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Determination of volatile compounds for the differentiation of PDO fortified wines with different ageing methods as a tool for controlling their authenticity

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ABSTRACT

The aim of this work was to study the differentiating volatile profiles of the Spanish protected designation of origin (PDO) fortified wines obtained by headspace solid phase microextraction in conjunction with gas chromatography-mass spectrometry and powerful chemometric tools, to finally identify the marker volatile compounds most related to fortified wine types. Results revealed a satisfactory discrimination, for the first time, of the different types of PDO fortified wines, involving only a reduced number of volatile compounds selected by chemometrics. Thus, 28 volatile compounds were responsible for the differentiation according to ageing type (biological, oxidative, or mixed) resulting useful markers for the identification of each specific type of fortified wine. Among them, some esters were strongly related to biological ageing, aldehydes and acids to oxidative ageing, and lactones to mixed ageing. These volatile molecules involved in their differentiation could explain the unique organoleptic characteristics or attributes of these PDO fortified wines.

1. Introduction

Spain is one of the main producers of high-quality wines. Particularly the fortified wines, which are elaborated in Andalusia, have acquired great prestige for being unique due to their production in a specific geographical area with traditional methods, the grape variety used, the climate and the soil. Such is their distinguishing feature achieved that they have been protected by the European Union with the indication 'Protected Designation of Origin' (PDO). Thus, there are four PDO of fortified wines in Andalucía ('Condado de Huelva', 'Jerez-Xérès-Sherry', 'Manzanilla-Sanlúcar de Barrameda', and 'Montilla-Moriles'). Furthermore, within each PDO, there are recognised different categories according to their particular winemaking conditions such as the ageing process (Fino and Manzanilla, Oloroso, Amontillado and Palo Cortado) (BOJA N° 34 de 16/02/2018a, BOJA N° 34 de 16/02/2018b, BOJA N° 70 de 12/04/2018).

The production of these wines is divided in two phases: firstly, production of the base wine through total or partial fermentation of the grape must and, secondly, wine ageing, which is the phase of ageing in wood barrels to which these wines are subjected after the addition of alcohol to raise their alcoholic degree (a process known as 'fortification') and to achieve the particular organoleptic qualities of their respective types of wine. The other particularity is that the ageing period, in addition to be mainly performed by the traditional system of 'criaderas and solera', can be of three kinds: biological ageing, by means of which the Fino and Manzanilla wines are obtained; oxidative ageing, which is the one that takes place in the Oloroso wines; and the combination of both ageing methods from which Amontillado and Palo cortado wines are obtained.

The wines submitted to biological ageing are fortified until they reach an alcoholic content of 15.5°. This enables the formation of the so-called 'velo de flor', which is a film of veil yeast that grows spontaneously on the wine surface, under which the wines age, preventing oxidation. This phenomenon is undoubtedly one of the main reasons for the uniqueness of these wines, as the action of the metabolism of these yeasts causes significant changes in the wine and, therefore, in its definitive organoleptic characteristics. In oxidative ageing, the wines are fortified until they reach an alcohol content of at least 17°, above which biological activity becomes impossible, even for the veil yeasts, and they aged exposed to the direct action of oxygen, which can be seen visually by the gradual darkening of the wine's colour (BOJA N°34 de 16/02/2018a, BOJA N°34 de 16/02/2018b, BOJA N°70 de 12/04/2018). There is also the mixture of both ageing process from which Amontillado and Palo Cortado wines are produced. In the case of Amontillado wine, it is

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well established that there is a first step of biological ageing under the 'velo de flor' that can entail several years (usually 5), followed by an oxidative step after the fortification of the wine and stoppage of the *flor* yeast growth (usually 2–3 years). However, Palo Cortado is a wine that, despite going through both ageing methods, the first step, biological ageing, used to last only between 6 months and 2 years because the oenologists of the winery detect its potential for Palo Cortado and then add alcohol to 17 alcoholic degrees avoiding the growth and starting oxidative ageing (BOJA N°34 de 16/02/2018a, BOJA N°34 de 16/02/2018b, BOJA N°70 de 12/04/2018). This genuine and rare production process has always been surrounded by a bunch of mystery, which has brought with it, together with the high prices, several falsifications, and adulterations. Surprisingly, there are scarce research that studied the volatile profile of Palo Cortado wines, and even less with the application of chemometric techniques.

The great diversity of high-quality wines on the market and the increase in their demand makes it is necessary to characterize them to establish quality and authenticity control parameters, thus protecting and assuring consumers that the product they are purchasing has the quality and characteristics declared. Although a multitude of wine parameters can be analysed, one of the most important, whose characterisation can be used to guarantee its authenticity, is the aroma. Wine in general, and particularly fortified wines, are very complex products, containing a wide variety of organic and inorganic substances in addition to water and alcohol. The focus on the aroma has been object of study for the characterization of these products since it is considered one of the most relevant quality criteria for wine (Zea et al., 2001, 2010a). It depends on the volatile compounds profile of the wine, having a primary origin, the grape used; secondary origin, fermentation by yeast and production process; and finally, tertiary origin, the ageing process. Therefore, by studying them, it is possible to characterise and differentiate wines based on the main sources of variation (raw material, production process and ageing). Even though, some authors have previously studied the volatile profile of some of these fortified wines (García-Moreno et al., 2021; Moyano et al., 2002; Zea et al., 2010b; Zea et al., 2001), they have some limitations. Hence, some of them considered a limited number of samples, which were sometime not controlled samples or without PDO, or studied only one type of fortified or PDO wine and not all at the same time, or even they did not employ chemometric tools or multivariate data analysis.

Gas chromatography-mass spectrometry (GC–MS) has been the most widely-used technique for analysing volatile compounds in wine and its derivates, after a proper extraction method, being headspace-solid phase microextraction (HS-SPME) one of the employed one for analysing the volatile profile of wine and wine vinegars (Morales et al., 2020; Panighel & Flamini, 2014; Ríos-Reina et al., 2019; Ubeda et al., 2017). It offers important advantages such as the non-use of extraction solvents and its capability of carrying out the extraction and concentration steps simultaneously in a short time. As wine is a complex matrix and its analysis by GC–MS provides a large amount of information, the treatment of the generated data is a difficult task. To deal with it, the combination of GC–MS with chemometric techniques such as PARAFAC2 has demonstrated to reduce the problems associated with GC–MS analysis of complex mixtures and to obtain the maximum information of the volatile profile for distinguishing between samples (Johnsen et al., 2017; Ríos-Reina et al., 2023). Thus, different software and algorithms have been developed that apply chemometric techniques useful to process complex data sets from GC–MS analysis quickly, easily and efficiently, such as the Deconvolution and Identification System called PARADISe® (Johnsen et al., 2017).

In this context, the aim of this work was to study and compare the characteristic volatile profile of the different fortified wines from each Spanish PDO by a reliable and simple method such as headspace solid phase microextraction (HS-SPME) in conjunction with gas chromatography-mass spectrometry (GC–MS) and powerful chemometric tools, to differentiate the Andalusian PDO fortified wines, being the first time that all the fortified wine types and PDOs are studied together. For that, a strategy based on different steps was developed: the processing of the profile or volatile fingerprint using chemometric techniques such as Parallel Factor Analysis (PARAFAC2), followed by principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), to finally selecting and identifying the marker volatile compounds most related to the each PDO fortified type of wine.

2. Materials and methods

2.1. PDO fortified wines under study

A total of 104 samples of fortified wines from the different Andalusian PDOs were collected from the Regulatory Councils of each PDO: 'Condado de Huelva', 'Jerez-Xérès-Sherry', 'Manzanilla -Sanlúcar de Barrameda', and 'Montilla-Moriles'. Within each PDO, the different types of fortified wines produced and marketed were considered: Fino (FI), Manzanilla (MA), Amontillado (AM), Palo Cortado (PC) and Oloroso (OL). Table 1 shows the number of samples of each type of wine belonging to each PDO, and the different types according to ageing process, as well as the codes used in this study. The number of samples for each PDO and type included in the study was limited according to the authentication and certification of the samples by the corresponding Regulatory Councils.

2.2. Reagents and solvents

As internal standard (IS) a solution of a commercial standard 4-methyl-2-pentanol in 10 mL methanol was used, both from Merck (Darmstadt, Germany), preserved at −20 °C until use. Sodium chloride (NaCl) from Sigma-Aldrich (Madrid, Spain) was also used to increase the salting out in the vial and facilitate the passage of volatile compounds into the headspace of the vial. The linear retention index (LRI) was calculated by injecting a series of straight-chain n-alkanes C10 to C40 (50 mg/L in n-hexane) purchased from Fluka (Madrid, Spain). Moreover, available standards of volatile compounds used for identification were bought from different commercial sources (Merck, Darmstadt, Germany and Sigma-Aldrich, Madrid, Spain).

2.3. Headspace extraction of volatile compounds by solid phase microextraction and analysis by gas chromatography and mass spectrometry (HS-SPME-GC–MS)

The basic conditions of sample extraction were based on previous reported validated method for wines (Ubeda et al., 2017), which was slightly adapted to increase the efficiency of the method. The method was as follows: 7.5 mL of wine, 1.5 g of NaCl and 10 μL of internal

Table 1
Samples of each type of wine considered in this study according to each PDO.

Ageing type	Wine type	Code	DOP	Number of samples
Oxidative	*Oloroso*	OL	*Jerez*	9
			Condado de Huelva	8
			Montilla-Moriles	8
Biological	*Fino*	FI	*Jerez*	9
			Condado de Huelva	5
			Montilla-Moriles	34
	Manzanilla	MA	*Sanlúcar de Barrameda*	5
Biological + Oxidative	*Amontillado*	AM	*Jerez*	10
			Montilla-Moriles	9
	Palo Cortado	PC	*Jerez*	5
			Montilla-Moriles	2

standard (IS) were placed into a 20 mL glass vial which was positioned in the thermostatized autosampler tray at 20 °C for HS-SPME sampling. Then, samples were incubated for 5 min at 40 °C with an agitation speed of 300 rpm. After the incubation, the volatile compounds were extracted from the headspace of the vial by exposing a 1 cm Carboxen/DVB/PDMS 50/30-μm SPME fibre (Supelco, Bellefonte, USA) during 40 min at 45 °C, and desorbed using the splitless mode with an injector temperature of 250 °C for 180 s.

GC–MS analysis was carried out using an 8890 Agilent GC system coupled with an Agilent 5977B quadrupole mass spectrometer (Agilent, Santa Clara, CA, US)). A J&W CPWax-57CB capillary column (50 m × 0.25 mm) and with a film thickness of 0.25 μm was employed (Agilent, Santa Clara, CA, US), with helium carrier gas at a flow rate of 1 mL/min. The oven temperature program was as follows: 35 °C for 1 min and subsequently raised to 160 °C at 2.5 °C/min (holding for 1 min), and then to 220 °C at 5 °C/min, being the final GC time of 64 min. The electron ionization mass spectra (29 to 300 amu) were acquired in full scan mode at 70 eV. The samples were analysed in duplicate, and blank runs using empty glass vial were performed after each analysis. All data were recorded using MS ChemStation software (Agilent).

2.4. Data processing, identification of volatile compounds and statistical analysis

The analysis of the chromatographic profiles was carried out in several steps, starting with the use of Parallel Factor Analysis (PARAFAC2) applied by the software PARADISe® to integrate the areas of volatile compounds and identified them, followed by classification analysis by partial least squares discriminant analysis (PLS-DA) for feature selection using the variables with importance in the projection (VIPs) obtained. Moreover, as PLS-DA was not used with a classificatory purpose due to the limited number of samples in some of the categories, exploratory analyses using principal component analysis (PCA) were performed with the volatile compounds selected to better explore the grouping and relationship with the volatiles.

PARADISe® is a powerful methodology for analysing complex chromatographic data based on PARAFAC2, which offers the advantage of being effective in the simultaneous deconvolution of the pure mass spectra of peaks and co-eluting compounds while robustly calculating their peak areas for all samples, as well as in the identification of these peaks using the resolved mass spectra and the NIST MS library (Johnsen et al., 2017). It is also effective in correcting for baseline and noise. These advantages provide an opportunity to identify minor compounds in complex volatile profiles such as those of fortified wines, as well as it facilitates and speeds up statistical analyses.

For using the program, different steps need to be taken. First, the chromatographic data were converted to netCDF format and exported to AIA format by MSD ChemStation (version F.01.01.2317). Then, selection of intervals along the chromatogram is needed. In this study, 176 intervals were selected to run the modelling of each interval using the PARAFAC2 algorithm, setting the maximum number of components to 7 per interval and applying the non-negativity constraint. To select the correct number of components for each model, and to check that the model is correct, two parameters were carefully optimized, the percentage of fit and the percentage of core-consistency, trying to achieve values as close as possible to 100% for each parameter. After modelling, the area value of a total of 346 peaks were obtained and their tentatively identification was provided by the NIST MS Search database (v.2.0). Then, the peak area values provided for each compound were normalized dividing them by the area value of the internal standard (i.e., obtaining the relative areas). In this study quantification of peaks was not considered since the aim was a non-targeted approach for a comparison and differentiation of the samples, leading to a somewhat more targeted approach by selecting relevant peaks.

Thus, the following step was to reduce the number of compounds due to within this set of compounds, there were volatile compounds, but there were also possible contaminants, noise, etc., which must be eliminated. Moreover, as the aim of this work was the differentiation of fortified wines and the selection of possible volatile markers, and not their complete characterization *per se,* already found in the literature, it was decided to carry out a methodology for data reduction and compound selection strategy. It was based on the selection of variables with importance in the projection (VIPs) using Partial Least Squares Discriminant Analysis (PLS-DA) according to the types of fortified wines. Hence, PLS-DA was not developed directly for a classification approach, due to the limited number of samples in some of the cases, but it was applied to study variables with predictive importance (VIP) for data reduction and selection. For that, a sequential PLS-DA classification models were developed by selecting in each one the volatile compounds with high VIP value, due the VIPs are scaled in such a way that all predictors that have a VIP ≥ 1 are considered relevant (Mehmood et al., 2012). In this study, to greatly reduce the large number of compounds obtained, variables whose VIP was ≥ 2 (VIPs ≥ 2) were selected in each of the PLS-DA models. PLS-DA models were validated by means of cross-validation (venetian blinds). The identification was assessed by using version 2.0 of the standard NIST library and the linear retention index (LRI) of reference standards, when available, or data in the literature. Significant differences between data were obtained by analysis of variance (ANOVA) followed by a post hoc comparison test (Tukey's test) using INFOSTAT software (FCA, Universidad Nacional de Córdoba, Argentina), principal component analysis (PCA) and PLS-DA were conducted using PLS Toolbox 7.9.5 (Eigenvector Research Inc., Wenatchee, WA, USA) working in a MATLAB 2016a environment (Mathworks) and circle packing heatmap was plotted by https://www.bioinformatics.com.cn/en. Prior to any modelling, the data were autoscaled.

3. Results and discussion

3.1. Data reduction and selection of the differential volatile compounds for each type of PDO fortified wine

After analysing the total set of samples, the chromatograms of all the samples were processed by the software PARADISe®. As it was described in the materials and method section, a data matrix of 208x346 was provided by PARADISe®, including the area values of the volatile compounds and a tentative identification. After normalizing the area values by the IS, a process of feature or variable reduction using VIP scores was repeated two times, hence obtaining three different PLS-DA models: one with the total deconvoluted peaks (346), called from now on *PLS-DA346*; another with 101 volatile compounds which were the VIP compounds in the *PLS-DA346* model, called from now on *PLS-DA101*; and a third one with 28 volatile compounds defined as VIP in the *PLS-DA101* model, called from now on *PLS-DA28*.

The objective of this strategy of sequential PLS-DA models was to evaluate how many variables or features could be removed from the model without reducing the discriminant performance achieved. For that reason, it should be noted that only compounds relevant to the differentiation were selected in the selection of the VIPs. Therefore, other compounds that were present in the samples but did not make a difference between the types of fortified wine were not considered, since, as indicated, the study was not merely a characterization of each type of wine separately, but rather a differentiation of samples. Hence, although the volatile profile of these wines has been studied by other authors (Moyano et al., 2002; Valcárcel-Muñoz et al., 2022; Zea et al., 2001), to our knowledge, this is the first time that this strategy, i.e., going from untargeted to targeted approach by means of the use of VIP values and PLS-DA, has been carried out with the volatile composition of all the types of fortified wines together, and with the aim of selecting possible volatile markers with the VIP approach. Moreover, although classification was not the aim of the PLS-DA model, the classification and cross-validation results obtained for all the models showed promising classification results (Table 2), presenting for the first time the

Table 2
Classification results for calibration (Cal.) and cross-validation (CV) obtained by developing a PLS-DA model with the total of 346 resolved peaks provided by PARADISe (4VL, 48.63% explained variance); a PLS-DA model with 101 VIP volatile compounds (6 LVs and 76.56% explained variance) and a PLS-DA model with 28 VIP volatile compounds (8 LVs and 58.57% explained variance).

		PLS-DA346[a] (346 volatile compounds)					PLS-DA101 (101 volatile compounds)					PLS-DA28 (28 volatile compounds)				
Types of fortified wines according to their ageing		AM	FI	MA	OL	PC	AM	FI	MA	OL	PC	AM	FI	MA	OL	PC
Cal	% Sens.[b]	84.2	96.8	**100**	94.0	**100**	**100**	**96.9**	**100**	96.0	**100**	94.7	**96.9**	**100**	**98.0**	**100**
	% Spec.	94.1	92.0	84.3	94.9	95.4	97.1	94.6	93.4	93.7	96.4	**97.6**	**94.9**	**94.9**	**97.5**	**97.9**
	% Error	10.8	5.58	7.82	5.53	2.32	**1.47**	**4.76**	3.28	5.16	1.80	3.80	5.13	**2.52**	**2.26**	**1.03**
CV[c]	% Sens.	84.2	95.8	100	94.0	100	94.7	95.8	**100**	94.0	**100**	**94.7**	**96.9**	**100**	**96.0**	**100**
	% Spec.	94.1	92.0	85.9	92.4	93.8	96.5	**94.6**	92.4	93.7	95.9	**97.6**	92.9	**93.4**	**96.8**	**96.9**
	% Error	10.8	6.10	7.07	6.79	3.09	4.39	4.76	3.78	6.16	2.06	**3.80**	5.13	**3.28**	**3.58**	**1.54**

Note: [a]The best classification values for each wine type were highlighted in bold. [b]Sens: sensitivity; Spec: specificity. [c]CV: Venetian blinds cross-validation.

possible usefulness of the proposed methodology to classify fortified wines according to the fortified type.

Once the first PLS-DA model (*PLS-DA346*) was performed with the 346 peaks for the selection of the compounds which VIP values ≥ 2, whose classification results are shown in Table 2, the number of compounds was reduced from 346 to 101 VIPs. Moreover, by performing the second PLS-DA with the 101 VIPs, it can be also seen that the reduction of volatile compounds from 346 to 101 clearly improved the classification results in most of the cases by increasing the percentage of sensitivity and specificity of both calibration and cross-validation (e.g., from 84.2% of sensitivity in AM to 100%) and decreasing the percentage of error of each wine type (e.g., from 10.4% to 1.47% in calibration of AM) (Table 2). The identification of these 101 selected volatile compounds was checked by their LRI and they were grouped into their corresponding chemical families. The mean and standard deviation of their relative peak area values according to each type of wine, as well as Tukey test results, are shown in Table 3, whose discussion will be done in the following section. In addition, when a PCA was performed with this dataset (208x101), this already showed a grouping of the samples according to the ageing type (**Fig. S1**).

However, with the aim of further reducing and selecting those volatile compounds that could potentially be markers of each type of fortified wine and to move towards a more targeted approach, subsequently, a second PLS-DA analysis was carried out with the 101 compounds selected in the *PLS-DA101* model, and once again, the volatile compounds with a VIP ≥ 2 were selected, being in this case 28. Then, to check that the reduction of compounds did not worsen the differentiation, thus finding the best and simplest classification model with fewer variables, a new PLS-DA model (*PLS-DA28*) was built with the 28 volatile compounds previously selected. Once again, an improvement in the classification results was obtained by this second reduction of compounds (Table 2), i.e., with only the 28 selected compounds, especially for the AM, MA, OL, and PC categories, which means that despite the important reduction in features, the statistical performance of the resulting model was not affected. The LRI, chemical families and odour description of these 28 volatile compounds is presented in **Table S1**. This process of variable reduction using VIP scores by repetition of PLS-DA models, and the selection of VIPs as markers, has been successfully applied in other previous works (Rodríguez-Hernández et al., 2023). This approach has been often used for the analysis of data such as metabolome or volatilome (Freire et al., 2021), with the aim of selecting features from multiple potential compounds which could be considered as important markers.

3.2. Study of the differences between each type of fortified wine according to the possible volatile markers selected

Among the 28 volatile compounds selected as possible markers of each wine (highlighted in Table 3 and summarized in **Table S1**), different chemical families were distinguished: esters, alcohols, aldehydes, acids, ketones, and lactones, and a group called others that included acetals, terpenes and norisoprenoids, among others. Some differences in the chemical families could be observed among wine types by studying the percentage of relative areas for each one (Fig. 1) and by studying the circle packing plots (Fig. 2), which show the differences in the volatiles dominating in relative area content between the different wine types. On the one hand, samples that have been submitted to oxidation (AM, PC and OL) showed a higher presence of differentiating compounds of the aldehydes' family in the proportion of the 28 volatile compounds selected (Fig. 1, Fig. 2). The relevance of aldehydes in the differentiation of wines with oxidative ageing was expected since aldehydes are formed because of alcohol oxidation (Moyano et al., 2002; Waterhouse et al., 2016; Zea et al., 2001). On the other hand, wines with only biological ageing, such as FI and MA, showed a higher percentage of relative areas for ester compounds within these 28 selected volatile compounds (Fig. 1, Fig. 2). *Flor* yeasts have been shown to increase the content of ethyl esters (Zea et al., 1995) being consistent with our results that are differentiation markers of biological ageing. Moreover, interesting differences could be also observed between the FI and MA samples, mainly with respect to the acid family, being higher in FI samples, and in the alcohol family, in higher amounts in MA samples. Differences among the group of samples with oxidative ageing was also observed, such as the higher relative areas of acids in OL samples than in those samples with the combination of both ageing types, and slightly higher presence of esters in AM than in PC samples (Fig. 1, Fig. 2), highlighting their previous biological ageing.

These compositional differences and similitudes can be easily observed by building a principal component analysis (PCA) with the 28 variables selected (Fig. 3). Fig. 3**a** shows the distribution of the analysed samples (scores) depicted in the function of their ageing type for PC1 and PC2, which accounted for 30.0% and 19.6% of the variance, respectively. As can be seen in Fig. 3**a**, the scores plot of the first two principal components (PCs) showed a grouping of the samples according to their type of ageing: biological (FI and MA, in green and blue colour) mainly in the negative side of the PC1; oxidative (OL, in light blue colour) in the negative side of PC2; and biological and oxidative (AM and PC) mainly in the positive part of PC1. As can be observed, wines submitted to two different kinds of ageing (oxidative and biological) were arranged in the plane more dispersed than the other types of wines that have only undergone one type of ageing. This fact difficulted the interpretation but despite this, it seems that samples were distributed among the y-axis (PC2). Thus, PC2 distributed samples from the oxidative ageing with high positive values y-axis of OL (in light blue colour) to the samples with mixture ageing type, with PC (pink colour) situated closer to OL and transitioning to AM (red colour), that was in the middle of the plot. At the bottom of the y-axis, with negative PC2 values, fortified wines with biological ageing are placed. Very similar samples such as FI and MA (in green and blue colour respectively) did not achieve a good separation, for that reason, the wines were studied separately to deeply understand the results. Thus, three groups were

Table 3
Means and standard deviations of the relative areas of the 101 selected volatile compounds according to wine type, linear retention index (LRI), identification and chemical family, as well as results of the Tukey statistical analysis.

				Biological							Oxidative				Biological + Oxidative						
				FI[d]			MA				OL				AM			PC			
LRI[b]	ID[c]	Chemical family	Volatile compounds[a]	mean	±SD[e]	WT[f]	mean	±SD	WT	*AT*[g]	mean	±SD	WT	*AT*	mean	±SD	WT	mean	±SD	WT	*AT*
1274	B	acid	3-Cyclohexene-1-carboxylic acid	0.59	0.30	b	0.81	0.15	c	*b*	0.11	0.20	a	*a*	0.16	0.22	a	0.07	0.06	a	*a*
1451	A	acid	Acetic acid	1.85	2.01	a	0.19	0.20	a	*a*	5.75	2.31	bc	*b*	4.84	2.53	b	7.00	1.31	c	*b*
1485	C	acid	2-Thiopheneacetic acid	0.11	0.06	a	0.11	0.03	a	*a*	0.08	0.07	a	*a*	0.20	0.12	b	0.13	0.04	a	*b*
1802	C	acid	2-Phenoxypropionic acid	0.41	0.26	a	0.51	0.26	a	*a*	1.06	0.39	b	*b*	1.23	0.58	b	1.83	0.63	c	*c*
1824	C	acid	4-Amino-1,5-pentandioic acid	0.02	0.02	a	0.01	0.01	a	*a*	0.11	0.06	b	*b*	0.10	0.08	b	0.10	0.04	b	*b*
1837	C	acid	Ethanediamide	0.05	0.06	a	0.07	0.05	ab	*a*	0.42	0.35	c	*b*	0.27	0.31	bc	0.86	0.45	d	*b*
1986	A	acid	**Heptanoic acid**	0.08	0.04	b	0.03	0.01	a	*a*	0.06	0.03	ab	*a*	0.06	0.03	ab	0.07	0.03	b	*a*
2155	A	acid	**Sorbic acid**	0.17	0.19	b	0.00	0.00	a	*b*	0.43	0.27	c	*c*	0.04	0.04	ab	0.03	0.07	ab	*a*
2213	A	acid	**Decanoic acid**	1.50	1.45	ab	0.61	1.21	a	*a*	3.75	2.46	c	*b*	1.24	1.06	ab	2.31	1.04	b	*a*
1157	A	alcohol	1-Butanol	1.06	0.45	ab	0.76	0.12	a	*b*	0.76	0.38	a	*a*	1.49	0.87	b	1.24	0.46	b	*c*
1311	B	alcohol	2-Ethylbutanol	0.22	0.20	b	0.26	0.08	b	*b*	0.06	0.04	a	*a*	0.31	0.26	b	0.17	0.07	ab	*b*
1331	A	alcohol	3-Methyl-1-pentanol	0.31	0.12	b	0.23	0.05	ab	*b*	0.17	0.05	a	*a*	0.29	0.17	b	0.24	0.07	ab	*b*
1386	A	alcohol	**(Z)-3-Hexen-1-ol**	0.16	0.12	a	0.34	0.09	b	*a*	0.18	0.09	a	*a*	0.17	0.15	a	0.14	0.09	a	*a*
1463	A	alcohol	**1-Heptanol**	0.15	0.07	a	0.22	0.07	b	*a*	0.18	0.09	ab	*a*	0.22	0.12	b	0.20	0.04	ab	*b*
1487	B	alcohol	4-Nonanol	0.09	0.08	b	0.07	0.03	ab	*b*	0.03	0.02	a	*a*	0.10	0.11	b	0.06	0.03	ab	*b*
1518	B	alcohol	**(*S*)-3-Ethyl-4-methylpentanol**	0.13	0.18	a	0.42	0.11	b	*a*	0.17	0.14	a	*a*	0.25	0.24	a	0.27	0.15	ab	*b*
1527	B	alcohol	2-Nonanol	0.05	0.03	a	0.05	0.02	a	*a*	0.20	0.08	c	*b*	0.15	0.07	b	0.32	0.13	d	*b*
1547	C	alcohol	E-2-Caren-4-ol	0.04	0.08	a	0.09	0.05	ab	*a*	0.12	0.15	b	*b*	0.05	0.05	ab	0.06	0.05	ab	*a*
1672	B	alcohol	1-Nonanol	0.28	0.13	a	0.32	0.14	ab	*a*	0.61	0.39	c	*c*	0.39	0.19	ab	0.49	0.19	bc	*b*
1731	A	alcohol	**Methionol**	0.23	0.09	b	0.31	0.12	c	*b*	0.07	0.07	a	*a*	0.07	0.08	a	0.06	0.04	a	*a*
1749	B	alcohol	4-Methyl-4-heptanol	0.05	0.05	ab	0.03	0.01	a	*b*	0.02	0.02	a	*a*	0.12	0.11	c	0.08	0.02	bc	*c*
1773	B	alcohol	1-Decanol	0.07	0.07	ab	0.03	0.02	a	*a*	0.17	0.12	c	*b*	0.08	0.04	ab	0.12	0.04	bc	*a*
1385	A	aldehyde	**Nonanal**	0.21	0.12	a	0.41	0.16	b	*a*	0.40	0.17	b	*b*	0.43	0.26	b	0.43	0.18	b	*b*
1453	A	aldehyde	**Furfural**	2.31	2.65	a	2.43	0.90	a	*a*	25.62	9.16	b	*b*	32.15	13.09	b	42.59	13.39	c	*c*
1514	A	aldehyde	**Benzaldehyde**	1.56	1.98	a	1.15	0.25	a	*a*	29.02	12.20	b	*b*	29.87	11.66	b	47.65	12.82	c	*c*
1567	A	aldehyde	5-Methylfurfural	0.21	0.16	a	0.13	0.05	a	*a*	3.82	1.43	b	*b*	3.34	1.55	b	5.44	2.49	c	*b*
1605	B	aldehyde	1-Ethyl-1H-pyrrole-2-carbaldehyde	0.03	0.03	a	0.04	0.01	a	*a*	0.13	0.07	b	*b*	0.18	0.16	b	0.35	0.16	c	*c*
1702	C	aldehyde	3-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-2-propenal	0.29	0.19	a	0.33	0.18	a	*a*	0.74	0.31	bc	*b*	0.68	0.37	b	0.96	0.27	c	*b*
1779	B	aldehyde	Benzenebutanal	0.03	0.04	ab	0.00	0.00	a	*a*	0.07	0.08	bc	*b*	0.11	0.11	c	0.01	0.00	a	*b*
1956	B	aldehyde	**(E)-3-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-2-propenal**	0.14	0.09	a	0.10	0.04	a	*a*	0.15	0.05	a	*a*	0.32	0.16	c	0.23	0.08	b	*b*
1058	A	ester	Ethyl butyrate	3.84	2.09	a	2.95	0.90	a	*a*	2.85	1.67	a	*a*	6.91	4.45	b	4.47	0.85	a	*b*
1070	A	ester	**Ethyl 2-methylbutyrate**	2.86	2.11	a	1.92	0.84	a	*b*	1.36	0.92	a	*a*	5.61	4.35	b	3.13	1.55	a	*c*
1081	A	ester	**Ethyl isovalerate**	6.86	4.26	ab	4.65	1.90	ab	*b*	3.78	1.95	a	*a*	11.92	8.48	c	7.84	3.06	b	*c*
1191	B	ester	Isopentyl isobutyrate	0.44	0.31	bc	0.34	0.18	abc	*b*	0.15	0.09	a	*a*	0.54	0.49	c	0.26	0.09	ab	*b*
1227	A	ester	Ethyl hexanoate	34.62	15.18	a	45.38	17.72	a	*a*	35.70	18.79	a	*a*	43.84	27.70	a	32.92	9.03	a	*a*
1252	A	ester	**Ethyl pyruvate**	0.02	0.01	a	0.02	0.00	a	*a*	0.12	0.05	b	*b*	0.14	0.08	b	0.13	0.03	b	*b*
1289	A	ester	2-Methylbutyl isovalerate	0.19	0.13	a	0.11	0.09	a	*a*	0.11	0.08	a	*a*	0.39	0.37	b	0.23	0.14	a	*b*
1298	B	ester	Methyl 2-hydroxy-2-methylbutanoate	0.02	0.02	a	0.03	0.01	a	*a*	0.04	0.03	a	*b*	0.09	0.04	b	0.12	0.07	c	*c*
1325	A	ester	Ethyl heptanoate	0.25	0.14	a	0.24	0.06	a	*a*	0.35	0.13	a	*b*	0.62	0.27	b	0.81	0.27	c	*c*
1381	A	ester	**Methyl octanoate**	0.07	0.05	a	0.10	0.06	b	*a*	0.14	0.10	ab	*b*	0.09	0.05	ab	0.09	0.03	ab	*ab*
1400	B	ester	Ethyl 2-hydroxybutanoate	0.02	0.03	a	0.01	0.00	a	*a*	0.03	0.02	a	*a*	0.13	0.10	b	0.12	0.09	b	*b*
1413	B	ester	Ethyl glycolate	0.01	0.01	a	0.01	0.00	a	*a*	0.07	0.03	b	*b*	0.08	0.07	b	0.19	0.11	c	*c*
1421	B	ester	**Ethyl 2-hydroxyisovalerate**	0.65	0.68	a	0.46	0.19	a	*a*	0.35	0.25	a	*a*	1.76	1.21	b	1.44	0.49	b	*b*
1433	A	ester	Ethyl octanoate	55.90	27.74	a	89.29	43.15	b	*a*	104.75	53.29	b	*b*	83.56	48.84	ab	102.50	30.23	b	*b*
1484	B	ester	**Ethyl diethoxyacetate**	0.01	0.01	a	0.00	0.00	a	*a*	0.09	0.04	b	*b*	0.11	0.04	b	0.18	0.05	c	*c*
1505	B	ester	Methyl 3-methoxypropionate	0.10	0.09	a	0.11	0.05	a	*a*	0.51	0.40	b	*b*	0.39	0.43	b	1.12	0.44	c	*b*

(*continued on next page*)

Table 3 (*continued*)

				Biological							Oxidative				Biological + Oxidative						
				FI[d]			MA				OL				AM			PC			
LRI[b]	ID[c]	Chemical family	Volatile compounds[a]	mean	±SD[e]	WT[f]	mean	±SD	WT	*AT*[g]	mean	±SD	WT	*AT*	mean	±SD	WT	mean	±SD	WT	*AT*
1533	B	ester	**Ethyl 2-hydroxy-4-methylvalerate**	1.36	1.47	a	0.86	0.24	a	*a*	0.67	0.50	a	*a*	3.55	2.76	b	2.74	0.91	b	*b*
1543	B	ester	**Ethyl dl-2-hydroxycaproate**	1.77	1.51	ab	2.04	0.39	ab	*b*	0.98	0.39	a	*a*	3.28	2.01	c	2.84	0.93	bc	*c*
1576	C	ester	Diethyl ethylmalonate	0.04	0.06	a	0.02	0.02	a	*a*	0.03	0.02	a	*a*	0.15	0.16	b	0.11	0.06	b	*b*
1606	B	ester	Ethyl levulinate	0.03	0.02	a	0.02	0.00	a	*a*	0.29	0.24	b	*b*	0.27	0.29	b	0.20	0.08	b	*b*
1612	B	ester	Ethyl 2-furoate	0.80	0.57	a	0.91	0.14	a	*a*	1.46	0.55	b	*b*	2.13	0.82	c	2.56	1.03	c	*c*
1631	B	ester	Ethyl methyl succinate	0.07	0.03	a	0.04	0.01	a	*a*	0.19	0.08	b	*b*	0.20	0.14	b	0.22	0.07	b	*b*
1638	A	ester	**Ethyl decanoate**	1.36	0.77	b	0.53	0.09	a	*a*	1.24	0.35	b	*a*	2.38	1.14	c	2.37	0.65	c	*b*
1661	A	ester	Ethyl benzoate	0.75	0.43	a	0.56	0.16	a	*a*	2.58	0.96	b	*b*	3.38	1.31	b	6.03	2.64	c	*c*
1682	B	ester	Diethyl succinate	49.15	17.82	a	43.29	8.61	a	*a*	91.93	21.15	b	*b*	107.74	49.88	b	151.34	38.71	c	*c*
1712	C	ester	Ethyl 1-methyl-4-oxo-2-cyclohexene-1-carboxylate	0.01	0.01	a	0.01	0.00	a	*a*	0.04	0.04	ab	*b*	0.05	0.05	b	0.17	0.13	c	*c*
1755	C	ester	Butyl isobutyl succinate	0.02	0.02	a	0.01	0.01	a	*a*	0.20	0.09	b	*b*	0.37	0.37	c	0.67	0.38	d	*c*
1759	B	ester	Butyl ethyl succinate	0.03	0.02	a	0.02	0.01	a	*a*	0.11	0.04	b	*b*	0.15	0.06	c	0.23	0.08	d	*c*
1778	C	ester	Ethyl 2-formyl-2-methyl-4-pentenoate	0.01	0.01	a	0.01	0.00	a	*a*	0.11	0.10	b	*b*	0.12	0.13	b	0.47	0.28	c	*c*
1782	B	ester	Diethyl glutarate	0.61	0.32	a	0.45	0.20	a	*a*	1.06	0.34	b	*b*	1.93	0.97	c	2.48	0.64	d	*c*
1858	C	ester	Diethyl 2-hydroxy-3-methylsuccinate	0.25	0.16	a	0.21	0.17	a	*a*	1.31	0.58	b	*b*	1.65	1.00	b	3.12	1.65	c	*c*
1993	C	ester	Ethyl 3-hydroxy-3-methylbutanoate	0.07	0.06	a	0.03	0.01	a	*a*	0.28	0.15	b	*b*	0.51	0.33	c	0.87	0.36	d	*c*
2034	B	ester	**Isopropyl myristate**	0.18	0.14	b	0.07	0.03	a	*b*	0.37	0.17	c	*c*	0.07	0.04	a	0.10	0.04	ab	*a*
2047	B	ester	**Diethyl malate**	0.26	0.18	ab	0.21	0.18	a	*a*	4.54	2.27	c	*c*	1.52	1.44	b	5.96	3.30	d	*b*
2051	B	ester	Methyl 3-hydroxydecanoate	0.00	0.00	a	0.00	0.00	a	*a*	0.04	0.03	b	*b*	0.03	0.04	b	0.11	0.06	c	*b*
2121	B	ester	Ethyl pentadecanoate	0.00	0.01	a	0.00	0.00	a	*a*	0.01	0.01	a	*a*	0.03	0.04	b	0.00	0.00	a	*b*
2182	B	ester	Ethyl 5-oxooxolane-2-carboxylate	0.37	0.18	a	0.46	0.34	a	*a*	1.21	0.44	b	*b*	1.00	0.55	b	2.07	0.87	c	*b*
2238	B	ester	(+)-Diethyl L-tartrate	0.11	0.11	a	0.12	0.12	a	*a*	0.60	0.46	b	*b*	0.86	0.57	b	1.17	0.63	c	*c*
1350	B	ketone	3-Nonanone	0.01	0.01	a	0.01	0.00	a	*a*	0.17	0.21	b	*c*	0.09	0.06	ab	0.13	0.07	b	*b*
1383	B	ketone	2-Nonanone	0.16	0.14	a	0.48	0.66	b	*a*	0.70	0.51	b	*c*	0.49	0.24	b	0.65	0.19	b	*b*
1395	C	ketone	5,6-Dihydro-4-(2,3-dimethyl-2-buten-1-yl)–2H-pyran-2-one	0.17	0.15	a	0.19	0.15	a	*a*	0.14	0.11	a	*a*	0.43	0.40	b	0.24	0.15	a	*b*
1489	C	ketone	2-Hydroxy-1-phenylbutan-1-one	0.11	0.05	ab	0.12	0.02	ab	*a*	0.10	0.04	ab	*a*	0.09	0.04	a	0.12	0.03	b	*a*
1498	B	ketone	Acetylfuran	0.22	0.13	a	0.15	0.02	a	*a*	0.53	0.22	b	*b*	0.90	0.41	c	1.09	0.50	c	*c*
1528	B	ketone	4-Undecanone	0.01	0.01	a	0.01	0.00	a	*a*	0.06	0.03	b	*b*	0.12	0.10	c	0.12	0.05	c	*c*
1618	C	ketone	1,1-Diethoxypropan-2-one	0.11	0.10	a	0.12	0.04	a	*a*	0.39	0.25	b	*b*	0.33	0.28	b	0.77	0.29	c	*b*
1646	B	ketone	3-Nonen-5-one	0.02	0.04	a	0.02	0.03	a	*a*	0.90	1.20	b	*c*	0.29	0.29	a	0.83	0.38	b	*b*
1647	A	ketone	Acetophenone	0.08	0.05	a	0.10	0.05	a	*a*	0.45	0.26	b	*b*	0.45	0.42	b	0.74	0.25	c	*b*
1692	B	ketone	2-Nonen-4-one	0.01	0.01	a	0.01	0.01	a	*a*	0.11	0.09	b	*c*	0.05	0.04	a	0.11	0.03	b	*b*
1756	C	ketone	7-Ethyl-2-methyl-4-undecanone	0.10	0.08	abc	0.09	0.02	ab	*b*	0.04	0.02	a	*a*	0.16	0.12	c	0.12	0.04	bc	*c*
1789	C	ketone	Benzocyclobuten-1(2H)-one	0.03	0.04	a	0.05	0.03	a	*a*	0.12	0.12	ab	*b*	0.17	0.21	b	0.62	0.33	c	*c*
1629	A	lactone	γ-Butyrolactone	0.64	0.27	a	0.50	0.08	a	*a*	0.59	0.30	a	*a*	1.10	0.56	b	1.18	0.30	b	*b*
1724	B	lactone	**4-Ethoxy-γ-butyrolactone**	0.01	0.00	a	0.01	0.00	a	*a*	0.12	0.12	b	*b*	0.15	0.16	b	0.26	0.12	c	*b*
1727	B	lactone	γ-Ethoxybutyrolactone	0.01	0.01	a	0.01	0.01	a	*a*	0.11	0.07	b	*b*	0.09	0.09	b	0.23	0.11	c	*b*
2037	B	lactone	**γ-Nonalactone**	0.01	0.01	a	0.01	0.00	a	*a*	0.03	0.03	a	*b*	0.04	0.05	a	0.15	0.10	b	*c*
2062	B	lactone	Solerone	0.04	0.06	a	0.01	0.00	a	*a*	0.07	0.07	a	*a*	0.23	0.28	b	0.05	0.01	a	*b*
1156	C	other	4,5-Dimethyl-2-pentadecyl-1,3-dioxolane	0.36	0.32	a	0.28	0.07	a	*a*	2.39	1.38	b	*b*	4.22	3.07	c	5.59	3.15	c	*c*
1243	C	other	1,1-Diethoxy-2-methylpropane	0.06	0.05	a	0.05	0.01	a	*a*	0.47	0.28	b	*b*	0.61	0.35	b	0.87	0.34	c	*c*
1278	C	other	**LRI1278**	1.49	0.53	a	1.52	0.31	a	*a*	2.50	0.72	b	*c*	1.55	0.55	a	3.27	0.95	c	*b*
1308	B	other	2,2-Diethyl-1,3-dioxolane	0.02	0.02	a	0.01	0.00	a	*a*	0.15	0.07	b	*b*	0.36	0.33	c	0.27	0.07	bc	*c*
1420	C	other	Valeric anhydride	0.05	0.06	a	0.03	0.01	a	*a*	0.11	0.05	b	*b*	0.18	0.08	c	0.24	0.12	d	*c*
1445	A	other	E-Linalool oxide (furanoid)	0.19	0.14	ab	0.33	0.05	c	*b*	0.11	0.07	a	*a*	0.27	0.20	bc	0.31	0.16	c	*c*
1467	B	other	**Nerol oxide**	0.06	0.08	a	0.22	0.08	b	*a*	0.08	0.07	a	*a*	0.08	0.11	a	0.08	0.07	a	*a*
1522	B	other	Vitispirane	4.89	3.54	ab	7.71	3.96	b	*ab*	6.14	5.57	ab	*b*	4.05	3.48	a	2.99	2.17	a	*a*
1549	B	other	Linalool	0.11	0.14	b	0.04	0.03	ab	*b*	0.01	0.02	a	*a*	0.01	0.01	a	0.01	0.01	a	*a*
1582	B	other	**Methyl thymol**	0.01	0.03	a	0.14	0.21	b	*a*	0.02	0.02	a	*a*	0.02	0.03	a	0.03	0.04	a	*a*

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Table 3 (*continued*)

				Biological						Oxidative				Biological + Oxidative						
				FI[d]			MA			OL				AM			PC			
LRI[b]	ID[c]	Chemical family	Volatile compounds[a]	mean	±SD[e]	WT[f]	mean	±SD	WT	*AT*[g]	mean	±SD	WT	*AT*	mean	±SD	WT	mean	±SD	WT	*AT*
1593	B	other	**Isothymol methyl ether**	0.04	0.14	a	1.17	2.41	b	*a*	0.03	0.03	a	*a*	0.08	0.15	a	0.07	0.09	a	*a*
1739	A	other	TDN (1, 1, 5-Trimethyl-1, 2-dihydronaphthalene)	3.01	2.73	ab	4.81	3.07	ab	*a*	6.19	7.12	b	*b*	2.61	2.36	a	2.37	1.18	a	*a*
1819	B	other	(E)-1-(2,3,6-trimethylphenyl) buta-1,3-diene (TPB)	0.09	0.10	a	0.08	0.03	a	*a*	0.25	0.19	b	*b*	0.06	0.06	a	0.27	0.07	b	*a*
1835	C	other	1,1-Diethoxyacetone	0.03	0.03	a	0.01	0.00	a	*a*	0.25	0.22	c	*c*	0.21	0.30	bc	0.09	0.04	ab	*b*
1936	C	other	(1-Ethyl-1-propenyl) benzene	0.02	0.02	a	0.02	0.01	a	*a*	0.51	0.23	b	*b*	0.41	0.25	b	0.90	0.30	c	*b*
2246	B	other	2,5-Dihydrothiophene	2.38	2.33	ab	2.22	1.35	ab	*b*	0.66	0.61	a	*a*	3.52	3.42	b	2.73	1.22	b	*c*

Notes: [a]Volatile compounds in bold are the 28 volatile compounds with VIP value ≥ 2 obtained by the *PLS-DA101* model. [b]LRI: linear retention index. [c]ID: identification: A, LRI agreed with standards, and mass spectrum with NIST mass spectral data base; B, mass spectrum agreed with NIST mass spectral data base and LRI agreed with the literature; C, mass spectrum agreed with mass spectral data base and LRI was not described in literature for polar column or did not match. [d]FI: Fino, MA: Manzanilla, OL: Oloroso, AM: Amontillado, PC: Palo Cortado. [e]SD: standard deviation. [f]WT and [g]AT: Tukey test results. Different lower-case letters in different "WT" columns indicate significant differences according to Tukey's test ($p < 0.05$) among fortified wine types. Different cursive lower-case letters in different "*AT*" columns indicate significant differences according to Tukey's test ($p < 0.05$) among ageing types.

organized: 1) wines produced by biological ageing (FI and MA); 2) wines produced by oxidative ageing (OL); 3) wines produced by mixed ageing (AM and PC).

Furthermore, looking at the loadings plot (Fig. 3**b**), it could be seen that one of the reasons of the differentiation of the samples is that those with oxidative ageing, either only oxidative or biological and oxidative, seemed to show a richer volatile profile, i.e., showing a higher relation of most of the volatile compounds than wines produced only biologically, being most of the selected volatile compounds in the positive side of PC1. Moreover, it could be also seen in the loadings plot the higher relation of esters and aldehydes with OL, AM and PC types, and acids with biological samples, being in line with the above discussion.

3.2.1. *Differentiation of wines produced by biological ageing: Fino and Manzanilla*

The most extensive literature on fortified wines belongs to studies on the Fino wine type (Zea et al., 2007, 2010a, 2010b). Our results highlighted, as shown in Fig. 1 and Fig. 2, that the esters group has a higher presence in the fortified wines produced by biological ageing. This is mainly because of the *flor* yeasts metabolic activity and because they may undergo autolysis, releasing fatty acids among other molecules to the wine and contributing to esters synthesis (Charpentier et al., 2004). However, by looking at the total mean of relative areas and not the percentage of each type, wines with mixed ageing showed higher mean relative area for the selected esters (**Fig. S2**). This was mainly due to the huge relative area that presented some esters from fixed or non-volatile acids (Table 3). In contrast, several other esters achieved a VIP score above 2 in the PLS-DA model and could be defined as specific markers of biological ageing (highlighted in bold letters in Table 3**,** and summarized in **Table S1**), which were mainly derived from volatile acids. This is the case of ethyl 2-methyl butyrate, ethyl isovalerate, and isopropyl myristate, showing higher contents with respect to the oxidative ageing, however, lower amounts than the mixed ageing. This could be explained by the fact that those compounds, mainly formed by biological ageing, are concentrated during the oxidative period of ageing that the mixed fortified wines suffer. These compounds have been described with a fruity odour contribution (**Table S1**), which is an aroma that has shown to be strongly related to the biological ageing process due to the *flor* yeast activity (Zea et al., 2007).

On the contrary, some of the esters with a VIP ≥ 2, stood out because of their relatively low amounts in the biological samples compared to the other types of wines. This was the case of ethyl pyruvate, ethyl diethoxyacetate and diethyl malate, which presented relatively higher areas for wines with oxidation ageing (Fig. 2), and which could contribute to the wine with caramellike notes (**Table S1**). These results agree with those from Zea et al. (2001) who determined diethyl malate and pyruvate in much lower quantities in Fino-type wines compared to Oloroso-type wines (Zea et al., 2001). In fact, in another work carried out also by Zea et al. (2007), evaluating volatile compounds in Fino wines along ageing time, ethyl pyruvate was found with an odour activity value (OAV) < 0.1 in all the ageing times analysed (Zea et al., 2007), meaning that the concentration of that compound in Fino wines was relatively low compared to its odour threshold, that is, it might not significantly contribute to the overall aroma or flavor perception of these wines. As was previously discussed, oxidative ageing seems to enhance the esterification of non-volatile acids, giving rise to these esters, in contrast to the majority of esters in biological samples, which are derived from volatile acids.

Regarding the group of alcohols, FI and specially MA, presented a relatively highlighted presence of alcohols in comparison to the other wines (Fig. 1, Fig. 2, Table 3). This had been previously assigned due to the *flor yeast* metabolic activity and autolysis releasing amino acids, precursors of higher alcohols (Zea et al., 1995). Among the alcohols with VIPs ≥ 2 in the PLS-DA2, it was found that heptanol, (Z)-3-hexen-1-ol, (S)-3-ethyl-4-methylpentanol and methionol were present in MA in significantly higher amounts than in the rest of the wines (Table 3). It

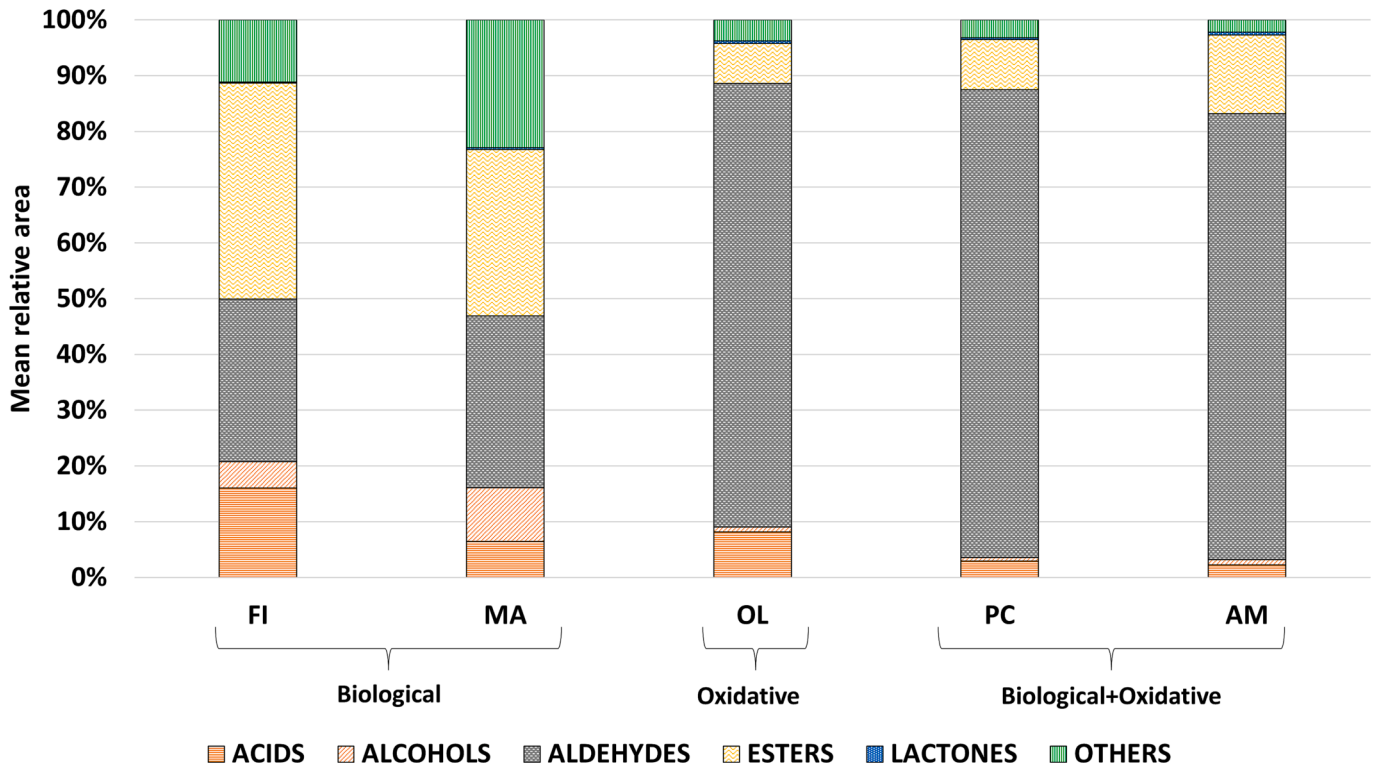

Fig. 1. Volatile profile of each type of fortified wine with respect to the mean of relative areas (%) considering the 28 VIP volatile compounds selected as differentiators after the second PLS-DA analysis. FI: Fino; MA: Manzanilla; OL: Oloroso; AM: Amontillado; PC: Palo Cortado.

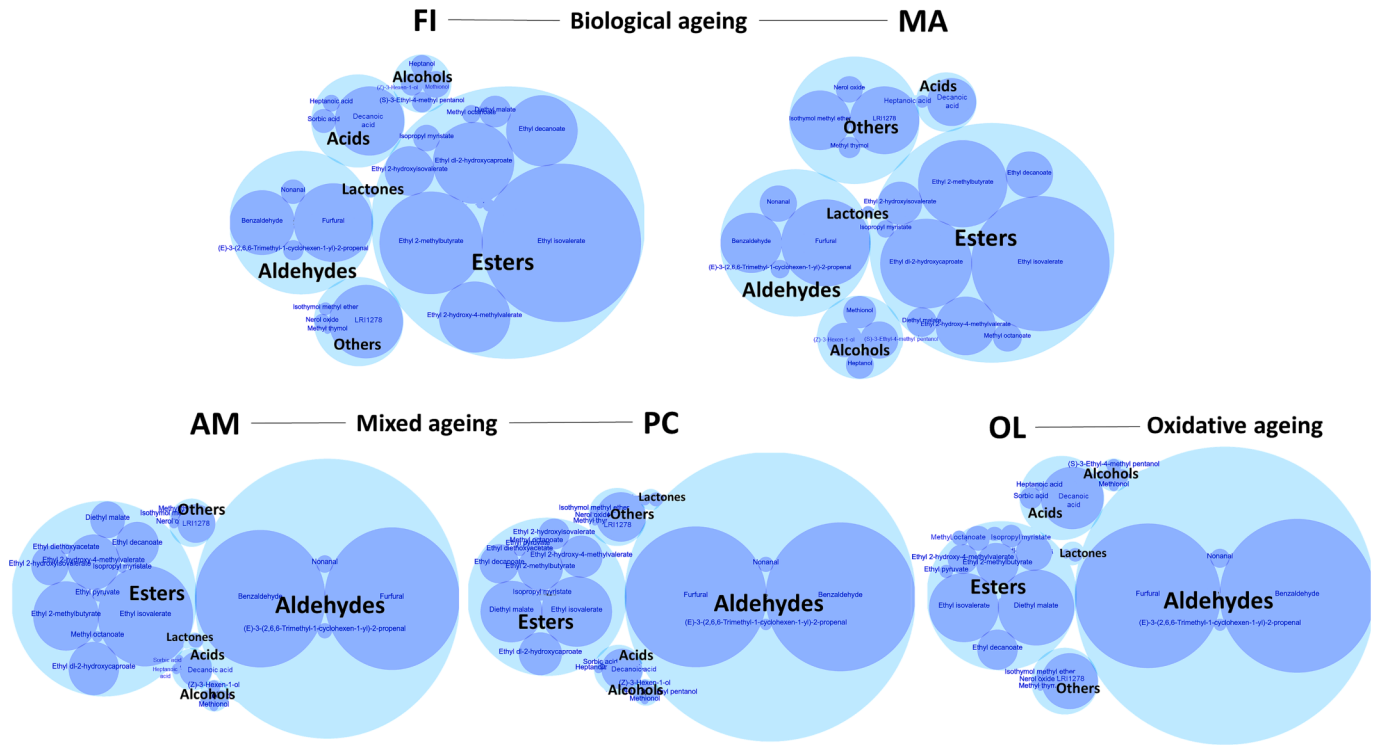

Fig. 2. Circle packing plots of the volatile profiles of each type of wine obtained by including only the 28 VIP volatile compounds selected from the second PLS-DA model. FI: Fino; MA: Manzanilla; OL: Oloroso; AM: Amontillado; PC: Palo Cortado.

should be noted that, although higher alcohols tend to have very high perception thresholds (above 100 mg/L) and are therefore unlikely to contribute directly to wine flavour, these specific alcohols have been shown to have the lowest odour thresholds among alcohols, between 0.5 and 2.5 mg/L (e.g., for methionol and heptanol, respectively) (Zea et al., 2007). Among them, heptanol and (Z)-3-hexen-1-ol have been described with green and fresh odour notes (**Table S1**), while the methionol is considered an *off-flavor* with attributes of potato and cauliflower giving

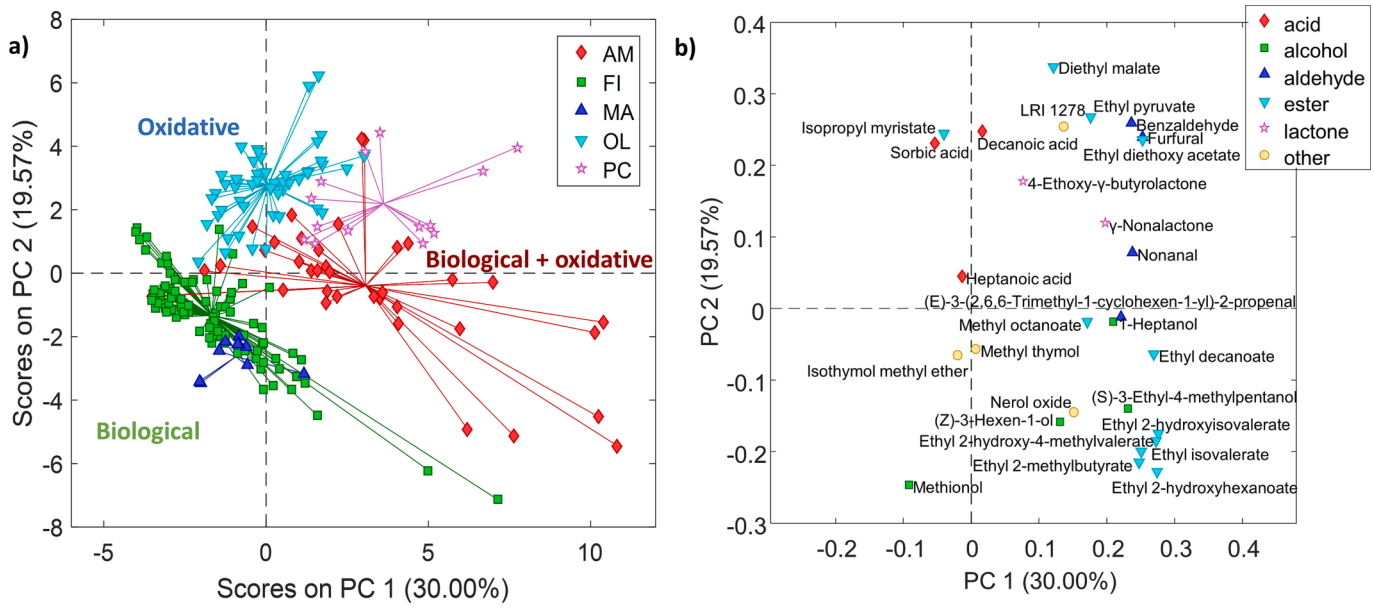

Fig. 3. Scores (a) and loadings (b) plots of the PCA model performed with the total of samples and the 28 VIP volatile compounds obtained from the second PLS-DA model. Samples coloured according to their type of ageing. AM: Amontillado (red); FI: Fino (green); MA: Manzanilla (dark blue); OL: Oloroso (clear blue); PC: Palo Cortado (pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vegetal notes to the wine (Zea et al., 2007). Thus, this compound was also found in high relative areas in FI samples with respect to the other types of wine, being significatively higher its presence in biological samples than in the rest (Table 3). This compound has shown to present concentrations in Fino wines above its odour threshold (Moyano et al., 2002), appears after 2.5 years of ageing, and continuously increases in a great extent during this stage of the production process (Zea et al., 2007). Our results revealed this compound as the strongest marker of biological ageing (Table 3) since the rest of the wines, in which oxidation has taken place, it was present in significantly lower amounts. The high production of methionol in biologically aged wines is expected since the *flor* yeasts have the difficult task of prevailing in a medium poor in available nitrogen, this results in the yeasts having to consume amino acids present in the wine to survive, methionine in this case, giving rise to methionol through the Ehrlich pathway (Panighel & Flamini, 2014). Likewise, the low presence of methionol in oxidized wines could be due to the oxidation to methional (Waterhouse et al., 2016), or to the esterification of acetates. It is also very dependent on the yeast strain employed to produce Fino wines (Morales et al., 2020). Similar results had been shown in the study of Moyano et al. (2010) after analysing the volatile profile during the sequential biological and oxidative ageing typical of the Amontillado wine, showing that after the fortification and beginning of the oxidative stage, methionol was not detected (Moyano et al., 2010). Conversely to these logical results, Zea et al. (2001), reported that Oloroso and Amontillado wines had higher significant quantities of methionol than Fino wines (Zea et al., 2001).

Regarding the other chemical families, although the group of acids and the group of others showed a high percentage in the volatile profile of biological samples (Fig. 2), the volatile compounds included in those two groups were more relevant in the differentiation of FI and MA, as will be discussed later.

Thus, considering that the elaboration process of Fino and Manzanilla is basically the same with the origin of the grape and the location of the cellar as the only variables, Jerez de la Frontera and Sanlúcar de Barrameda, respectively, located both in Cádiz (Andalusia), there has been an increased interest to define chemical differences among them. Surprisingly, as far as we know, no studies have been carried out to deepen this aspect. For this purpose, a PCA was performed with the 28 selected volatile compounds including only wines from these two types (Fig. 4**a and b**). It was found that plotting of PC2 vs PC4 in the scores plot, it could be seen a trend of grouping of the samples into both types of wines (Fig. 4**a**). Manzanillas were in the upper left quadrant defined by (Z)-3-hexen-ol, methyl octanoate, ethyl decanoate, methyl thymol, isothymol methyl ether, among others. These last two compounds caught our attention among the others. Methyl thymol and isothymol methyl ether have been described in the essential oil of plants such as Thyme (Asllani & Toska, 2003; Jan et al., 2020), as well as in some wines (Ruiz-Bejarano, et al., 2016). Their aroma description is herbal/green (**Table S1**) and are structurally related to the monoterpene thymol and this last, in turn, with p-cymene and carvacrol, which have been found in grapes and wines (Perestrelo et al., 2014; Ruiz-Bejarano et al., 2013; Zea et al., 2007). They have been also described as volatile compounds that can suffer a migration from the cork to the wine (Díaz-Maroto, et al., 2023). These compounds would be among the more useful to differentiate among these two types of fortified wines (Fig. 2), showing significant differences in their relative values (Table 3). Moreover, two esters resulted suitable for this differentiation since the significantly higher presence of ethyl decanoate in FI and methyl octanoate, again with a green odour character (**Table S1**), in MA were notably useful (Table 3). In addition, two acids also showed significant differences between FI and MA, heptanoic acid and sorbic acid, with relative areas significantly higher in FI wines.

3.2.2. *Differentiation of wines produced by oxidative ageing: Oloroso*

The analysis of wines produced exclusively with oxidative ageing presented significant differences with respect to the wines biologically aged. Among the 28 VIPs selected, decanoic and sorbic acids showed significatively higher relative values in OL samples than in the other types (Table 3). It should be highlighted that OL samples are usually aged during longer periods of time than biological samples, whose ageing time is shorter due to the early disappearance of the 'velo de flor', and this could explain the higher presence of some compounds in this type of samples. Thus, as expected, some aldehydes also increased with ageing, mainly due to the oxidation of alcohols, among other factors such as the degradation of the wood lignin that takes place during the ageing, as well as the concentration phenomena during ageing (García-Moreno et al., 2021). The higher presence of aldehydes in OL samples represented one of the main differences with respect to biological ageing

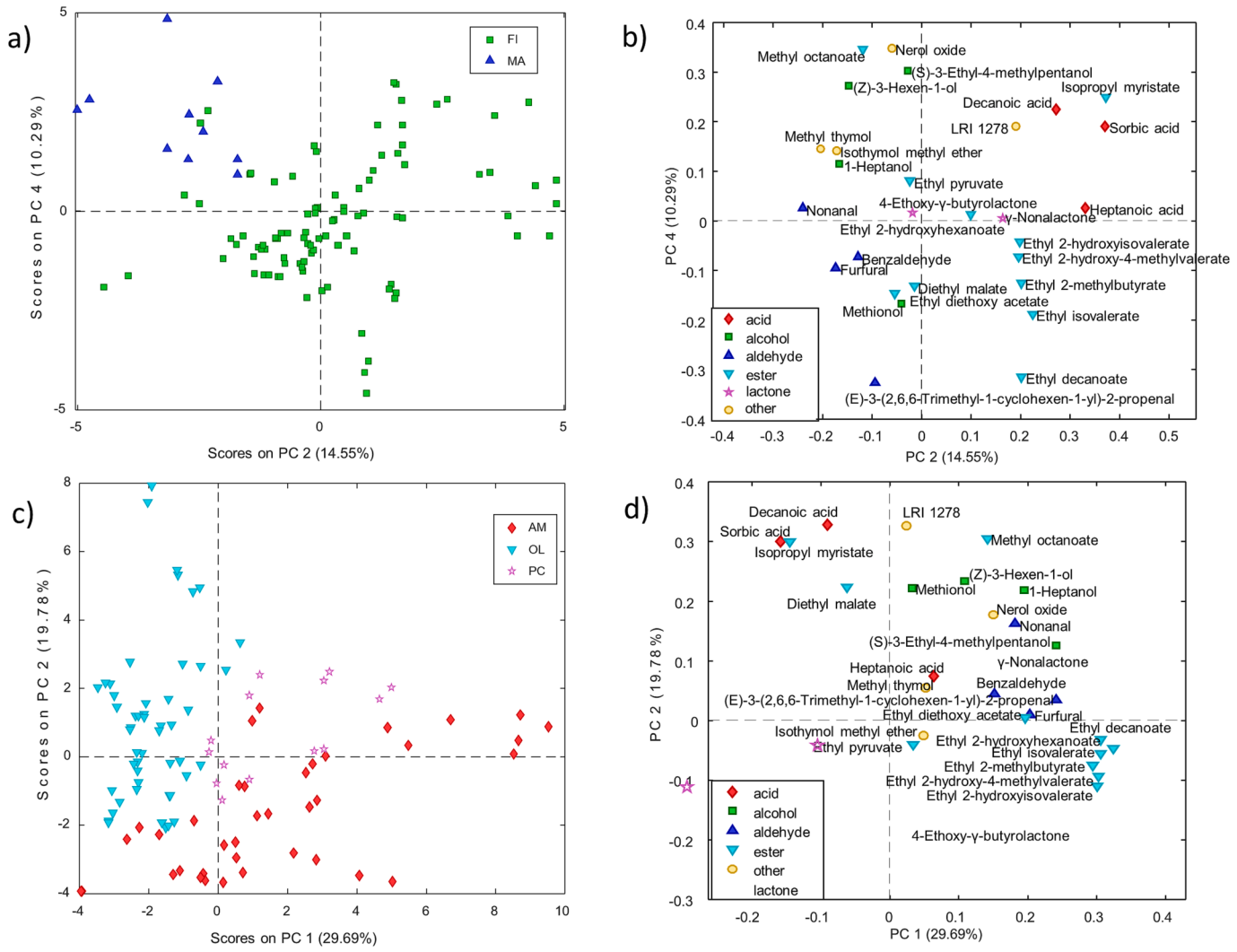

Fig. 4. Scores (a) and loadings (b) plots of the PCA model performed with biological samples (Fino-FI, and Manzanilla-MA), and the 28 VIP volatile compounds obtained from the second PLS-DA model; scores (c) and loadings (d) plots of the PCA model performed with oxidative and mix samples (Amontillado-AM, Palo Cortado-PC and Oloroso-OL), and the 28 VIP volatile compounds obtained from the second PLS-DA model.

(Fig. 1, Fig. 2), which could be partially explained by the fact that OL samples suffer a longer period of ageing than biological wines. Three of them were VIPs, which were benzaldehyde, nonanal and furfural. The last one, together with 5-methylfurfural belongs to the furfural family and showed the highest relative areas in the OL with respect to the MA and FI, biologically aged (Table 3). These furfurals are due to Maillard reactions and the extraction from the wood (Guerrero-Chanivet et al., 2020), and had been found to increase in the OL wines (García-Moreno et al., 2021; Zea et al., 2001). Furfural is a strong differentiating compound among biological and oxidative ageing as can be observed in Fig. 2, since furfural was found in a significative much lower amount in FI and MA, in fact, this compound has been reported to appear after 1.5 years in Fino wines (Zea et al., 2007) and is not even perceived until 4.5 years of ageing (Zea et al., 2010b). In addition, among the aldehydes, benzaldehyde showed a significantly higher content in oxidative samples compared to biologically aged wines since this aldehyde is another typical compound from the oxidative process in wine (Ferreira et al., 1997). These aldehydes have been described with an aroma description of nuts such as walnuts or almonds (**Table S1**).

Regarding the esters, it was observed higher contents of some of them in OL samples, such as ethyl pyruvate and diethyl malate, but also lower contents of others as were the cases of ethyl 2-methyl butyrate and ethyl isovalerate (Table 3), which was also observed by other authors (García-Moreno et al., 2021; Zea et al., 2001). It has been seen that esters formed by the esterification of volatile acids has shown to be strongly affected during oxidation and tend to decrease in this type of samples, while they are more presented in biological wines (Patrianakou & Roussis, 2013). Thus, oxygen exposure during ageing of OL samples plays a crucial role in promoting the oxidative degradation of fatty acid esters. Moreover, the degradation of fatty acid esters in fortified wines could be also influenced by the acidity of the sample. Thus, higher acidity levels, typically indicated by lower pH values, promote the hydrolysis and oxidation of esters (Makhotkina & Kilmartin, 2012). In contrast, in wine types such as OL, it has been observed increases of esters formed by non-volatile acids, such as succinic acid. In this context, is interesting to remark the great presence of diethyl succinate and diethyl tartrate in oxidative samples (Table 3), also described by Zea et al. (2001) (Zea et al., 2001), which could proceed from the longer aging time under OL samples are submitted in comparison to biological samples which usually are less aged.

Ketones were also found in higher amounts in OL compared to FI and MA, such as 2-nonanone and 3-nonanone and the tentatively identified 3-nonen-5-one, and 2-nonen-4-one (Table 3). These carbonyls use to be formed during the oxidation of alcohols therefore their higher presence in OL wines is justified by the process itself (Waterhouse et al., 2016). Finally, TDN (1, 1, 5-Trimethyl-1, 2-dihydronaphthalene), was found in

the highest quantity in OL wines. This compound is present in the grape in its glycosidic precursor form, linked to a sugar moiety. The high presence of TDN in other oxidized wines such as Porto wines has been also reported by other authors, showing an increasing in concentration during ageing (Silva Ferreira et al., 2003). Thus, the higher presence of TDN in OL wines could be explained not only by the low pH of these samples, which favours its formation, but above all by the concentration effect produced both by the precursors and the TDN itself during their longer ageing, also favoured by the presence of oxygen (Silva Ferreira & Guedes de Pinho, 2004).

Moreover, OL samples also showed differences in comparison to the other two types of fortified samples that had an oxidative period, AM and PC, as it could be observed in the scores plot of the PCA model made without the biologically aged wines, FI and MA, and the 28 selected volatile compounds **(**Fig. 4**c)**. This difference could be derived from the significant higher relative values in OL than in AM and PC for volatile compounds already mentioned such as decanoic and sorbic acid, isopropyl myristate and diethyl malate, as could be observed in the loadings plot (Fig. 4**d**) and in the statistical results in Table 3.

3.2.3. Differentiation of wines produced by mixed ageing: Amontillado and Palo Cortado

In this section, the results of the markers found in two different types of wines that have had biological and oxidative ageing at some stage of their production process will be discussed. As explained before, PC samples was halfway between AM and OL samples, which agrees with the process subjected. By visualising the volatile profile of the 28 selected volatile compounds in Fig. 2, this similarity can be observed with both. Thus, as can be also seen in the scores plot of the PCA (Fig. 4**c**), a suitable separation was achieved mainly between pure oxidative (OL) and mixed samples (AM and PC), although PC was right between both kinds of wines, being closer to AM samples. This revealed that, despite the shorter period of biological ageing in comparison to AM, it seems that significantly affected their volatile profile.

Loadings plot (Fig. 4**d**) undoubtedly points locate AM wines highly related to esters composition, some of them connected to biological ageing such as ethyl 2-methylbutyrate, ethyl isovalerate and ethyl 2-hydroxyhexanoate, and others even in significantly higher quantities than those found in FI and MA, such as ethyl 2-hydroxyisovalerate and ethyl 2-hydroxy-4-methylvalerate, being potential AM markers (Table 3). On the contrary, other authors (Zea et al., 2001, 2008) did not observe this specific trend in the esters group, however, they analysed a smaller number of samples and compounds. The prominent presence of esters in these AM wines, higher than in the PC samples, could make sense because they are subjected to a longer biological ageing in terms of time than the PC samples, which produces a higher amount of alcohols because of the *flor* yeast, which are then condensed with acids in the medium to create esters, which would concentrate during the oxidation stage. Moreover, sensory description analysis of AM wines has a very remarkable fruity component that would make sense with these results (**Table S1**) (Marcq & Schieberle, 2015). In the case of PC wines, also a remarkable presence of esters was found, however to a lesser extent than in AM wines (Table 3). Thus, despite being most of the esters in common with AM, in the case of PC is convenient to highlight the significantly increased presence of diethyl succinate, ethyl benzoate and diethyl glutarate compared to AM and also with OL. Similar results were obtained by Valcárcel et al. (2022) after the analysis of OL and PC wines, showing that diethyl succinate concentration in PC wines was higher and that it increased along the ageing time. Precursors of this typical ageing marker are succinic acid and ethanol (Valcárcel-Muñoz et al., 2022). Succinic acid is usually produced by *Saccharomyces cerevisiae* via the reductive tricarboxylic acid cycle under microaerobic or anaerobic conditions (Ito et al., 2014), which are found in biological ageing with the *flor* yeast. This, joint with the increment of ethanol in the media, could also favour the formation of this volatile marker of PC wines.

Moving along the ester's family, lactones are found, which are cyclic esters with odour descriptions of the aromatic range of caramel/peach/coconut quite different from the fresh fruit attributes of ethyl and acetate esters (Waterhouse et al., 2016). In general, it was observed higher amounts of lactones such as butyrolactone and 4-ethoxy-γ-butyrolactone in both AM and PC wines than in biological wines and also slightly higher than OL, together with solerone in AM and γ-nonalactone in PC samples (Table 3). Lactones can have a varietal origin, be extracted from the wood, or even be synthesized due to media conditions (Miller et al., 2022). Our results point to solerone as a possible marker of AM wines due to a significant higher presence in these samples than in the rest. It is a typical lactone found in sherry wines being associated with oxidation processes having a controversial impact on sherry wines aroma (Martin et al., 1991). Besides, the origin of γ-nonalactone, highly related (Fig. 4**d)** and significantly more present (Table 3) in PC wines, has been assigned to the grape variety (Fan et al., 2010), to oxidation processes (Mislata et al., 2020), and to the yeasts (Hernández-Orte et al., 2008), among others. The combination of a first biological ageing step followed by a second oxidative step could increase the concentration of lactones in AM and PC wines.

In addition, as it could be observed in Table 3, the amount of acetic acid in the PC wines was the highest among all the wines analysed, even more than OL, but not significantly, maybe partially due to the special biochemical changes and microbiological processes involved in the sensory deviation of 'sobretablas' wines during biological aging, which leads to the origin of special or rare Palo Cortado wines, different from the FI, MA and AM (Palacios et al., 2018). This is consistent with the fact that PC has a shorter biological aging period than FI, MA or AM, due to the fact that *flor* yeast decreases volatile acidity by acetic acidity, and also aging increases acidity by aging losses. (Valcárcel-Muñoz et al., 2022). Just as in the case of acetic acid, several aldehydes such as furfural, 5-methyl furfural and benzaldehyde appeared in wines with mixed ageing in higher amounts than the other wines, with PC wines accounting for the highest quantity. This relationship can be also observed in the loadings plot of Fig. 4**d**. Furthermore, the volatile markers of PC that locate the wine samples grouped in the PCA confirm the typicity and special characteristics of this unique wine.

4. Conclusions

In conclusion, this research has revealed, for the first time, that the volatile differentiating fingerprint can be used to discriminate the different types of PDO fortified wines according to the ageing type. Thus, by employing a strategy that focused on a reduced number of volatile compounds, the study achieved satisfactory discrimination of the wines both in calibration and cross-validation. Among the 345 volatile compounds initially detected, a subset of 28 compounds was identified by a multivariate approach as having the highest potential for differentiation among biological, oxidative, or mixed type of ageing process. Biological ageing was characterized by specific esters and a prominent marker, methionol, while markers such as methyl thymol and isothymol methyl ether distinguished between Fino and Manzanilla. Oxidative ageing was marked by higher levels of benzaldehyde, nonanal, furfural, ethyl pyruvate, diethyl malate and TDN compared to the biological samples, along with decanoic and sorbic acid, isopropyl myristate, and diethyl malate in comparison to mixed wines. Mixed ageing (biological followed by oxidative), exhibited volatile compounds like lactones in general and solerone in Amontillado and γ-nonalactone in Palo Cortado wines. Additionally, specific esters such as ethyl 2-hydroxyisovalerate and ethyl 2-hydroxy-4-methylvalerate in Amontillado, and diethyl succinate, ethyl benzoate and diethyl glutarate in Palo Cortado, proved useful as volatile markers for their identification. Furthermore, this approach highlighted the unique characteristics of Palo Cortado wines and revealed volatile compounds responsible for differentiating PDOs such as Manzanilla and Fino. Limitations of the present work such as the number of samples and the standardization of ageing times, let a wide range of possibilities for future research.

CRediT authorship contribution statement

Cristina Ubeda: Conceptualization, Methodology, Investigation, Writing – original draft. **David Cortejosa:** Investigation, Methodology, Data curation. **M. Lourdes Morales:** Visualization, Writing – review & editing. **Raquel M. Callejón:** Visualization, Writing – review & editing, Supervision. **Rocío Ríos-Reina:** Data curation, Formal analysis, Writing – original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113320.

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