

Grape musts differentiation based on selected aroma compounds using SBSE-GC-MS and statistical analysis

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Summary

Fifty-one aroma compounds in musts from 'Muscat Ottonel', 'Aligoté', 'Muscat of Alexandria' and 'Pedro Ximénez' white grapes have been determined, three of them identified for the first time in grapes. Two fingerprints for each cultivar, based in 6 groups of aroma compounds before and after acidic hydrolysis of the musts were obtained by Multiple Variable Analysis. Only 17 aroma compounds before and 21 after hydrolysis, were selected by their high discriminating power. The Principal Component Analysis carried out with data of these selected compounds provided two components explaining 85.11 % of the overall variance for free aroma compounds and 87.58 % for those obtained after hydrolysis of musts, allowing an objective differentiation of each cultivar.

Key words: grape; aroma compounds; differentiation; statistical analysis.

Abbreviations: AL: 'Aligoté'; CAS: Chemical Abstracts Service; CIS: Cooled injection system; DOP: Denomination of Origin Protected; GC: Gas chromatography; GI: Geographical indication; LRI: Linear retention index; MA: 'Muscat of Alexandria'; MO: 'Muscat Ottonel'; MS: Mass spectrometry; MSA: Multiple sample comparison; MSD: Mass selective detector; MVA: Multivariate Analysis; NIST: National Institute of Standards and Technology; PC: Principal component; PCA: Principal component analysis; PX: 'Pedro Ximénez'; SBSE: Stir Bar Sorptive Extraction; TDU: Thermal desorption unit.

Introduction

The aroma of wine is a mixture of volatile compounds originating from the grape-must (varietal and pre-fermentative aromas), from yeast during alcoholic fermentation (fermentative aromas) and from the aging processes (post-fermentative aromas). In this way, the information provided by analysis of aroma compounds, allows the establishment of objective criteria to certify the origin of the raw materials and wines from a specific Denominations of Origin Protected (DOP) or Geographical Indication (GI), to define the wine types and to differentiate among the brands in a particular wine type (ADDOR and GRAZIOLI 2002, MILDNER-SZKUDLARZK and JELEN 2008, DALL'ASTA *et al.* 2011, WEL-

DEGERGIS *et al.* 2011 and MORENO-GARCÍA *et al.* 2013). The main components of varietal wine aroma are the monoterpenols, more abundant in Muscat varieties, the C¹³-norisoprenoids in Chardonnay, methoxy-pyrazines in 'Cabernet', mercaptanes in 'Sauvignon', volatile phenols in 'Traminer' and dimethyl sulphide in 'Syrah', but these compounds could also contribute significantly to the aroma of several other varieties (VILANOVA and OLIVEIRA 2012). Other volatiles (mainly aldehydes and alcohols with 6 carbon atoms) have a pre-fermentative origin and are formed by enzymatic action along the grape crushing. All these aroma compounds are present in the free form, with high volatility and in the non-volatile bound form, as glyco- or cysteine-conjugates, which can undergo acid or enzymatic hydrolysis, releasing free volatile compounds and enhancing the must and wine aromas (ZOECKLEIN *et al.* 1997, KANG *et al.* 2010, VILANOVA *et al.* 2012, GENOVESE *et al.* 2013).

Stir bar sorptive extraction (SBSE) is the most advanced technique successfully applied to aroma compound determination in aqueous matrices, grape-musts and wine (ARBULU *et al.* 2013, GÓMEZ *et al.* 2012, PEDROZA *et al.* 2010, CASTRO *et al.* 2008, TREDoux *et al.* 2008 and MARÍN *et al.* 2005). Also, advanced statistical analysis, applied to data obtained by chemical analysis of aroma compounds are used for classification, being the multiple sample comparison (MSC), multiple variable analysis (MVA) and Principal Component Analysis (PCA) the most used (ROCHA *et al.* 2006, COZZOLINO *et al.* 2009, SAURINA 2010, ROBINSON *et al.* 2011, MORENO-GARCÍA *et al.* 2013).

This work aimed at establishing a straightforward procedure for the objective differentiation of grape varieties based on the determination by SBSE and Gas-Chromatography-Mass Spectrometry of a few selected aroma compounds and the use of statistical tools.

Material and Methods

Cultivars and grape-growing areas: Four white grape varieties were used: 'Aligoté' (AL) and 'Muscat Ottonel' (MO) from Iasi (Romania) and 'Muscat of Alexandria' (MA) and 'Pedro Ximénez' (PX) from Montilla-Moriles (Spain) DOP grape-growing areas. MO and MA are considered aromatic grape varieties whereas the AL and PX are neutral varieties, according to the organoleptic properties of their respective musts and wines.

MO is a younger and less-distinguished member of the Muscat family; it is widely cultivated in Austria, Romania and Alsace because it has a high resistance to the cold. MA is an ancient grape variety used both as table grape and for wine production in some regions, including Malaga and Montilla – Moriles DOP's, which make high-quality dessert wines. AL is used to make dry white wines, with significant plantings in much of Eastern Europe including Romania, Russia, Ukraine, Hungary, Moldova and Bulgaria. PX is known for its role in the elaboration of raisin sweet wines from Montilla-Moriles, Malaga and Jerez-Xeres-Sherry DOP's (Southern Spain) and also is used to make sherry-wine types under biological aging.

The Montilla-Moriles grape-growing area is located to the Southwest of Spain (37.58° N, 4.63° W) and can be framed in the climatic region V of the Winkler and Amerine classification (LÓPEZ *et al.*, 1988). The Iasi area is located in the NE of Romania (47.15° N, 27.59° E) and can be framed in the region I of the same classification (ȚARDEA and ROTARU 2003). All vineyards were conducted according to the "good practices" used in both DOP and under non-irrigated growing conditions. Three lots of grapes for each variety were picked at their industrial maturity under optimum sanitary conditions and were frozen at a temperature of -18 °C until they were crushed.

Grape-musts obtention: Every lot of frozen grapes from each variety was thawed overnight at 4 °C in a refrigerator and was crushed with a laboratory stainless steel press (Ferrari, Italy) without breaking seeds. All the must samples were homogenized, centrifuged at 5,000 rpm for 5 min and subjected immediately to analysis of the Brix degree, pH, titratable acidity and sugars. Several fractions of 100 mL were frozen at -18 °C in separate recipients for the analysis of volatile compounds. Samples of must from each variety were subjected to triplicate analysis.

Oenological variables: Total soluble solid was determined by a hand refractometer (Atago, Tokyo, Japan), pH by a pH-meter (Crison, Barcelona, Spain) and titratable acidity was quantified according to European Union Official Methods (CEE, 1990).

Extraction of aroma compounds by Stir Bar Sorptive Extraction: The non-polar aroma compounds were extracted with a polydimethylsiloxane (PDMS) coated stir bar (Twister®) with 0.5 mm film thickness, 10 mm length (Gerstel GmbH, Mülheim und der Ruhr, Germany), according TREDOUX *et al.* (2008). For free aroma fraction, a volume of 1 mL must was added to a 10 mL vial, then 0.1 mL of an internal standard solution (0.4464 mg·L⁻¹ of ethyl nonanoate in pure ethanol) and a volume of solution (ethanol 12 % (v/v), tartaric acid 2.6 g·L⁻¹ and potassium bitartrate, 2.2 g·L⁻¹, pH 3.5) were added to the vial to complete 10 mL. Twister was added to the vial and the extraction process was carried out in a magnetic stirrer (Variomag Multipoint 15, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) during 100 min at 1,200 rpm at room temperature (22 °C). Subsequently, the Twister was transferred to a desorption tube, which was placed in a Thermal Desorption Unit (TDU) from Gerstel GmbH & Co., (Mülheim und der Ruhr, Germany), for GC-MS analysis. The acidic hydrolysis of the bound aroma

compounds was carried out at pH 2.5, (70 °C, 2 hours) by adding a volume of 2 M citric acid to 1 mL of grape-must (PEDROZA *et al.* 2010). After hydrolysis, the samples were cooled at room temperature and the same protocol used for free fraction extraction was applied. According to these authors, this procedure has as an advantage that the obtained results are referred to both free and bound fractions and can be considered as the total content of grape must in aroma compounds that can be revealed along the wine-making processes (fermentation, conservation or ageing) under the acidic wine conditions.

Gas chromatography–mass spectrometry (GC–MS) of aroma compounds: The aroma compounds were transferred from the Twister in TDU, at initial temperature 35 °C (0.1 s), 120 °C·min⁻¹ to 280 °C (10 min) and 16 mL·min⁻¹ helium flow in splitless mode, to a Cooled Injection System (CIS-4) from Agilent Technologies, Palo Alto California, USA) provided with an inlet liner packed with Tenax® (3 x 2 mm). The CIS-4 was programmed at an initial temperature of 25 °C (0.05 s) 12 °C·s⁻¹ to 280 °C (7 min); helium inlet flow, 16 mL·min⁻¹. The 7890A GC and MSD 5975C (Agilent Technologies, Palo Alto California, USA) was equipped with a fused silica capillary column HP-5MS (30 m, 0.25 mm, 0.25 μm) from Agilent Technologies, Delaware, USA. Oven initial temperature was set at 50 °C (2 min), then 4 °C·min⁻¹ to 190 °C (10 min) MSD in electron impact mode (EI) at 70 eV, a mass range from 35 to 550 Da and 150 °C was used. Three replicates for each must sample were analyzed.

Peak identification of the aroma components was achieved by comparison of mass spectra with mass spectral data from the Wiley7 and NIST08 libraries and confirmation with GC linear retention indices (LRI, VAN DER DOOL and KRATZ 1963) of standards compiled in the NIST WEB BOOK OF CHEMISTRY (2014). Compounds were also effectively identified by comparing its mass spectrum with those of available commercial authentic substances (see Tab. 2). Total ion chromatogram peak area for each component was measured and divided by the peak area of the internal standard to obtain their respective relative areas, that were used for statistical data analysis.

Chemical reagents: A C7-C40 hydrocarbon mixture in hexane (Sigma-Aldrich) was used for the determination of LRI. All reagents and pure commercial substances were provided by Sigma-Aldrich, Fluka and Merck, as is detailed in Tab. 2.

Statistical analysis: Statgraphics® Centurion XVI Software package (Stat Points Technologies, Inc. Warrenton, Virginia, USA) was used for Multiple Sample Comparison analysis (MSC), Multiple Variable Analysis (MVA) and principal component analysis (PCA).

Results and Discussion

Oenological characteristic: Musts obtained from AL and MA have pH values below 4.0 whereas those from MO and PX are around 4.14. Also, AL and MA show higher values for titratable acidity than the remain-

Table 1

Enological characteristics of musts obtained from 4 grape varieties. Mean concentrations and standard deviations (n = 3 samples and triplicate analysis)

Must composition	Aligoté		Muscat Ottonel		Muscat of Alexandria		Pedro Ximenez	
pH	3.61 ^a	0.01	4.15 ^c	0.02	3.923 ^b	0.006	4.14 ^c	0
Titrateable acidity (g/L) [†]	5.65 ^d	0.05	3.47 ^a	0.06	5.02 ^c	0.07	3.78 ^b	0
Soluble solids (°Bx)	19.43 ^b	0.05	23.0 ^d	0.1	18.93 ^a	0.06	21.3 ^c	0.1
Sugars (g/L)	191.9 ^b	0.5	226 ^d	1	179.8 ^a	0.6	207 ^c	1
Ethanol potential (%v/v)	11.4 ^b	0.1	13.45 ^d	0.07	10.68 ^a	0.03	12.3 ^c	0.06

[†] Expressed as tartaric acid. a,b,c,d Different superscripts in the same row indicate statistical differences at the 0.05 P-value according to the Fisher's least significant difference (LSD) method.

ing varieties, in a clear correspondence with their pH values. Total soluble solids, fermentable sugars and probable ethanol content are directly related variables, showing MO the higher values followed by PX, AL and MA, (Tab. 1). The ratio: sugars/titrateable acidity is considered as an index of the industrial ripeness status of the grapes, showing MO and PX values of 65 and 55, respectively, while MA and AL show 36 and 34. These indices and all the values shown in Tab. 1 reveal how the grapes of the four varieties were harvested in an adequate ripe stage for making table wines.

Musts differentiation by aroma compounds: Table 2 lists the aroma compounds identified in musts having a match quality greater than or equal to 75, obtained by comparing its mass spectrum with those in the NIST08 and Wiley7n spectrum libraries, and a LRI value equal to those described in the literature and collected in the NIST website (2014). Compounds are also confirmed by comparison of their mass spectrum with those of authentic substances commercially available, which are detailed in Tab. 2. In this way, 51 compounds were identified, 14 among them were classified as terpenes and norisoprenoids, 7 as aldehydes and ketones, 2 as alcohols, 7 as benzene compounds, 8 as organic acids, and finally 13 were classified as esters. This classification is based on their functional groups and chemical structures, providing as an additional advantage the uniformity of aroma descriptors among compounds in the same group. In this way, terpenes and norisoprenoids usually have floral odors, the aldehydes and ketones a green, herbaceous odor, the alcohols show a vinous, sometimes herbaceous, green odor, the benzene compounds a chemical, phenolic balsamic odor, the fatty acids have a rancid or sweat, unpleasant odor and finally the esters group have a pleasant fruit-like odor. Three aroma compounds have been identified for the first time in grape musts: (E,E)-2,6-dimethyl-1,3,5,7-octatetraene (cosmene, CAS number 460-01-5), a monoterpene previously isolated in lavender by TSCHIGGERL and BUCAR 2010, *Akebia trifoliata* by XIAO *et al.* 2013 and *Lilium* by ZHANG *et al.* 2013; the 2-cyclopenten-1-one, 2-hydroxy-, (CAS 10493-98-8) found in roasted coffee by NIEMELA 1988, and *Aphanamixis grandifolia* by LIU *et al.* 2010 and the 3-buten-2-one,1-(2,3,6-trimethylphenyl), (CAS 10493-98-8) found in brandy by ZHAO *et al.* 2008. Cosmene and 3-buten-2-one,1-(2,3,6-trimethylphenyl) were found in the

aroma fraction obtained after hydrolysis of musts from MA and AL respectively and the 2-cyclopenten-1-one, 2-hydroxy-, in the free aroma fraction of PX musts.

Tab. 3 and 4, shows the mean, standard deviations and the homogeneous groups (HG), at $p \leq 0.05$ confidence level, of the relative areas obtained for 51 aroma compounds identified in musts before and after their acidic hydrolysis and grouped in the 6 chemical families. Among these compounds, only 32 were found as free form and 18 of them are present at high levels in musts from AL, 23 in MO, 11 in MA and 21 in PX. After hydrolysis, the number of compounds increases to 41, showing AL 21 compounds, MO 28, MA 25 and PX 23. The increase obtained for MA variety, is fundamentally due to those compounds included in the terpenes and nor-isoprenoids and the esters groups. The differences obtained among varieties can be summarized by summing the areas obtained for the aroma compounds enclosed in each of the 6 previously defined groups and using the obtained values as variables for MVA. The sunray plots obtained for free and hydrolyzed aroma compounds are shown in Fig. 1 (A, B) where each grape variety is represented by a hexagon that is defined by the 6 rays corresponding to the 6 considered variables. The distance from the center to each vertex of the hexagon corresponds to the value of each variable, being the end of the ray the mean value plus three standard deviations and the center of hexagon the mean minus three standard deviations.

As can be seen in Fig. 1A, the four studied varieties show different free aroma compounds profiles. AL is different from the other varieties by their values in free alcohols and esters groups, MO by terpenes and nor-isoprenoids, jointly with aldehydes and ketones and alcohols, MA by aldehydes and ketones and acids and finally, PX by the aldehydes and ketones, benzenic compounds, acids and esters groups. With respect to the aroma compounds obtained after the acidic hydrolysis of musts (Fig. 1B), PX shows the most regular hexagon, with an ester content higher than its remaining groups; MA also has a very regular hexagon, with esters content higher than those of PX and the musts from AL have higher values in benzene compounds and acids. Finally, MO shows again higher values in terpenes and norisoprenoids, aldehydes and ketones and alcohol families. With respect to their respective free forms pattern, MO changes its profile by increasing terpenes and norisoprenoids and acids groups, AL increases in the aldehydes

Table 2
Aroma compounds identified in musts

No.	Compound name	LRI ^a	LRI ^b	CAS	Odor descriptor
1	Linalool ²	1093	1097	78-70-6	Flower, lavender, lemon and lime-like with a slight herbal and floral nuance
2	Geraniol	1260	1258	106-24-1	Sweet, floral, fruity, rose, citrus
3	Limonene ¹	1033	1031	138-86-3	Citrus, herbal, sweet
4	Ocimenol	1169	NF	5986-38-9	Fresh citrus, lemon, lime, cologne, sweet, mace spice
5	<i>Beta</i> -ocimene	1043	1046	3779-61-1	Citrus, herb, flower, sweet
6	Ocimen quintoxide	1051	1049	7416-35-5	Woody, citrus, lime,
7	<i>Gamma</i> -Terpinene	1063	1062	99-85-4	Citrus, lime-like, oily, green, tropical fruity
8	(<i>E</i>)- <i>Beta</i> -Damascenone	1391	1388	23726-93-4	Apple, rose, honey, tobacco, sweet
9	Nerol oxide	1160	1153	1786-08-9	Green, vegetative, floral, leafy and waxy with an herbal, minty depth
10	Dehydroxylinalool oxide	1012	1007	13679-86-2	Woody, piney, spicy, minty with a green citrus nuance
11	Nerol ²	1234	1229	106-25-2	Lemon, bitter, green, fruity
12	(<i>Z</i>)-Citral ¹	1247	1237	106-26-3	Sweet, citral, lemon peel
13	Vitispirane	1285	1279	65416-59-3	Fruity, floral, earthy, woody
14	(<i>E,E</i>)-2,6-Dimethyl-1,3,5,7-octatetraene (Cosmene)	1127	1134	460-01-5	Floral. Isolated in lavender; <i>Akebia trifoliata</i> , liliium,
15	2-Cyclopenten-1-one, 2-hydroxy-	928	926	10493-98-8	Found in roasted coffee and <i>Aphanamixis grandifolia</i> . No descriptor available
16	Furfural ¹	836	839	98-01-1	Sweet, woody, bread, caramel, with a slight phenolic nuance
17	Hexanal ³	806	806	66-25-1	Green, woody, vegetative, apple, grassy, citrus and orange
18	2-Hexenal ¹	856	854	505-57-7	Fresh green, leafy, fruity with rich vegetative nuances
19	Octanal	1008	1004	124-13-0	Green, citrus, orange peel
20	Nonanal	1109	1107	124-19-6	Fat, citrus, green
21	Decanal ¹	1211	1207	112-31-2	Waxy, fatty, citrus and orange peel with a slight green melon nuance
22	1-Hexanol ²	871	867	111-27-3	Green, fruity, apple-skin, oily
23	2-Furanmethanol	858	864	98-00-0	Alcoholic, chemical, musty, sweet, caramel, bread, coffee
24	Benzaldehyde ¹	964	959	100-52-7	Sweet, bitter almond, oily, cherry, nutty, woody
25	Benzophenone ³	1635	1621	119-61-9	Balsam, rose, metallic, powdery geranium
26	Phenol ³	985	989	108-95-2	Phenolic, plastic, rubber
27	Phenol, 2,4-bis(1,1-dimethylethyl)	1519	1513	96-76-4	Phenolic
28	3,6-Dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran	1191	1178	70786-44-6	Herbal dill
29	Naphthalene, 1,2-dihydro- 1,1,6-trimethyl-	1359	1354	30364-38-6	Licorice, petroliferous
30	3-Buten-2-one, 1-(2,3,6-trimethylphenyl)	1621	NF	10493-98-8	Found in brandy. No descriptor available.
31	Nonanoic acid ²	1273	1280	112-05-0	Waxy, cheese cultured dairy
32	n-Decanoic acid ²	1374	1380	334-48-5	Rancid, sour, fatty, citrus
33	Dodecanoic acid ¹	1567	1570	143-07-7	Metal, mild, fatty, coconut, bay oil
34	Tetradecanoic acid	1767	1769	544-63-8	Waxy, fatty, soapy, creamy, cheesy, with a good mouth feel
35	Pentadecanoic acid	1866	1868	1002-84-2	Waxy
36	n-Hexadecanoic acid	1964	1969	57-10-3	Waxy, creamy, fatty, soapy
37	<i>Cis</i> -9-Hexadecenoic acid	1953	1953	373-49-9	Waxy, creamy, fatty, soapy
38	9,12-Octadecadienoic acid	2167	2170	60-33-3	Faint, fatty
39	1-Butanol, 3-methyl-, acetate	881	884	123-92-2	Sweet, fruity, banana, solvent
40	2-Phenylethyl acetate	1263	1257	103-45-7	Fruity, rose, sweet, honey, tropical
41	Hexyl acetate ²	1019	1013	142-92-7	Fruity, green, fresh, sweet, banana peel, apple and pear;
42	Hexyl butanoate ¹	1198	1194	2639-63-6	Fruity, green, sweet, apple, waxy,
43	Ethyl heptanoate ¹	1104	1098	106-30-9	Fruity, pineapple, banana and strawberry with a spicy, oily nuance
44	Ethyl octanoate ²	1203	1196	106-32-1	Sweet, waxy, fruity and pineapple with creamy, fatty, mushroom and cognac notes
45	Ethyl decanoate ²	1402	1397	110-38-3	Waxy, fruity, sweet, apple
46	Octanoic acid, 2-methyl-ethyl ester	1233	NF	30982-02-6	Floral, sweet
47	Phenethyl butanoate ¹	1448	1440	103-52-6	Fruity, floral, green with a tropical winey nuance
48	Phenethyl isobutanoate ¹	1374	1371	103-48-0	Heavy, honey, floral, aldehydic with floral nuances
49	Phenethyl phenyl acetate	1920	NF	102-20-5	Honey, floral, green, rose, cocoa, hay
50	Hexyl hexanoate	1388	1385	6378-65-0	Sweet, fruity and green with tropical notes
51	Isoamyl butanoate ¹	1061	1054	106-27-4	Fruity, green, apricot, pear, banana

LRI: Linear retention index according VAN DER DOOL and KRATZ (1963) in a HP-5MS capillary column (30 m/0.25 mm/0.25 μ m, He); a calculated values. b data collected from the NIST Webbook of Chemistry (2014). NF: not found in comparable conditions. Superscripts indicate the authentic pure compounds used for co-elution and provided by: ¹Sigma Aldrich, ²Fluka and ³Merck.

Table 3

Relative area, standard deviations (n = 3 samples and triplicate analysis) and homogeneous groups for free aroma compounds identified in musts

No.		Aligoté			Muscat Ottonel			Muscat of Alexandria			Pedro Ximenez		
		Mean	SD	HG	Mean	SD	HG	Mean	SD	HG	Mean	SD	HG
1	Terpenes & isoprenoids	NF		a	1.53	0.03	b	NF		a	NF		a
2		NF		a	0.10	0.02	b	NF		a	NF		a
3		0.030	0.002	b	0.041	0.002	d	NF		a	0.036	0.002	c
11		NF		a	0.13	0.01	b	NF		a	NF		a
12		NF		a	0.018	0.002	b	NF		a	NF		a
15		Aldehydes & ketones	NF		a	NF	NF	a	NF		a	0.026	0.004
16	NF			a	0.002	0.002	b	0.013	0.003	b	0.024	0.002	c
17	NF			a	0.02	0.01	ab	NF		a	0.04	0.02	b
19	0.023		0.003	b	0.05	0.01	c	NF		a	0.06	0.01	c
20	0.072		0.007	a	0.122	0.002	b	0.11	0.02	b	0.17	0.03	c
21	0.041		0.003	a	0.058	0.005	b	0.068	0.002	c	0.079	0.001	d
22	Alcohols	0.189	0.005	c	0.139	0.007	b	NF		a	NF	NF	a
24	Benzenic compounds	NF		a	NF		a	0.013	0.001	b	0.038	0.003	c
25		0.019	0.001	b	0.021	0.001	c	NF		a	0.054	0.001	d
27		NF		a	0.034	0.001	b	0.11	0.01	c	0.158	0.008	d
28		0.087	0.003	c	0.052	0.006	b	NF		a	NF		a
31	Acids	0.038	0.002	c	0.033	0.002	b	0.034	0.002	b	0.028	0.001	a
32		NF		a	0.015	0.002	b	0.022	0.0008	c	NF		a
36		0.078	0.003	b	0.0382	0.0004	a	0.12	0.02	c	0.172	0.006	d
38		NF		a	NF		a	NF		a	0.136	0.008	b
39	Esters	NF		a	NF		a	0.016	0.001	b	NF		a
40		0.016	0.001	b	NF		a	NF		a	0.050	0.002	c
41		0.112	0.002	d	0.018	0.002	b	NF		a	0.050	0.006	c
42		0.023	0.004	b	0.030	0.002	c	NF		a	0.020	0.001	b
43		0.058	0.003	b	NF		a	NF		a	0.12	0.01	c
44		0.0571	0.0008	a	0.057	0.001	a	0.095	0.004	b	0.099	0.009	b
45		0.019	0.002	b	0.020	0.002	b	NF		a	NF		a
46		0.022	0.002	b	NF		a	NF		a	NF		a
47		NF		a	NF		a	NF		a	0.015	0.001	b
48		0.013	0.001	b	0.015	0.001	b	0.022	0.004	c	NF		a
49		NF		a	NF		a	NF		a	0.044	0.006	b
50	0.022	0.001	b	0.024	0.003	b	0.020	0.003	ab	0.018	0.001	a	

Terpenes and nor-isoprenoids: 1-14; aldehydes and ketones: 15-21; alcohols: 22-23; benzenic compounds: 24-30; fatty acids: 31-38; esters: 39-51. SD = Standard Deviation; HG= Homogeneous Groups; a,b,c,d different letters in the same row indicate statistical differences among groups at 0.05 *P*-value, according to Fisher's least significant difference method. NF = not found.

and ketones, benzenic compounds and acid groups, while MA increases the ester groups and PX changes drastically its profile. These results show how the content in different families of aroma compounds is dependent on the grape variety and that the MVA statistical treatment can be considered as an objective procedure to obtain the fingerprint on musts for each grape variety based on analytical data.

Muscat and Muscat-like wines are characterised by their content in monoterpenes, in both free and bound forms, nevertheless, some bound aromas, particularly the glycosidic combinations, are known as the most important group responsible for the varietal attributes of wines obtained from neutral or non-floral grapes (FRANCIS *et al.* 1996, BUREAU *et al.* 2000, LÓPEZ *et al.* 2004 and MARTÍNEZ-GIL *et al.* 2012). The acidic or enzymatic hydrolysis of these bound-forms yields the corresponding odor-active compounds, enhancing the varietal aroma compounds of the wine. In this sense the MVA provides a visual overview of the evolution of these compounds that is achieved through the wine making process, at the acidic pH of must

and wine, for each grape variety by means of their respective fingerprinting.

The HG shown in Tabs 3 and 4 for the mean of each compound, were obtained by applying a multiple comparison procedure and the least significant difference (LSD) method, with the aim to establish which means are significantly different from the others, at a significance level of $p \leq 0.05$. Following this criterion, only 6 free aroma compounds, numbered as 3, 21, 25, 27, 36 and 41, show 4 HG, in accordance with those grape varieties studied, and 11 compounds (numbers 16, 19, 20, 22, 24, 28, 31, 32, 42, 43 and 48) show significant differences among 3 varieties. All these 17 compounds have been selected as variables to establish a statistical and objective procedure for grape must differentiation. Also, only 21 compounds, obtained after acidic hydrolysis (Tab. 4), were selected to differentiate among the grape varieties. From them, 9 showed 4 HG (numbers 3, 6, 16, 27, 35, 36, 40, 43 and 46) and others 12 compounds showed 3 HG (1, 5, 8, 9, 10, 20, 21, 22, 34, 41, 44 and 51). Two PCA, in the standardized data

Table 4

Relative area, standard deviations (n = 3 samples and triplicate analysis) and homogeneous groups for aroma compounds after acidic hydrolysis (free and bound forms)

No.	Aligoté			Muscat Ottonel			Muscat of Alexandria			Pedro Ximenez		
	Mean	SD	HG	Mean	SD	HG	Mean	SD	HG	Mean	SD	HG
1	NF		a	0.89	0.04	c	0.34	0.01	b	NF		a
3	NF		a	0.34	0.03	d	0.08	0.01	c	0.043	0.004	b
4	NF		a	0.09	0.01	b	NF		a	NF		a
5	NF		a	0.09	0.01	c	0.061	0.003	b	NF		a
6	0.046	0.004	b	1.75	0.04	d	0.57	0.03	c	NF		a
7	NF		a	0.13	0.02	b	NF		a	NF		a
8	0.100	0.002	ab	0.18	0.02	c	0.0814	0.0005	a	0.11	0.01	b
9	0.059	0.002	a	1.7	0.1	c	0.402	0.005	b	NF		a
10	NF		a	0.80	0.03	c	0.149	0.009	b	NF		a
13	NF		a	0.12	0.02	b	NF		a	NF		a
14	NF		a	NF		a	0.078	0.002	b	NF		a
16	0.022	0.004	b	0.045	0.003	d	0.017	0.001	a	0.035	0.002	c
17	NF		a	NF		a	NF		a	0.023	0.005	b
18	0.064	0.007	b	0.056	0.004	b	NF		a	NF		a
19	0.079	0.004	a	0.13	0.03	b	0.06	0.02	a	0.058	0.003	b
20	0.56	0.05	b	0.74	0.04	c	0.24	0.05	a	0.31	0.02	a
21	0.21	0.02	c	0.19	0.03	bc	0.12	0.01	a	0.175	0.005	b
22	0.25	0.02	c	0.18	0.01	b	NF		a	NF		a
23	NF		a	NF		a	NF		a	0.023	0.001	b
24	NF		a	NF		a	NF		a	0.031	0.002	b
25	0.046	0.006	a	0.044	0.001	a	0.045	0.003	a	0.051	0.009	b
26	NF		a	NF		a	NF		a	0.015	0.002	b
27	0.84	0.05	d	0.69	0.03	c	0.14	0.01	a	0.19	0.02	b
29	0.61	0.07	b	0.21	0.03	a	NF		a	0.20	0.01	a
30	0.034	0.007	b	NF		a	NF		a	NF		a
31	0.04	0.01	a	0.04	0.01	a	0.043	0.008	a	0.04	0.01	a
33	NF		a	0.08	0.02	b	NF		a	NF		a
34	0.3	0.2	c	0.2	0.1	b	0.10	0.01	a	0.11	0.04	a
35	0.08	0.05	d	0.06	0.04	c	0.032	0.002	b	NF		a
36	0.4	0.1	d	0.27	0.06	c	0.126	0.007	a	0.21	0.04	b
37	0.7	0.5	b	NF		a	NF		a	NF		a
38	NF		a	0.14	0.02	b	NF		a	NF		a
40	NF		a	0.029	0.002	b	0.04	0.01	d	0.035	0.005	c
41	NF		a	NF		a	0.055	0.003	c	0.039	0.003	b
42	0.039	0.009	b	NF		a	NF		a	0.032	0.004	b
43	0.081	0.007	b	NF		a	0.221	0.007	d	0.103	0.004	c
44	0.095	0.006	a	0.159	0.009	c	0.109	0.002	b	0.10	0.01	a
45	NF		a	0.031	0.002	b	NF		a	NF		a
46	NF		a	0.057	0.004	d	0.030	0.006	c	0.025	0.007	b
48	NF		a	NF		a	0.023	0.001	b	0.023	0.003	b
51	0.027	0.001	b	NF		a	0.036	0.003	c	NF		a

Terpenes and nor-isoprenoids: 1-14; aldehydes and ketones: 15-21; alcohols: 22-23; benzenic compounds: 24-30; fatty acids: 31-38; esters: 39-51. SD = Standard Deviation; HG = Homogeneous Groups; a,b,c,d different letters in the same row indicate statistical differences among groups at 0.05 *P*-value, according to Fisher's least significant difference method. NF = not found.

option, have been performed using the selected free and hydrolyzed aroma compounds and the obtained results are plotted in Figs 2A and 2B respectively. This multivariate statistical procedure has been successfully used in enology to determine whether a specific wine belongs indeed to the claimed grape variety, its origin, the ageing type and time, year of vintage, etc. (VANDEGINSTE *et al.* 1997, ALEXANDRE *et al.* 2002, HEÄBERGE *et al.* 2003, VILLIERS *et al.* 2005, TREDoux *et al.* 2008, GARCÍA-MARINO *et al.* 2011 and MORENO-GARCÍA *et al.* 2013).

According to Fig. 2A, two PCs are selected by their eigenvalue (8.83 for PC1 and 5.63 for PC 2), explaining the 51.97 % and 33.14 % respectively of the total variance. In this Figure, each compound is plotted by means

of their corresponding vector, whose projection over each axis shows their contribution to the respective component. In this way, the 6 free aroma compounds numbered 16, 20, 21, 24, 27 and 36 [furfural; nonanal; decanal; benzaldehyde, phenol,2,4-bis(1,1-dimethylethyl) and n-hexadecanoic acid] have a positive coefficient higher than 0.3, while compounds 22, 28 and 31 have a negative coefficient, being all these 9 compounds the most contributing compounds to PC1. The 8 free compounds (limonene; octanal; benzophenone, n-decanoic acid, hexyl acetate, hexyl butanoate, phenethyl isobutyrate and ethyl heptanoate) show the highest contribution to the PC2. The musts are grouped in accordance to their grape variety by their PC1 and PC2 scores: musts from PX are located in the lower right corner

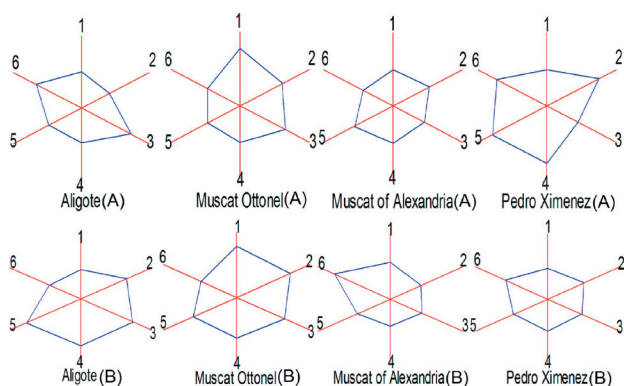


Fig. 1: Grape musts fingerprint obtained by multivariate data analyses of aroma compounds as free forms (A series) and after acidic hydrolysis (B series). Each ray in the hexagon corresponds to one group of aroma compounds: 1: terpenes and nor-isoprenoids, 2: aldehydes and ketones, 3: alcohols, 4: benzenic compounds, 5: acids; 6: esters. The distance from the center to each vertex corresponds to the value of each group. The end of the ray is the mean value plus three standard deviations and the centre the mean minus three standard deviations.

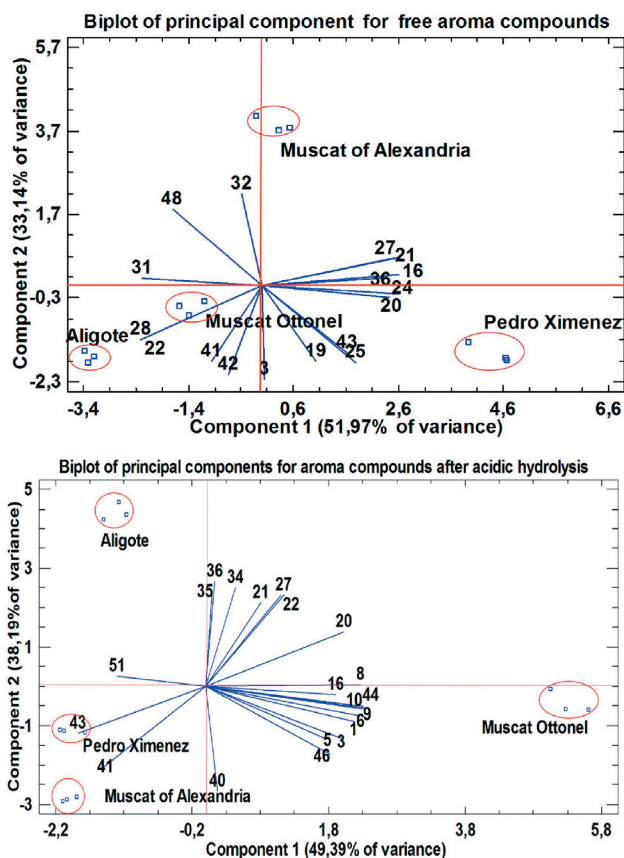


Fig. 2: Principal component analyses of selected aroma compounds as free forms (A) and after acidic hydrolysis (B). For compound names see Tab. 2.

of the Figure 2A, being differentiated by their higher score on PC1; musts from MA are differentiated from the remaining musts by their positive score in PC2; musts from MO show scores close to 0 and the samples from AL have the lowest score on PC1 and PC2. The free compounds contributing with a positive coefficient to PC1 have the odor descriptors: sweet, brown, woody, bready (16); fat,

citrus, green (20); waxy, fatty, citrus (21); sweet, bitter almond (24); phenolic (27); waxy, creamy, fatty, soapy (36); differentiating the PX musts from the rest. Also, n-decanoic acid (32) and phenethyl isobutyrate (48) are the most important compounds contributing to the differentiation of MA with rancid, sour and heavy, honey, floral, odor descriptor respectively. Finally MO and AL with negative values for PC1 and PC2, are differentiated fundamentally by the aroma compounds having odor descriptors: citrus, herbal (3); fruity, green, sweet, apple (42); fruity, green, fresh, sweet, banana (41); green, fruity, apple (22); herbal dill (28); waxy, cheese (31).

The PCA performed with the 21 compounds selected after acidic hydrolysis of musts provided two principal components: PC1 with an eigenvalue of 10.37 and PC2 with 8.32, explaining 49.39 % and 38.19 % of the total variance, respectively. According to the Fig. 2B, musts from MO are located to the right, showing the highest score in PC1 and values near to 0 in PC2, AL musts are located to the upper left corner, with the highest scores in PC2, while PX and MA have negative scores for PC1 and PC2, showing MA the lowest scores. The most important compounds contributing to PC 1 with a positive sign are (E)-beta-damascenone (8), nerol oxide (9), dehydroxylinalool oxide (10), ethyl octanoate (44); ocimen-quintoxide (6); linalool (1); limonene (3); β -ocimene (5); nonanal (20); furfural (16); and octanoic acid 2-methylethyl ester (46). Only 3 compounds show negative coefficients for PC1: ethyl heptanoate (43); hexyl acetate (41) and isoamyl butanoate (51). These 14 compounds contributing to PC1 have agreeable odor descriptors with floral and fruity nuances and contribute to differentiate the MO musts from the others, which have similar scores in this PC1. Compounds as hexadecanoic acid (36); pentadecanoic acid (35); tetradecanoic acid (34); decanal (21); phenol-2,4-bis(1,1-dimethylethyl) (27) having unpleasant odors (waxy, fatty, soap, phenolic) and only two, as are 1-hexanol (22) and 2-phenylethyl acetate (40), with green and honey as more pleasant odor descriptors, are the ones most influencing PC2.

The results obtained by means of MVA and PCA before and after of acidic hydrolysis of musts show the potential in aroma compounds for each cultivar that could be revealed trough the winemaking process at the acid pH of must and wines. In this respect, SEFTON *et al.*, (1994) suggest that transformations taking place along wine making include relevant chemical rearrangements in acid media that are better predicted by acid hydrolysis. Also, LOSCOS *et al.* 2009, established that the “natural hydrolysis” carried out by yeast during the fermentation has the lowest potential to release volatile compounds from their precursors, whereas enzymatic hydrolysis is the most efficient but the most different too, which implies that the predictive ability of the enzymatic hydrolysis is rather poor comparing to the acidic hydrolysis, for a more adequate measure of the aroma potential of grapes for winemaking.

The two PCA performed in this work for specific free and hydrolyzed aroma compounds, confirm, in accordance with REBOLO *et al.* (2000), that the use of all available variables is unnecessary and undesirable, because the consideration of variables with no discriminating information

only introduces “background noise” in the pattern recognition process. The differentiation of musts based on the statistical selection of some few “key compounds” with high discrimination power and the application of multivariate statistical methods has the main advantage that it is an objective method and does not need an expensive collection of pure compounds in order to obtain the actual concentration values for each one.

Conclusions

Fifty-one aroma compounds (32 in free form and 41 after acid hydrolysis) have been identified in musts from 'Muscat Ottonel', 'Aligoté', 'Muscat of Alexandria' and 'Pedro Ximénez' grape varieties and three compounds are described for the first time.

Two fingerprints for each cultivar based in six aroma compound families before and after acidic hydrolysis of musts are obtained by Multiple Variable Analysis.

Seventeen compounds in free form and 21 after acidic hydrolysis of musts were selected by a Multiple Sample Comparison analysis by their discrimination power. The Principal Component Analysis carried out with these compounds allows to group the must samples according to the variety on the basis of two PC's.

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