME Optimization and Validation

Extraction efficiency in SPME not only depends on the polarity and thickness of the stationary phase, but is also affected by other variables such as agitation, time, temperature, or the addition of salts to the sample [28]. As a first step, we compared the performance of different fiber coatings for the extraction of volatile compounds from strawberry: polydimethylsiloxane (PDMS), polyacrylate (PA), polydimethylsiloxane/divinylbenzene (PDMS/DVB), carboxen/polydimethylsiloxane (CAR/PDMS), and divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). For this purpose, generic extraction conditions were applied based on our previous experience and literature: equilibration time, 15; extraction time, 30 min; extraction temperature, 50 °C; weight of sample, 10 g; weight of background electrolyte, 10%. The extraction efficiency was evaluated in terms of the total number of compounds extracted and their peak areas. The results evidence that, although different fibers were best suited for the extraction of certain compounds, the best overall extraction efficiency was obtained by using DVB/PDMS coatings, in line with previous studies [29–31]. Furthermore, we also checked that the HS volume did not have a significant influence on the extraction process if maintained in the range 30–50% of the total volume, thus providing suitable reproducibility and extraction recovery.

To investigate the effect of other variables in the adsorption/desorption process, a two-level screening fractional factorial experimental design ($2^{(7-3)}$) with three replicates in the center point was carried out. This screening experimental design evidenced a significant positive influence of sample weight and adsorption temperature on the extraction efficacy, which reached a plateau at the higher experimental conditions tested in this study. Accordingly, these parameters were subsequently optimized by using a central composite experimental design (CCD, with α = 1.414). To this end, sample weight and extraction temperature were tested in the ranges 6.5–12.5 g and 30–80 °C, respectively. All the other variables were kept at levels providing the best responses according to the Pareto diagram (Figure 1): equilibration time, 30 min; extraction time, 20 min; desorption time, 5 min; desorption temperature, 240 °C; weight of salt, 10%.

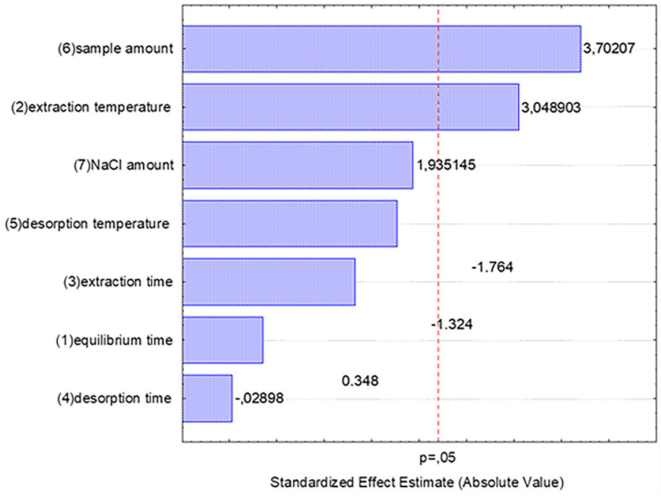


Figure 1. Pareto diagram showing the effect of factors investigated on the extraction efficiency.

By applying the response surface methodology, the estimated optimum values for extraction temperature and sample weight were 65.4 °C and 11.4 g, respectively (Figure 2). The adequacy of the model was evaluated by using the coefficient of determination (R^2), and assessing the regression fitness. The model showed statistical valid fitness ($p < 0.05$ for the regression F test) and non-significant lack of fit ($p > 0.05$). In addition, the coefficient of determination was estimated as 0.419.

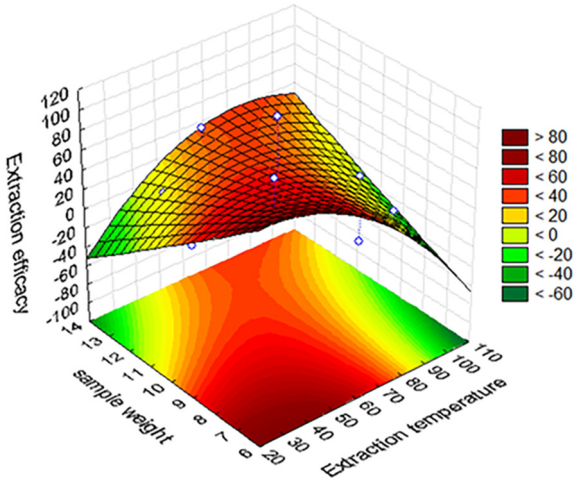


Figure 2. Response surface plots showing the effect of temperature and sample weight on the extraction efficiency according to the central composite design (CCD).

To assess the precision of the extraction method previously optimized, nine independent extractions were carried out from the same batch of fruit puree at the optimum conditions. The extracts were

prepared in three non-consecutive days, and replicated three times within each day. Repeatability was evaluated as the relative standard deviation of the ANOVA within-condition variance (S^2_W) considering the different days as the variation source. On the other hand, intermediate precision was evaluated from between-condition variance (S^2_B) and within-condition variance (S^2_W): $S^2_R = S^2_W + S^2_B$ [32]. The results evidence satisfactory repeatability, in the range 3.2% (ethyl butanoate) to 13.5% (hexanal), and intermediate precision, in the range 6.3% (geraniol) to 22.1% (hexanal). The limits of detection (LODs) and limits of quantification (LOQs) were calculated as the lowest concentration giving an average signal-to-noise (S/N) ratio above 3 and 10, respectively, by evaluating the background noise in blank samples. LOD values ranged from 0.42 μg mL^{-1} (for geraniol) to 3.53 μg mL^{-1} (for cis-nerolidol), whereas LOQs ranged from 1.41 μg mL^{-1} (for geraniol) to 11.78 μg mL^{-1} (for cis-nerolidol), expressed as IS equivalents. The analysis of standard solutions and spiked strawberry samples demonstrated the absence of significant degradation processes during the extraction (i.e., the signal intensity for standards dissolved in solvent that were analyzed without performing HS-SPME was comparable with that obtained after the extraction of the same standard solutions and spiked strawberry samples).

3.2. Characterization of the Strawberry Volatile Profile

The HS-SPME-GC-FID methodology previously optimized was applied to characterize the volatile composition of three strawberry varieties grown in soilless system (Figure 3). In total, 24 compounds were identified in the samples under study, including esters, aldehydes, alcohols, acids, lactones, terpenes and furanones.

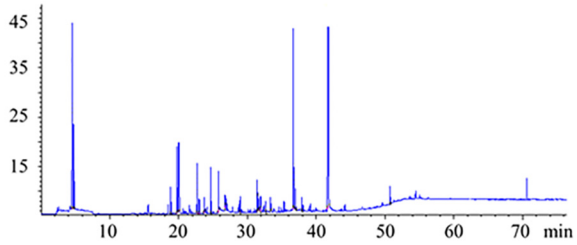


Figure 3. Typical chromatogram obtained by headspace solid phase micro-extraction coupled with gas chromatography (HS-SPME-GC) analysis of a Camarosa strawberry sample.

The concentrations of these volatile compounds in the three strawberry cultivars investigated are listed in Table 1. Esters were the most represented chemical class, with seven different compounds detected in, at least, two of the investigated cultivars. This agrees with previous works reporting that methyl and ethyl esters of butanoic and hexanoic acids are major volatiles in strawberry, responsible for its fruity and floral aroma [1,6,14,33]. According to the literature, terpenes (e.g., cis-nerolidol) and furans (e.g., furaneol, mesifurane) are also important contributors to the typical strawberry-like aroma [1,8,34], which were also detected in the present study at high concentrations.

The results evidence that the volatile profile is significantly different between strawberry varieties, both qualitatively and quantitatively, in line with previous studies reported in literature (Table 2). Esters were the most abundant chemical class in the Camarosa cultivar (22%), followed by terpenes (19.1%) and acids (12.2%). On the other hand, Candonga and Festival varieties were richer in terpenes (31.6 and 34.8%, respectively), followed by esters (24.7 and 26.4%, respectively). Specifically, methyl butyrate, hexyl hexanoate, linalool, geraniol and furaneol were the most abundant volatile compounds, whereas methyl anthranilate was not detected in any of the analyzed samples. As shown in Table 1, the Camarosa variety was characterized by significantly lower levels of most of the volatiles investigated, including esters (e.g., ethyl butanoate, ethyl hexanoate, hexyl hexanoate), terpenes (e.g., linalool, geraniol), and furanones (e.g., furaneol). Ethyl hexanoate was not detected in any of the samples analyzed for this variety, and mesifuran was only found in one of the samples analyzed, in line with the results obtained by Ubeda et al. for some strawberry varieties (Fuentepina, Camarosa, Candonga, Sabrina) [7]. Conversely, cis-nerolidol was majorly detected in Camarosa strawberries, at levels similar

to those previously reported [35]. On the contrary, Festival samples showed the highest total content of esters, terpenes and furanones (Table 1), although some specific compounds were not detected (e.g., hexanoic acid, benzyl alcohol, benzaldehyde, cis-3-hexenyl acetate). It should be noted that furaneol concentration in Festival cultivar was almost five times higher than in the Camarosa variety and almost double that in Candonga, being this volatile molecule a key aroma compound in strawberry according to the literature [1,7,8,33,34]. Finally, the volatile levels in Candonga strawberries were, in general, between those found for the other two cultivars investigated. All these results therefore suggest that Festival strawberries potentially have a more aromatic profile than Candonga and, especially, Camarosa ones, with higher concentrations for most of the differential volatile compounds listed in Table 1. However, these results should be validated by comparing these concentrations with sensory thresholds.

Table 1. Volatile composition of three strawberry varieties grown in a soilless system (expressed as the mean ± standard deviation of 2-octanol equivalents, μg kg^{-1}) and *p*-values obtained by ANOVA (N = 5 per study group).

	Camarosa	**Candonga**	**Festival**	***p*-Value**
Esters				
methyl butanoate	142.21 ± 14.65 ^{a}	112.72 ± 10.59 ^{b}	99.63 ± 7.92 ^{b}	0.0002
ethyl butanoate	14.37 ± 1.62 ^{a}	28.01 ± 2.31 ^{a}	48.06 ± 3.14 ^{b}	0.01
methyl hexanoate	24.51 ± 2.75 ^{a}	62.31 ± 3.20 ^{b}	57.64 ± 4.04 ^{b}	0.0001
ethyl hexanoate	ND	19.26 ± 3.31 ^{a}	28.48 ± 4.31 ^{b}	0.0006
hexyl hexanoate	95.45 ± 16.59 ^{a}	150.47 ± 24.00 ^{a}	303.79 ± 94.39 ^{b}	0.0063
cis-3-hexenyl acetate	40.71 ± 9.10	42.52 ± 8.37	ND	NS
trans-2-hexenyl acetate	24.77 ± 1.90	33.66 ± 3.39	26.44 ± 2.33	NS
Aldehydes				
hexanal	26.41 ± 9.51	18.24 ± 4.45	26.43 ± 6.54	NS
trans-2-hexen-1-al	19.75 ± 2.24 ^{ab}	6.78 ± 0.92 ^{a}	21.96 ± 4.44 ^{b}	0.0055
benzaldehyde	29.21 ± 6.53	21.68 ± 4.85	ND	NS
Alcohols				
1-hexanol	38.25 ± 2.10	21.83 ± 4.07	34.77 ± 2.19	NS
trans-2-hexen-1-ol	30.94 ± 12.24	31.96 ± 15.13	50.76 ± 18.35	NS
benzyl alcohol	12.68 ± 2.09	6.60 ± 1.47	ND	NS
Terpenes				
linalool	75.08 ± 14.63 ^{a}	135.00 ± 21.74 ^{b}	188.74 ± 32.02 ^{b}	0.0053
geraniol	151.63 ± 29.96 ^{a}	426.43 ± 35.72 ^{b}	537.73 ± 122.01 ^{b}	0.0001
cis-nerolidol	51.77 ± 7.60 ^{a}	5.91 ± 1.32 ^{b}	12.69 ± 2.46 ^{b}	0.0001
nerol	7.59 ± 1.70 ^{a}	7.73 ± 1.73 ^{a}	4.33 ± 0.86 ^{b}	0.0053
Furanones				
mesifurane	27.83 ± 6.22	33.84 ± 5.08	39.80 ± 8.12	NS
furaneol	96.56 ± 24.29 ^{a}	210.38 ± 47.73 ^{b}	456.97 ± 117.81 ^{c}	0.00001
Acids				
2-methylbutanoic acid	30.71 ± 5.67 ^{a}	64.72 ± 16.26 ^{b}	46.23 ± 6.14 ^{a}	0.0017
3-methylbutanoic acid	109.32 ± 22.48 ^{a}	89.26 ± 15.81 ^{a}	4.63 ± 0.92 ^{b}	0.0001
hexanoic acid	43.01 ± 9.60	29.04 ± 6.42	ND	NS
Lactones				
γ-nonalactone	9.89 ± 2.21	11.69 ± 2.61	ND	NS
Δ-decalactone	4.83 ± 1.08	20.36 ± 4.55	ND	NS

Superscript letters within each row indicate significant differences between groups marked with different letters, according to the post-hoc LSD test ($p < 0.05$). ND: not detected, NS: not significant ($p > 0.05$).

Table 2. Summary of previous works investigating the influence of the variety in the strawberry volatile profile.

Varieties Investigated	Findings	Reference
35	Thirty-one volatile compounds correlated to strawberry flavor intensity, particularly esters, terpenes and furans.	[1]
4	Key odorants identified were furaneol, γ-decalactone, ethyl butanoate, ethyl hexanoate, ethyl 3-methylbutanoate, diacetyl and hexanoic acid. The aroma of Fuentepina and Candonga varieties presented mainly green notes, whereas the aromatic notes in Camarosa and Sabrina varieties were mainly sweet.	[7]
12	The most abundant volatile sulfur compounds in strawberry are methanethiol, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide, being methanethiol the predominant aromatic compound. Festival and Florida Radiance presented higher thioester concentrations., whereas Dover, Rosa Linda and Florida Belle were characterized by relatively high sulfide and low thioester concentrations.	[10]
9	Esters, such as methyl butanoate, pentyl acetate and methyl hexanoate, characterized the aroma of ripe strawberries, and allow discriminating between cultivars.	[11]
16	Great diversity of the volatile patterns in F. vesca accessions in comparison with F. × ananassa cultivars.	[33]
5	The content of volatiles varied depending on the cultivars, but in general ethyl butanoate, mesifurane, ethyl hexanoate, ethyl 3-methylbutanoate, hexyl acetate and γ -dodecalactone had the highest odor activity values.	[35]

3.3. Chemometrics Approaches to Identify Chemical Descriptors of Strawberry Cultivar

To investigate the potential of volatile metabolites as chemical descriptors to discriminate between strawberry cultivars grown in soilless systems, various complementary chemometrics approaches were employed in the present study. Principal component analysis (PCA) was first applied for a rapid exploration and visualization of the data trends. The PCA model obtained, with three principal components (PC), was able to explain up to 69.4% of the total variance. As shown in the corresponding scores plot (Figure 4A), the three strawberry varieties were slightly separated along the first principal component, but significant intra-cultivar variability was also observed. The PC1 was positively associated with levels of ethyl butanoate, ethyl hexanoate, hexyl hexanoate, linalool, geraniol and furaneol (Figure 4B). That is, the volatile content was in general higher in Festival samples, located in the right side of the PCA plot with positive loadings, in line with results presented in Table 1. On the other hand, the main contributors to PC2 were trans-2-hexen-1-ol, 1-hexanol, methyl butanoate, mesifurane and cis-nerolidol, thereby being responsible for the variability detected within Camarosa strawberries and, consequently, their wide spread on PC2 (Figure 4A).

In a second step, linear discriminant analysis (LDA) was applied to build classification models for the predefined groups and to select the variables with higher discriminant power. For this purpose, the dataset was randomly divided into two groups, i.e., training and validation sets, accounting for 80% and 20% of the total number of samples, respectively. The training set was used to build the model, whereas the validation set enabled the testing of its performance. To validate the recalling rate (effectiveness of classification in the training set) and the prediction ability (effectiveness of classification in the validation set) of the LDA model, both training and validation sets were repeated at least ten times with different constitutions. The average percentage of correctly classified cases in the recalling and prediction tests obtained from these 10 runs was used as a measure of the method performance. Using this method, two significant discriminant functions (roots) were obtained by stepwise LDA (Wilks' lambda p-values 0.0000 and 0.0001, respectively), which explained 68% and 32% of the total variability of the data, respectively. As shown in Table 3, the most discriminant variables were geraniol, hexyl hexanoate, and trans-2-hexen-1-al.

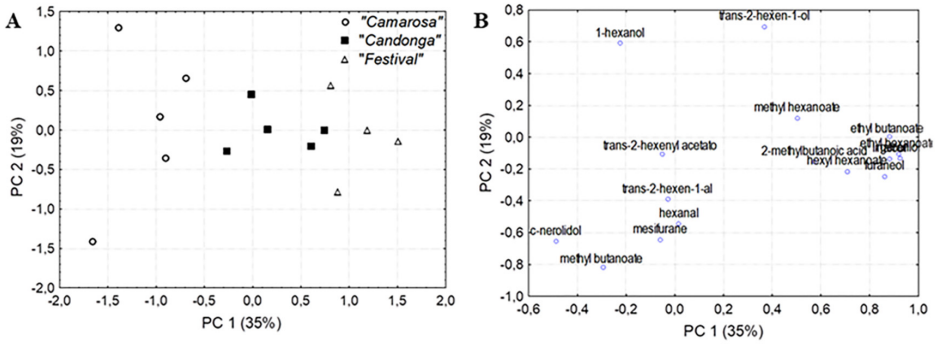


Figure 4. Principal component analysis (PCA) scores plot showing the distribution of samples in the space defined by the two first principal components (**A**); and a PCA loadings plot showing the contribution of each variable to the two first principal components (**B**).

Table 3. Summary of the statistical performance for the linear discriminant analysis (LDA) model.

		Root 1	**Root 2**
Canonical correlation		0.9808	0.9597
Eigenvalue		25.33009	11.66195
Cum. Prop		0.68474	1.00000
variables	*F*-value [a]	correlation of variables with roots	
geraniol	23.26316	−0.450750	0.217442
hexyl hexanoate	18.57001	−0.299080	−0.093153
trans-2-hexen-1-al	6.17330	−0.021863	−0.235022

[a] significant at $p < 0.001$.

This LDA model yielded satisfactory classification (92.18%) and prediction (75.68%) rates, thereby allowing a clear discrimination among the study groups, as illustrated in Figure 5. The first discriminant function enabled the separation of the three varieties, mainly Festival and Camarosa cultivars with negative and positive loadings for root 1, respectively. The primary variables responsible for the discrimination of Festival strawberries with respect to the other two varieties were geraniol and hexyl hexanoate, with negative loadings in root 1 (Table 3). On the other hand, Candonga samples were orthogonally separated in the second root. This second discriminant function was positively associated with geraniol and negatively with trans-2-hexen-1-al, which could therefore serve as chemical descriptors for the Candonga cultivar.

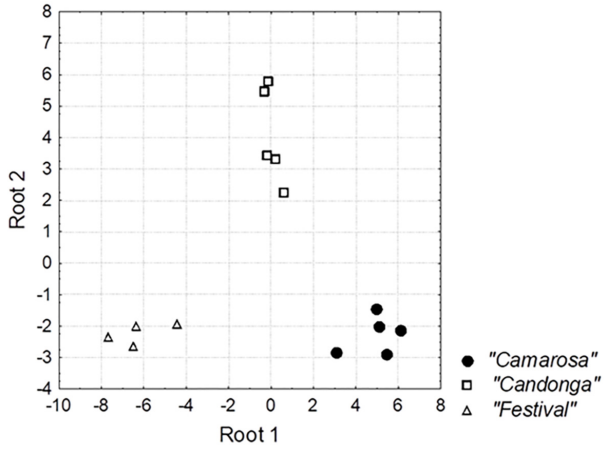


Figure 5. Linear discriminant analysis (LDA) scatterplot showing the distribution of samples in the space defined by the two discriminant functions.

4. Conclusions

In this study, we aimed to investigate the varietal differences in the volatile profile of strawberries cultivated in soilless systems. To this end, we have developed, in the present study, a method based on headspace solid phase micro-extraction coupled with gas chromatography (HS-SPME-GC-FID) for characterizing the volatile fraction of strawberry. This methodology enabled the detection of 24 aroma-related compounds in strawberry fruits grown in a soilless system. Furthermore, we observed that the levels of these metabolites were highly influenced by genotypic factors, with the Festival cultivar having higher concentrations for most of the differential volatile compounds here assayed. The application of complementary chemometrics approaches suggested that some of these molecules, namely geraniol and hexyl hexanoate, might be employed as chemical descriptors to discriminate between different strawberry cultivars.

Author Contributions: Conceptualization, Á.F.-R.; methodology, A.S. and Á.F.-R.; software, R.G.-D. and Á.F.-R.; validation, R.G.-D. and Á.F.-R.; formal analysis, I.A.; investigation, R.G.-D., A.S., I.A. and Á.F.-R.; resources, A.S. and Á.F.-R.; data curation, Á.F.-R.; writing—original draft preparation, Á.F.-R.; writing—review and editing, R.G.-D., A.S., I.A. and Á.F.-R.; visualization, R.G.-D. and Á.F.-R.; supervision, Á.F.-R.; project administration, Á.F.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors thank López-Medina (Department of Agroforestry Science, University of Huelva, Spain) for kindly providing the strawberry samples analyzed in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Schwieterman, M.L.; Colquhoun, T.A.; Jaworski, E.A.; Bartoshuk, L.M.; Gilbert, J.L.; Tieman, D.M.; Odabasi, A.Z.; Moskowitz, H.R.; Folta, K.M.; Klee, H.J.; et al. Strawberry flavor: Diverse chemical compositions, a seasonal influence, and effects on sensory perception. *PLoS ONE* **2014**, *9*, e88446. [CrossRef]
2. Lu, H.; Ban, Z.; Wang, K.; Li, D.; Li, D.; Poverenov, E.; Luo, Z. Aroma volatiles, sensory and chemical attributes of strawberry (*Fragaria* × *ananassa* Duch.) achenes and receptacle. *Int. J. Food Sci. Technol.* **2017**, *52*, 2614–2622. [CrossRef]
3. Schieberle, P.; Hofmann, T. Evaluation of the character impact odorants in fresh strawberry juice by quantitative measurements and sensory studies on model mixtures. *J. Agric. Food Chem.* **1997**, *45*, 227–232. [CrossRef]
4. Urrutia, M.; Rambla, J.L.; Alexiou, K.G.; Granell, A.; Monfort, A. Genetic analysis of the wild strawberry (*Fragaria vesca*) volatile composition. *Plant Physiol. Biochem.* **2017**, *121*, 99–117. [CrossRef] [PubMed]
5. Haugeneder, A.; Trinkl, J.; Härtl, K.; Hoffmann, T.; Allwood, J.W.; Schwab, W. Answering biological questions by analysis of the strawberry metabolome. *Metabolomics* **2018**, *14*, 145. [CrossRef] [PubMed]
6. Pérez, A.; Sanz, C. Strawberry flavor. In *Handbook of Fruit and Vegetable Flavors*; Hui, Y.H., Ed.; Wiley: Hoboken, NJ, USA, 2010; pp. 431–449.
7. Ubeda, C.; San-Juan, F.; Concejero, B.; Callejón, R.M.; Troncoso, A.M.; Morales, M.L.; Ferreira, V.; Hernández-Orte, P. Glycosidically bound aroma compounds and impact odorants of four strawberry varieties. *J. Agric. Food Chem.* **2012**, *60*, 6095–6102. [CrossRef] [PubMed]
8. Song, C.; Hong, X.; Zhao, S.; Liu, J.; Schulenburg, K.; Huang, F.C.; Franz-Oberdorf, K.; Schwab, W. Glucosylation of 4-Hydroxy-2,5-Dimethyl-3(2H)-Furanone, the key strawberry flavor compound in strawberry fruit. *Plant Physiol.* **2016**, *171*, 139–151. [CrossRef]
9. Olbricht, K.; Ulrich, D.; Weiss, K.; Grafe, C. Variation in the amounts of selected volatiles in a model population of *Fragaria* × *ananassa* Duch. As influenced by harvest year. *J. Agric. Food Chem.* **2011**, *59*, 944–952. [CrossRef]
10. Du, X.; Whitaker, V.; Rouseff, R. Changes in strawberry volatile sulfur compounds due to genotype, fruit maturity and sample preparation. *Flavour Fragr. J.* **2012**, *27*, 398–404. [CrossRef]

11. Vandendriessche, T.; Vermeir, S.; Mayayo Martinez, C.; Hendrickx, Y.; Lammertyn, J.; Nicolaï, B.M.; Hertog, M.L.A.T.M. Effect of ripening and inter-cultivar differences on strawberry quality. *LWT* **2013**, *52*, 62–70. [CrossRef]
12. Perdones, A.; Escriche, I.; Chiralt, A.; Vargas, M. Effect of chitosan-lemon essential oil coatings on volatile profile of strawberries during storage. *Food Chem.* **2016**, *197*, 979–986. [CrossRef]
13. Dong, J.; Zhong, C.F.; Wang, G.; Chang, L.; Sun, J.; Sun, R.; Zhang, H.L.; Li, R.; Wei, Y.Q.; Zheng, S.Q.; et al. Comparative study on fruit volatiles of different day-neutral strawberry cultivars in autumn and winter. *Sci. Agric. Sin.* **2019**, *52*, 2309–2327.
14. Ulrich, D.; Kecke, S.; Olbricht, K. What do we know about the chemistry of strawberry aroma? *J. Agric. Food Chem.* **2018**, *66*, 3291–3301. [CrossRef] [PubMed]
15. Ajwa, H.A.; Klose, S.; Nelson, S.D.; Minuto, A.; Gullino, M.L.; Lamberti, F.; Lopez-Aranda, J.M. Alternatives to methyl bromide in strawberry production in the United States of America and the Mediterranean region. *Phytopathol. Mediterr.* **2003**, *42*, 220–244.
16. Recamales, A.F.; López Medina, J.; Hernanz, D. Physicochemical characteristics and mineral content of strawberries grown in soil and soilless system. *J. Food Qual.* **2007**, *30*, 837–853. [CrossRef]
17. Hernanz, D.; Recamales, A.F.; Meléndez-Martínez, A.J.; González-Miret, M.L.; Heredia, F.J. Assessment of the differences in the phenolic composition of five strawberry cultivars (*Fragaria ananassa* Duch.) grown in two different soilless system. *J. Agric. Food Chem.* **2007**, *55*, 1846–1852. [CrossRef] [PubMed]
18. Hernanz, D.; Recamales, A.F.; Meléndez-Martínez, A.J.; González-Miret, M.L.; Heredia, F.J. Multivariate statistical analysis of color-anthocyanin relationships in different soilless-grown strawberry genotypes. *J. Agric. Food Chem.* **2008**, *56*, 2735–2741. [CrossRef]
19. Akhatou, I.; González-Domínguez, R.; Fernández-Recamales, Á. Investigation of the effect of genotype and agronomic conditions on metabolomic profiles of selected strawberry cultivars with different sensitivity to environmental stress. *Plant Physiol. Biochem.* **2016**, *101*, 14–22. [CrossRef]
20. Akhatou, I.; Sayago, A.; González-Domínguez, R.; Fernández-Recamales, Á. Application of targeted metabolomics to investigate optimum growing conditions to enhance bioactive content of strawberry. *J. Agric. Food Chem.* **2017**, *65*, 9559–9567. [CrossRef]
21. Akhatou, I.; Fernández-Recamales, A. Nutritional and nutraceutical quality of strawberries in relation to harvest time and crop conditions. *J. Agric. Food Chem.* **2014**, *62*, 5749–5760. [CrossRef]
22. Akhatou, I.; Fernández-Recamales, Á. Influence of cultivar and culture system on nutritional and organoleptic quality of strawberry. *J. Sci. Food Agric.* **2014**, *94*, 866–875. [CrossRef] [PubMed]
23. González-Domínguez, R.; Sayago, A.; Akhatou, I.; Fernández-Recamales, Á. Multi-chemical profiling of strawberry as a traceability tool to investigate the effect of cultivar and cultivation conditions. *Foods* **2020**, *9*, 96. [CrossRef] [PubMed]
24. Dasenaki, M.E.; Thomaidi, N.S. Quality and authenticity control of fruit juices—A review. *Molecules* **2019**, *24*, 1014. [CrossRef] [PubMed]
25. Morales, M.L.; Callejón, R.M.; Ordóñez, J.L.; Troncoso, A.M.; García-Parrilla, M.C. Comparative assessment of software for non-targeted data analysis in the study of volatile fingerprint changes during storage of a strawberry beverage. *J. Chromatogr. A* **2017**, *1522*, 70–77. [CrossRef]
26. Yang, X.; Peppard, T. Solid-phase microextraction for flavor analysis. *J. Agric. Food Chem.* **1994**, *42*, 1925–1930. [CrossRef]
27. Ferreira, S.L.C.; Silva Junior, M.M.; Felix, C.S.A.; da Silva, D.L.F.; Santos, A.S.; Santos Neto, J.H.; de Souza, C.T.; Cruz Junior, R.A.; Souza, A.S. Multivariate optimization techniques in foods analysis. A review. *Food Chem.* **2019**, *273*, 3–8. [CrossRef]
28. Pawliszyn, J. *Solid Phase Microextraction: Theory and Practice*; Wiley–VCH: New York, NY, USA, 1997.
29. Almenar, E.; Hernández-Muñoz, P.; Gavara, R. Evolution of selected volatiles in chitosan-coated strawberries (*Fragaria* × *ananassa*) during refrigerated storage. *J. Agric. Food Chem.* **2009**, *57*, 974–980. [CrossRef]
30. Vandendriessche, T.; Nicolai, B.M.; Hertog, M.L.A.T.M. Optimization of HS SPME Fast GC-MS for high-throughput analysis of strawberry aroma. *Food Anal. Methods* **2013**, *6*, 512–520. [CrossRef]
31. Kafkas, E.; Kafkas, S. Identification of strawberry (*Fragaria* × *ananassa* 'Rubygem') volatiles using various SPME fibres by GC/MS. *Acta Hortic.* **2017**, *1156*, 689–694. [CrossRef]

32. González, A.G.; Herrador, M.A. Accuracy profiles from uncertainty measurements. *Talanta* **2006**, *70*, 896–901. [CrossRef]
33. Ulrich, D.; Olbricht, K. Diversity of volatile patterns in sixteen *Fragaria vesca* L. accessions in comparison to cultivars of *Fragaria* × *ananassa*. *J. Appl. Bot. Food Qual.* **2013**, *86*, 37–46.
34. Prat, L.; Espinoza, M.I.; Agosin, E.; Silva, H. Identification of volatile compounds associated with the aroma of white strawberries (*Fragaria chiloensis*). *J. Sci. Food Agric.* **2014**, *94*, 752–759. [CrossRef] [PubMed]
35. Jetti, R.R.; Yang, E.; Kurnianta, A.; Finn, C.; Qian, M.C. Quantification of selected aroma-active compounds in strawberries by headspace solid-phase microextraction gas chromatography and correlation with sensory descriptive analysis. *J. Food Sci.* **2007**, *72*, 487–496. [CrossRef] [PubMed]

foods

MDPI

Article

Development of Head Space Sorptive Extraction Method for the Determination of Volatile Compounds in Beer and Comparison with Stir Bar Sorptive Extraction

José E. Ruvalcaba [1], Enrique Durán-Guerrero [2,*], Carmelo G. Barroso [2] and Remedios Castro [2]

[1] Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, Av. Dr. Manuel Nava #6, Zona Universitaria, 78210 San Luis Potosí, Mexico; joseleazar77@gmail.com

[2] Analytical Chemistry Department, Faculty of Sciences-IVAGRO, University of Cadiz, Agrifood Campus of International Excellence (ceiA3), Polígono Río San Pedro, s/n, Puerto Real, 11510 Cádiz, Spain; carmelo.garcia@uca.es (C.G.B.); remedios.castro@uca.es (R.C.)

* Correspondence: enrique.duranguerrero@uca.es; Tel.: +34-956-016-456

Received: 31 January 2020; Accepted: 24 February 2020; Published: 27 February 2020

Abstract: A headspace sorptive extraction method coupled with gas chromatography–mass spectrometry (HSSE–GC–MS) was developed for the determination of 37 volatile compounds in beer. After optimization of the extraction conditions, the best conditions for the analysis were stirring at 1000 rpm for 180 min, using an 8-mL sample with 25% NaCl. The analytical method provided excellent linearity values (R^2 > 0.99) for the calibration of all the compounds studied, with the detection and quantification limits obtained being low enough for the determination of the compounds in the beers studied. When studying the repeatability of the method, it proved to be quite accurate, since RSD% values lower than 20% were obtained for all the compounds. On the other hand, the recovery study was successfully concluded, resulting in acceptable values for most of the compounds (80–120%). The optimised method was successfully applied to real beer samples of different types (ale, lager, stout and wheat). Finally, an analytical comparison of the optimised HSSE method, with a previously developed and validated stir bar sorptive extraction (SBSE) method was performed, obtaining similar concentration values by both methods for most compounds.

Keywords: headspace sorptive extraction; beer; volatile compounds; stir bar sorptive extraction

1. Introduction

Beer is one of the oldest and most widely consumed alcoholic beverages in the world, and the third most popular drink after water and tea [1]. Beer is usually made from malted barley, but other grains such as wheat, corn, or rice can also be used. During the brewing process, the fermentation of sugars from starch produces ethanol and carbon dioxide [2]. Most modern beers are brewed in the presence of hops, which add bitterness and aroma to the finished beer, as well as acting as a natural preservative and stabilising agent. Other possible flavourings that can be used in addition to or instead of hops are gruit (herbal mixture), herbs, or fruits.

The final aroma and taste of a particular beer is the result of hundreds of aromatically active compounds, which are produced during the course of the brewing process. However, the vast majority of the compounds are produced during the fermentation phase and are mainly metabolic intermediates or yeast by-products. Higher alcohols, esters and vicinal diketones, which determine the final quality of each beer, are some of the key compounds produced by the yeast [3]. While higher alcohols and esters are positive compounds that produce a pleasant aroma, vicinal diketones are often considered

off-flavours. In addition, yeast metabolism generates other types of compounds, such as organic acids, sulphur compounds and aldehydes [3].

The aroma of the different beers is one of the most important aspects related to the final quality of the product. In addition, it is important to keep the off-flavours within certain limits in order to achieve a pleasant final aroma and thus obtain good consumer acceptance. It is, therefore, important to develop analytical methodologies that are sensitive, accurate, rapid and uncomplicated, and that are also capable of quantifying the volatile compounds responsible for each beer specific aroma. In the past decades, the development of automated and miniaturised sample preparation methods, which reduce or eliminate solvent consumption, has been a dominant trend in analytical chemistry.

Stir bar sorptive extraction (SBSE) is a sample preparation technique developed by Baltussen et al. [4] and is a solvent-less enrichment technique by means of sorptive extraction. In 2000, one year after SBSE was developed, sorptive extraction was applied to headspace by Tienpont et al. [5] and Bicchi et al. [6] under the name of headspace sorptive extraction (HSSE). Both techniques are based on the sorption of the analytes into a thick layer of polydimethylsiloxane (PDMS) that covers a magnetic stirring bar with a glass cover. The analytes are extracted either by introducing the PDMS stirring bar directly into the liquid sample (SBSE) or by placing it in the headspace of the sample for a period of time (HSSE). The efficiency of the extraction process depends on the polarity of the analytes and is more efficient with compounds of medium–low polarity because of PDMS's low polar character. However, due to the large amount of PDMS used for the stirring bars, both techniques present much higher recoveries than other similar ones (such as solid-phase micro extraction, SPME) for compounds with a low polarity [7].

In recent years, different applications of SBSE coupled with GC–MS for the aroma characterization of beers have appeared in the literature [8–13], but nevertheless, HSSE has been significantly less employed for this purpose. However, this latter technique has been successfully used for the determination of off-flavours in aged beers [14] and for the study of volatile compounds derived from hops, with similar results to those obtained when using SBSE [15]. Although immersion techniques are generally more sensitive, headspace extraction has the advantage that it reduces the risk of contamination and increases the lifetime of the stirring bar as well as being more representative of the aroma perceived by consumers [16].

The objective of this research is to develop a new solventless methodology for the analysis of volatile compounds in beers, employing HSSE. This is the first time that the extraction conditions for HSSE are optimised for the analysis of an extensive number of volatile compounds responsible for the aroma of beer and belonging to different chemical families. The optimised method was analytically validated and successfully applied to different types of beer (ale, lager, stout and wheat). A comparison with the SBSE method previously developed by this research group [13] has also been conducted, and it has been demonstrated that the results obtained by the two analysis techniques are generally comparable. So, with this new HSSE methodology, it is possible to characterise the aromatic profile of beers in a reliable way, employing a minimal amount of sample and decreasing the degradation of stirring bars.

2. Materials and Methods

2.1. Chemical and Reagents

A total of 37 volatile compounds from different chemical families were studied and are presented in Table 1 along with their retention times, chemical family and quantifying ions.

4-Methyl-2-pentanol (retention time: 21.9 min) and 2-octanol (retention time: 30.7 min) were used as internal standards and each volatile compound studied was referred to as one of the two standards (Table 1).

All the standards used in the study presented purity levels above 99% and were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Volatile compounds studied, retention times, internal standard employed, chemical family and MS fragment employed for the quantification.

Volatile Compound	Retention Time (min)	Internal Standard *	Chemical Family	SIM
isobutyl acetate	15.16	A	branched alkyl ester	43
ethyl butyrate	16.33	A	ethyl ester (linear chain)	43
ethyl isovalerate	17.70	A	branched alkyl ester	88
hexanal	18.20	A	aldehyde (linear chain)	44
ethyl pentanoate	20.11	B	ethyl ester (linear chain)	88
isopentyl acetate	20.11	B	branched alkyl ester	43
3-methyl-1-butanol	23.38	A	alcohol (branched chain)	55
ethyl hexanoate	24.35	B	ethyl ester (linear chain)	88
hexyl acetate	25.65	A	linear alkyl acetate ester	43
octanal	26.89	A	aldehyde (linear chain)	41
6-methyl-5-hepten-2-one	27.85	A	ketone (linear)	43
ethyl heptanoate	27.89	B	ethyl ester (linear chain)	88
1-hexanol	28.25	A	alcohol (linear chain)	56
heptyl acetate	29.28	B	linear alkyl acetate ester	43
nonanal	30.07	B	aldehyde (linear chain)	57
ethyl octanoate	31.56	A	ethyl ester (linear chain)	88
heptanol	31.94	A	alcohol (linear chain)	70
isopentyl hexanoate	32.96	A	branched alkyl ester	70
octyl acetate	32.99	B	linear alkyl acetate ester	43
benzaldehyde	34.49	A	aldehyde (aromatic)	77
linalool	35.22	B	alcohol (alkene chain)	41
isobutyric acid	35.69	B	carboxylic acid (branched chain)	41
octanol	35.69	B	alcohol (linear chain)	41
2,3-dihydrobenzofurane	37.42	A	cyclic ether	91
ethyl decanoate	39.02	B	ethyl ester (linear chain)	88
benzenoic acid ethyl ester	39.83	A	aromatic alkyl ester	105
1-decanol	43.16	B	alcohol (linear chain)	41
phenylethyl acetate	45.14	B	aromatic alkyl ester	104
β-damascenone	45.97	A	ketone (cyclic)	69
guaiacol	46.24	B	cyclic ether	81
ethyl dodecanoate	46.35	A	ethyl ester (linear chain)	88
benzopropanoic acid ethyl ester	47.46	B	aromatic alkyl ester	104
hexanoic acid 2-phenylethyl ester	47.46	B	aromatic alkyl ester	104
isobutyric acid phenethyl ester	48.21	A	aromatic alkyl ester	104
nerolidol	52.94	B	alcohol (alkene chain)	41
octanoic acid	52.94	A	carboxylic acid (linear chain)	60
β-phenylethyl-2-methylbutyrate	56.78	A	aromatic alkyl ester	104

* A: 4-methyl-2-pentanol; B: 2-octanol. SIM: selected ion monitoring.

2.2. Stardards Preparation

Standard solutions of all the studied volatile compounds were prepared at a concentration of about 100 mg/L in a synthetic beer matrix (5% ethanol–water mixture). For calibration purposes, six concentration levels were prepared using the standard solutions in duplicate, in the range of 0.04 to 2000 μg/L, which allowed to determine the volatile compounds found in the real samples. For the recovery study, increasing concentrations in duplicate (20, 40, 100 and 200 μg/L) of the standards were added to a lager beer sample.

The internal standard solutions were prepared in synthetic beer matrices at a concentration of 2300 mg/L in the case of 4-methyl-2-pentanol and 104 mg/L in the case of 2-octanol. All the solutions were stored at 4 °C until their use.

2.3. Beer Samples

For the experimental design, the repeatability study and the recovery study, lager beer was used. Then, ten beers of different styles (4 lagers, 2 ales, 2 stouts and 2 wheats) were analysed according to the optimised HSSE method. All the beers used in the study were purchased from local markets and kept refrigerated at 4 °C until analysis. All the analyses were carried out in duplicate.

2.4. Head Space Sorptive Extraction

Once the method had been optimised, 8 mL of beer was placed in a 20-mL vial designed for headspace analysis, supplied by Gerstel (Mülheim an der Ruhr, Germany). Then, 25% salt (*w/v*), a small stirring bar and 16 µL of each internal standard were added. The extraction bar, commercially known as Twister® (Gerstel), was placed in an adapted holder with a small opening in its bottom to keep it in the upper part of the vial. Then the vial was sealed by encapsulating it with an aluminium cap and a PTFE/silicone septum. The twisters used were made of 10 mm long and 0.5 mm thick PDMS. The vial was placed on an agitator plate at 1000 rpm for 180 min. When the extraction process was completed, the vial was opened and the twister was washed with distilled water for 20 seconds and then dried using paper and placed on a glass liner for its subsequent chromatographic analysis. No conditioning or cleaning of the twisters were performed after each analysis.

2.5. Instrumentation

The sampling system consisted of a thermal desorption unit (TDS-2) equipped with multipurpose sampler (MPS) and a programmed temperature vaporization (PTV) cooled injector system (CIS-4) by Gerstel. The thermal desorption unit was operated in splitless mode. The desorption temperature was set up to climb from 40 to 300 °C at 60 °C/min and 10 minutes holding time, with a helium flow of 75 mL/min. The desorbed analytes were then cryofocused in the CIS using liquid nitrogen at −140 °C. The CIS was set up to climb from −140 to 300 °C at a 10 °C/s rate before GC–MS analysis.

For the GC–MS analysis of the samples, a 7890 gas chromatography system coupled with a 5975C inert mass spectrometry detector (Agilent Technologies, Palo Alto, CA, USA) was employed. The capillary column was a DB-Wax (J&W Scientific, Folsom, CA, USA), 60 m × 250 µm × 0.25 µm. Helium as a carrier gas was maintained at 1 mL/min flow. The GC oven was started at 35 °C, held at that temperature for 10 min and then ramped up to 100 °C at a 5 °C/min rate, then the temperature was increased to 210 °C at a 3 °C/min rate and finally held at that temperature for 40 minutes. The compounds were identified by comparing the mass spectra obtained with those in Wiley 7N (Wiley Registry of Mass Spectral Data, 7th Edition, 2000, John Wiley & Sons, NJ, Hoboken, USA) and by comparing their retention times and mass spectra with commercial standards.

2.6. Comparative Study against Stir Bar Sorptive Extraction

For the comparative study against SBSE, four additional beers (one from each type) were analysed using HSSE and SBSE in duplicate. The testing conditions for SBSE were those developed in an earlier study by our research team [13]. A comparison was made between the concentration values obtained by HSSE and SBSE of all the compounds studied.

2.7. Statistic Tools

An experimental design was conducted to optimise the extraction conditions. The total chromatographic area obtained and the number of peaks were considered as the experimental response. A full factorial design 3^2 was used to determine which factors had a significant effect on the response. The statistics program used to carry out this study was Statgraphic Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA).

The concentration data of the volatile compounds studied were subjected to analysis of variance (ANOVA) and a subsequent post-hoc analysis of means comparison (Tukey's test) at a 5% significance level. The statistic application StatSoft GmbH, Hamburg, Germany was used to perform this statistical study.

3. Results and Discussion

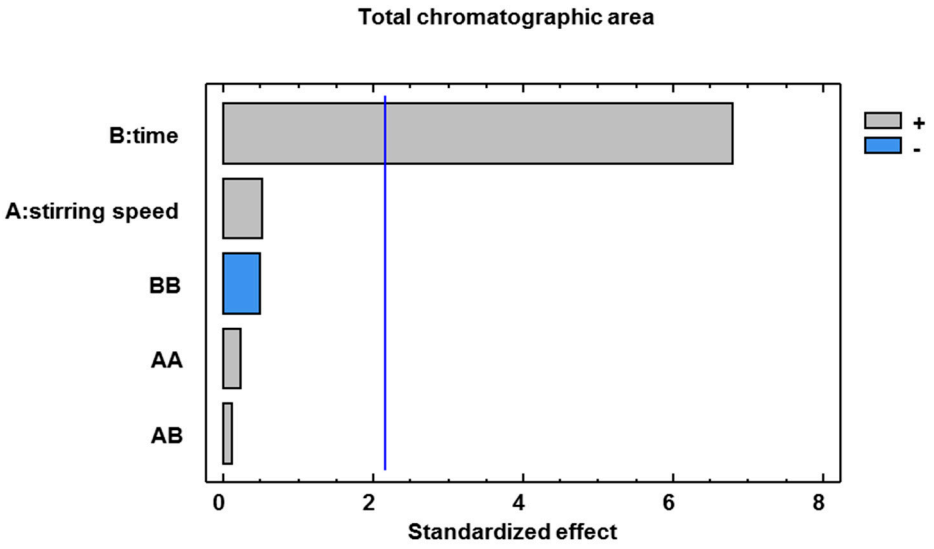
3.1. Optimisation of the Extraction Conditions

Potential factors that may affect HSSE are sample amount, extraction time, agitation speed, salt addition, among others [16]. Although the extraction temperature is a parameter that may affect the process, it was not taken into account in this study. Other authors have indicated that, although an increase in temperature shortens the time required to reach equilibrium, it also increases the solubility of the analytes in water, which means that the amount extracted by the stirring bar may decrease [17]. As our study seeks to obtain maximum sensitivity rather than processing speed, the analyses were performed at room temperature.

Even though the sample volume normally has a positive effect on the extraction of volatile compounds in food samples by PDMS, i.e., the larger the sample volume, the more efficient the extraction [18–21], in the specific case of HSSE, other researchers have concluded that sample volume is not significant with respect to extraction [16,22]. In our case, the maximum volume allowed by the experimental device was taken, namely, 8 mL. In any case, the sample volume used for this technique is considerably smaller than that used for SBSE, where the optimised volume was 50 mL [13].

Adding salt has been proven to be an important variable to improve the extraction of volatile compounds from beer by SBSE [13] and from other water matrices such as vinegar [18] or orange juice [23]. The addition of salt to the medium changes the ionic strength of the medium, which in turn affects the relative polarity of the compounds and thus the extraction of the compounds by the (low-polar) PDMS polymer. Consequently, a value of 25% was set for our study. Other authors employed similar values of salt for the extraction of volatile compounds in beers by SPME [24].

For the study of both variables "extraction time" and "stirring speed" a factorial 3^2 design was carried out. Ten experiments were performed (in duplicate) by modifying their extraction time (30–180 min) and stirring speed (500–2000 rpm) in order to observe their influence on the experimental responses "total chromatographic area" and "number of chromatographic peaks". Both experimental responses are related to the total amount of volatile compounds extracted. The data were evaluated by ANOVA at a 5% significance level. The main effects and interactions observed can be seen in the Pareto charts in Figure 1.



(A)

Figure 1. *Cont.*

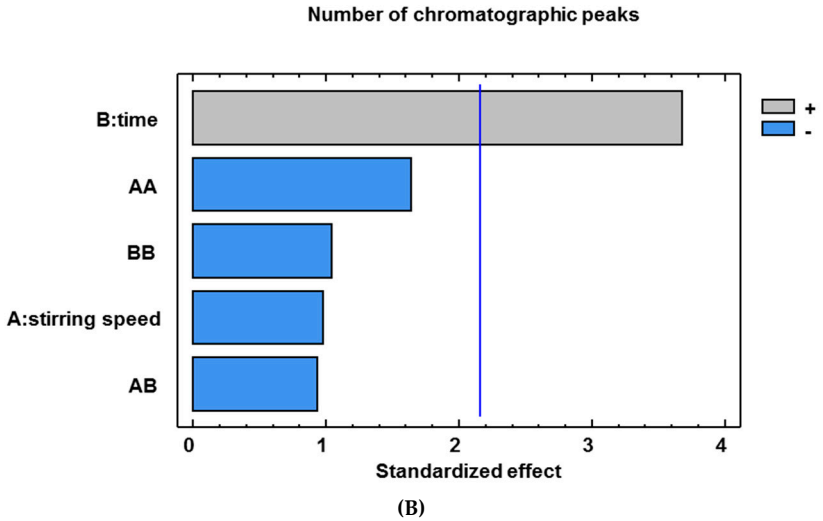


Figure 1. Pareto charts for total chromatographic area (A) and number of chromatographic peaks (B) as experimental responses.

Regarding both the number of chromatographic peaks as well as the total area, it can be observed that extraction time is the only factor with a significant positive effect ($p < 0.05$; Figure 1). Therefore, it can be deduced from this fact that longer extractions result in the extraction of a greater number of compounds and a greater amount of the same. The optimum value proposed by our design was over 200 minutes, but for operational reasons, it was set at 180 minutes.

Stirring speed did not reveal any significant influence and was therefore set at the intermediate value of 1000 rpm.

3.2. Analytical Validation of the Optimised Method

Thirty-seven volatile compounds were studied for calibration, employing the optimal conditions that had been obtained after the optimisation process. Table 2 shows the outcome of the calibration for the compounds studied. As can be seen, virtually all the determination coefficients obtained (R^2) were higher than 0.99.

The limits of detection (LOD) and the limits of quantification (LOQ) were calculated based on the calibration curves according to the following formulae:

$LOD = 3SI/b$

$LOQ = 10SI/b$

SI = standard deviation of the intercept of the regression line

b = slope from the regression line

Table 2. Analytical features of the headspace sorptive extraction (HSSE) method for the studied compounds.

Volatile Compound	Slope	Intercept	R^2	LOD (µg/L)	LOQ (µg/L)	Recovery (%)	Inter-tw Precision * (%)	Inter-Day Precision ** (%)
isobutyl acetate	0.0017	0.0728	0.9998	25.48	84.95	96.22	15.53	9.12
ethyl butyrate	0.0006	0.0150	0.9980	2.77	9.26	110.44	13.70	8.19
ethyl isovalerate	0.0010	0.0015	0.9999	0.30	1.02	101.89	4.65	7.45
hexanal	0.00008	0.0066	0.9902	12.76	42.54	100.39	10.52	19.43
ethyl pentanoate	0.0030	0.0077	0.9998	0.85	2.84	104.02	4.86	13.81
isopentyl acetate	0.0096	0.3258	0.9970	19.63	65.45	-	9.98	5.91
3-methyl-1-butanol	0.00003	0.0053	0.9961	68.16	227.20	-	16.47	9.51
ethyl hexanoate	0.0065	0.2460	0.9979	10.80	36.01	103.18	9.85	5.32
hexyl acetate	0.0071	−0.0148	0.9990	0.96	3.21	105.25	5.71	6.03
octanal	0.0016	−0.0005	0.9991	1.38	4.60	103.00	17.35	17.54
6-methyl-5-hepten-2-one	0.0014	0.0047	0.9993	1.85	6.16	132.87	12.66	8.11
ethyl heptanoate	0.0115	−0.0018	0.9980	1.10	3.66	94.84	9.61	2.67
1-hexanol	0.0001	0.0010	0.9997	0.93	3.12	90.88	13.05	11.30
heptyl acetate	0.0231	0.0055	0.9994	0.25	0.85	110.07	6.14	17.38
nonanal	0.0038	0.0438	0.99905	7.68	25.62	113.91	4.73	16.39
ethyl octanoate	0.0052	0.0222	0.9990	4.18	13.94	86.06	12.30	5.67
heptanol	0.0002	0.0016	0.9978	10.80	36.01	81.26	-	-
isopentyl hexanoate	0.0050	0.0002	0.9992	0.01	0.05	93.60	12.73	12.24
octyl acetate	0.0286	−0.1174	0.9989	0.95	3.16	88.38	14.42	10.14
benzaldehyde	0.0001	0.0050	0.9962	0.35	1.16	104.75	6.78	3.16
linalool	0.0021	0.0220	0.9990	2.64	8.80	77.16	11.73	15.30
isobutyric acid	0.0015	0.0157	0.9997	1.59	5.30	87.10	13.78	4.33
octanol	0.0015	0.0116	0.9996	0.72	2.41	86.57	6.43	16.03
2,3-dihydrobenzofurane	0.0013	−0.0031	0.9708	0.16	0.54	90.13	-	-
ethyl decanoate	0.0060	0.0283	0.9994	0.31	1.04	98.03	8.99	9.69
benzenoic acid ethyl ester	0.0026	−0.0137	0.9989	0.24	0.82	81.76	11.98	10.26
1-decanol	0.0019	0.0283	0.9994	6.42	21.41	<60	7.21	10.65
phenylethyl acetate	0.0020	0.1798	0.9914	5.95	19.85	111.73	9.30	6.23
β-damascenone	0.0020	−0.0091	0.9982	1.09	3.63	77.96	11.52	7.64
guaiacol	0.00004	0.0029	0.9907	1.69	5.65	-	-	-
ethyl dodecanoate	0.0003	0.0018	0.9987	0.62	2.07	82.29	17.59	19.34
benzopropanoic acid ethyl ester	0.0016	0.0030	0.9997	1.23	4.11	96.74	16.90	18.59
hexanoic acid 2-phenylethyl ester	0.0018	0.0001	0.9995	0.78	2.60	88.61	-	-
isobutyric acid phenethyl ester	0.0007	−0.0043	0.9953	2.56	8.53	<60	14.38	10.18
nerolidol	0.0003	0.0214	0.9938	15.31	51.03	96.28	14.49	17.07
octanoic acid	0.0003	0.0134	0.9997	11.44	38.14	130.67	13.26	15.33
β-phenylethyl-2-methylbutyrate	0.0009	0.00003	0.9993	0.36	1.21	<60	11.78	17.15

* Coefficient of variation (%) calculated for 5 replicates using 5 different twisters the same day; ** coefficient of variation (%) calculated for 5 replicates in 5 different days using the same twister; LOD: limit of detection; LOQ: limit of quantification.

Most of the compounds studied presented acceptable LOD and LOQ values and were low enough to be quantified in beer samples. However, some of the results obtained in this case were slightly higher than those obtained using the SBSE method [13]. In addition, a total of 52 compounds had been studied using this method, compared to the 37 compounds studied in this particular case. This result should be considered logical since, when a headspace method is used, sensitivity decreases compared to that of submerged extractions, which means that the number of compounds detected in the real samples may be lower. However, the results obtained in headspace are typically more representative of what the consumer perceives by smelling the beer. In addition, HSSE is usually more sensitive than other headspace methodologies such as HS-SPME because of the higher amount of polymer available, but extractions are usually longer. Other authors quantified 19 volatile compounds in beers employing HS-SPME, with 30 minutes of extraction [25]. Giannetti et al. [26] performed SPME extractions of 10 minutes, but no quantification was carried out.

The accuracy of our method was evaluated through inter-twister precision and inter-day precision. Inter-twister precision was evaluated by extracting five replicates from a single lager sample on the same day, using different twisters. The repeatability of the method on different days (inter-day precision) was evaluated by extracting five beer samples on five consecutive days, using the same twister for the extractions. The coefficients of variation of the detected compounds were calculated (Table 2). The results obtained were lower than 20%, which are generally accepted values for this type of technique and thus corroborate the high precision level of the methodology employed. Similar values were obtained for the determination of volatile compounds in beers when SBSE was the technique used [13]. Other methodologies such as SPME also provide similar values of precision [27].

In addition, the recovery values of the compounds studied in a larger sample were calculated after different concentration values were added (Table 2). Of the 37 compounds studied, only seven were outside the generally accepted values, with recovery values outside the range 80–120%, even though some of them presented values close to this range (linalool: 77.16%; β-damascenone: 77.96%; octanoic acid: 130.67%). On the other hand, the recovery values for three other compounds could not be calculated (Table 2) since they did not show a linear correlation between the added concentrations and their experimental responses. Other authors [24] obtained better values of recovery for these compounds employing SPME, but with polyacrylate (PA) as extracting polymer and they found better responses when they compared PA to PDMS fibres. The almost single current use of PDMS for HSSE could be a limitation due to the low polarity of this polymer.

Finally, in order to demonstrate the applicability of the optimised method, different real samples of different types of beer (4 lager, 2 ale, 2 stout and 2 wheat) were analysed in duplicate. The results obtained are shown in Table 3. This table also presents the significant differences between the compounds' concentrations following the application of the Tukey test ($\alpha = 0.05$). As can be seen, the compound with the highest concentration in all the types of beer was 3-methyl-1-butanol, followed by ethyl octanoate, ethyl hexanoate, isopentyl acetate, nerolidol, phenylethyl acetate and octanoic acid. Most of these compounds have demonstrated to be odour-active compounds for the aroma of beers [28]. Similar results had been found in previous studies where different extraction techniques had been used [8,13,25,27,29]. However, other compounds such as benzaldehyde, guaiacol, linalool, hexanoic acid 2-phenylethyl ester or heptanol were only identified in some of the beer types. Also, volatile compounds such as hexanal or nonanal, normally considered as off-flavours [14], were not detected in any of the samples studied. Other authors [14] also found no detectable concentrations of hexanal in the samples of beers that had been treated with light and heat. As can be seen, our method has proven to be suitable for the analysis of volatile compounds in beers.

Table 3. Concentrations (μg/L) determined of volatile compounds in beers by HSSE–GC–MS.

Beer Sample	Lager (*N* = 8)		Wheat (*N* = 4)		Stout (*N* = 4)		Ale (*N* = 4)	
Volatile Compound	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
isobutyl acetate	<LOQ a	-	128.05 b	67.07	<LOQ a	-	<LOQ a	-
ethyl butyrate	107.85 a	25.26	88.81 a	8.32	103.94 a	29.18	145.21 a	44.62
ethyl isovalerate	4.08 a	4.59	<LOQ	-	4.01 a	2.46	2.04 a	0.96
hexanal	ND	-	ND	-	ND	-	ND	-
ethyl pentanoate	13.24 a	7.66	40.78 b	18.46	13.74 a	1.99	6.33 a	4.96
isopentyl acetate*	2.61 b	0.45	4.31 c	0.87	0.75 a	0.26	1.47 a	0.78
3-methyl-1-butanol*	51.32 a	12.37	39.82 a	17.15	38.94 a	8.18	64.89 a	24.07
ethyl hexanoate	307.14 a	259.04	64.90 a	8.01	284.65 a	247.13	361.33 a	97.23
hexyl acetate	7.33 b	3.07	6.86 ab	1.46	3.43 a	0.75	3.74 ab	0.87
octanal	5.54 a	3.02	<LOQ	-	8.39 a	6.14	5.02 a	4.07
6-methyl-5-hepten-2-one	6.36 a	5.12	<LOQ	-	26.22 a	27.64	12.72 a	12.32
ethyl heptanoate	<LOQ	-	<LOQ	-	12.25 a	12.74	6.02 a	4.80
1-hexanol	11.26 a	2.97	12.45 a	9.50	56.79 a	57.27	33.79 a	19.39
heptyl acetate	<LOQ	-	1.13 a	0.06	3.41 b	0.23	2.13 ab	1.79
nonanal	ND	-	ND	-	ND	-	ND	-
ethyl octanoate	139.08 a	52.82	103.08 a	20.90	529.03 a	514.51	762.58 a	655.03
heptanol*	ND	-	ND	-	1.25 a	1.09	4.12 b	0.71
isopentyl hexanoate	0.35 a	0.21	0.53 a	0.12	0.49 a	0.65	1.35 b	0.26
octyl acetate	5.49 b	0.62	5.25 b	0.21	4.16 a	0.04	4.37 a	0.21
benzaldehyde	7.87 a	3.12	ND	-	ND	-	5.80 a	2.32
linalool	<LOQ	-	ND	-	100.13 a	3.45	106.42 a	26.66
isobutyric acid	6.05 a	3.28	5.66 a	0.11	<LOQ	-	17.81 b	3.00
octanol	5.47 a	2.55	8.64 a	0.27	6.76 a	0.41	15.83 b	8.10
2,3-dihydrobenzofurane	3.48 a	1.66	3.23 a	0.98	3.01 a	0.62	ND	-
ethyl decanoate	50.07 a	47.31	57.11 ab	5.72	66.44 ab	44.73	154.61 b	87.21
benzenoic acid ethyl ester	5.87 a	0.49	5.95 a	0.25	6.43 ab	0.53	7.17 b	0.46
1-decanol	47.28 a	23.98	<LOQ	-	64.99 a	46.59	<LOQ	-
phenylethyl acetate	360.93 b	98.68	378.90 b	26.03	31.31 a	20.49	141.57 a	76.76
β-damascenone	4.63 a	0.10	4.84 a	0.12	7.37 b	0.11	6.99 b	1.91
guaiacol	22.92 a	22.80	ND	-	69.46 b	5.14	ND	-
ethyl dodecanoate	17.38 a	19.98	2.36 a	0.51	19.13 a	11.48	11.99 a	11.93
benzopropanoic acid ethyl ester	8.51 a	3.12	4.30 a	2.40	5.77 a	3.39	15.17 b	4.94
hexanoic acid 2-phenylethyl ester	<LOQ	-	ND	-	4.87 a	5.03	3.63 a	2.13
isobutyric acid phenethyl ester	12.20 ab	2.73	9.09 a	0.79	10.26 a	2.47	15.58 b	3.57
nerolidol	380.29 ab	179.77	172.73 a	54.20	192.11 a	119.94	621.57 b	174.28
octanoic acid	199.88 ab	118.62	88.90 a	10.53	95.84 a	68.18	288.87 b	69.42
β-phenylethyl-2-methylbutyrate	3.83 a	4.76	2.69 a	1.20	<LOQ	-	<LOQ	-

* mg/L; SD: standard deviation; ND: not detected; <LOQ: below limit of quantitation; For each compound, different letters indicate significant differences according to Tukey's test (α = 0.05).

3.3. Comparative Study against SBSE

To thoroughly test whether the optimised HSSE methodology was providing similar results to the SBSE methodology, a duplicate analysis of four beers (one of each type studied) was carried out using both methodologies. The concentrations obtained by both methodologies were plotted against each other and a linear regression was performed so as to obtain a line equation ([SBSE] = a [HSSE] + b) for each of the compounds studied. The slope of the calculated lines indicates the level of similarity of the data obtained, where a slope value equal to 1 corresponds to a perfect similarity between both methodologies. The data obtained from the linear regression are shown in Table 4. In some cases, the analysis was not feasible since some compounds were not detected by both techniques when applied to the same beer samples. It can be seen that most of the compounds that were determined presented similar concentration values by both HSSE and SBSE. Therefore, it has been demonstrated that the HSSE methodology developed in this study provides similar results to those obtained by the SBSE methodology previously developed by this research group [13].

Table 4. Parameters of the linear regression obtained by representing concentration values determined with HSSE against concentration values determined with SBSE: [SBSE] = slope [HSSE] + intercept.

Volatile Compound	Slope	Intercept	R^2
isobutyl acetate	0.9069	6.2751	0.9989
ethyl butyrate	1.2508	−18.6980	0.9473
ethyl isovalerate	1.0774	-0.1628	0.9911
hexanal	-	-	-
ethyl pentanoate	1.1131	−0.8687	0.9964
isopentyl acetate	1.0094	−37.4939	0.9998
3-methyl-1-butanol	0.9250	7651.0458	0.8338
ethyl hexanoate	0.9942	0.3938	0.9954
hexyl acetate	1.9794	11.1685	0.9593
octanal	0.9057	0.7722	0.7429
6-methyl-5-hepten-2-one	1.0106	0.0371	0.9999
ethyl heptanoate	0.9804	0.0140	0.9994
1-hexanol	1.0336	−0.3775	0.9795
heptyl acetate	0.9967	0.0030	0.9999
nonanal	-	-	-
ethyl octanoate	0.9909	−0.5271	0.9864
heptanol	-	-	-
isopentyl hexanoate	1.6954	21.1004	0.2844
octyl acetate	0.9503	0.1023	0.9985
benzaldehyde	0.8895	0.6306	0.9999
linalool	1.1181	1.1259	0.9746
isobutyric acid	-	-	-
octanol	0.9963	0.4574	0.9221
2,3-dihydrobenzofurane	-	-	-
ethyl decanoate	1.1743	−8.3197	0.9636
benzenoic acid ethyl ester	1.0740	−1.6923	0.5956
1-decanol	0.9603	0.7992	0.9963
phenylethyl acetate	1.0524	39.9797	0.8937
β-damascenone	-	-	-
guaiacol	1.0057	0.3731	0.9999
ethyl dodecanoate	1.0780	−1.1354	0.9565
benzopropanoic acid ethyl ester	0.9646	0.0722	0.9950
hexanoic acid 2-phenylethyl ester	0.9977	0.0395	0.9976
isobutyric acid phenethyl ester	0.1649	−0.2036	0.5740
nerolidol	1.0683	175.1769	0.6893
octanoic acid	-	-	-
β-phenylethyl-2-methylbutyrate	1.0219	−0.1420	0.9898

4. Conclusions

It is clear that HSSE is an appropriate method to determine the different types of volatile compounds in beers. The results that have been obtained are similar to those achieved by other more widely accepted techniques, such as SBSE. However, HSSE presents some advantages, such as a significantly lower degradation of the stirring bars when compared to the submersion method used in SBSE. Furthermore, the volume of the samples is considerably reduced and also the aroma detected by HSSE is considered as more representative of what consumers perceive when they smell a particular beer. Even though it is true that sensitivity levels are slightly lower, the detection and quantification limits that have been obtained allow us to determine a significant number of the volatile compounds that are present in beer samples from different types.

Author Contributions: Conceptualization, R.C.; methodology, E.D.-G.; software, E.D.-G. and R.C.; validation, E.D.-G. and R.C.; formal analysis, J.E.R.; investigation, J.E.R.; resources, C.G.B.; data curation, J.E.R.; writing—original draft preparation, E.D.-G.; writing—review and editing, E.D.-G. and R.C.; visualization, R.C.; supervision, E.D.-G.; project administration, C.G.B.; funding acquisition, C.G.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Nelson, M. *The Barbarian's Beverage: A History of Beer in Ancient Europe*; Taylor and Francis Inc.: Abingdon, UK, 2004.
2. Barth, R. *The Chemistry of Beer: The Science in the Suds*; Wiley: Hoboken, NJ, USA, 2013.
3. Pires, E.J.; Teixeira, J.A.; Brányik, T.; Vicente, A.A. Yeast: The soul of beer's aroma—A review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1937–1949. [CrossRef] [PubMed]
4. Baltussen, E.; Sandra, P.; David, F.; Cramers, C. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: Theory and principles. *J. Microcolumn Sep.* **1999**, *11*, 737–747. [CrossRef]
5. Tienpont, B.; David, F.; Bicchi, C.; Sandra, P. High capacity headspace sorptive extraction. *J. Microcolumn Sep.* **2000**, *12*, 577–584. [CrossRef]
6. Bicchi, C.; Cordero, C.; Iori, C.; Rubiolo, P.; Sandra, P. Headspace Sorptive Extraction (HSSE) in the headspace analysis of aromatic and medicinal plants. *HRC J. High Resolut. Chromatogr.* **2000**, *23*, 539–546. [CrossRef]
7. David, F.; Sandra, P. Stir bar sorptive extraction for trace analysis. *J. Chromatogr. A* **2007**, *1152*, 54–69. [CrossRef]
8. Castro, L.F.; Ross, C.F. Determination of flavour compounds in beer using stir-bar sorptive extraction and solid-phase microextraction. *J. Inst. Brew.* **2015**, *121*, 197–203. [CrossRef]
9. Horák, T.; Čulík, J.; Kellner, V.; Čejka, P.; Hašková, D.; Jurková, M.; Dvořák, J. Determination of selected beer flavours: Comparison of a stir bar sorptive extraction and a steam distillation procedure. *J. Inst. Brew.* **2011**, *117*, 617–621. [CrossRef]
10. Ochiai, N.; Sasamoto, K.; David, F.; Sandra, P. Solvent-assisted stir bar sorptive extraction by using swollen polydimethylsiloxane for enhanced recovery of polar solutes in aqueous samples: Application to aroma compounds in beer and pesticides in wine. *J. Chromatogr. A* **2016**, *1455*, 45–56. [CrossRef]
11. Sharp, D.C.; Qian, Y.; Clawson, J.; Shellhammer, T.H. An exploratory study toward describing hop aroma in beer made with American and European Hop Cultivars. *BrewingScience* **2016**, *69*, 112–122.
12. Stefanuto, P.H.; Perrault, K.A.; Dubois, L.M.; L'Homme, B.; Allen, C.; Loughnane, C.; Ochiai, N.; Focant, J.F. Advanced method optimization for volatile aroma profiling of beer using two-dimensional gas chromatography time-of-flight mass spectrometry. *J. Chromatogr. A* **2017**, *1507*, 45–52. [CrossRef]
13. Ruvalcaba, J.E.; Durán-Guerrero, E.; Barroso, C.G.; Castro, R. Development of a stir bar sorptive extraction method to study different beer styles volatile profiles. *Food Res. Int.* **2019**, *126*, 108680. [CrossRef] [PubMed]
14. Marsili, R.T.; Laskonis, L.C.; Kenaan, C. Evaluation of PDMS-based extraction techniques and GC-TOFMS for the analysis of off-flavor chemicals in beer. *J. Am. Soc. Brew. Chem.* **2007**, *65*, 129–137. [CrossRef]
15. Richter, T.M.; Eyres, G.T.; Silcock, P.; Bremer, P.J. Comparison of four extraction methods for analysis of volatile hop-derived aroma compounds in beer. *J. Sep. Sci.* **2017**, *40*, 4366–4376. [CrossRef] [PubMed]
16. Hevia, K.; Castro, R.; Natera, R.; González-García, J.A.; Barroso, C.G.; Durán-Guerrero, E. Optimization of Head Space Sorptive Extraction to Determine Volatile Compounds from Oak Wood in Fortified Wines. *Chromatographia* **2016**, *79*, 763–771. [CrossRef]
17. Camino-Sánchez, F.J.; Rodríguez-Gómez, R.; Zafra-Gómez, A.; Santos-Fandila, A.; Vílchez, J.L. Stir bar sorptive extraction: Recent applications, limitations and future trends. *Talanta* **2014**, *130*, 388–399. [CrossRef]
18. Guerrero, E.D.; Marín, R.N.; Mejías, R.C.; Barroso, C.G. Optimisation of stir bar sorptive extraction applied to the determination of volatile compounds in vinegars. *J. Chromatogr. A* **2006**, *1104*, 47–53. [CrossRef]
19. Delgado, R.; Durán, E.; Castro, R.; Natera, R.; Barroso, C.G. Development of a stir bar sorptive extraction method coupled to gas chromatography-mass spectrometry for the analysis of volatile compounds in Sherry brandy. *Anal. Chim. Acta* **2010**, *672*, 130–136. [CrossRef]
20. Vasile-Simone, G.; Castro, R.; Natera, R.; Masino, F.; Barroso, C.G.; Durán-Guerrero, E. Application of a stir bar sorptive extraction method for the determination of volatile compounds in different grape varieties. *J. Sci. Food Agric.* **2017**, *97*, 939–948. [CrossRef]

21. Maruti, A.; Durán-Guerrero, E.; Barroso, C.G.; Castro, R. Optimization of a multiple headspace sorptive extraction method coupled to gas chromatography-mass spectrometry for the determination of volatile compounds in macroalgae. *J. Chromatogr. A* **2018**, *1551*, 41–51. [CrossRef]
22. Callejón, R.M.; González, A.G.; Troncoso, A.M.; Morales, M.L. Optimization and validation of headspace sorptive extraction for the analysis of volatile compounds in wine vinegars. *J. Chromatogr. A* **2008**, *1204*, 93–103. [CrossRef]
23. Herrera, C.; Castro, R.; García-Barroso, C.; Durán-Guerrero, E. Development of a stir bar sorptive extraction method for the determination of volatile compounds in orange juices. *J. Sep. Sci.* **2016**, *39*, 3586–3593. [CrossRef] [PubMed]
24. Nešpor, J.; Karabín, M.; Hanko, V.; Dostálek, P. Application of response surface design to optimise the chromatographic analysis of volatile compounds in beer. *J. Inst. Brew.* **2018**, *124*, 244–253. [CrossRef]
25. Nešpor, J.; Andrés-Iglesias, C.; Karabín, M.; Montero, O.; Blanco, C.A.; Dostálek, P. Volatile Compound Profiling in Czech and Spanish Lager Beers in Relation to Used Production Technology. *Food Anal. Methods* **2019**, *12*, 2293–2305. [CrossRef]
26. Giannetti, V.; Boccacci Mariani, M.; Torrelli, P.; Marini, F. Flavour component analysis by HS-SPME/GC–MS and chemometric modeling to characterize Pilsner-style Lager craft beers. *Microchem. J.* **2019**, *149*, 103991. [CrossRef]
27. Ocvirk, M.; Mlinarič, N.K.; Košir, I.J. Comparison of sensory and chemical evaluation of lager beer aroma by gas chromatography and gas chromatography/mass spectrometry. *J. Sci. Food Agric.* **2018**, *98*, 3627–3635. [CrossRef] [PubMed]
28. Kishimoto, T.; Noba, S.; Yako, N.; Kobayashi, M.; Watanabe, T. Simulation of Pilsner-type beer aroma using 76 odor-active compounds. *J. Biosci. Bioeng.* **2018**, *126*, 330–338. [CrossRef]
29. Coelho, E.; Magalhães, J.; Pereira, F.B.; Macieira, F.; Domingues, L.; Oliveira, J.M. Volatile fingerprinting differentiates diverse-aged craft beers. *LWT* **2019**, *108*, 129–136. [CrossRef]

foods

MDPI

Article

Fatty Acid Profiling for the Authentication of Iberian Hams According to the Feeding Regime

Raúl González-Domínguez [1,2,*,†], Ana Sayago [1,2] and Ángeles Fernández-Recamales [1,2,*]

1 Department of Chemistry, Faculty of Experimental Sciences, University of Huelva, 21007 Huelva, Spain; ana.sayago@dqcm.uhu.es
2 International Campus of Excellence CeiA3, University of Huelva, 21007 Huelva, Spain
* Correspondence: raul.gonzalez@dqcm.uhu.es (R.G.-D.); recamale@dqcm.uhu.es (Á.F.-R.); Tel.: +34-959-219-975 (R.G.-D.); +34-959-219-958 (Á.F.-R.)
† Current address: Biomarkers & Nutrimetabolomics Laboratory, Department of Nutrition, Food Sciences and Gastronomy, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain.

Received: 19 December 2019; Accepted: 1 February 2020; Published: 3 February 2020

Abstract: The quality and sensory characteristics of Iberian ham are closely related to the pig feeding regime. These are mainly due to the inclusion or not of acorns into the diet, which significantly increases the content of monounsaturated fatty acids in this food product. In this work, the fatty acid profile from subcutaneous fat samples was evaluated and modeled with various chemometric approaches as a potential tool for authentication of Iberian ham from three categories according to the rearing system: "Jamón de Bellota", "Jamón de Cebo de Campo", and "Jamón de Cebo". The application of artificial neural networks provided satisfactory classification and prediction rates, with oleic acid being the most important variable driving this differentiation.

Keywords: Iberian ham; authentication; feeding; fatty acids; oleic acid; artificial neural network

1. Introduction

The Mediterranean diet, mainly based on the consumption of vegetables, fresh fruits, cereals, fish and moderate quantities of wine, has been associated with reduced incidence of cerebral and cardiovascular diseases. The main source of fat in the traditional Mediterranean diet is olive oil, characterized by high contents of oleic acid, while the intake of meat should be limited. However, it should be noted that the Iberian pig, an autochthonous breed from southwest Spain, has lower content of saturated fatty acids and higher monounsaturated profile than other meats [1]. In this vein, it has been demonstrated that the moderate consumption of this meat elicits similar effects to those provoked by olive oil on the lipid profile and over cardiovascular risk factors [2,3]. Particularly, dry-cured hams are important sources of proteins, iron, phosphorous, B-vitamins, and other valuable nutrients, which determine the high nutritional value of this food product [4].

Iberian ham has a characteristic taste and flavor, highly appreciated by consumers over the world, which are mainly attributed to volatile compounds produced from lipid oxidation [5–7]. Multiple factors can influence the lipid composition of pig meat, thus significantly affecting the organoleptic quality of derived products, but it has been sufficiently proven that breed and the type of diet received by the pigs at the end of their fattening period before slaughtering are the main players [8,9]. The traditional exploitation system of Iberian pigs is called "Montanera", an extensive feeding regime based on pasture and acorns over approximately the last three months of their lives. Acorns are rich in monounsaturated fatty acids [1], so the quality and market value of ham greatly depend on the inclusion or not of this fruit in the diet during these last three months. Thus, according to the type of feed, the Spanish legislation establishes three main categories for Iberian ham: "Jamón de

Bellota", from Iberian pigs exclusively fed with natural pasture and acorn, "Jamón de Cebo de Campo", receiving mixed feeding with concentrate feeds and complemented with natural pasture and acorn, and "Jamón de Cebo", exclusively fed with concentrate feeds [10].

Considering the great commercial value of Iberian ham and the crucial role of pig feeding on its final quality, numerous efforts have been made to develop sensitive analytical approaches for characterizing the chemical composition of this product. Most of the published literature is based on investigating the lipid profile of Iberian ham, particularly focusing on fatty acids, which have demonstrated a great potential to differentiate Iberian hams according to the feeding regime [11–16], but also by studying other components such as triacylglycerols [13,16] or the entire lipidomic profile [17,18]. In this context, the use of advanced chemometric tools to manage the complex data sets generated by using these analytical approaches is also of utmost importance. Various pattern recognition techniques have been frequently employed in food authenticity research, such as linear discriminant analysis, or partial least squares discriminant analysis [19,20], but the application of novel machine learning tools is gaining importance in recent years, including artificial neural network modeling, random forest, and support vector machines [21].

On this basis, the main aim of the present study was to investigate the fatty acid profile from subcutaneous fat as a potential authentication tool to discriminate Iberian hams according to the pig rearing system. To this end, the performance of various pattern recognition techniques, including linear discriminant analysis (LDA) and artificial neural network (ANN) modeling, was compared with the aim of obtaining suitable models for classification purposes. This work thus demonstrates that fatty acids can be potential descriptors for Iberian ham authentication and fraud detection, which is of great importance in the food industry due to the high commercial value of this product.

2. Materials and Methods

2.1. Iberian Ham Samples

Sixty-three Iberian ham samples produced under the Protected Designation of Origin "Jamón de Huelva" were used in this study. Three study groups were considered: "Jamón de Bellota" (N = 25), "Jamón de Cebo de Campo" (N = 23), and "Jamón de Cebo" (N = 15). Samples of subcutaneous fat were collected before the dry-curing process by longitudinal cutting in the tail insertion area from the coxal region. The skin and muscle were removed, and the fat was minced and blended.

2.2. Lipid Extraction and Transesterification

Lipid extraction and transesterification was performed according to the normative UNE-EN ISO 5508:1996. For lipid extraction, 10 g of subcutaneous fat was mixed with 10 mL of diethyl ether and homogenized during 10 min. Then, extracts were filtered through 0.45 μm filters and the solvent was removed by rotatory vacuum evaporation. For transesterification, 20 mg of the resulting solid residue was accurately weighed and dissolved with 4 mL of n-hexane. Afterwards, 200 μL of 2 M methanolic potassium hydroxide was added to the extract, and after mild shaking, the mixture was left to stand for 30 min until total clarification. The upper layer containing fatty acid methyl esters was finally transferred to injection vials.

2.3. Determination of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAMEs) were analyzed by using a Hewlett-Packard 6890 Plus gas chromatograph equipped with split/splitless injector and flame ionization detector (FID). Separation was performed on a DB-23 capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness) coated with polar stationary phase (50% cyanopropyl, 50% methyl polysiloxane) (Agilent Technologies, Santa Clara, CA, USA). The GC conditions were as follows: oven temperature, 190 °C isothermal for 20 min; injector temperature, 250 °C; detector temperature, 275 °C; carrier gas, helium; carrier gas flow rate, 1.5 mL/min flow rate; flow split, 85:1; injection volume, 1.0 μL. Data were collected using the HP

workstation, and fatty acid methyl esters were identified by comparing retention times with those from reference standard mixtures: lauric, myristic, palmitic, palmitoleic, margaric, heptadecenoic, stearic, oleic, linoleic, linolenic, arachidic, and gadoleic acids. The results were expressed as percentage of the total fatty acid methyl ester content determined in this study (i.e., sum of 12 FAMEs). Method repeatability was assessed by analyzing ten times the same extract, while reproducibility was estimated by analyzing ten extracts from the same subcutaneous tissue sample.

2.4. Data Analysis

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were first applied for preliminary exploration of data, and ANOVA was employed to find significant differences among the study groups. Then, linear discriminant analysis (LDA) and artificial neural network (ANN) were performed to build classification models. For supervised multivariate analysis (i.e., LDA and ANN), the dataset was randomly divided into two groups, a training set used to build the model, and a prediction set to test its performance. The percentage of samples taken for the prediction set was about 25%. To validate the recalling rate (effectiveness of classification in the training set) and the prediction ability (effectiveness of classification in the prediction set) of the methods applied, both training and prediction sets were repeated ten times for different constitutions. The average of hits obtained in the recalling and prediction from these ten runs was used as a measure of the classification performance. Regarding ANN modeling, several architectures were built by applying the multi-layer perceptron trained by the error back-propagation algorithm. In all cases, ANNs were two-layer neural networks with log-sigmoidal and bias transfer function, with three output neurons and as many input neurons as variables, and with a priori indeterminate number of hidden neurons. The output from the network was adjusted to a zero/one response. For back-propagation training, initial weights were taken randomly between −0.1 and 0.1, maximal epochs were 1000, and learning rate and momentum were fixed to 0.2 and 0.5, respectively, and were kept constant during training. The root-mean-squared (RMS) errors were computed to assess the effectiveness of the training. All statistical analyses were conducted using Statistica 8.0 software (StatSoft, Tulsa, OK, USA).

3. Results and Discussion

3.1. Fatty Acid Profiling of Iberian Hams

Following extraction and transesterification, the total fatty acid content from Iberian ham subcutaneous fat samples was profiled by using gas chromatography coupled to flame ionization detector (GC-FID). This method enabled the fast determination of 12 fatty acids with suitable analytical performance, as shown in Table 1. Repeatability and reproducibility, expressed as percentage of relative standard deviation (RSD), were below 0.2% and 0.4% for major fatty acids (percentage of total FAME content above 5%), while for minor species, these parameters were lower than 10%, in line with previous inter-laboratory studies [22].

Descriptive statistical analysis (i.e., mean, minimum, and maximum values, expressed as percentage of total FAME content) for these 12 fatty acids quantified in Iberian ham samples under study is also summarized in Table 1. Results were in good agreement with those reported by other authors for "Jamón de Huelva" samples [14,23], and for other Iberian ham samples (e.g., "Guijuelo", "Dehesa de Extremadura") [14]. As can be seen, the predominant fatty acids in all samples were oleic, palmitic, and stearic acids, which represented almost 85% of the total fatty acid content. Similarly to previous studies, total percentages of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids were 33%, 57%, and 9%, respectively [14]. Interestingly, this higher proportion of MUFAs, coming from the intake of acorns during the pig fattening period [24], has been previously described as a characteristic feature of Iberian ham compared with dry-cured hams from white pigs [13] and pigs reared in intensive systems [25].

Table 1. Analytical performance of the gas chromatography-flame ionization detector (GC-FID) method in terms of repeatability and reproducibility. Mean, minimum, and maximum concentrations (expressed as percentage of total fatty acid methyl ester, FAME, content) found in Iberian ham samples under study.

Fatty Acids	Retention Time	Repeatability (RSD, %)	Reproducibility (RSD, %)	Mean	Min.	Max.
Lauric acid (C12:0)	3.63	8.7	9.3	0.07	0.05	0.08
Myristic acid (C14:0)	4.28	1.1	1.4	1.32	1.11	1.51
Palmitic acid (C16:0)	5.52	0.1	0.1	21.39	19.53	23.57
Margaric acid (C17:0)	5.73	1.0	0.8	0.32	0.25	0.45
Stearic acid (C18:0)	6.41	3.3	5.2	10.07	7.90	12.19
Arachidic acid (C20:0)	6.78	2.9	5.6	0.18	0.14	0.23
Palmitoleic acid (C16:1*n*-7)	7.71	0.2	0.35	2.23	1.70	2.94
Heptadecenoic acid (C17:1*n*-7)	8.25	0.04	0.09	0.32	0.24	0.43
Oleic acid (C18:1*n*-9)	9.02	0.1	0.4	53.29	49.97	56.40
Gadoleic acid (C20:1*n*-11)	10.21	2.2	2.6	1.55	1.22	2.00
Linoleic acid (C18:2*n*-6)	11.55	3.2	6.3	8.74	6.99	11.29
Linolenic acid (C18:3*n*-3)	12.32	0.5	1.9	0.51	0.36	0.86

Min., minimum; Max., maximum.

3.2. Characterization of Iberian Ham Fatty Acid Profiles According to the Feeding Regime

The fatty acid composition (expressed as mean ± standard deviation of the percentage of total FAME content) of subcutaneous fat from Iberian hams grouped according to the three fattening systems were subjected to ANOVA to look for significant differences (Table 2). "Jamón de Bellota" hams were characterized by higher percentage of oleic, gadoleic, and total content of monounsaturated fatty acids, while lauric, myristic, palmitic, palmitoleic, and stearic acids were increased in "Jamón de Cebo" samples, in agreement with previous studies [13,14,17,18]. The fatty acid concentrations in "Jamón de Cebo de Campo" ranged between the values observed in "Jamón de Bellota" and "Jamón de Cebo", as expected since this feeding regime is based on mixed feeding with concentrate feeds and natural pasture and acorns. Proportions of polyunsaturated fatty acids were similar in the three feeding systems considered, in agreement with previous studies [1,11].

Complementarily, principal component analysis (PCA), and hierarchical cluster analysis (HCA) were also applied for a preliminary data exploration and for visualizing data trends. When PCA was applied, four principal components with eigenvalues higher than 1.0 were extracted (Kaiser criterion), accounting for 87.2% of the total variance. Three overlapped clusters can be observed in the two-dimensional scores plot defined by the two first principal components (Figure 1), with "Jamón de Bellota" samples situated on the left side of the plot, "Jamón de Cebo" samples characterized by positive scores on PC1 (39.78% of total variability explained), and "Jamón de Cebo de Campo" samples clustered between these two groups. Variables contributing to this clustering (loading values higher than 0.7) were oleic acid (negative values in PC1), lauric, myristic, palmitic, and palmitoleic acids (positive values in PC1). Only two variables presented significant loadings on PC2 (19.44% of

total variability explained): arachidic and gadoleic acids. This grouping of samples along PC1 clearly reflects the effect of diet on the fatty acid composition of subcutaneous fat, from higher proportions of oleic acid in "Jamón de Bellota" as a result of feeding with acorns, to increased content of SFA in "Jamón de Cebo" [26].

Table 2. Fatty acid composition of subcutaneous fat from Iberian hams (expressed as mean ± standard deviation of the percentage of total FAME content).

	"Jamón de Bellota"	"Jamón de Cebo "	"Jamón de Cebo de Campo"
C12:0	0.064 ± 0.004 ^{a}	0.071 ± 0.004 ^{b}	0.067 ± 0.005 ^{a}
C14:0	1.27 ± 0.07 ^{a}	1.40 ± 0.08 ^{b}	1.33 ± 0.08 ^{c}
C16:0	20.52 ± 0.62 ^{a}	22.66 ± 0.67 ^{b}	21.51 ± 0.70 ^{c}
C17:0	0.31 ± 0.03	0.33 ± 0.06	0.31 ± 0.04
C18:0	9.52 ± 0.58 ^{a}	10.87 ± 0.67 ^{b}	10.09 ± 0.57 ^{c}
C20:0	0.18 ± 0.02	0.19 ± 0.02	0.19 ± 0.02
Total saturated	31.87 ± 1.05 ^{a}	35.61 ± 1.13 ^{b}	33.50 ± 1.06 ^{c}
C16:1*n*-7	2.09 ± 0.28 ^{a}	2.38 ± 0.30 ^{b}	2.28 ± 0.31 ^{ab}
C17:1*n*-7	0.32 ± 0.05	0.34 ± 0.06	0.32 ± 0.05
C18:1*n*-9	54.80 ± 0.65 ^{a}	50.96 ± 0.69 ^{b}	53.18 ± 0.64 ^{c}
C20:1-11	1.63 ± 0.16 ^{a}	1.44 ± 0.14 ^{b}	1.54 ± 0.15 ^{ab}
Total monounsaturated	58.83 ± 0.85 ^{a}	55.12 ± 0.69^{b}	57.31 ± 0.68 ^{c}
C18:2*n*-6	8.77 ± 0.85	8.80 ± 1.01	8.68 ± 0.97
C18:3*n*-3	0.53 ± 0.10	0.47 ± 0.09	0.51 ± 0.06
Total polyunsaturated	9.30 ± 0.90	9.27 ± 1.05	9.91 ± 1.02

Superscript letters within each row indicate significant differences between groups sharing the same letter according to Tukey HSD test ($p < 0.05$).

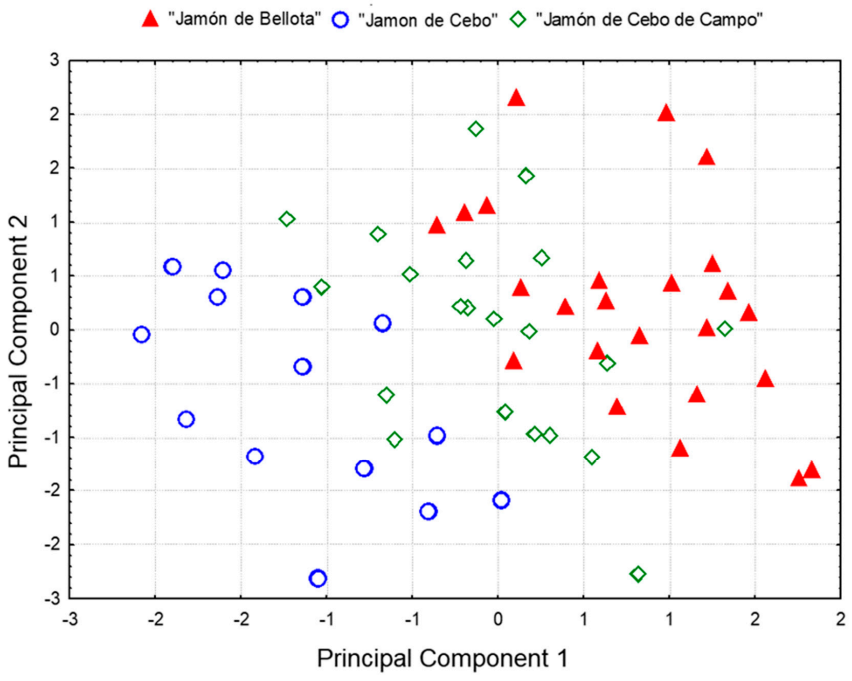


Figure 1. Principal component analysis (PCA) scores plot showing the distribution of samples from the three study groups in the plane defined by the two first principal components.

Similar results were observed when HCA (Ward's method of agglomeration and Euclidean distances) was applied to the same dataset for evaluating similarity between samples. Although the composition of the different clusters is not so evident, three main groups can be identified in the dendrogram represented in Figure 2. One first group contained nearly all "Jamón de Cebo" and some "Jamón de Cebo de Campo" samples, while a second group was mainly composed by "Jamón de Bellota" samples (14 "Jamón de Bellota", 6 "Jamón de Cebo de Campo", 1 "Jamón de Cebo"). The third cluster was more heterogeneous, containing both "Jamón de Cebo de Campo" (13) and "Jamón de Bellota" (11), as well as some "Jamón de Cebo" samples (4).

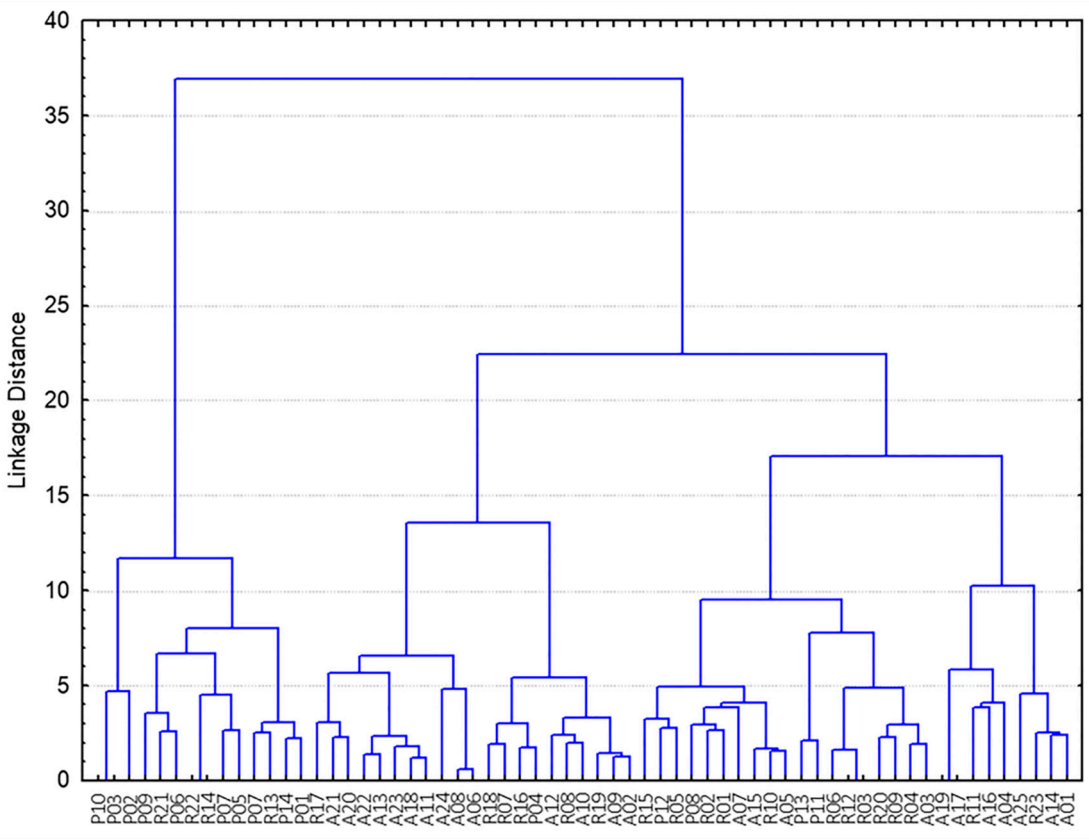


Figure 2. Hierarchical cluster analysis (HCA) dendrogram showing the distribution of samples from the three study groups. P: "Jamón de Cebo", R: "Jamón de Cebo de Campo", A: "Jamón de Bellota".

3.3. Classification Models for Iberian Ham Authentication

Multiple supervised pattern recognition procedures and machine learning algorithms have been recently proposed in food research to solve authentication problems for various foods with high commercial value, such as olive oil [27–29], strawberry [30–32], or wine [33,34]. In this study, two statistical multivariate classification methods were tested with the aim of building classification models for discriminating Iberian ham varieties. Linear discriminant analysis (LDA) was first employed to obtain suitable classification rules for assigning categories to samples, being oleic and margaric acids the most discriminant variables. A correct global classification of 96% was obtained by using the training set ("Jamón de Bellota" $N = 15$, "Jamón de Cebo de Campo" $N = 15$, "Jamón de Cebo" $N = 10$), which was successfully validated in the prediction set (89.2% success rate). All study groups showed good percentages of correctly classified cases (Table 3).

Table 3. Statistical performance for linear discriminant analysis (LDA) and artificial neural network (ANN) classification methods.

	LDA	ANN						
		MLP 12 × 3 × 3	MLP 12 × 4 × 3	MLP 12 × 8 × 3	MLP 12 × 2 × 2 × 3	MLP 4 × 8 × 7 × 3	MLP 7 × 2 × 2 × 3	MLP 1 × 4 × 3
Classification Rate (%)								
"Jamón de Bellota"	96	100	100	93.3	100	93.3	100	100
"Jamón de Cebo"	100	100	100	100	100	100	100	100
"Jamón de Cebo de Campo"	93.9	100	92.3	93.3	100	100	100	100
Prediction Rate (%)								
"Jamón de Bellota"	96	100	100	75	100	100	66.7	100
"Jamón de Cebo"	96	100	100	100	100	100	100	100
"Jamón de Cebo de Campo"	76.8	100	75	100	100	66.7	100	100

MLP, multilayer perceptron.

Finally, artificial neural network (ANN) modeling was also applied for classifying Iberian hams, for which different neural network structures, or multilayer perceptrons (MLP), were compared. As a first step, three-layer networks with different number of nodes in the hidden layer ($12 \times 3 \times 3$, $12 \times 4 \times 3$, $12 \times 8 \times 3$) and four-layer network with two nodes in each of the two hidden layers ($12 \times 2 \times 2 \times 3$) were employed. Previous results from PCA were also considered to reduce the number of inputs into the MLP and take into account possible correlation among variables, so that the four main principal components were used as inputs ($4 \times 8 \times 7 \times 3$). Furthermore, two additional MLPs were also built by using the seven most discriminant variables in the PCA as inputs ($7 \times 2 \times 2 \times 3$) or only the most discriminant one ($1 \times 4 \times 3$). Table 3 shows the percentage of correct classification and prediction for all these networks, which gives satisfactory classifications with maximum errors of 15%. It should be noted that several of these models yielded 100% classification and prediction rates for the three study groups, clearly surpassing the performance provided by LDA, probably due to the intrinsically non-linear nature of the class distribution. To assess the effectiveness of the training by applying the back propagation method, RMS errors between the actual and desired network outputs are listed in Table 4 for the training, cross-validation, and tests sets for all these models. Interestingly, the best results were obtained by applying the $1 \times 4 \times 3$ MLP structure, in line with findings from LDA.

Table 4. Calculated root-mean-squared (RSM) errors for the seven multilayer perceptron (MLP) architectures compared in the study.

	MLP 12 × 3 × 3	MLP 12 × 4 × 3	MLP 12 × 8 × 3	MLP 12 × 2 × 2 × 3	MLP 1 × 4 × 3	MLP 7 × 2 × 2 × 3	MLP 4 × 8 × 7 × 3
Training set	0.063	0.133	0.015	0.036	0.001	0.045	0.170
Verification set	0.085	0.169	0.448	0.087	0.018	0.231	0.286
Test set	0.115	0.3678	0.443	0.481	0.091	0.112	0.352

4. Conclusions

To sum up, it can be concluded that the fatty acid profile of subcutaneous fat is strongly influenced by the feeding regime, with oleic acid being the most important variable driving this differentiation. This therefore represents a first step towards the development of a suitable tool for Iberian ham authentication. In this work, we demonstrated the need of applying advanced chemometric tools to efficiently determine the pig rearing system. The combination of classical multivariate statistical models and artificial neural network provided good performance for pattern recognition and classification. These results could be a starting point for the implementation of quality control assays in the food industry for fraud detection and authentication of Iberian hams.

Author Contributions: Conceptualization, Á.F.-R.; methodology, A.S. and Á.F.-R.; software, Á.F.-R.; validation, A.S. and Á.F.-R.; formal analysis, A.S. and Á.F.-R.; investigation, R.G.-D., A.S. and Á.F.-R.; resources, A.S. and Á.F.-R.; data curation, R.G.-D., A.S. and Á.F.-R.; writing—original draft preparation, Á.F.-R.; writing—review and editing, R.G.-D., A.S. and Á.F.-R.; visualization, R.G.-D., A.S. and Á.F.-R.; supervision, Á.F.-R.; project administration, Á.F.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cava, R.; Ruiz, J.; López-Bote, C.; Martín, L.; García, C.; Ventanas, J.; Antequera, T. Influence of finishing diet on fatty acid profiles of intramuscular lipids, triglycerides and phospholipids in muscles of the Iberian pig. *Meat Sci.* **1997**, *45*, 263–270. [CrossRef]
2. García Rebollo, A.J.; Maciá Botejara, E.; Ortiz Cansado, A.; Morales Blanco, P.J.; Martín Bellido, M.; Fallola Sánchez, A.; Mena Arias, P.; Campillo Alvarez, J.E. Effects of consumption of meat product rich in monounsaturated fatty acids (the ham from the Iberian pig) on plasma lipids. *Nutr. Res.* **1998**, *18*, 743–750. [CrossRef]
3. Márquez-Contreras, E.; Vázquez-Rico, I.; Baldonedo-Suárez, A.; Márquez-Rivero, S.; Jiménez, J.; Machancoses, F.; Morano-Báez, R.; León-Justel, A. Effect of moderate and regular consumption of Cinco Jotas acorn-fed 100% Iberian ham on overall cardiovascular risk: A cohort study. *Food Sci. Nutr.* **2018**, *6*, 2553–2559. [CrossRef] [PubMed]
4. Ivanovic, J.; Baltic, M.Z.; Janjic, J.; Markovic, R.; Baltic, T.; Boskovic, M.; Djordjevic, J.; Mrdovic, B.; Jovanovic, D. Health aspects of dry-cured ham. *Meat Technol.* **2016**, *5*, 43–50.
5. Andrés, A.I.; Cava, R.; Ventanas, J.; Thovar, V.; Ruiz, J. Sensory characteristics of Iberian ham: Influence of salt content and processing conditions. *Meat Sci.* **2004**, *68*, 45–51. [CrossRef]
6. Narváez-Rivas, M.; Gallardo, E.; León-Camacho, M. Analysis of volatile compounds from Iberian hams: A review. *Grasas Aceites* **2012**, *63*, 432–454.
7. García-González, D.L.; Tena, N.; Aparicio, R. Contributing to interpret sensory attributes qualifying Iberian hams from the volatile profile. *Grasas Aceites* **2009**, *60*, 277–283. [CrossRef]
8. Fuentes, V.; Ventanas, S.; Ventanas, J.; Estévez, M. The genetic background affects composition, oxidative stability and quality traits of Iberian dry-cured hams: Purebred Iberian versus reciprocal Iberian × Duroc crossbred pigs. *Meat Sci.* **2014**, *96*, 737–743. [CrossRef]
9. Pérez-Palacios, T.; Antequera, T.; Durán, M.L.; Caro, A.; Rodríguez, P.G.; Ruiz, J. MRI-based analysis, lipid composition and sensory traits for studying Iberian dry-cured hams from pigs fed with different diets. *Food Res. Int.* **2010**, *43*, 248–254. [CrossRef]
10. Ministerio de Agricultura, Alimentación y Medio Ambiente. Real Decreto 4/2014, de 10 de enero, por el que se aprueba la norma de calidad para la carne, el jamón, la paleta y la caña de lomo ibérico. *Boletín Oficial del Estado* **2014**, *10*, 1569–1585.
11. Ruiz, J.; Cava, R.; Antequera, T.; Martín, L.; Ventanas, J.; López-Bote, C.J. Prediction of the feeding background of Iberian pigs using the fatty acid profile of subcutaneous, muscle and hepatic fat. *Meat Sci.* **1998**, *49*, 155–163. [CrossRef]
12. Timón, M.L.; Martín, L.; Petrón, M.J.; Jurado, Á.; García, C. Composition of subcutaneous fat from dry-cured Iberian hams as influenced by pig feeding. *J. Sci. Food Agric.* **2002**, *82*, 186–191. [CrossRef]
13. Petrón, M.J.; Muriel, E.; Timón, M.L.; Martín, L.; Antequera, T. Fatty acids and triacylglycerols profiles from different types of Iberian dry-cured hams. *Meat Sci.* **2004**, *68*, 71–77. [CrossRef] [PubMed]
14. Fernández, M.; Ordóñez, J.A.; Cambero, I.; Santos, C.; Pin, C.; de la Hoz, L. Fatty acid compositions of selected varieties of Spanish dry ham related to their nutritional implications. *Food Chem.* **2007**, *101*, 107–112. [CrossRef]
15. Recio, C.; Martín, Q.; Raposo, C. GC-C-IRMS analysis of FAMEs as a tool to ascertain the diet of Iberian pigs used for the production of pork products with high added value. *Grasas Aceites* **2013**, *64*, 181–190. [CrossRef]
16. Bayés-García, L.; Tres, A.; Vichi, S.; Calvet, T.; Cuevas-Diarte, M.A.; Codony, R.; Boatella, J.; Caixach, J.; Ueno, S.; Guardiola, F. Authentication of Iberian dry-cured ham: New approaches by polymorphic fingerprint and ultrahigh resolution mass spectrometry. *Food Control* **2016**, *60*, 370–377. [CrossRef]

17. Narváez-Rivas, M.; Gallardo, E.; León-Camacho, M. The changes in gliceridic fractions of sweaty fat and weight loss during ripening time of Iberian dry-cured ham. *Food Res. Int.* **2013**, *54*, 1657–1669. [CrossRef]
18. González-Dominguez, R.; García-Barrera, T.; Gómez-Ariza, J.L. Iberian ham typification by direct infusion electrospray and photospray ionization mass spectrometry fingerprinting. *Rapid Commun. Mass Spectrom.* **2012**, *26*, 835–844. [CrossRef]
19. Berrueta, L.A.; Alonso-Salces, R.M.; Héberger, K. Supervised pattern recognition in food analysis. *J. Chromatogr. A* **2007**, *1158*, 196–214. [CrossRef]
20. Roberts, J.J.; Cozzolino, D. An Overview on the Application of Chemometrics in Food Science and Technology-An Approach to Quantitative Data Analysis. *Food Anal. Methods* **2016**, *9*, 3258–3267. [CrossRef]
21. Danezis, G.P.; Tsagkaris, A.S.; Brusic, V.; Georgiou, C.A. Food authentication: State of the art and prospects. *Curr. Opin. Food Sci.* **2019**, *10*, 22–31. [CrossRef]
22. García-Olmo, J.; De Pedro, E.; Garrido, A.; Paredes, A.; Sanabria, C.; Santolalla, M.; Salas, J.; García-Hierro, J.R.; Gonzalez, I.; García-Cachan, M.D.; et al. Determination of the precision of the fatty acid analysis of Iberian pig fat by gas chromatography. Results of a mini collaborative study. *Meat Sci.* **2002**, *60*, 103–109.
23. Garrido-Fernández, A.; León-Camacho, M. Assessing the effect of season, montanera length and sampling location on Iberian pig fat by compositional data analysis and standard multivariate statistics. *Food Chem.* **2019**, *295*, 377–386. [CrossRef] [PubMed]
24. Jiménez-Colmenero, F.; Ventanas, J.; Toldrá, F. Nutritional composition of dry-cured ham and its role in a healthy diet. *Meat Sci.* **2010**, *84*, 585–593. [CrossRef] [PubMed]
25. Gilles, G. Dry cured ham quality as related to lipid quality of raw material and lipid changes during processing: A review. *Grasas Aceites* **2009**, *60*, 297–307. [CrossRef]
26. Ruiz, J.; López Bote, C. Improvement of dry-cured hams quality by lipid modification through dietary means. In *Research Advances in the Quality of Meat and Meat Products*; Toldra, F., Ed.; Research Singpost: Kerala, India, 2002; pp. 255–271.
27. Sayago, A.; González-Domínguez, R.; Beltrán, R.; Fernández-Recamales, Á. Combination of complementary data mining methods for geographical characterization of extra virgin olive oils based on mineral composition. *Food Chem.* **2018**, *261*, 42–50. [CrossRef]
28. Sayago, A.; González-Domínguez, R.; Urbano, J.; Fernández-Recamales, Á. Combination of vintage and new-fashioned analytical approaches for varietal and geographical traceability of olive oils. *LWT* **2019**, *111*, 99–104. [CrossRef]
29. González-Domínguez, R.; Sayago, A.; Morales, M.T.; Fernández-Recamales, Á. Assessment of Virgin Olive Oil Adulteration by a Rapid Luminescent Method. *Foods* **2019**, *8*, 287. [CrossRef]
30. Akhatou, I.; Sayago, A.; González-Domínguez, R.; Fernández-Recamales, Á. Application of targeted metabolomics to investigate optimum growing conditions to enhance bioactive content of strawberry. *J. Agric. Food Chem.* **2017**, *65*, 9559–9567. [CrossRef]
31. Akhatou, I.; González-Domínguez, R.; Fernández-Recamales, Á. Investigation of the effect of genotype and agronomic conditions on metabolomic profiles of selected strawberry cultivars with different sensitivity to environmental stress. *Plant Physiol. Biochem.* **2016**, *101*, 14–22. [CrossRef]
32. González-Domínguez, R.; Sayago, A.; Akhatou, I.; Fernández-Recamales, Á. Multi-chemical profiling of strawberry as a traceability tool to investigate the effect of cultivar and cultivation conditions. *Foods* **2020**, *9*, 96. [CrossRef] [PubMed]
33. Versari, A.; Laurie, V.F.; Ricci, A.; Laghi, L.; Parpinello, G.P. Progress in authentication, typification and traceability of grapes and wines by chemometric approaches. *Food Res. Int.* **2014**, *60*, 2–18. [CrossRef]
34. González-Domínguez, R.; Sayago, A.; Fernández-Recamales, Á. Metabolomics: An Emerging Tool for Wine Characterization and the Investigation of Health Benefits. In *Engineering Tools in the Beverage Industry. Volume 3: The Science of Beverages*, 1st ed.; Grumezescu, A., Holban, A.M., Eds.; Woodhead Publishing: Duxford, UK, 2019; Volume 3, pp. 315–350.

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland
Tel. +41 61 683 77 34
Fax +41 61 302 89 18
www.mdpi.com

Foods Editorial Office
E-mail: foods@mdpi.com
www.mdpi.com/journal/foods

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34
Fax: +41 61 302 89 18

www.mdpi.com

ISBN 978-3-0365-1667-7