Aroma characterization of grape juice enriched with grapevine by-products using thermomaceration

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ABSTRACT

The need to reuse grapevine (*Vitis vinifera* L.) by-products avoiding negative environmental impact demands the search for new valorization methods like thermomaceration. The combination of grapevine by-products and grape must may be an alternative to get hold of additional aroma. The objective of this study was to assess the aroma potential of grape pomace, grapevine leaves and canes for País (PA) and Lachryma Christi (LC) cultivars to enrich grape must. Fifty aroma compounds were identified in the samples using stir bar sorptive extraction followed by thermal desorption, gas chromatography and mass spectrometry. 'País' must showed high contents of esters with a characteristic fruity odor, while benzenoids prevail in LC must giving a phenolic, balsamic odor. Thermomaceration increased contents of 13 free and bound volatile compounds, as well as new compounds (isoeugenol, phenol, vanillin and 2-ethyl hexanol) appeared in enriched juice (EJ). Cluster analysis showed differences among PA, LC and EJ samples. Principal component analysis was successfully applied to discriminate grape juice samples ($p < 0.05$) being D-limonene, 5-hydroxymethyl-2-furfural and hexadecanoic acid the compounds that contributed most to the differentiation and increase of free and hydrolyzed aroma compounds after thermomaceration. Both grape-grapevine by-products and juice processing conditions affected the generation and increase of free and bound aroma compounds after thermomaceration of grape must.

Key words: Aroma compounds, aroma enrichment, chemometrics, grape must, thermomaceration, *Vitis vinifera*.

INTRODUCTION

Despite the existence of many *Vitis vinifera* L. cultivars in wine producing countries, only a few cultivars account for the vast majority of worldwide wine production. Ancient grapevine cultivars played a significant role in many viticulture regions, but nowadays these cultivars cannot fulfil current quality standards of fine wines. Therefore, it is crucial to look for new opportunities for these wine grapes by the development of new grape berry based products. Considering the increased consciousness of many consumers about the importance of functional foods in their diet, the production of natural grape juice from undervalued, ancient grapevine cultivars may be an excellent opportunity for the viticulture sector. The consumption of pure fruit juices appreciated due to their healthy, natural and tasty characteristics is an easy and convenient way to secure one of the five portions of fruits and vegetables following the recommendations of the World Health Organization. Concerning the positive health benefits of the consumption of grape juice, an annual growing demand for grape juice of 2.75% is expected with an estimated average volume of 2.22 L per capita in 2020 (Statistica, 2016).

In addition to grape juice production, the generation of agro-industrial by-products, such as skins, seeds and stems, potential sources of health promoting phytochemicals, which can make up to 30% (w/w) of raw material, has led to a growing concern in manufacturers (Teixeira et al., 2014). On the other hand, the valorization potential of these by-products as a source of aroma compounds for grape juice has been almost unexploited until now, which offers new opportunities. Grape skins have been found to contain more than half the free and glycoconjugated volatile compounds present in grape berries, being particularly rich in monoterpenes, norisoprenoids and aldehydes (Noguerol-Pato et al., 2012; Pedroza et al., 2013; Slegers et al., 2015). Grapevine matter other than grapes is a rich source of volatile compounds. Petioles contain terpenes with increased floral aroma (Ward et al., 2015); grapevine leaves are a source of carotenoids regarded as precursors of norisoprenoids (Baumes, 2009), while woody parts supplying volatile phenols (Weldegergis et al., 2011).

Cold maceration using dehydrated grape skin residues from the juice industry has been proposed by Pedroza et al. (2013) as a new enological tool to compensate color and aroma degradation in red wines. Moreover, skin maceration on its own juice in presence of sulfur dioxide using glycolytic enzymes to improve the availability of bound floral aroma compounds from berry

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Received: 3 February 2017.
Accepted: 6 June 2017.
doi:10.4067/S0718-58392017000300234

skins may protect and improve flavor complexity (Pedroza et al., 2010; Noguerol-Pato et al., 2012). Additionally, heating and pH adjustment are appropriate techniques in juice processing to induce flavor in grape must by the hydrolysis of bound aroma precursors. However, hot break processing should be used carefully, especially with immature fruit, as it may result in the persistence of green aromas in grape juice (Iyer et al., 2010). Recently, thermomaceration has been used as a green extraction technique to fortify grape juice with antioxidant compounds from grape pomace, grapevine leaves and canes (Aguilar et al., 2016). Nevertheless, the potential of winery and grapevine by-products as a source of aroma compounds has still received small attention.

Therefore, the aim of this study was to assess the aroma potential of grape pomace, grapevine leaves and canes from País and Lachryma Christi cultivars to enrich grape musts using chemometrics. Additional knowledge about the volatile composition of grape musts with and without thermomaceration regarded as free and bound glycoside terpenoids, norisoprenoids, benzenoids, aldehydes, ketones, alcohols, acids and esters offers a means of evaluation of the possibilities of both grape cultivars to be used as raw material different to wine making.

MATERIALS AND METHODS

Cultivars and grape-growing areas

Grapes, leaves and canes from *Vitis vinifera* L. 'País' (PA), a red cultivar, and 'Lachryma Christi' (LC), a Teinturier cultivar, were provided by two vineyards located in the Itata Valley (36°33' S, 72°10' W and 36°30' S, 72°05' W, respectively), San Nicolás, Chile, between April and June 2014. 'País' is an ancient Spanish cultivar also called 'Moscatel Negro', a Spanish homonym of 'Muscat' and synonym of 'Listán Prieto', now restricted to the Canary Islands in Europe. Moreover, it is known as 'País', Uva Negra Vino, Viña Blanca and Viña Negra in Chile, Criolla Chica in Argentina, Rosa del Peru and Negra Corriente in Peru, Misión in Mexico, Mission in United States, and Hariri in Morocco (Milla Tapia et al., 2007). 'Lachryma Christi' is a dyer or 'Teinturier', which accumulates red, purple pigments in pulp and skin, and commonly used for blending with pale red wine to give an intensive red color.

Grape must and enriched juice preparation

Healthy grapes were collected without reaching complete industrial maturity (sugar contents 169 g L^{-1}, pH 3.03 and total acidity 3.64 g L^{-1}), then destemmed, crushed (PAS.0540, Bertuzzi, Brugherio, Italy) and pressed (D.64625, Willmes, Bensheim, Germany) to get must and pomace (skins and seeds). Afterwards pomace and must, previously treated by ultrasound-assisted cold pasteurization (Ultrasonic Cleaner 2800, Branson Ultrasonics, Danbury, Connecticut, USA) at 100 W for 30 min, were stored at -20 °C before processing and analysis. After vintage, autumn leaves and canes were cut and stored at -20 °C until processing. Compounds from grape pomace, grapevine leaf and cane samples were extracted by thermomaceration as previously reported (Aguilar et al., 2016). After filtration using 20-25 μm nylon filter bags, extracts of pomace, leaves and canes were mixed in a proportion of 40:20:40% (v/v/v), respectively. Enriched grape juice (EJ) compromised a blend of 250 mL extracts of both 'País' and 'Lachryma Christi' grapes, followed by heating (63 °C for 30 min) and storage at room temperature until analysis.

Extraction and chemical analysis of aroma compounds

Free aroma fractions of grape musts and enriched juice were concentrated by stir bar sorptive extraction (SBSE) using a Twister coated with polydimethylsiloxane followed by thermal desorption (TD) and gas chromatography (GC) coupled to mass spectrometry (MS), using a previously validated method (Vararu et al., 2015). The analytical platform of SBSE-TD-GC-MS included: a thermal desorption unity (Gerstel GmbH, Mülheim an der Rühr, Germany) and an Agilent 7890A gas chromatograph equipped with a mass selective detector (5975, Agilent Technologies, Wilmington, Delaware, USA). The fused silica capillary column used was a HP-5MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness) from Agilent Technologies. Ethyl nonanoate was used as internal standard for determining the relative area from the total ion chromatogram peak area for each compound. Resulting data set is a semi-quantitative measure of the content of each compound. Further details about the extraction procedure, GC and mass detector operation conditions are given by Vararu et al. (2015).

Bound aroma compounds of grape musts and enriched juice were hydrolyzed by the addition of 2 M citric acid to pH 2.5 according to Pedroza et al. (2010). After hydrolysis, the samples were cooled at room temperature using the same protocol as free aroma fraction analysis to determine the total content of aroma compounds (free and bound fractions).

Identification of the volatile compounds was done by comparing mass spectra with mass spectral data from the Wiley7N and NIST08 libraries, and confirmed by comparing the linear retention indices (LRI) calculated according to Vararu et al. (2015) with those standards compiled in the NIST Chemistry WebBook (NIST, 2016). Some compounds were also identified by comparing their mass spectrum with those from commercial standards available in our laboratory.

Chemical standards and reagents

A C7-C40 mixture in hexane (Sigma-Aldrich Quimica SL, Madrid, Spain) was used for LRI determination. Pure water was obtained from a Milli-Q purification system (Millipore, Madrid, Spain). All reagents and standards were of analytical grade and supplied by Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Riedel de Haën (Seelze, Germany) or Sigma-Aldrich.

Statistical analyses

The data of peak areas of three replicates for target compounds relative to the internal standard peak area, calculated after SBSE-TD-GC-MS analysis, were averaged and standard deviation (SD) was calculated. Data were previously normalized ($\log_{10} x$) and subjected to scaling techniques to avoid differences between units for each quantified compound (Seisonen et al., 2016). ANOVA and Fisher's least significant difference (LSD) method applied to the relative peak areas at 5% significance level ($p < 0.05$) were used in order to identify homogeneous groups (HG) and to determine the suitability of volatile compounds data for the differentiation between must and juice samples. Cluster analysis (CA) was carried out by calculating Euclidean distance for previously selected aroma compound data using Ward's minimum variance method to identify similarity among must and juice samples. Multiple variable analysis (MVA) was done by using the sum of the relative peak area for each individual compound for the major chemical groups as variable in order to characterize samples by finger printing their volatile compounds. Principal component analysis (PCA) was performed based on the relative area of selected aroma compounds as variables by selecting a number of linear combinations that account for most of the variability in data to visualize differences among the samples. The software package Statgraphics Plus, 2.0 (STSC Inc., Rockville, Maryland, USA) was used for statistical analysis.

RESULTS AND DISCUSSION

Identification of volatile compounds

Table 1 lists the mass spectrum match quality (MS Q) value, LRI calculated at experimental conditions, those compiled in the Wiley and NIST libraries and the Chemical Abstracts Service number of volatile compounds detected in must and juice samples of PA and LC grapes. Moreover, the providers of pure reagents are listed in this table. Establishing MS Q values higher than 75 and LRI differences lower than 13 units as evaluation criteria, the identification of each compound can be considered as correct (Welke et al., 2014a). Fifty compounds were detected in the characteristic volatile profile of PA and LC samples, among them 34 species were unambiguously identified (Table 1). These compounds could be divided in nine chemical classes with their characteristic aroma descriptors: Terpenoids (two compounds), norisoprenoids (three compounds), lactones (two compounds), aldehydes and ketones (six compounds), alcohols (six compounds), benzenoids (nine compounds), alkenes (one compound), acids (eight compounds) and esters (13 compounds). The identified components have been reported before in grape and grape-derived products (Weldegergis et al., 2011; Ferrandino et al., 2012; Galano et al., 2015; Ghaste et al., 2015; González-Barreiro et al., 2015; Vararu et al., 2015; 2016; Yuan and Qian, 2016; Wang et al., 2017).

Enrichment of aroma compounds

Thermomaceration of grape pomace, grapevine leaves and canes in must was able to increase the content of free furan-derived (2(5*H*)-furanone (6), 5-hydroxymethyl-2-furfural (13) and 2-furanmethanol (14)) and benzene-derived (phenol (21), 2-methoxy-4-vinylphenol (24), vanillin (25) and isoeugenol (26)) compounds (Table 2). Volatile benzenoids identified as isoeugenol, phenol and vanillin, were absent in the aroma fractions of PA and LC musts, so their origin in enriched grape juice can be attributed to thermomaceration of grape pomace, grapevine leaves and canes. On the other hand, total content of the following free and bound volatile compounds increased in enriched juice compared to grape musts: D-limonene (1), 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (4), 5-hydroxymethyl-2-furfural (13), 2-ethyl hexanol (16), benzaldehyde (20), isoeugenol (26), hexadecanoic acid (36) and 9,12-octadecadienoic acid (37) (Table 3). Except D-limonene, 5-hydroxymethyl-2-furfural and isoeugenol, the other species are mainly present in glycosylated combinations. Amongst them, 2-ethyl hexanol is a new formed alcohol compound, detected after thermomaceration in enriched juice.

Thermal processing conditions may induce chemical reactions or inactivate enzymatic transformations affecting organoleptic properties of grape juice. Furan derivatives may be formed from sugars and furfural during heating in presence of woody material contributing to the burnt sugar-like aroma of enriched juice (Galano et al., 2015; Yuan and Qian, 2016). In addition, hot-press juice processing increased significantly the concentration of herbaceous odorant C6 alcohols (hexanol, *cis*-3-hexenol and *trans*-2-hexenol) (Iyer et al., 2010). C6 alcohols that resulted from enzymatic degradation of unsaturated fatty acids or from glycoside aroma precursors were derived mainly from grapes, but also related to the presence of woody parts and leaves during grape crushing (Baumes, 2009; Yuan and Qian, 2016). Both grape skins and other matter than grapes can provide important fractions of total volatile compounds present that may affect sensory and chemical profiles of grape juice. In particular, skins contain relatively high concentrations of free monoterpenes, norisoprenoids, aldehydes and volatile phenols (Noguerol-Pato et al., 2012). Free volatile phenols like vanillin and isoeugenol may be directly recovered from grape skins (Noguerol-Pato et al., 2012; Slegers et al., 2015) or formed as by-products of the lignin breakdown during thermomaceration of wood related material (Martínez-Gil et al., 2011; Weldegergis et al., 2011). Additionally, grapevine leaves may release terpenoids and benzenoids into grape juice (Ward et al., 2015). As a general rule, it is known that glycosylated terpenes are not odorant and form the hidden aroma of grapes. Therefore, their extraction into must during thermomaceration followed by their hydrolysis and conversion into odor-active aglycones during storage can play an important role in the development of enriched juice flavor. However, the increase and de novo synthesis of free and bound aroma compounds after thermomaceration

Table 1. Aroma compounds in grape musts and enriched juice identified by GC-MS and confirmed by linear retention index (LRI)[1] values.

Nr	Compound	Prv[2]	MS Q[3]	LRI[a]	LRI[b]	CAS[4]	Odor descriptor[5]
	Terpenoids						
1	D-Limonene	S	99	1027	1027	5989-27-5	Citrus, sweet, herbal[III]
2	*trans*-Geranylacetone		95	1451	1454	3796-70-1	Green, fruity, rose, waxy, woody, pear, guava[V, VI]
	Norisoprenoids						
3	Vitispirane		98	1279	1271	65416-59-3	Floral[III]
4	Naphthalene, 1,2-dihydro-1,1,6-trimethyl- (TDN)		97	1351	1355	30364-38-6	Licorice[III]
5	β-Damascenone		97	1384	1388	23726-93-4	Honey, sweet[III]
	Lactones						
6	2(*5H*)-Furanone	S	80	913	916	497-23-4	Buttery[IV]
7	γ-Dodecalactone		80	1677	1681	2305-05-7	Fatty, fruity, peach, sweet[VI]
	Aldehydes and ketones						
8	Furfural	F	93	831	835	98-01-1	Sweet, woody, bready, caramel[III]
9	Octanal	S	85	1002	1001	124-13-0	Waxy, citrus, orange[IV]
10	2-Nonanone		86	1090	1091	821-55-6	Fruity, sweet, waxy, soapy, cheese, herbal, coconut[V, VI]
11	Nonanal	S	91	1103	1102	124-19-6	Waxy, citrus, cucumber[III, IV]
12	Decanal	S	91	1204	1206	112-31-2	Sweet, citrus, orange, waxy[IV]
13	5-Hydroxymethyl-2-furfural	S	75-91	1225	1230	67-47-0	Fatty, buttery, musty, waxy, caramel[VI]
	Alcohols						
14	2-Furanmethanol	F	97	851	853	98-00-0	Chemical, musty, sweet, caramel, bread, coffee[III]
15	1-Hexanol	F	83	865	867	111-27-3	Green, fruity, apple-skin, oily[III]
16	2-Ethyl hexanol		87	1027	1027	104-76-7	Citrus, fresh, floral, oily, sweet[V, VI]
17	Phenethyl alcohol	F	91	1111	1114	60-12-8	Floral, sweet, fresh, bready[II]
18	Decanol	F	90	1271	1272	112-30-1	Sweet, fatty[V]
19	Dodecanol		90	1472	1475	112-53-8	Flowery in low concentration[V]
	Benzenoids						
20	Benzaldehyde	S	93	958	959	100-52-7	Bitter almond, walnut, smoked[III]
21	Phenol	M	87	979	981	108-95-2	Sweet, tar-like[III, V]
22	Guaiacol	S	76-92	1087	1086	90-05-1	Smoke, sweet, medicine[V]
23	2,4-Dimethylbenzaldehyde		95	1183	1181	15764-16-6	Naphthyl, cherry, almond, spice, vanilla[VI]
24	2-Methoxy-4-vinylphenol	S	94	1311	1313	7786-61-0	Spicy, clove, peanut, woody[I, VI]
25	Benzaldehyde, 4-hydroxy-3-methoxy (*vanillin*)	S	95	1396	1395	121-33-5	Vanilla[I]
26	Isoeugenol	S	95	1448	1456	5932-68-3	Spicy[I]
27	2,4-Di-*tert*-butylphenol		97	1511	1513	96-76-4	Bilberry, phenolic[III, VI]
28	Benzophenone	M	96	1625	1623	119-61-9	Balsam, rose, metallic, powdery geranium[III]
	Alkenes						
29	1-Tetradecene		95	1390	1389	1120-36-1	Mild, waxy[IV]
	Acids						
30	Octanoic acid	F	80	1171	1176	124-07-2	Fatty, waxy, rancid, vegetable, cheese[II]
31	Nonanoic acid	S	91	1269	1273	112-05-0	Waxy, cheese[III]
32	*n*-Decanoic acid	F	97	1365	1368	334-48-5	Rancid, sour, fatty, citrus[III]
33	Dodecanoic acid	S	98	1560	1566	143-07-7	Fatty, coconut, bay oil[III]
34	Tetradecanoic acid	S	97	1757	1763	544-63-8	Waxy, fatty, soapy, coconut[III]
35	9-Hexadecenoic acid		97	1934	1942	2019-29-4	Waxy, creamy, fatty, soapy[III]
36	Hexadecanoic acid	S	99	1954	1959	57-10-3	Waxy, fatty[III]
37	9,12-Octadecadienoic acid		98	2145	2144	60-33-3	Fatty[III]
	Esters						
38	1-Butanol, 3-methyl-, acetate	F	86	875	884	123-92-2	Sweet, banana, fruity[III]
39	Hexyl acetate	S	86	1013	1013	142-92-7	Green, fruity, sweet, fatty, fresh, apple, pear[III]
40	Ethyl heptanoate	S	< 70	1098	1095	106-30-9	Fruity, pineapple, sweet, banana[III]
41	Ethyl octanoate	F	75-98	1196	1196	106-32-1	Waxy, sweet, musty, pineapple, fruity[III]
42	2-Phenethyl acetate	S	86	1256	1256	103-45-7	Fruity, rose, sweet, honey[IV]
43	Ethyl decanoate	F	99	1394	1397	110-38-3	Sweet, waxy, fruity, apple[III]
44	Ethyl dodecanoate	F	97	1593	1593	106-33-2	Sweet[V, VI]
45	2-Phenethyl hexanoate	S	86	1641	1639	6290-37-5	Sweet, honey, floral, waxy[VI]
46	Ethyl tetradecanoate	F	96	1792	1793	124-06-1	Sweet fruit, butter, fatty[VI]
47	2-Phenethyl octanoate	S	90	1845	1838	5457-70-5	Sweet, waxy, green cocoa, fruity[VI]
48	Methyl hexadecanoate		98	1921	1926	112-39-0	Waxy, fatty, orris[VI]
49	Ethyl E-11-hexadecenoate		96	1967	1974	1000245-7-9	Waxy, leather[VI]
50	Ethyl hexadecanoate	F	99	1990	1994	628-97-7	Waxy, fruity, creamy, milky[V, VI]

[1] LRI: Linear retention index in a HP-5MS capillary column (30 m/0.25 mm/0.25 μm, He). LRI[a]: Calculated values using van den Dool and Kratz equation. LRI[b]: Data collected from the NIST Chemistry WebBook.

[2] Prv: Standard providers. F: Fluka; M: Merck; S: Sigma-Aldrich.

[3] MS Q: Mass spectrum match quality. This number is a measure of the similarity of the mass spectrum of the compound obtained by sample analysis and those compiled in the NIST or Willey mass spectra libraries. Values near to 100 suggest that the mass spectrum is the same and corresponds to only one compound.

[4] CAS: Chemical Abstracts Service number.

[5] Reference I: Baba and Kumazawa, 2014; II: López de Lerma et al., 2012; III: Vararu et al., 2015; IV: Vararu et al., 2016; V: Welke et al., 2014b; VI: TGSC, 2017.

Table 2. Relative area, standard deviations (n = 3 samples and triplicate analysis) and homogeneous groups (HG) for free aroma compounds.

		'País' must		'Lachryma Christi' must		Enriched grape juice	
Nr	Compound	Mean ± SD	HG	Mean ± SD	HG	Mean ± SD	HG
1	Terpenoids	0.170 ± 0.010	b	0.340 ± 0.030	c	NF	a
2		0.118 ± 0.003	a	0.120 ± 0.010	a	0.130 ± 0.010	a
3	Norisoprenoids	0.210 ± 0.030	b	NF	a	NF	a
4		0.260 ± 0.010	b	NF	a	NF	a
5		0.154 ± 0.005	b	NF	a	NF	a
6	Lactones	0.120 ± 0.010	b	0.079 ± 0.005	a	0.170 ± 0.010	c
7		NF	a	0.114 ± 0.010	b	NF	a
8	Aldehydes and ketones	0.160 ± 0.010	b	0.100 ± 0.020	a	0.180 ± 0.020	b
9		0.064 ± 0.003	a	0.080 ± 0.010	b	0.078 ± 0.003	b
10		0.500 ± 0.030	b	NF	a	NF	a
11		0.090 ± 0.010	a	0.570 ± 0.040	b	0.550 ± 0.010	b
12		0.090 ± 0.010	a	0.330 ± 0.020	c	0.240 ± 0.020	b
13		0.006 ± 0.000	b	NF	a	0.130 ± 0.010	c
14	Alcohols	0.230 ± 0.010	b	0.170 ± 0.010	a	0.360 ± 0.010	c
15		0.212 ± 0.001	c	0.170 ± 0.010	b	NF	a
16		0.042 ± 0.001	b	0.040 ± 0.010	ab	0.034 ± 0.002	a
17		0.100 ± 0.010	b	NF	a	NF	a
18		0.080 ± 0.010	b	NF	a	NF	a
19		0.096 ± 0.003	a	0.140 ± 0.010	b	0.146 ± 0.001	b
20	Benzenoids	0.030 ± 0.002	a	0.040 ± 0.003	b	0.044 ± 0.003	b
21		NF	a	NF	a	0.080 ± 0.010	b
22		0.025 ± 0.002	a	0.035 ± 0.003	b	0.032 ± 0.001	b
23		0.180 ± 0.020	a	0.170 ± 0.010	a	0.180 ± 0.010	a
24		0.045 ± 0.002	b	NF	a	0.080 ± 0.010	c
25		NF	a	NF	a	0.016 ± 0.001	b
26		NF	a	NF	a	0.040 ± 0.003	b
27		0.100 ± 0.002	b	4.52 ± 0.19	c	0.088 ± 0.002	a
28		0.270 ± 0.010	b	0.210 ± 0.010	a	0.220 ± 0.010	a
29	Alkenes	0.100 ± 0.010	b	0.100 ± 0.010	b	0.076 ± 0.005	a
30	Acids	0.260 ± 0.020	b	0.170 ± 0.020	a	0.180 ± 0.020	a
31		0.120 ± 0.010	a	0.170 ± 0.010	b	0.160 ± 0.010	b
32		0.130 ±0.010	b	0.090 ± 0.020	a	0.080 ± 0.010	a
33		0.189 ± 0.005	ab	0.230 ± 0.020	b	0.170 ± 0.030	a
34		0.085 ± 0.001	a	0.140 ± 0.010	b	0.095 ± 0.005	a
35		NF	a	0.280 ± 0.040	b	NF	a
36		0.370 ± 0.040	a	1.99 ± 0.23	c	1.56 ± 0.10	b
37		NF	a	0.140 ± 0.070	b	0.181 ± 0.003	b
38	Esters	0.360 ± 0.020	b	NF	a	NF	a
39		0.150 ± 0.010	b	0.140 ± 0.020	b	NF	a
40		0.038 ± 0.001	c	0.035 ± 0.003	b	NF	a
41		0.055 ± 0.001	a	0.080 ± 0.010	b	0.056 ± 0.002	a
42		0.130 ± 0.004	b	NF	a	NF	a
43		0.270 ± 0.020	b	NF	a	NF	a
44		0.300 ± 0.010	b	NF	a	NF	a
45		0.296 ± 0.003	b	NF	a	NF	a
46		0.067 ± 0.004	b	NF	a	NF	a
47		0.740 ± 0.020	b	NF	a	NF	a
48		0.015 ± 0.001	a	0.070 ± 0.010	b	0.077 ± 0.001	b
49		0.640 ± 0.040	b	NF	a	NF	a
50		0.160 ± 0.010	b	NF	a	NF	a

For compound details see Table 1.
SD: Standard deviation; NF: not found.
Different letters in the same row indicate significant differences among groups at p = 0.05, according to Fisher's least significant difference method.

require further chemometric differentiation between must and juice samples, as well as a statistical selection of key components. This may help to distinguish between process and raw material effects in grape juice enrichment.

Differentiation of grape musts and juice

Taking into account the diversity of factors that affect the level of each volatile compound in must and juice samples, it is often difficult to interpret volatile data and to establish relationships between chemical constituents, sensory properties and process conditions. However, multivariate data analysis may be useful to group between must samples with and without thermomaceration, and to identify chemical groups and specific aroma compounds that contribute to odor perception of grape musts and enriched juice. Using the data of relative peak area of free aroma compounds, 10 compounds numbered as 1, 6, 12, 13, 14, 15, 24, 27, 36 and 40, show three homogeneous groups (HG) (Table 2). These compounds have been selected for statistical differentiation by cluster analysis (CA) among must and juice samples. On the other hand, nine compounds with numbers 1, 3, 5, 13, 20, 26, 36, 37 and 39, show

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

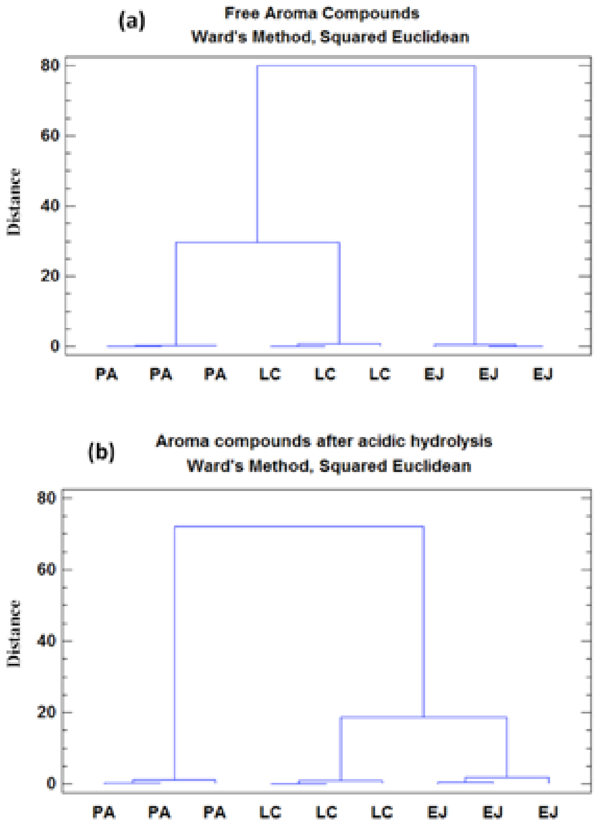
		'País' must		'Lachryma Christi' must		Enriched grape juice	
Nr	Compound	Mean ± SD	HG	Mean ± SD	HG	Mean ± SD	HG
1	Terpenoids	0.120 ± 0.010	a	0.160 ± 0.010	b	0.220 ± 0.030	c
2		0.120 ± 0.010	a	0.130 ± 0.010	ab	0.150 ± 0.010	b
3	Norisoprenoids	1.21 ± 0.05	c	0.570 ± 0.010	a	0.810 ± 0.030	b
4		0.240 ± 0.010	a	0.220 ± 0.020	a	0.680 ± 0.060	b
5		0.160 ± 0.010	b	0.270 ± 0.010	c	NF	a
6	Lactones	NF	a	0.110 ± 0.020	b	0.100 ± 0.010	b
8	Aldehydes and ketones	0.105 ± 0.003	a	0.160 ± 0.040	b	0.160 ± 0.010	b
9		0.070 ± 0.010	a	0.090 ± 0.010	b	0.070 ± 0.010	a
10		0.270 ± 0.030	b	NF	a	NF	a
11		0.480 ± 0.030	a	0.560 ± 0.030	ab	0.640 ± 0.100	b
12		0.150 ± 0.010	a	0.210 ± 0.010	b	0.240 ± 0.050	b
13		NF	a	0.110 ± 0.010	b	0.130 ± 0.020	c
14	Alcohols	0.131 ± 0.005	a	0.220 ± 0010	b	0.230 ± 0.020	b
15		0.210 ± 0.020	b	0.220 ± 0.010	b	NF	a
16		NF	a	NF	a	0.040 ± 0.020	b
17		0.110 ± 0.010	b	NF	a	NF	a
18		0.072 ± 0.004	b	NF	a	NF	a
19		0.078 ± 0.004	b	NF	a	0.100 ± 0.020	b
20	Benzenoids	0.060 ± 0.010	b	0.029 ± 0.001	a	0.390 ± 0.004	c
22		NF	a	0.039 ± 0.002	b	NF	a
23		0.148 ± 0.004	a	0.168 ± 0.008	b	0.170 ± 0.010	b
24		0.052 ± 0.000	a	0.091 ± 0.006	b	0.100 ± 0.010	b
25		NF	a	0.032 ± 0.004	b	0.040 ± 0.010	b
26		NF	a	0.048 ± 0.004	b	0.100 ± 0.010	c
27		0.082 ± 0.004	a	0.088 ± 0.004	a	0.080 ± 0.010	a
28		0.250 ± 0.030	a	0.246 ± 0.020	a	0.240 ± 0.020	a
29	Alkenes	0.050 ± 0.010	b	0.050 ± 0.010	b	NF	a
30	Acids	0.110 ± 0.010	a	0.130 ± 0.010	a	0.120 ± 0.010	a
31		0.090 ± 0.020	a	0.090 ± 0.010	a	0.120 ± 0.010	a
32		0.130 ± 0.020	b	0.050 ± 0.010	a	0.050 ± 0.010	a
33		0.210 ± 0.010	b	0.120 ± 0.020	a	0.180 ± 0.020	b
34		0.100 ± 0.003	a	0.110 ± 0.010	a	0.110 ± 0.020	a
35		NF	a	0.970 ± 0.080	b	NF	a
36		0.370 ± 0.040	a	0.850 ± 0.140	b	1.18 ± 0.31	c
37		NF	a	0.064 ± 0.002	b	0.090 ± 0.020	c
38	Esters	0.190 ± 0.020	b	NF	a	NF	a
39		0.200 ± 0.010	c	0.119 ± 0.004	b	NF	a
40		0.042 ± 0.004	a	0.070 ± 0.010	b	0.080 ± 0.010	b
41		0.160 ± 0.010	a	0.155 ± 0.000	a	0.170 ± 0.020	a
42		0.072 ± 0.004	b	NF	a	NF	a
48		0.060 ± 0.001	a	0.070 ± 0.002	b	0.080 ± 0.010	b

For compound details see Table 1.
SD: Standard deviation; NF: not found.
Different letters in the same row indicate significant differences among groups at p = 0.05, according to Fisher's least significant difference method.

significant differences ($p < 0.05$) among the three samples (PA, LC and EJ) according to the data of relative peak area of free and bound aroma compounds that have been selected for further differentiation among the samples (Table 3). According to Figure 1a, PA and LC musts show more similarity with regard to free aroma compounds compared to enriched juice. On the other hand, chemical composition of PA must for both free and bound aroma compounds is different compared to LC and EJ samples (Figure 1b). Thermomaceration of grape pomace, leaves and canes in must modifies sensory profile by free aroma compounds and to a less extent the global aroma compounds detected after hydrolysis. Apparently, thermomaceration is unable to provoke the hydrolysis of a large amount of bound aroma compounds.

After statistical treatment by MVA of analytical data of volatile compounds previously classified in chemical families, the resulting Sunray Plots (Statgraphics Plus, 2.0, STSC Inc., Rockville, Maryland, USA; Figure 2) yield a visual overview of free and total aroma development from grape musts to enriched juice. In here, each ray in the polygon corresponds to a chemical family. 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 center the mean minus three standard deviations. Concerning free aroma compounds, both PA and LC musts exhibit irregular polygons, while a relatively regular polygon structure was found for the EJ sample (Figure 2). These fingerprints show high ester values for PA. These compounds are related to odor descriptors that result into a pleasant fruit-like odor, while benzenoids prevail in LC are related to a phenolic, balsamic odor. Fingerprints differ also for aroma compounds after acidic hydrolysis. In this case, a more regular polygon structure can be observed for LC must (Figure 2). Fatty acids with an unpleasant rancid odor are predominant in LC and enriched

Figure 1. Dendrograms resulting from applying cluster analysis to the selected aroma compounds as: Free aroma compounds (a) and total content (b) of aroma compounds after acidic hydrolysis.

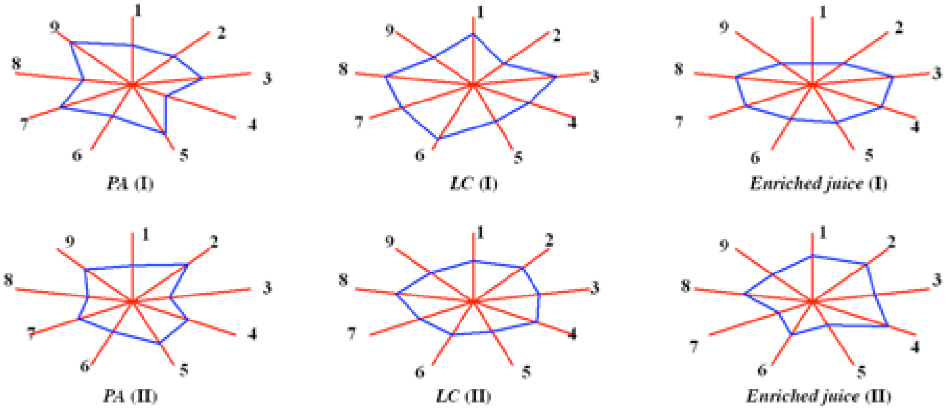


PA: 'País' must; LC: 'Lachryma Christi' must; EJ: Enriched grape juice.

juice, while norisoprenoids with a floral odor prevail in PA must. These results are in accordance with Vararu et al. (2015) who found that fingerprints depend upon cultivars and grape-growing area.

In order to clarify differences among grape musts and enriched juice, and identify constituents able to distinguish thermomaceration from control treatments, a principal component analysis was carried out using as grouping variables those aroma compounds with different homogeneous groups. According to Figure 3a, two principal components (PC) accounted for 99.10% of the total variance of free aroma compounds. For the first PC, four free aroma compounds (D-limonene (1), 1-hexanol (15), 2,4-di-*tert*-butylphenol (27) and ethyl heptanoate (40)) have been selected due to their positive weight coefficients higher than 0.3, while the compounds 2(5*H*)-furanone (6), 5-hydroxymethyl-2-furfural (13), 2-furanmethanol (14) and 2-methoxy-4-vinylphenol (24) selected because of their negative weight coefficients less than -0.3. For the second PC, decanal (12), 1-hexanol (15), 2,4-di-*tert*-butylphenol (27) and hexadecanoic acid (36) show the highest contribution in must characterization. Then, PC1 and PC2 scores group enriched grape juice and musts of both grape cultivars. 'Lachryma Christi' must differs from PA must and EJ sample by a higher score on PC1, while PA must is differentiated by its negative PC2 score (Figure 3a). Free compounds that considerably contribute to the aroma profile of LC must are 2,4-di-*tert*-butylphenol (27) with bilberry and D-limonene (1) with citrus, sweet and herbal odor descriptors. Free 5-hydroxymethyl-2-furfural (13) yields a significant contribution to the aroma profile of enriched grape juice, which indicates the importance of heat treatment for juice processing. The free aroma profile of PA must is more complex, but should include those compounds with a negative weight coefficient for PC2 (1-hexanol (15) and ethyl heptanoate (40)). On the other hand, PCA done with nine compounds selected after acidic hydrolysis of musts and juice provided two principal components: PC1 with an eigenvalue of 6.86 and PC2 with 1.97, explaining 76.25% and 21.88% of the total variance,

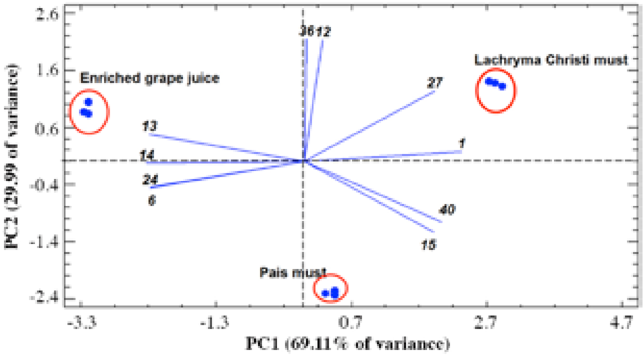
Figure 2. Grape musts fingerprint obtained by multivariate data analysis of aroma compounds as free forms (I series) and after acidic hydrolysis (II series), grouped by chemical families.



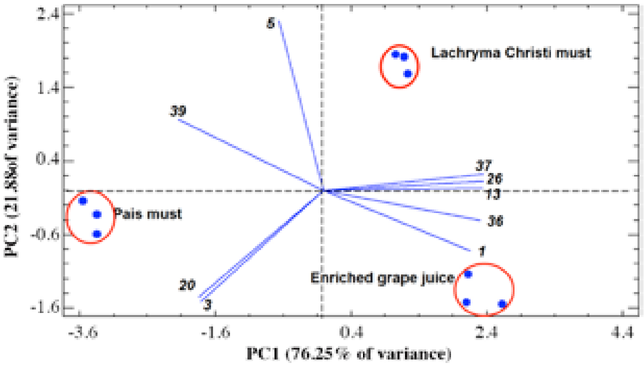
PA: 'País' must; LC: 'Lachryma Christi' must.
Each number corresponds to a chemical family: 1. Terpenoids; 2. Norisoprenoids; 3. Lactones; 4. Aldehydes and ketones; 5. Alcohols; 6. Benzenoid compounds; 7. Alkenes; 8. Acids; 9. Esters.

Figure 3. Principal component analyses of selected aroma compounds as free forms (a) and after acidic hydrolysis (b). For compound numbers see Table 1.

(a) Free Aroma Compounds (99.10% of total variance)



(b) Aroma compounds after acidic hydrolysis (98.13% of total variance)



respectively. The most important free and compounds after acidic hydrolysis that contribute to PC1 with a positive weight coefficient are D-limonene (1), 5-hydroxymethyl-2-furfural (13), isoeugenol (26), hexadecanoic acid (36) and 9,12-octadecadienoic acid (37). Just hexyl acetate (39) contributes to PC1 with a negative weight coefficient. The most important free and bound aroma compounds for PC2 are vitispirane (3), β-damascenone (5) and benzaldehyde (20) with weight coefficients of -0.446, 0.679 and -0.425, respectively. According to Figure 3b, LC and EJ are located to the right, showing a positive score for PC1, while PA is located at the left side. LC and EJ have opposite scores for PC2, while PA shows values near to zero for PC2. After hydrolysis, D-limonene (1) is the most characteristic aroma compound for enriched grape juice (Figure 3b). None of the selected aroma compounds after hydrolysis could be individualized and associated directly to LC or PA must. The results obtained by means of PCA after acidic hydrolysis show the complexity of aroma potential of PA and LC musts.

According to previous results chemometrics can be successfully applied to characterize grape juice samples after thermomaceration being D-limonene, 5-hydroxymethyl-2-furfural and hexadecanoic acid the compounds that contributed most to the free aroma fraction and the fraction obtained after hydrolysis. In addition, 5-hydroxymethyl-2-furfural, a key component of the free aroma fraction of enriched juice, seems to be related to juice processing conditions. Heating of monosaccharides under acidic conditions e.g. pasteurization of fruit juices gives rise to a large number of furan compounds, amongst them 5-hydroxymethyl-2-furfural (Belitz et al., 2009), which agrees with the results of this study (Table 2). Another key component is hexadecanoic acid that may become available after grape crushing due to the contact between must and skins, particularly, in case of 'Lachryma Christi' grapes. Esters of hexadecanoic acids are the major part of cuticular waxes of red grape skins (Mendes et al., 2013), which may be converted into the free acid form during juice processing and thermomaceration.

CONCLUSIONS

Since viticulturists are looking for new productive alternatives for ancient undervalued wine grape cultivars, this study provides new strategies for their valorization by revealing the chemical composition and aroma potential of grape musts and winery and grapevine by-products. In particular, chemometrics applied to aroma data using SBSE-TD-GC-MS with and without hydrolysis are very useful for the characterization of pure grape juices of País and Lachryma Christi cultivars and the resulting juice from thermomaceration. In addition, grape pomace, grapevine leaves and canes are valuable sources to yield aroma enrichment of grape juice or the generation of new aroma compounds. Juice processing conditions affect the rather complex aroma profile of enriched grape juice.

ACKNOWLEDGEMENTS

The authors wish to thank Viña Zamora for providing grape samples, and the Chilean Ministry of Education (FONDEF VIU 120010) and the European Union (Erasmus + KA1) for financial support.

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