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Aroma biogenesis and distribution between olive pulps and seeds with identification of aroma trends among cultivars

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ABSTRACT

The two constitutive parts of four cultivars (Arbequina, Picual, *Local* and Manzanilla de Sevilla) grown in Spain were separately analysed in order to establish the role of pulp and seed in the biogenesis of extra virgin olive oil (EVOO) aroma through the lipoxygenase (LOX) pathway. C6 and C5 volatile compounds responsible of EVOO aroma were produced by endogenous enzymes in both parts of olive fruits and the differences can be attributed to different enzymes distribution in pulp and seed. According to results, C6 and C5 volatile compounds have mainly their biogenesis in pulp (80–90%) *vs.* seed (20–10%), independently of the cultivar considered. A linear discriminant analysis was used to establish discriminant aroma compounds between pulp and seed related to the maturity index. A decrease in *trans*-2-hexen-1-al and an increase in 1-hexanol with ripeness were observed independently of the cultivar considered. Finally, Partial Least Squares (PLS) regression analysis between pulp and seed aroma compounds allowed to establish those volatile compounds that better describe each cultivar.

1. Introduction

Extra-virgin olive oil (EVOO) is the principal source of fat in the Mediterranean diet (Krichène, Allalout, Salvador, Fregapane, & Zarrouk, 2010). Spain is a traditionally olive-growing area of the Mediterranean producing and exporting high quality EVOO from a wide variety of cultivars. The Spanish olive grove is present in 34 of the 50 Spanish provinces and occupies an area of 2,509,677 ha. The areas of olive production in Spain – in descending order – are Andalusia (60.4%), Castilla-La Mancha (15.8%), Extremadura (10.2%), Catalonia (4.6%), Valencia (3.7%), Aragon (2.3%) and others (3.1%) like Galicia (AAO, 2011).

EVOO is highly appreciated by consumers due to their health benefits as well as their aromatic characteristics; this is the reason why is important to prevent off-flavours by different contamination processes (Gamazo-Vázquez, García-Falcón, & Simal-Gándara, 2003; Quinto-Fernández, Pérez-Lamela, & Simal-Gándara, 2003). Volatile compounds responsible for the oil aroma profiles, more than 100 components, are mainly formed from chemical and enzymatic oxidation. Endogenous plant enzymes, especially those involved through the lipoxygenase (LOX) pathway, are considered responsible for the positive oil aroma. Aliphatic C_6 and C_5 compounds – aldehydes, alcohols and their corresponding esters from LOX pathway – represent the most important fraction (80%) of volatile compounds of high-quality virgin olive oils from a quantitative point of view (Angerosa et al., 2004). The presence of other minor volatile compounds gives information about authentication of olive oils (Aparicio, Morales, & Alonso, 1997). However chemical oxidation and exogenous enzymes, usually from microbial activity, are associated with sensory defects (Morales, Luna, & Aparicio, 2005).

To our knowledge few studies have been published concerning the aroma distribution between pulp and seed in olives for oil extraction (Luaces, Pérez, & Sanz 2003; Servili et al., 2007). The aims of this work were to obtain the aroma distribution of the constitutive parts (pulp and seed) of different varieties of olive fruits – Arbequina, Picual, *Local* and Manzanilla de Sevilla – to define their role in the biogenesis of EVOO aroma and select those compounds that better describe each cultivar.

2. Materials and methods

2.1. Olive sampling and cultivar identification

For this study, four olive cultivars growing in different regions of Spain – Andalusia (S.W. Spain) and Galicia (N.W. Spain) – were selected such it was described in Table 1: Arbequina, Picual, *Local* and Manzanilla de Sevilla. From each cultivar and location five independent lots of olive fruits, approximately 3 kg each, were collected in December 2010 and immediately transported to the laboratory under refrigerated conditions. Once in the laboratory, the samples were stored at −80 °C until analysis.

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Table 1
Physical–chemical parameters of the olives studied, together with their weight percentage of pulp and seed.

Olive sample	Origin	Ripeness index[a]	Moisture (%)	Oil content (%, f.w.)	Industrial oil yield (%) by Abencor[a]	Pulp[b] (%)	Seed[c] (%)
Arbequina	Galicia	2.2	44.0	22.9	18.6	60.5	39.5
	Andalusia	1.5	59.0	14.7	11.1	74.0	26.0
Picual	Galicia	4.8	49.3	25.9	23.3	80.7	19.3
	Andalusia	3.7	50.0	18.5	[d]	73.2	26.8
Local	Galicia	5.4	45.1	28.3	24.8	80.9	19.1
Manzanilla de Sevilla	Galicia	4.2	57.7	17.7	14.6	81.7	18.3

[a] Significant linear regression equations for Industrial oil yield (%) and ripeness index: – Industrial oil yield (%) = 0.852 × oil content (%, f.w.); $r^2 = 0.997$ – Ripeness index = −0.181 × moisture (%) + 0.171 × pulp (%); $r^2 = 0.991$.
[b] ($n = 5$) Mean of samples.
[c] ($n = 5$) Mean of samples.
[d] The amount of olives collected was not sufficient for the calculation of this parameter.

Fingerprinting based on Simple Sequence Repeat (SSR) markers was employed for characterising the studied cultivars. Approximately 1 kg of olives from each cultivar was send to the Pomology Group at the Department of Agronomy at the University of Cordoba (Spain) to develop the molecular studies. Nine microsatellite primers (DCA01, DCA04, DCA09, DCA18, UDO15, UDO17, GAPU59, GAPU71B y GAPU89) were used for the analysis following the protocol described by Belaj, Muñoz-Díez, Baldoni, Satovic, and Barranco (2010). Correctly identified varieties included in the database of World Olive Germplasm Bank of Cordoba-Spain (WOGB), which represents the main repository of olive genotypes in Spain, were employed as reference samples.

One of the samples (called by the authors *Local* variety), did not match with any variety included in the database. Additionally, this cultivar was characterised using biometrical parameters of the fruit and endocarp. The methodology used in this characterization is based on the recommendations of the International Olive Council (COI, 1997) which refer the analysis of 32 different characteristics including the tree, leaves, flowering, fruits and endocarps. This work focuses only on the fruit and endocarp characterization (total of 19 parameters shown in Table 2). After fruits characterization, the endocarps were removed and subjected to the characterization shown in Table 1.

Table 2
Morphological characterization of *Local* variety.

Character	Level of expression
Fruit	
Weight	Medium
Shape in position (A)	Ovoid
Symmetry in position (A)	Slightly asymmetric
Position of maximum transverse diameter in position (B)	Central
Apex in position (A)	Rounded
Base in position (A)	Truncate
Nipple	Absent
Colour at full maturity	Black
Endocarp	
Weight	Medium
Shape in position (A)	Elliptic
Symmetry in position (A)	Asymmetric
Symmetry in position (B)	Symmetric
Position of maximum transverse diameter in position (B)	Towards apex
Apex in position (A)	Rounded
Base in position (A)	Pointed
Surface in position (B)	Rough
Number of grooves	Medium
Distribution of the grooves	Regular
Termination of the apex in position (A)	With mucro

2.2. Physical–chemical parameters of olive samples

2.2.1. General

Several physical–chemical parameters were determined as specified below and the obtained results are shown in Table 1. To evaluate them, the constitutive parts of the fruit were grouped as follows: pulp (sum of skin and flesh) and seed (sum of stone and seed).

2.2.2. Fruit ripeness

It was determined according to the method proposed by the International Olive Council (IOOC, 1984), based on the evaluation of the olive skin and pulp colours of the fruit.

2.2.3. Water content of olive fruit

It was determined by desiccation according to the UNE Spanish Standard method 55032:1973.

2.2.4. Fat content

It was determined by low-resolution pulsed nuclear magnetic resonance spectroscopy (NMR) according to ISO 8292-1:2010 method. The fat content was expressed as percentage of fresh olive paste weight (oil content, %, f.w.).

2.2.5. Industrial oil yield

The percentage of extractable oil was determined for each sample using the Abencor system (Martínez Suárez, Muñoz Aranda, Alba Mendoza, & Lanzón Rey, 1975). This system reproduces the industrial process (at laboratory scale) through three basic elements: hammer mill, thermomixer and centrifuge.

Significant linear regressions were found between industrial oil yield (%) and oil content (%, f.w.), as well as between ripeness index and moisture (%) and pulp (%), respectively (see Table 1).

2.3. Aroma determination in olive pulp and seed

2.3.1. Preparation of volatile standard solutions

Volatile compounds tested in olives were purchased from Sigma–Aldrich (St. Louis, MO, USA). Individual standard solutions for the volatile compounds were prepared in EtOH (about 20,000 μg/ml). Secondary standard solutions were also prepared by dilution in hexane of the individual standard solutions. Working solutions, containing target analytes, were prepared by mixing and diluting different amounts of secondary standard solutions in hexane. In the same manner a solution was prepared containing the internal standard (IS), 2-octanol.

Stripped oil, used as matrix for calibration, was prepared by vacuum pumping commercial refined sunflower oil purchased by Aceites Abril S.L. (Galicia). Calibration standard solutions were

prepared by spiking appropriate working solutions in the stripped refined sunflower oil.

2.3.2. Extraction of olive volatile compounds

Volatile compounds were extracted from the olives samples by DHS with an automatic sampler device, the Master DHS (DANI Instruments S.p.A., Cologno Monzene, Milan, Italy), following our previous works (Reboredo-Rodríguez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2012, 2013). Temperature of extraction was diminished to 40 °C (60 °C for olive oil) to avoid excessive water evaporation because olives contain high concentration of water (Sabatini, Mucciarella, & Marsilio, 2008).

To evaluate the aroma compounds production from the constitutive parts of the fruit, the same two groups – pulp and seed – were considered. Pulp and seed were crushed separately in all cases. A porcelain mortar was used for crushing olive seeds. Two grams of the crushed material (pulp or seed) was placed into a 20 ml vial with the internal standard (2-octanol). Finally, the vial was maintained at 25 °C for 15 min to develop LOX pathway compounds, and then, 2 ml of $CaCl_2$ saturated solution as an enzymatic inhibitor were added, following the protocol by Servili et al. (2007).

2.3.3. Identification and quantification of volatiles by GC–ITMS

Aroma compounds were separated and identified on a Trace GC gas chromatograph with a Polaris Q ion trap mass selective detector and interfaced to a PC computer running the software Xcalibur 1.4, from Thermo Finnigan (Rodano, Italy). Chromatographic separations were done with a ZB-WAX fused-silica capillary column (60 m × 0.32 mm ID, 0.50 μm film thickness) (Phenomenex, Torrance, CA, USA). The carrier gas, helium, was circulated at 1 ml/min in the constant flow mode. A split/splitless injector in the split mode was used (split ratio: 10). The injector temperature was 200 °C. The oven temperature programme was as follows: 40 °C for 5 min; 2 °C/min ramp to 125 °C; 10 °C/min ramp to 250 °C and holding for 5 min. The transfer line temperature was 250 °C, and the ion trap manifold temperature 200 °C. The ion energy for electron impact (EI) was set constantly at 70 eV. Identification of the aroma compounds was achieved by comparing the GC retention times and mass spectra over the mass range 35–300 amu for the samples with those for pure standards analysed under the same conditions. Mass detection was performed in the selected ion recording (SIR) mode for quantification. The results of the relative peak areas were calculated on the basis of the relative calibration curve for each compound and transformed in ng/g of fresh olive weight fruit.

2.4. Statistical treatment

Linear discriminant analysis (LDA) and linear regression were performed with the statistical software package Statgraphics Plus v. 5.1 (Manugistics, Rockville, MD, USA). Partial least squares (PLS) regression was implemented by using the statistical package Unscrambler v. 9.1 for Windows (CAMO Software, Oslo, Norway).

3. Results and discussion

3.1. Role of olive pulp and seed in the biogenesis of EVOO aroma

In order to establish the role of olive pulp and seed in the biogenesis of EVOO aroma, the constitutive parts of olive fruits from several cultivars described above (see Section 2) were separately analysed. As can be seen in Table 3, 17 compounds were determined in both parts of olive fruits and the differences could be attributed to different enzymes distribution in pulp and seed as it is discussed below.

3.1.1. Major volatile compounds

C6 and C5 saturated and unsaturated volatile compounds, responsible for the positive green sensory perceptions in olive oil, were the main compounds identified. They are mainly produced by endogenous olive enzymes, through the LOX pathway, and they are incorporated later into the oily phase.

LOX activity and corresponding contribution (%) of the constitutive parts of olive fruits for two different cultivars (Coratina and Frantoio) were determined by Servili et al. (2007). According to these results the contribution of LOX activity in pulp was higher (*ca.* 80%) than in seed (*ca.* 20%) for both cultivars. In the same way, C6 and C5 compounds had their biogenesis mainly in the olive pulp for the studied varieties such it can be seen in Table 3. C6 aldehydes content in crushed pulp was higher than in crushed seed and they represented the highest percentage (ranging from 79% to 93%) with respect to the total C6 aldehydes content (sum of pulp and seed) except for *Local* cultivar (59%). C6 alcohols content in crushed pulp represented the highest percentage (ranging from 79% to 93%) with respect to the total C6 alcohols content (sum of pulp and seed) except for *Local* and Manzanilla de Sevilla cultivars which percentages were greater (97% and 96%, respectively). Finally, C6 esters content in crushed pulp represented also the highest percentage (ranging from 84% to 94%) with respect to the total C6 esters content (sum of pulp and seed).

3.1.1.1. C6 aldehydes. The LOX pathway is initiated by the release of enzymes when olive fruit tissues are disrupted. In the first step of volatile compound formation, acyl hydrolase (AH, a group of enzymes that includes lipases, phospholipases and galactolipases) hydrolyses tryglicerides and phospholipids to release free fatty acids (Kalua et al., 2007). Then, free fatty acids are oxidised by lipoxygenase (LOX) and cleaved by hydroperoxide lyase (HPL) to yield aldehydes.

LOX in olive pulp is more active with linolenic acid (LnA, 100%) than with linoleic acid (LA, 61%) (Sánchez & Salas, 2000). As a consequence of the cleavage of 13-hydroperoxide from LA, hexanal is formed at lower concentration compared to the same biogenesis from LnA (resulting the unsaturated aldehyde *cis*-3-hexen-1-al) (Kalua et al., 2007). This unsaturated aldehyde is unstable and undergoes rapid isomerisation to stable *trans*-2-hexen-1-al (Williams, Salas, Sánchez, & Harwood, 2000) which represented between 75–94% of the C6 aldehydes in all varieties, except for *Local* cultivar (54%). *trans*-2-Hexen-1-al content, responsible for green and almond sensory notes (Aparicio, Calvente, & Morales, 1996), ranged from 302 ng/g for *Local* crushed pulp to 5225 ng/g for Arbequina crushed pulp. On the other hand, the less amount of hexanal related with the apple and green fruity attributes varied between 146 ng/g for Picual and 622 ng/g for Arbequina crushed pulps.

C6 aldehydes were also formed in crushed seed, coming from LOX pathway, at lower contents (*ca.* 7–21%) in comparison with the pulp except for *Local* cultivar (41%). Some authors pointed the much higher content of LA than LnA in olive seeds (Cortesi, Fiorino, & Rovellini, 1997). This fact could explain that hexanal and *trans*-2-hexen-1-al were present at the same order in seeds, not being *trans*-2-hexen-1-al the main compound now in all cultivars. Nevertheless, olive seed seems to contain enzymes other than HPL that also metabolise 13-hydroperoxides (the substrate of aldehydes) resulting also in a net decrease of unsaturated C6 aldehydes (Luaces et al., 2003), especially in olives grown in Galicia (Picual and *Local*).

3.1.1.2. C6 alcohols. C6 aldehydes are subsequently reduced by alcohol dehydrogenase (ADH) to alcohols (related to fruity, green, and grassy sensory notes). 1-Hexanol was formed from LA (being hexanal as substrate) and *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol

Table 3
Concentration of volatile compounds (ng/g of fresh olive weight fruit) in pulp (a) and seed (b) olives from different varieties and origin (Galicia and Andalusia).

	PIC1	PIC2	ARB1	ARB2	L	M
a. PULP (ng/g ± SD)						
Aldehydes						
trans-2-Hexen-1-al (LnA)	426 ± 72	591 ± 110	2952 ± 397	5225 ± 712	302 ± 65	2905 ± 348
trans-2-Pentenal (LnA)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Hexanal (LA)	146 ± 28	193 ± 12	187 ± 24	622 ± 160	257 ± 55	283 ± 84
Pentanal (LA)	n.d.	n.d.	<LOQ	n.d.	n.d.	180 ± 36
∑ Aldehydes	572	784	3139	5847	559	3368
Alcohols						
trans-2-Hexen-1-ol (LnA)	106 ± 10	n.d.	12 ± 3	40 ± 7	22 ± 3	148 ± 29
trans-3-Hexen-1-ol (LnA)	0.8 ± 0.2	n.d.	n.d.	n.d.	22 ± 5	<LOQ
cis-3-Hexen-1-ol (LnA)	262 ± 57	192 ± 39	37 ± 6	274 ± 41	2415 ± 382	618 ± 86
1-Penten-3-ol (LnA)	<LOQ	11 ± 4	<LOQ	<LOQ	<LOQ	<LOQ
1-Hexanol (LA)	359 ± 51	94 ± 11	47 ± 8	135 ± 27	1585 ± 237	266 ± 31
1-Pentanol (LA)	<LOQ	5.3 ± 2.2	<LOQ	<LOQ	48 ± 4	52 ± 8
trans-2-Penten-1ol (LnA)	<LOQ	n.d.	n.d.	n.d.	32 ± 7	n.d.
∑ Alcohols	728	302	96	449	4124	1084
Esters						
cis-3-Hexenyl acetate (LnA)	42 ± 8	278 ± 24	26 ± 3	127 ± 14	485 ± 55	471 ± 55
Hexyl acetate (LA)	<LOQ	44 ± 3	33 ± 3	143 ± 12	67 ± 6	237 ± 20
∑ Esters	42	322	59	270	552	708
Ketones						
3-Pentanone (LA)	<LOQ	<LOQ	<LOQ	n.d.	91 ± 6	34 ± 7
∑ Ketones	–	–	–	–	91	34
Terpenes						
α-Pinene	n.d.	n.d.	<LOQ	n.d.	n.d.	22 ± 2
p-Cymene	6.1 ± 0.9	n.d.	14 ± 0.1	n.d.	<LOQ	5.3 ± 0.8
Terpinolene	17 ± 4	n.d.	12 ± 0.7	n.d.	87 ± 19	28 ± 5
∑ Terpenes	23	–	26	–	87	55
∑ Total pulp compounds	**1365**	**1408**	**3320**	**6566**	**5413**	**5249**
b. SEED (ng/g ± SD)						
Aldehydes						
trans-2-Hexen-1-al (LnA)	38 ± 5	41 ± 9	254 ± 40	260 ± 40	115 ± 8	162 ± 19
trans-2-Pentenal (LnA)	<LOQ	4.3 ± 1.6	<LOQ	<LOQ	<LOQ	<LOQ
Hexanal (LA)	72 ± 8	42 ± 12	125 ± 21	192 ± 45	153 ± 27	129 ± 26
Pentanal (LA)	45 ± 9	116 ± 23	<LOQ	n.d.	116 ± 5	46 ± 5
∑ Aldehydes	155	203	379	452	384	337
Alcohols						
trans-2-Hexen-1-ol (LnA)	12 ± 1	13 ± 1	<LOQ	14 ± 3	4.6 ± 0.6	12 ± 1
trans-3-Hexen-1-ol (LnA)	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.
cis-3-Hexen-1-ol (LnA)	22 ± 2	44 ± 8	6.1 ± 0.2	43 ± 10	77 ± 11	24 ± 2
1-Penten-3-ol (LnA)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1-Hexanol (LA)	25 ± 3	20 ± 4	8.7 ± 2.0	12 ± 3	40 ± 6	13 ± 3
1-Pentanol (LA)	<LOQ	4.2 ± 0.6	<LOQ	<LOQ	13 ± 3	<LOQ
trans-2-Penten-1ol (LnA)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
∑ Alcohols	59	81	15	69	135	49
Esters						
cis-3-Hexenyl acetate (LnA)	6.0 ± 0.4	39 ± 7	5.4 ± 0.9	22 ± 1	81 ± 12	28 ± 4
Hexyl acetate (LA)	n.d.	10 ± 2	<LOQ	22 ± 3	23 ± 3	19 ± 3
∑ Esters	6.0	49	5.4	44	104	47
Ketones						
3-Pentanone (LA)	<LOQ	29 ± 8	<LOQ	n.d.	19 ± 2	8.0 ± 2.5
∑ Ketones	–	29	–	–	19	8.0
Terpenes						
α-Pinene	n.d.	n.d.	<LOQ	n.d.	n.d.	<LOQ
p-Cymene	1.9 ± 0.1	n.d.	8.9 ± 0.1	n.d.	<LOQ	1.0 ± 0.2
Terpinolene	3.7 ± 0.6	n.d.	7.2 ± 0.3	n.d.	<LOQ	1.9 ± 0.2
∑ Terpenes	5.6	–	16	–	–	2.9
∑ Total seed compounds	**226**	**362**	**415**	**565**	**642**	**444**
∑ Total olive compounds	**1591**	**1770**	**3735**	**7131**	**6055**	**5693**

PIC1: Picual from Galicia; PIC2: Picual from Andalusia; ARB1: Arbequina from Galicia; ARB2: Arbequina from Andalusia; L: *Local* variety; M: Manzanilla de Sevilla.

were formed from LnA (being *cis*-3-hexen-1-al and *trans*-2-hexen-1-al as substrates, respectively). They are mainly synthesized in olive pulp. Higher concentrations of *cis*-3-hexen-1-ol content respect to 1-hexanol were registered for all cultivars except for Picual and Arbequina from Galicia. *cis*-3-Hexen-1-ol content in crushed pulp ranged from 37 ng/g (Arbequina grown in Galicia) to 2415 ng/g

Table 4
Discriminant functions used to predict at which group the new observations (aroma compounds) belong to, together with the means of the value for each group.

Attributes	Function 1 (p = 0.000)	Function 2 (p = 0.000)	Mean		
			2	4	5
(a) Groups of pulp according to the maturity index (2, 4 and 5)					
Standardized coefficients (F = 15)					
trans-2-Hexen-1-al	+0.280	−1.292	**4358**	1783	407
cis-3-Hexenyl acetate	+3.032	+1.693	77	**353**	257
1-Hexanol	−8.569	+0.738	91	183	**934**
trans-3-Hexen-1-ol	+5.789	−1.957	0	0	**11**
(b) Groups of seed according to the maturity index (2, 4 and 5)					
Standardized coefficients (F = 15)					
3-Pentanone	−1.256	-1.216	0	**19**	10
trans-2-Hexen-1-al	−1.297	+0.266	**292**	109	82
1-Hexanol	+1.823	+0.768	12	21	**34**
(c) Groups of pulp vs. seed					
Standardized coefficients (F = 3)					
		Function 1 (p = 0.000)	Mean		
			Pulp	Seed	
Pentanal		−0.392	26	**53**	
trans-2-Hexen-1-al		+2.053	**2183**	170	
p-Cymene		+0.385	**4.2**	2.0	
Hexyl acetate		−2.780	**91**	14	
trans-2-Penten-1ol		−0.553	**4.5**	0.0	
cis-3-Hexenyl acetate		+3.022	**229**	33	
trans-2-Hexen-1-ol		+0.595	**55**	9.7	

Note: Function 1 and 2 significant at a 95% probability level.
In bold: the highest level for the descriptor.

(*Local* cultivar), meanwhile 1-hexanol ranged from 47 ng/g (Arbequina grown in Galicia) to 1585 ng/g (*Local* cultivar). The high alcohol content in pulp should be noted for the *Local* cultivar.

C6 alcohols were also identified in crushed seed in lower levels than in crushed pulp as a difference of the behaviour described by Luaces et al. (2003). Olive seeds seem to be a good source of ADH which is more specific for saturated C6 aldehydes than unsaturated aldehydes and they do not act on C5 alcohols (Luaces et al., 2003). This fact could explain that C5 aldehydes were not transformed into their respective C5 alcohols. However, 1-hexanol content (ranging from 8.7 to 40 ng/g) was lower or in the same order in all cultivars respect to *cis*-3-hexen-1-ol (ranging from 6.1 to 77 ng/g).

3.1.1.3. C6 esters. Alcohols produced by the action of ADH can form acetate esters by the enzyme alcohol acetyl transferase (AAT), resulting hexyl acetate and *cis*-3-hexenyl acetate. *cis*-3-Hexenyl acetate (fruity and grass notes) was the main acetate ester in crushed pulp except for Arbequina cultivar grown in Galicia; this could be attributed to the low AAT activity. This behaviour was also registered in crushed seeds. C6 ester content in crushed pulp ranged from 42 ng/g (Picual grown in Galicia) to 708 ng/g (Manzanilla de Sevilla). C6 ester content in seed ranged from 5.4 ng/g (Arbequina grown in Galicia) to 104 ng/g (*Local*).

3.1.1.4. C5 compounds. These compounds would be generated through an additional branch of the LOX pathway (Angerosa, Basti, Vito, & Lanza, 1999) but they were in minor content than C6. Only two alcohols (1-penten-3-ol and 1-pentanol) were identified in Picual grown in Andalusia crushed pulp; the latest alcohol was also identified in *Local* and Manzanilla de Sevilla cultivars together with 3-pentanone; the aldehyde pentanal was detected only in Local pulp. Two aldehydes (*trans*-2-pentenal and pentanal), one alcohol (1-pentanol) and one ketone (3-pentanone) were identified in crushed seed in some of the studied cultivars.

3.1.2. Minor volatile compounds

Other minor compounds, terpenes, were found in some cultivars and their low content accounted for 1–2% of total volatiles. Whilst other olive oil volatiles are greatly influenced by technological factors, the presence of terpenic hydrocarbons is determined by the variety and growing conditions of the olive trees (Zunin, Boggia, Salvadeo, & Evangelisti, 2005). Only two terpenes (*p*-cymene and terpinolene) were identified just in cultivars grown in Galicia, not in Andalusia. While terpinolene content was higher than *p*-cymene on both parts of Picual, *Local* and Manzanilla de Sevilla olives, these concentrations were quite similar in Arbequina olives. Vichi, Guadayol, Caixach, López-Tamames, and Buxaderas (2006) proposed the monoterpene composition of virgin olive may be taken into consideration to be studied as genetic or geographic markers of virgin olive oil origin.

3.2. Aroma compounds separating pulp and seed at different olive ripeness

A linear discriminant analysis (Table 4) was used to determine which variables (aroma compounds) were significant predictors of groups of samples, namely: groups of pulp according to the maturity index (2, 4 and 5), groups of seed according to the maturity index (2, 4 and 5), and groups of pulp *vs*. seed.

For the groups of pulp according to the maturity index (Table 4a), the linear discriminant analysis was based on a forward step algorithm with an *F-to-enter* = 15, and four variables were used in the analysis to reach a 100% correct classification: the alcohols 1-hexanol and *trans*-3-hexen-1-ol were clearly increasing with maturity index, whereas the aldehyde *trans*-2-hexen-1-al was decreasing; the ester *cis*-3-hexenyl acetate increased from ripeness index of 2–4, and slightly decreased after that. All are C6 compounds, what highlights the importance of these compounds in olive pulp ripeness.

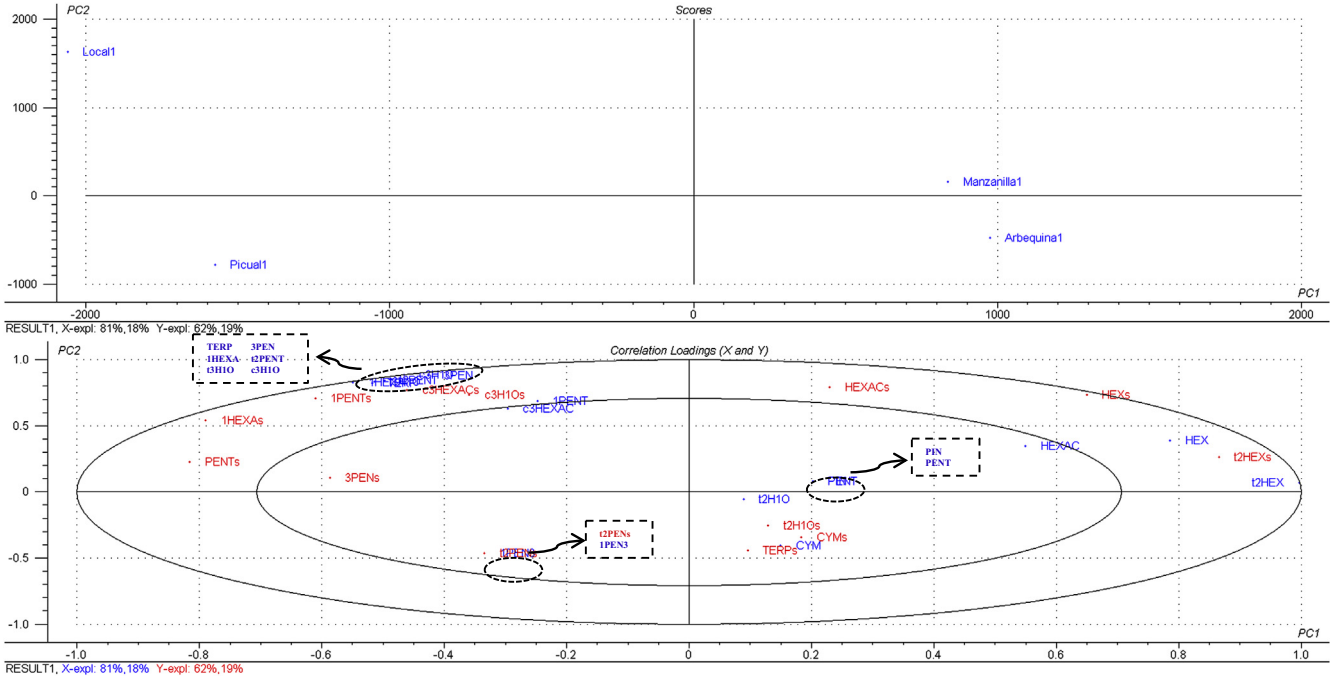

Fig. 1. PLS2: scores plot for olives (top), together with correlations between the loadings of *X* and *Y* variables (bottom). Acronyms used in the figure for pulp compounds (blue): t2HEX: *trans*-2-hexen-1-al; HEX: hexanal; PENT: pentanal; t2H1O: *trans*-2-hexen-1-ol; t3H1O: *trans*-3-hexen-1-ol; c3H1O: *cis*-3-hexen-1-ol; 1PEN3: 1-penten-3-ol; 1HEXA: 1-hexanol; 1PENT: 1-pentanol; t2PENT: *trans*-2-penten-1-ol; c3HEXAC: *cis*-3-hexenyl acetate; HEXAC: hexyl acetate; 3PEN: 3-pentanone; PIN: α-pinene; CYM: *p*-cymene; TERP: terpinolene and seed compounds (red): t2HEXs: *trans*-2-hexen-1-al; t2PENs: *trans*-2-pentenal; HEXs: hexanal; PENTs: pentanal; t2H1Os: *trans*-2-hexen-1-ol; c3H1Os: *cis*-3-hexen-1-ol; 1HEXAs: 1-hexanol; 1PENTs: 1-pentanol; c3HEXACs: *cis*-3-hexenyl acetate; HEXACs: hexyl acetate; 3PENs: 3-pentanone; CYMs: *p*-cymene; TERPS; terpinolene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For the groups of seed according to the maturity index (Table 4b), the linear discriminant analysis was based on a forward step algorithm with an *F-to-enter* = 15, and only three variables were required to reach a 100% correct classification: the aldehyde *trans*-2-hexen-1-al, just as in pulp, was decreasing with the ripening index; also 1-hexanol was increasing with the ripening index; in this case the C5 ketone, 3-pentanone, increased from ripeness index of 2–4, and slightly decreased after that.

As a conclusion, the decrease in *trans*-2-hexen-1-al content and the increase of 1-hexanol content, in both pulp and seed, with ripeness were observed, independently of the cultivar considered. These results are in accordance with previous studies developed in olive oil instead of olives:

- Kalua, Allen, Bedgood, Bishop, and Prenzler (2005) found that the volatile compounds *trans*-2-hexen-1-al, 1-hexanol, 1-penten-3-ol, and *cis*-2-penten-1-ol had a significant contribution toward the discrimination of maturity stages of six cultivars: Leccino, Barnea, Manzanilla, Mission, Corregiola, and Paragon.
- Gómez-Rico, Desamparados-Salvador, La Greca, and Fregapane (2006) reported that the most useful volatiles for classification of the Cornicabra extra virgin olive oil samples according to the stage of ripening of the fruit were 1-hexanol and *trans*-2-hexen-1-ol.

A linear discriminant analysis (Table 4c) was also used to determine which variables were significant predictors of both groups of samples, namely: pulp *vs*. seed from the studied olives. The discriminant analysis was based on a forward step algorithm with an *F-to-enter* = 3, and the only one standardized discriminating function needed seven variables to reach a 100% correct classification: *trans*-2-hexen-1-al >> *cis*-3-hexenyl acetate >> hexyl acetate > *trans*-2-hexen-1-ol were dominant in pulp *vs*. seed, being these four aroma compounds those clearly separating both groups. The thirteen times higher amount of the unsaturated aldehyde *trans*-2-hexen-1-al, main metabolite in pulp *vs*. seeds, is attributable to the much higher activity in the pulp of the HPL enzyme (Servili et al., 2007). The seven times higher amount of *cis*-hexenyl acetate in pulp *vs*. seeds proved a very high content of AAT in the pulp catalysing the production of this ester.

3.3. Aroma trends that best distinguish among the cultivars

Partial Least Squares (PLS) regression is a multivariate technique widely used to relate both sensory and aroma data sets or either of them (Cozzolino, Cynkar, Shah, Dambergs, & Smith, 2009). PLS2 modelling (Fig. 1) between the matrices of 17 aroma compounds determined by GC–ITMS in both pulp and seed of 30 olive samples (5 *Local* + 5 Manzanilla + 10 Picual + 10 Arbequina; Fig. 1-top) provided a two-factor model explaining 99% of the variance in *X* and 81% of that in *Y* (aroma compounds in pulp-blue- *vs*. seed-red; Fig. 1-bottom). The ensuing model was evaluated via the root mean square error for predictions (RMSEP), which was calculated to be lower than 10% for the *Y* data.

The central ellipsoid in Fig. 1-bottom indicates that all compounds between the borders of both circles were correlated variables ($r > 0.700$) to explain variation in the aroma compounds in pulp and seed. In the upper-right quadrant, significant positive correlations ($r > 0.700$) were found between the pleasant volatile compounds hexyl acetate (HEXAC), hexanal (HEX) and *trans*-2-hexen-1-al (t2HEX) from pulp and seed, which could describe olives from Manzanilla de Sevilla cultivar; these volatiles could provide an apple-like character to the oils. In the lower-right quadrant, the two compounds *p*-cymene (CYM) and *trans*-2-hexen-1-ol (t2H1O) were not significantly correlated between pulp and seed, but are those that could better describe Arbequina variety

providing a grass-like character. In the upper-left quadrant, *cis*-3-hexen-1-ol (c3H1O), *cis*-3-hexenyl acetate (c3HEXAC), 1-hexanol (1HEXA), 1-pentanol (1PENT) were some of the aroma compounds correlated in pulp and seed that could better describe the Galician *Local* cultivar providing a distinctive banana-like character. In the lower-left quadrant, the aroma components that were not significantly correlated between pulp and seed, but that better could characterise Picual olives were 1-penten-3-ol (1PEN3) from pulp and *trans*-2-pentenal (t2PEN) from seed, compounds which could provide a bitter, grass-like character. A sensory descriptive analysis would be required to ensure the main character of the different olive oils.

4. Conclusions

The analysis of crushed pulp and seed, separately, allows to establish the role of both constitutive parts of olive in the biogenesis of EVOO aroma. Independently of the cultivar considered and according to these results, the main volatile compounds coming from LOX pathway had their biogenesis in pulp. Total volatile content was highly variable between the cultivars studied. The volatile compounds: pentanal, *trans*-2-hexen-1-al, *p*-cymene, hexyl acetate, *trans*-2-penten-1ol, *cis*-3-hexenyl acetate and *trans*-2-hexen-1-ol allowed to classify olive fruits according to their constitutive parts, pulp and seed. A Linear Discriminant Analysis was employed to identify aroma compounds in the constitutive parts of olive fruit (pulp and seed) that are responsible for maturity index. The major indicators of ripeness in olive pulp were the volatile compounds *trans*-2-hexen-1-al, 1-hexanol, *trans*-3-hexen-1-ol, and *cis*-3-hexenyl acetate, while in the olive seed were *trans*-2-hexen-1-al, 1-hexanol and 3-pentanone. A Partial Least Squares regression allowed to establish positive correlations between aroma compounds from pulp and seed and also a first attempt to identify the specific aroma trends that best distinguish among the four cultivars studied.

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