1 **Epicuticular wax in developing olives (*Olea europaea*) is highly dependent on cultivar and**
2 **fruit ripeness**
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ABSTRACT

The epicuticular wax (EW) layer is located on the surface of most of plants organs. It provides the cuticle with most of its properties and is the primary barrier against biotic and abiotic stress. Despite the importance of *Olea europaea* cultivation, few studies have characterized the EW covering leaves and olives, which could be involved in resistance to both infection and environmental conditions. In the present study, wide-ranging screening was carried out using direct-injection electrospray ionisation coupled to high-resolution mass spectrometry to analyse EW in developing olives of nine varieties. The proportions of EW fractions (wax esters (WEs), diacylglycerols, triacylglycerols (TAGs), triterpenic acids and aldehydes) strongly depended on the olive cultivar, and in only a few cases were they influenced by the sampling date. The specific compositions of the major fractions, WEs and TAGs, were strictly related to the cultivar, while the degree of unsaturation and the chain length of the WEs evolved throughout the four weeks prior to the olive colour turning.

Keywords: epicuticular wax; olive; cultivar; ripening; high-resolution mass spectrometry.

Highlights

- The proportions of EW fractions strongly depended on the olive cultivar
- The composition of the main EW fractions was also related to the olive cultivar
- The degree of unsaturation and the chain length of the wax esters evolved over time
- Major differences between cultivars concerned the EW phenolic fraction
- EW cultivar differences could explain the different biotic and abiotic resistance

INTRODUCTION

Epicuticular wax (EW) is the complex monomeric mixture that forms the highly hydrophobic layer which covers the polymeric cutin structure on the surface of most plant organs.[1-2] As this layer is located at the interface between the plant and the environment, it provides the cuticle with most of its properties. EW is the primary barrier against biotic and abiotic stress, and for this reason it is particularly important during fruit development.[3] EW contributes to the prevention of water loss and determines the wettability of the plant surface;[4-5] it protects against incident radiation by favouring light reflection;[5-7] it shields from bacterial and fungal pathogens[8-9]; and it plays a significant role in host-plant recognition by insects.[5,10-11] It has further been shown that wax constituents can influence insect behaviour regarding oviposition.[12-14]

The chemical composition of EW greatly affects the physical properties of plant surfaces,[3] and our understanding of the effect of EW composition on different biological functions deserves to be extended. In turn, EW can be influenced by environmental conditions such as ambient temperature, irradiation and moisture,[15-16] as well as by genetic factors, as evidenced by the diversity of EW composition in different plant species and varieties.[17-18]

Olea europaea cultivation is of great importance in the Mediterranean Basin, but relatively few studies have characterized the EW covering olive leaves [19-20] and olives;[17,20-22] these studies would be useful to elucidate the role of EW in the adaptation of olive trees to Mediterranean agro-climatic conditions, or its possible function in resistance towards plagues and pathogens.

The EW of ripe, healthy olive is composed of alkanes, alcohols, aldehydes (ALDs), alkyl (AEs) and benzyl (BEs) esters, triacylglycerols (TAGs), fatty acids (FAs), triterpenic acids (TAs) and alcohols, among others.[17,20-23] At present, little is known of EW compositional differences in olives from distinct varieties,[19] and at different stages of maturity .[17] Most of the information available is related to the amount of wax esters (WEs) in the oils obtained from olives,[21,24-26] and little about EW composition at early stages of olive development. At the stage of olives

turning colour, the barrier between the plant and the environment becomes much more important, because this period corresponds to high levels of temperature and UV irradiance, together with summer storms that increase humidity in the olive canopy, resulting in very appropriate conditions for the development of pests and diseases. As EW chemical composition can significantly influence the properties of the olive surface, characterization of EW according to the olive cultivar and the stage of maturity could help us to better understand differences among olive cultivars in tolerance or resistance to biotic and abiotic factors.

With the aim of documenting the diversity of EW composition on olive fruits and to provide a starting point for further understanding of the mechanisms that determine olive adaptation and resistance to environmental stresses during olive development, a wide-ranging screening was carried out using a rapid and efficient analytical method developed recently.[23]

The EW and olive physical characteristics of nine olive varieties grown in the same geographical area were screened and monitored for four weeks, corresponding to the developmental stage prior to the olives changing colour.

MATERIAL AND METHODS

Chemicals. Reagents were of mass spectrometry grade. Dichloromethane, methanol and hexane, (MS SupraSolv®) were purchased from Merck (Darmstadt, Germany). Ammonium formate was from by Sigma-Aldrich (St. Louis, MO, USA). Nitrogen (Alphagaz, 99.999%, Air Liquide) was used in the Orbitrap-Exactive as the nebulization gas.

Plant material.

Healthy olive fruits of nine varieties pertaining to the collection of IRTA-Mas de Bover (Constantí, Spain), grown in the same parcel were studied. Cultivars were selected to cover the full range of ripening from very early (majority of fruits reache MI=3 during the second half of october): 'Grossal Vimbodí' (1), 'Empeltre' (2) and 'Palomar'(3); early (fruits reach MI=3 during the first half of november): 'Sevillenca' (4) and 'Menya'(5); medium (fruits reach MI=3 during second half of november): 'Arbequina' (6) and 'Picual' (7); late (fruits reach MI=3 during firs

half of december): 'Morrut' (8); and very late (fruits reach MI=3 during second half of december): 'Llumet'(9), as described by Tous and Romero.[27]

Olives were hand-picked at four different dates in correspondence with fruit modifications prior to the colour turning stage (maturity index changing from MI=0 to 1): 01, 08, 18 and 28 of august of 2011. At each sampling point, 2 trees of each cultivar were sampled, for a total of n=72 olive samples. Trees were 20 years old, planted at 4 x 7 m layout and grown under drip irrigation. The orchard was sited at latitude 41.172 N and longitude 1.169 ºE with 100 m altitude. The climate is tipical Meditarranean with 500 mm annual rainfall, concentrated mainly in April-May and in September. Fertilization and cultural practices are the usual in the area. Neither pesticides, nor fungicides were applied during the years of the trial. Sampled fruits were not affected by fungus and fruits bitted by olive fly were excluded from the sampling.

Extraction of epicuticular waxes. Extraction was carried out according to Vichi et al.[23] with some modifications. Each single olive was placed into a screw cap tube, covered with 3 mL of solvent and vortex-stirred during 1 min. Hexane : dichloromethane 90:10 (v/v) was the extraction solvent chosen, because it appeared as the best compromise for the extraction of weakly polar compounds, such as glyceride compounds and EAs, in positive mode, and secoiridoids in negative mode.

Before analysis, the extract was diluted 1:100 with dichloromethane : methanol (70:30).

Direct-Electrospray-Ultra high resolution mass spectrometry (ESI-UHRMS)

Flow injection analysis (FIA) of 5 µL of the samples was carried out with an Orbitrap-Exactive (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray source (H-ESI II). The LC system consisted of a Surveyor MS Plus pump (Thermo Fisher Scientific, San Jose, California). The mobile phase was methanol : dichloromethane 80:20 with ammonium formate 20 mM at a flow of 50 µL/min.

The ionisation were according to Vichi et al.[23] : mass spectra were acquired in full scan positive and negative ionization modes produced by spray voltages of 3.00 and -3.00 kV, capillary voltages 35 and -35V, tube lens 90 and -90V and skimmer voltages 18 and -18V, respectively. The sheath gas flow rate was set at 20 au (arbitrary units) and the aux gas flow rate was 2 au. Capillary and heater temperatures were fixed at 275 °C and 30 °C, respectively. The mass range was set to *m/z* 120-1200 and ultrahigh resolving power defined as R: 100,000 (*m/z* 200, FWHM) was set.

The mass peaks considered were single charged ions with relative intensities ≥0.01% and absolute intensity 10^3. These peaks were exported to peak lists and from these lists feasible elemental formulae were generated. Lists of possible candidate formulae from a mass measurement were obtained by setting restrictive criteria: C ≤ 150, H ≤ 400, O ≤ 10, N ≤ 1 and RDBE ≥ -1.5. The maximum mass error tolerance was fixed at 2 ppm. The molecular formulae calculation was performed with Xcalibur 2.1 (Thermo Fisher Scientific, Bremen, Germany), and the identification of EW compound classes was performed as previously described.[23]

Olive fruit morphological and physical parameters

Colour measurement. At the stage of the sampling, prior to the colour turning stage, fuits were green and some of them started to change to yellow. In order to gain precision in the definition of so slight differences, the colour was analysed by the CIELAB colorimetric system. A spectrophotometer Minolta CN3500D (Osaka, Japan) was used to assess the colour of the olive surface. The olive colour was expressed as chromatic ordinates *a**, *b** and *L**.

Fruit weight, length and diameter. The olive weight and dimensions were determined systematically for each fruit analysed.

Statistics

The SAS package (V9.2) was used for the statistical analysis of data concerning fruit parameters and EW composition, which were subjected to factorial analysis of variance according to olive cultivar and sampling period. Duncan's multiple range test was applied for post-hoc

153 classification of olive varieties. Moreover, Pearson correlation of each variable as a function of
154 the sampling period was assessed in order to identify general trends. For all the statistical
155 analyses performed, differences were considered significant at $p \leq 0.05$. Cluster analysis
156 (centroid method) was applied to the average data in order to study the relationships between
157 varieties, due to their EW composition.
158
159 **RESULTS AND DISCUSSION**
160 **Epicuticular wax profiles of olives prior to the colour turning stage**
161 The conditions for the extraction of EW from ripe olives[23] (MI≥3) had to be optimized for the
162 analysis of unripe olives (MI≤1). The higher amount of phenolic compounds in the extracts
163 from unripe olives suppresses ionisation of other species, such as glycerides, WEs, and even
164 TAs. For this reason, different solvents were tested to favour the extraction of less polar
165 compounds, to the detriment of the phenolic substances that cause ionisation suppression. As
166 expected, the molecular composition of olive EW extracts was highly influenced by the
167 extraction solvent used (**Supplementary material S1**). A weakly polar mixture was chosen as
168 the solvent for the extraction of EW from the olive surface because it appeared to be the best
169 compromise for the extraction of weakly polar compounds, such as glyceride compounds and
170 WEs, in positive mode, and secoiridoids in negative mode. TAs and FAs were detected in both
171 positive and negative ionisation mode. In order to minimize the influence of the composition
172 of the phenolic fraction on TAs and FAs data, their signals were obtained in positive mode.
173 As previously reported for ripe olives,[23] the positive ESI spectra of EW organic extracts (**Fig.1a**)
174 showed elemental compositions attributable to diacylglycerols (DAGs), TAGs, FAs, TAs,
175 hydrocarbons (HCs), dicarboxylic fatty acids (DFAs), and WEs (**Table 1**) including benzyl esters
176 (BEs), geranylgeranyl esters (GEs), and alkyl and phytyl esters (AEs) (**Table 2**); all detected as
177 NH_4^+ adducts. The highest abundance was observed for WEs and TAGs, which accounted for
178 approximately 80% of the spectrum signal. The analysis of unripe olives (MI≤1) under these

conditions allowed us to detect molecules within a large range of chain length and unsaturation. In particular, EW profiles in positive ESI mode showed AEs with FA chains from C29 to C44, with 0 to 3 double bonds; BEs with saturated and monounsaturated FAs from C20 to C31; GEs with monounsaturated and saturated FAs from C18 to C22 (**Table 2**), and TAGs from C40 to C60 and from 0 to 7 double bonds in the FA chains (**Table 3**). Moreover, the following species, which are reported in **Table 1** as the sum of the compounds, were detected: DAGs from C25:0 to C41:1; HCs from C21:1 to C30:1; ALDs from C16:0 to C28:0, and DFAs from C22:0 to C25:0.

The main components identified in the negative ESI spectra (**Fig. 1b**) had exact masses matching the molecular formulae of FAs, TAs, and phenolic and secoiridoid compounds, comprised those of oleuropein and ligstroside derivatives, elenolic acid and hydroxytyrosyl acetate (**Table 4**).

Relation between epicuticular wax profile, olive cultivar and maturity

The objective of characterizing olive EW was to find differences in the olive-environment barrier which could contribute to our understanding of the distinct varietal resistance to biotic and abiotic factors. The evolution of the EW profile during olive ripening was monitored in the nine varieties, from the beginning to the end of August, corresponding to modifications prior to olive colour turning, the upsurge of climatic stress and the onset of plagues attack.[28] During this period, morphological parameters, such as olive weight, length and diameter, and olive surface colour, were also recorded in order to estimate the progression of ripening (**Table 1**). The chromatic ordinates *L*, a** and *b** were strictly correlated to the sampling period, and thus with the degree of ripening of the olives. The differences between the *L*, a* and b** values of the distinct varieties can be attributed to the different maturation of the olives within the sampling period. In fact, 'Grossal Vimbodí' and 'Empeltre', which are described as very early ripening varieties, showed the highest *L** and *b** values, corresponding to the beginning of colour turning from dark green to yellow.

EW and olive cultivar

The nine olive varieties studied showed significant differences in the relative amounts of total WEs, TAs, DAGs, TAGs, ALDs and phenols (**Table 1**). 'Grossal Vimbodí', 'Sevillenca', 'Menya' and 'Morrut' varieties were characterized by the highest proportions of WEs, while varieties 'Picual', 'Arbequina', 'Palomar' and 'Empeltre' were the richest in TAGs. The highest proportions of DAGs and TAs were found in 'Arbequina', and in 'Sevillenca' and 'Picual', respectively. 'Picual' EW presented the highest proportions of phenols, detected in the negative ESI spectrum. No significant differences were observed in the relative amounts of total epicuticular HCs, DFAs or FAs in the distinct varieties.

Not only the relative amount, but also the specific composition of the main fractions of the EW were considered (WEs, TAGs in positive ESI mode, and phenols in negative ESI mode), and they were strictly related to the olive cultivar (**Tables 2-4**). Within WEs, the proportions of the main classes of WE: BEs, GEs, and AEs (including phytyl esters) are shown in **Table 2**. BEs contributed the highest signal and were proportionally more abundant in the varieties 'Sevillenca' and 'Menya'; while AEs were more abundant in the cultivar 'Arbequina'. Moreover, small but significant differences were found in the composition of each WE and TAG fraction, according to the degree of unsaturation and the carbon number of their FA constituents (**Tables 2** and **3**). Regarding WEs (**Table 2**), the main BEs were those containing C26 and C28 FAs, and were most abundant in 'Picual' and 'Morrut,' and in 'Llumet', respectively. Saturated BEs were considerably higher than monounsaturated BEs, which were slightly higher in 'Arbequina' and 'Empeltre' olives. Only saturated AEs were significantly different in the olive varieties analysed, with 'Arbequina' olives the richest. AEs with carbon number from C40 to C44 were higher in 'Grossal Vimbodí' and 'Arbequina' varieties; while AEs from C34 to C36 were higher in the other varieties. Different AEs proportions linked to the olive cultivar were recently reported in olive oil.[25] Saturated and C18 GEs were higher in 'Menya', while monounsaturated and C22 GEs were higher in 'Morrut' and 'Arbequina'.

Regarding TAGs (**Table 3**), highly polyunsaturated species were more abundant in 'Llumet' olives, while di-unsaturated and tri-unsaturated TAGs were more abundant in 'Grossal Vimbodí' and 'Menya, and in Palomar, respectively'. 'Menya' and 'Grossal Vimbodí' were the richest in C40-45 and C50-51 TAGs, respectively; 'Arbequina' and 'Llumet' the richest in C52-53 TAGs; and 'Palomar' the richest in C54-55 TAGs.

Finally, the major compositional differences related to the olive cultivar were observed in the phenolic fraction (**Table 4**). The major compound of the EW phenolic fraction in all the varieties was oleuropein aglycon, and the EW of 'Picual' and 'Morrut' olives showed the highest proportion of this compound. 'Llumet', 'Grossal Vimbodí', 'Empeltre' and 'Menya' presented the highest proportions of ligstroside aglycon, decarboxymethyl oleuropein aglycon, methyl elenolic acid and elenolic acid, respectively.

The compositional differences observed in the EW of the nine olive varieties grown in the same geographical area could explain the different surface micromorphology, in particular the crystalloid structures, reported for olives of distinct varieties.[29] This ultrastructure could determine the resistance of each cultivar to several biotic and abiotic factors. In this regard, cluster analysis of EW components resulted in two main groups of varieties (**Fig. 2**). The variables which mainly explained the grouping were: phenols (r PRIN1=+0.702 and r PRIN2=+0.414), oleurepein aglycone (r PRIN1=+0.396 and r PRIN2=+0.227), AEs (r PRIN1=+0.217 and r PRIN2=-0.354), BEs (r PRIN1=-0.227 and r PRIN2=+0.367), TAGs (r PRIN1=+0.206 and r PRIN2 = -0.292), elenoic acid (r PRIN1=-0.279) and wax esters (r PRIN1=-0.211 and r PRIN2=+0.258). The first cluster includes 'Llumet', 'Palomar', 'Picual', 'Morrut' and 'Arbequina' varieties, which were described as less susceptible to the olive fly attack.[28] The second cluster includes 'Empeltre', 'Sevillenca', 'Grossal Vimbodí' and 'Menya' varieties, which are characterized by a severe incidence of this plague.[28] These results suggest certain relationship between EW composition and varietal resistance to external conditions, but

256 further research is crucial to elucidate the role of the EW in olive varietal resistance to plagues
257 and environmental conditions.

258 *EW during olive ripening*

259 The evolution of the EW profile of the olives was monitored in both positive and negative ESI
260 modes during the period prior to the olive colour turning. This is the first report of the
261 evolution of the EW profile during olive development. The relative proportions of the EW
262 classes showed only a few significant modifications: the relative amount of HCs, ALDs and TAs
263 (**Table 1**). The first were inversely correlated with time, as shown by the Pearson coefficient;
264 while the ALD proportion increased slightly over time. Only the reduction of TAs during olive
265 ripening had previously been reported.[20] Regarding the proportion of phenols, DAGs and TAGs,
266 although significant differences were observed between the sampling dates, no correlation
267 with the sampling time was observed.

268 The proportions of the distinct WE classes (BEs, AEs and GEs), remained unchanged over the
269 sampling period (**Table 2**); as did most of the TAGs according to chain length and the degree of
270 unsaturation (**Table 3**). In the latter case, a slight increase of long-chain and poly-unsaturated
271 TAGs with respect to shorter-chain and di-unsaturated species was observed. The most
272 outstanding modifications during olive development concerned the degree of unsaturation
273 and the chain length of WEs (**Table 2**). In particular, olive development was accompanied by a
274 decrease of monounsaturated and an increase of saturated AE and BE species; as well as by a
275 clear decrease of short-chain AEs (from C29 to C37) in favour of longer-chain AEs (from C40 to
276 C44), as shown by the Pearson coefficient. Also, in the case of GEs, the long-chain species C22
277 GE increased over time. Finally, an increase of C26 BE and unsaturated GEs in detriment of
278 C23, C27 and C30 BEs and saturated GEs, respectively, was observed over time.

279 Regarding the composition of the phenolic fraction, the proportion of oleuropein aglycon
280 increased at the expense of decarboxymethyl forms of ligstroside and oleuropein aglycons
281 (**Table 4**).

In conclusion, analysis of the EW of developing olives reveals the presence of various fractions consisting of compounds from different chemical families. The proportions of these fractions depended strongly on the olive cultivar, and only in a very few cases were they influenced by the sampling date during the period prior to the colour turning stage. The specific composition of the main fractions, WEs, TAGs and phenols, was also strictly related to the olive cultivar. Moreover, the degree of unsaturation and the chain length of WEs evolved throughout the four weeks prior to the olive colour turning. In particular, olive development was accompanied by: a decrease of monounsaturated and an increase of saturated AE and BE species; a clear decrease of shorter-chain AEs; an increase of longer-chain AEs and GEs, as well as of C26 BEs; and an increase of the proportion of oleuropein aglycon in detriment of decarboxymethyl secoiridoid aglycons.

The different proportions of each EW fraction and their specific composition according to the olive cultivar, as well as the modifications of WE acyl chains length and unsaturation over time, should have an effect on the physical properties of the EW layer and influence the microstructural characteristics of the olive surface.

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372 **Figure legends**

373 **Figure 1:** Positive (a) and negative (b) ESI-UHRMS spectra of 'Sevillenca' olive EW extract.

374 Elemental formulae, RDBE (rings and double bonds equivalents) and mass error are shown. R:

375 100 000 (m/z 200, FWHM).

376 **Figure 2.** Cluster analysis of EW compositional data.

Table 1. Fruit parameters and main EW fractions detected in positive and negative ESI HRMS spectrum, their factorial analysis of variance according to olive cultivar and sampling period, post-hoc classification of olive cultivar by Duncan's multiple range test (different letters indicate significant differences), and Pearson correlation of each variable as a function of sampling period. The highest means are evidenced in bold.

	cultivar[a]									ANOVA[b]			correlation[c]	
	1	2	3	4	5	6	7	8	9	var[d]	period[e]	var*period[f]	Pearson coeff.	*p*
Fruit parameters														
*L**	**41.8a**	41.0ab	31.2e	36.4cd	37.0bcd	38.4bc	34.7c	35.9cd	38.6bc	<0.001	<0.001	<0.001	0.735	<0.001
*a**	-12.6d	-12.5cd	**-9.7a**	-12.9d	-11.2b	-12.1bcd	-11.5bc	-11.4b	-12.8d	<0.001	<0.001	<0.001	-0.717	<0.001
*b**	**25.8a**	**26.3a**	16.8f	21.9bcd	20.7cd	23.0abc	17.7ef	19.8ed	24.2ab	<0.001	<0.001	<0.001	0.717	<0.001
Lenght (mm)	15.7e	19.8bc	15.9e	18.0cd	17.8d	14.1f	**22.3a**	20.9ab	15.9e	<0.001	<0.05	NS[g]	0.245	<0.05
Diameter (mm)	13.4bc	12.7cd	13.3bc	12.0d	9.6f	11.6de	14.3ab	**15.1a**	10.6ef	<0.001	<0.001	NS	0.415	<0.001
Weight (g)	1.8b	1.9b	2.0b	1.5bc	1.1d	1.2cd	**2.6a**	**2.5a**	1.1d	<0.001	<0.001	NS	0.280	0.017
Proportions of EW classes (ESI+ mode)														
% HCs	7.2	4.0	6.5	5.7	8.8	6.9	7.3	6.5	6.7	NS	<0.010	NS	-0.291	<0.01
% ALDs	1.6cde	1.9abc	1.1e	1.8bcd	1.1de	1.9abc	**2.4ab**	**2.6a**	1.8bcd	<0.001	<0.001	0.001	0.233	<0.05
% FAs	0.9	1.0	0.9	1.1	1.3	1.3	0.8	1.3	1.2	NS	NS	NS	NS	NS
% WEs	**44.1a**	23.6b	21.2b	**40.7a**	**39.7a**	17.9b	22.6b	**40.2a**	27.9ab	0.001	NS	NS	NS	NS
% TAs	1.7ab	1.0b	0.8b	**2.8a**	2.0ab	1.6ab	**2.8a**	0.7b	1.4b	<0.01	<0.001	NS	-0.454	<0.001
% DFAs	0.4	0.4	0.3	0.4	0.6	0.3	0.3	0.4	0.3	NS	NS	NS	NS	NS
% DAGs	2.5b	2.8b	2.4b	3.0b	3.2b	**4.1a**	2.3b	3.1b	2.3b	<0.01	<0.01	NS	NS	NS
% TAGs	41.6c	**65.3a**	**66.8a**	44.5bc	43.4bc	**65.9a**	**61.5ba**	45.3bc	58.3abc	<0.01	<0.01	NS	NS	NS
Proportions of EW classes (ESI- mode)														
% phenols	38.1cd	25.0d	73.7ab	41.6cd	28.7d	53.1bc	**92.3a**	74.0ab	74.1ab	<0.001	<0.001	0.025	NS	NS

[a]: olive cultivar (1: Grossal Vimbodí; 2: Empeltre; 3: Palomar; 4: Sevillenca; 5: Menya; 6: Arbequina; 7: Picual; 8: Morrut; 9: Llumet); [b]; factorial analysis of variance; [c]: Pearson correlation; [d]: cultivar; [e]: period; [f]: interaction between cultivar and period; [g]: non-significant (p>0.05).

Table 2. WE fractions detected in positive ESI HRMS spectrum, their factorial analysis of variance according to olive cultivar and sampling period, post-hoc classification of olive cultivar by Duncan's multiple range test (different letters indicate significant differences), and Pearson correlation of each variable as a function of sampling period. The highest means are evidenced in bold.

	cultivar[a]									ANOVA[b]			correlation[c]	
	1	2	3	4	5	6	7	8	9	var[d]	period[e]	var*period[f]	Pearson coeff.	*p*
Wax esters proportions														
% BEs	77.7ab	68.1b	65.9b	**82.5a**	**81.6a**	47.2c	65.8b	75.0ab	76.3ab	<0.001	NS[g]	NS	NS	NS
% AEs	21.0bc	30.2b	32.4b	16.5c	17.4c	**50.3a**	32.3b	23.6bc	22.1bc	<0.001	NS	NS	NS	NS
% GEs	0.4	0.6	0.7	0.6	0.4	0.8	0.6	0.6	0.6	NS	NS	NS	NS	NS
Benzyl esters according to insaturation (%)														
% BEs monounsaturated	3.1b	**8.9a**	3.9b	2.5b	4.0b	**7.1a**	2.9b	2.7b	2.6c	<0.001	<0.05	<0.001	-0.179	<0.05
% BEs saturated	**96.9a**	91.1b	**96.1a**	**97.5a**	**96.0a**	92.9b	**97.1a**	**97.3a**	**97.4a**	<0.001	<0.05	<0.001	0.179	<0.05
Benzyl esters according to chain length (%)														
% BEs C20	0.13e	0.39b	0.34bc	0.15e	0.18de	**0.61a**	0.11e	0.21cde	0.32bcd	<0.001	NS	<0.01	NS	NS
% BEs C21	0.03	0.03	0.04	0.04	0.05	0.06	0.03	0.02	0.02	NS	NS	NS	NS	NS
% BEs C22	**2.0a**	0.3c	0.3c	0.5bc	0.4bc	0.9bc	0.8bc	1.0b	0.3c	<0.001	NS	<0.001	NS	NS
% BEs C23	0.59bc	0.44bcd	0.39bcd	0.36cd	0.24d	**0.89a**	0.64b	0.39bcd	0.26d	<0.001	<0.001	<0.05	-0.250	<0.05
% BEs C24	**13.6a**	5.1c	3.9c	4.6c	4.8c	5.5c	9.4b	10.3b	3.8c	<0.001	NS	<0.001	NS	NS
% BEs C25	2.6c	3.0b	2.4c	2.4c	1.8d	1.8d	**3.9a**	**3.6a**	2.3c	<0.001	NS	<0.001	NS	NS
% BEs C26	53.4b	51.8bcd	48.6d	50.1bcd	52.6bc	49.7cd	**58.4a**	**59.1a**	45.3e	<0.001	<0.05	<0.001	0.264	<0.05
% BEs C27	3.7e	8.1ab	**9.2a**	7.9ab	4.7e	5.1de	6.2cd	8.1ab	7.6bc	<0.001	<0.001	NS	-0.241	<0.05
% BEs C28	18.8d	23.1c	26.4b	26.6b	26.4b	24.6bc	14.4e	12.7e	**30.7a**	<0.001	NS	<0.001	NS	NS
% BEs C29	2.0	3.7	4.9	3.9	2.4	4.3	2.9	2.6	3.9	NS	NS	NS	NS	NS
% BEs C30	2.6bc	3.3b	3.0b	3.1b	**5.6a**	**5.1a**	1.3bc	1.5c	**4.8a**	<0.001	<0.01	NS	-0.265	<0.05
% BEs C31	0.11c	0.39b	0.30bc	0.24bc	0.26bc	**0.69a**	0.18c	0.21bc	0.25bc	<0.01	<0.05	<0.001	NS	NS
Alkyl esters according to unsaturation (%)														
% AEs tri-unsaturated	6.6	8.0	6.7	5.9	6.7	6.3	6.4	7.5	5.7	NS	NS	NS	NS	NS
% AEs di-unsaturated	37.9	39.1	35.8	36.6	36.2	31.6b	35.3	35.3	32.3	NS	NS	NS	NS	NS
% AEs mono-unsaturated	46.7	46.1	48.5	47.8	47.8	41.9	48.5	44.8	47.6	NS	0.001	<0.05	-0.445	<0.001
% AEs saturated	8.9bc	6.9c	9.0bc	9.9bc	9.4bc	**20.6a**	9.8bc	12.5b	14.5ab	<0.01	0.001	NS	0.471	<0.001
Alkyl esters according to chain length (%)														
% AEs C29	1.7	1.7	1.7	2.2	2.5	1.4	1.1	1.5	2.0	NS	<0.05	NS	-0.214	<0.05
% AEs C30	3.6bc	3.7bc	4.0bc	4.9ab	**6.0a**	4.0bc	2.9c	3.8bc	5.0ab	<0.01	<0.001	NS	-0.323	<0.01
% AEs C31	2.9	2.9	3.0	3.5	3.4	2.6	2.5	2.9	3.2	NS	<0.001	NS	-0.286	<0.01
% AEs C32	5.6	6.1	6.6	6.0	7.0	6.0	5.5	7.4	6.7	NS	<0.001	<0.05	NS	NS
% AEs C33	4.2	4.4	4.4	5.0	4.4	4.3	4.7	4.6	5.0	NS	<0.05	NS	-0.210	<0.05
% AEs C34	9.0d	9.7bcd	**11.3a**	10.4abc	10.6ab	9.2cd	10.2abcd	10.2abcd	11.0ab	<0.05	<0.01	<0.001	-0.254	<0.05
% AEs C35	7.1c	8.1ba	**8.4a**	**8.3a**	7.9abc	7.2bc	**8.2a**	7.7abc	7.9abc	<0.01	<0.001	<0.001	-0.294	<0.01

% AEs C36	17.1b	**20.2a**	**20.0a**	**18.4a**	**19.0a**	16.9b	**19.4a**	**19.1a**	**19.2a**	<0.001	<0.01	<0.001	-0.204	<0.05
% AEs C37	8.1c	**9.4a**	9.2ab	8.3bc	8.5abc	8.3bc	9.2ab	9.1ab	8.7abc	<0.01	<0.001	<0.001	-0.237	<0.05
% AEs C38	12.8bc	**14.6a**	14.2ab	12.4c	13.7abc	12.8bc	14.5a	13.8abc	12.9bc	<0.01	<0.05	NS	NS	NS
% AEs C39	5.4	5.9	5.9	5.3	5.4	5.4	5.9	5.9	5.1	NS	<0.001	<0.05	NS	NS
% AEs C40	**11.2a**	8.0c	7.6c	8.2c	7.1c	**10.8a**	9.4b	7.8c	7.5c	<0.001	<0.001	<0.001	0.370	<0.001
% AEs C41	2.1	2.1	1.8	1.7	1.7	2.7	2.1	2.3	2.0	NS	<0.001	NS	0.358	<0.001
% AEs C42	**7.9a**	2.7bc	1.7c	4.8b	2.3bc	**7.9a**	3.7bc	3.0bc	3.4bc	<0.001	<0.001	<0.001	0.325	<0.05
% AEs C43	0.4	0.2	0.1	0.1	0.1	0.2	0.3	0.4	0.1	NS	<0.001	NS	0.404	<0.001
% AEs C44	**1.0a**	0.4bc	0.2c	0.4bc	0.4bc	0.7ab	0.4bc	0.5bc	0.3c	<0.001	<0.001	<0.001	0.398	<0.001
Geranylgeranyl esters according to unsaturation (%)														
% GEs saturated	80.3bc	81.4bc	82.6bc	92.0ab	**94.5a**	74.4c	84.6abc	75.3c	93.2ab	<0.05	<0.05	NS	-0.216	<0.05
% GEs unsaturated	19.7ab	18.6ab	17.4ab	8.0bc	5.5c	**25.6a**	15.4abc	**24.7a**	6.8bc	<0.05	<0.05	NS	0.216	<0.05
Geranylgeranyl esters according to chain length (%)														
% GEs C18	59.9bcd	58.7bcd	66.2 abcd	76.1ab	**80.6a**	56.4cd	68.0abcd	51.6d	75.4abc	<0.05	NS	NS	NS	NS
% GEs C20	20.3	22.8	15.5	15.1	14.5	19.4	17.4	23.8	16.6	NS	NS	NS	NS	NS
% GEs C22	19.8ab	18.5ab	18.4ab	8.8bc	4.9c	**24.2a**	14.6abc	**24.6a**	8.0bc	<0.01	<0.05	NS	0.216	<0.05

[a]: olive cultivar (1: Grossal Vimbodí; 2: Empeltre; 3: Palomar; 4: Sevillenca; 5: Menya; 6: Arbequina; 7: Picual; 8: Morrut; 9: Llumet); [b]; factorial analysis of variance; [c]: Pearson correlation; [d]: cultivar; [e]: period; [f]: interaction between cultivar and period; [g]: non-significant (p>0.05).

Table 3. TAG fraction detected in positive ESI HRMS spectrum, factorial analysis of variance according to olive cultivar and sampling period, post-hoc classification of olive cultivar by Duncan's multiple range test (different letters indicate significant differences), and Pearson correlation of each variable as a function of sampling period. The highest means are evidenced in bold.

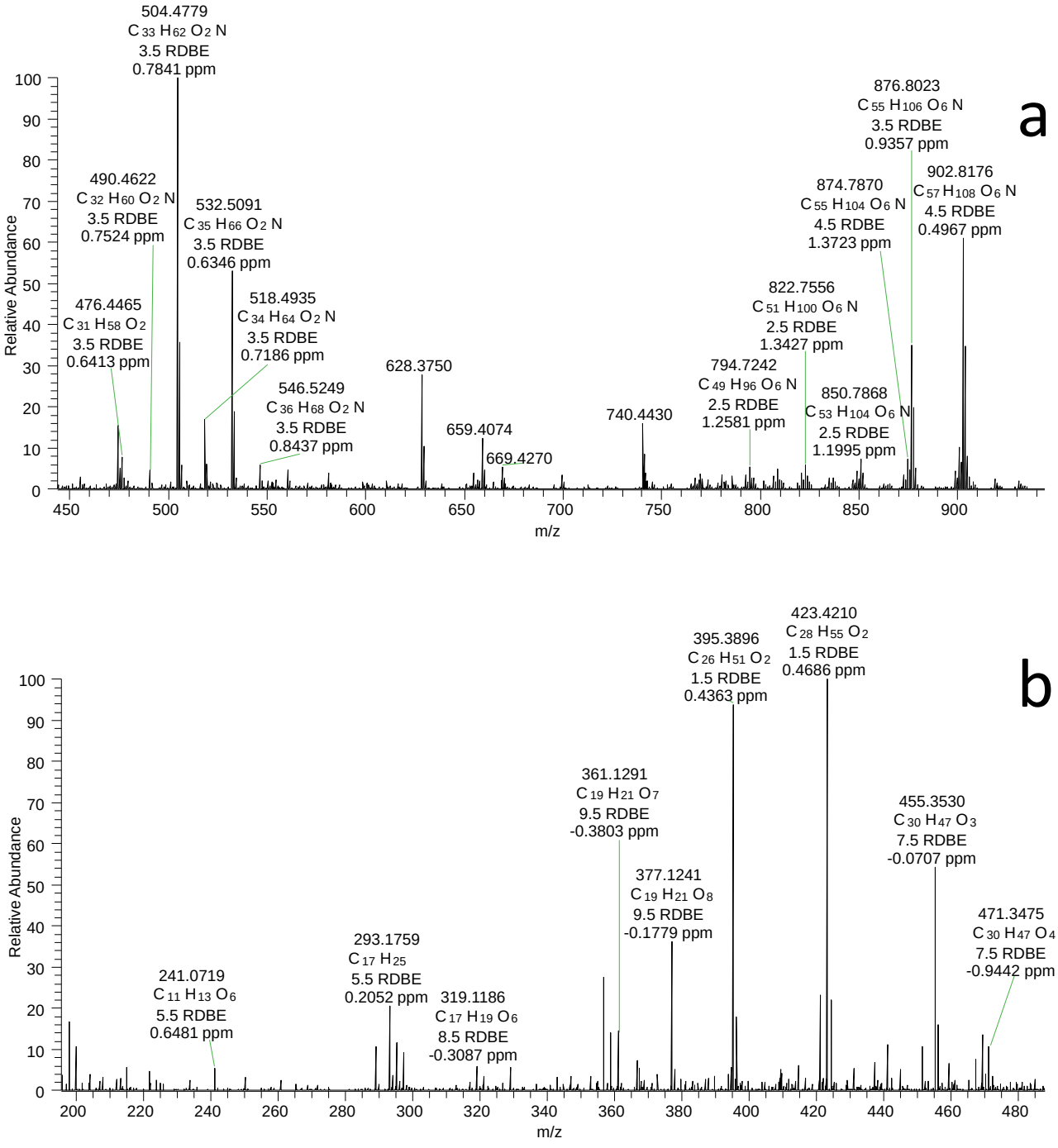
	cultivar[a]									ANOVA[b]			correlation[c]	
	1	2	3	4	5	6	7	8	9	var[d]	period[e]	var*period[f]	Pearson coeff.	*P*
Triacylglycerols according to unsaturation (%)														
% TAGs hexa-unsaturated	0.8bc	0.5cd	0.5cd	0.8bc	0.3d	0.5cd	0.5cd	1.0b	**1.9a**	<0.001	<0.001	NS[g]	0.445	<0.001
% TAGs penta-unsaturated	3.3b	2.3bc	2.8b	3.1b	1.6c	2.8b	2.5bc	2.8b	**5.3a**	<0.001	NS	NS	0.202	<0.05
% TAGs tetra-unsaturated	9.4	9.5	9.2	10.4	7.3	11.4	6.2	9.9	12.3	NS	NS	NS	NS	NS
% TAGs tri-unsaturated	31.6bc	37.9ab	**41.1a**	34.5abc	31.8bc	36.1abc	38.8ab	29.2c	35.7abc	<0.01	NS	<0.05	NS	NS
% TAGs di-unsaturated	**31.2a**	30.4ab	29.4bc	29.3bc	**31.7a**	30.1ab	30.8ab	28.3b	27.7b	<0.001	<0.05	NS	-0.286	<0.01
% TAGs mono-unsaturated	18.1	14.7	13.1	16.9	20.4	14.6	15.3	21.6	13.2	NS	NS	NS	NS	NS
% TAGs saturated	5.5	4.6	3.8	4.8	6.7	4.4	5.7	7.1	3.9	NS	NS	NS	NS	NS
Triacylglycerols according to chain length (%)														
% TAGs C40-C45	8.1bc	6.2bc	5.1c	9.7abc	**12.6a**	6.6bc	7.5bc	10.3ab	5.5c	<0.05	<0.05	NS	-0.272	<0.05
% TAGs C46-C49	21.2	16.0	14.9	18.9	26.5	15.7	17.4	27.8	13.9	NS	NS	NS	NS	NS
% TAGs C50-C51	**11.1a**	9.4abc	8.0c	9.6abc	10.9ab	10.0abc	8.8bc	10.5ab	9.4abc	<0.01	NS	NS	NS	NS
% TAGs C52-C53	27.0ab	28.0ab	26.2ab	24.8abc	21.3bc	**29.7a**	25.9ab	18.6c	**29.1a**	<0.05	NS	NS	NS	NS
% TAGs C54-C55	28.6bc	36.5abc	**41.2a**	33.3abc	26.7c	35.3abc	37.3abc	28.6bc	38.4ab	<0.01	NS	<0.05	NS	NS
% TAGs C56-C57	2.6	3.0	3.3	3.3	3.1	2.3	2.9	2.9	2.4	NS	NS	NS	NS	NS
% TAGs C58-C60	0.6	0.6	0.6	0.6	0.5	0.6	0.6	0.8	0.6	NS	<0.01	NS	0.332	<0.01

[a]: olive cultivar (1: Grossal Vimbodí; 2: Empeltre; 3: Palomar; 4: Sevillenca; 5: Menya; 6: Arbequina; 7: Picual; 8: Morrut; 9: Llumet); [b]; factorial analysis of variance; [c]: Pearson correlation; [d]: cultivar; [e]: period; [f]: interaction between cultivar and period; [g]: non-significant (p>0.05).

Table 4. Phenol fraction detected in negative ESI HRMS spectrum, factorial analysis of variance according to olive cultivar and sampling period, post-hoc classification of olive cultivar by Duncan's multiple range test (different letters indicate significant differences), and Pearson correlation of each variable as a function of sampling period. The highest means are evidenced in bold.

	cultivar[a]									ANOVA[b]			correlation[c]	
	1	2	3	4	5	6	7	8	9	VAR[d]	period[e]	var*period[f]	Pearson coeff.	*P*
% Hydroxytyrosyl acetate	4.0	13.6	14.3	1.7	1.3	3.6	0.6	0.7	0.8	NS[g]	NS	NS	NS	NS
% Elenolic acid	19.3ab	16.4abc	1.3bc	8.6bc	**29.5a**	3.1bc	2.7bc	5.7bc	7.5bc	<0.01	NS	<0.01	NS	NS
% methyl elenolic acid	4.3b	**14.8a**	0.2b	0.6b	2.5b	0.03b	0.04b	0.1b	0.5b	<0.001	NS	NS	NS	NS
% Decarboxymethyl ligstroside aglycone	0.9	0.2	1.3	<0.01	<0.01	0.01	0.02	0.2	<0.01	NS	<0.05	<0.01	-0.313	<0.01
% Decarboxymethyl oleuropein aglycone	**21.7a**	0.6c	15.3ab	5.5bc	0.7c	8.6bc	0.9c	1.2c	1.6c	<0.001	<0.05	NS	-0.365	<0.001
% Ligstroside aglycone	4.5b	4.3b	7.7b	7.5b	4.8b	1.4b	4.8b	4.5b	**27.2a**	<0.001	NS	NS	NS	NS
% Oleuropein aglycone	45.3d	50.1d	59.8cd	76.0abc	61.2bcd	83.3ab	**90.9a**	**87.8a**	62.2bcd	<0.001	<0.01	<0.05	0.209	<0.05

[a]: olive cultivar (1: Grossal Vimbodí; 2: Empeltre; 3: Palomar; 4: Sevillenca; 5: Menya; 6: Arbequina; 7: Picual; 8: Morrut; 9: Llumet); [b]; factorial analysis of variance; [c]: Pearson correlation; [d]: cultivar; [e]: period; [f]: interaction between cultivar and period; [g]: non-significant (p>0.05).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C57 H108 O6 N
4.5 RDBE
0.4967 ppm
874.7870
C55 H104 O6 N
4.5 RDBE
1.3723 ppm
490.4622
C32 H60 O2 N
3.5 RDBE
0.7524 ppm
532.5091
C35 H66 O2 N
3.5 RDBE
0.6346 ppm
822.7556
C51 H100 O6 N
2.5 RDBE
1.3427 ppm
476.4465
C31 H58 O2
3.5 RDBE
0.6413 ppm
518.4935
C34 H64 O2 N
3.5 RDBE
0.7186 ppm
628.3750
794.7242
C49 H96 O6 N
2.5 RDBE
1.2581 ppm
850.7868
C53 H104 O6 N
2.5 RDBE
1.1995 ppm
546.5249
C36 H68 O2 N
3.5 RDBE
0.8437 ppm
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659.4074
669.4270
Relative Abundance
m/z
b
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1.5 RDBE
0.4686 ppm
395.3896
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1.5 RDBE
0.4363 ppm
361.1291
C19 H21 O7
9.5 RDBE
-0.3803 ppm
455.3530
C30 H47 O3
7.5 RDBE
-0.0707 ppm
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C19 H21 O8
9.5 RDBE
-0.1779 ppm
471.3475
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7.5 RDBE
-0.9442 ppm
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C17 H25
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0.2052 ppm
241.0719
C11 H13 O6
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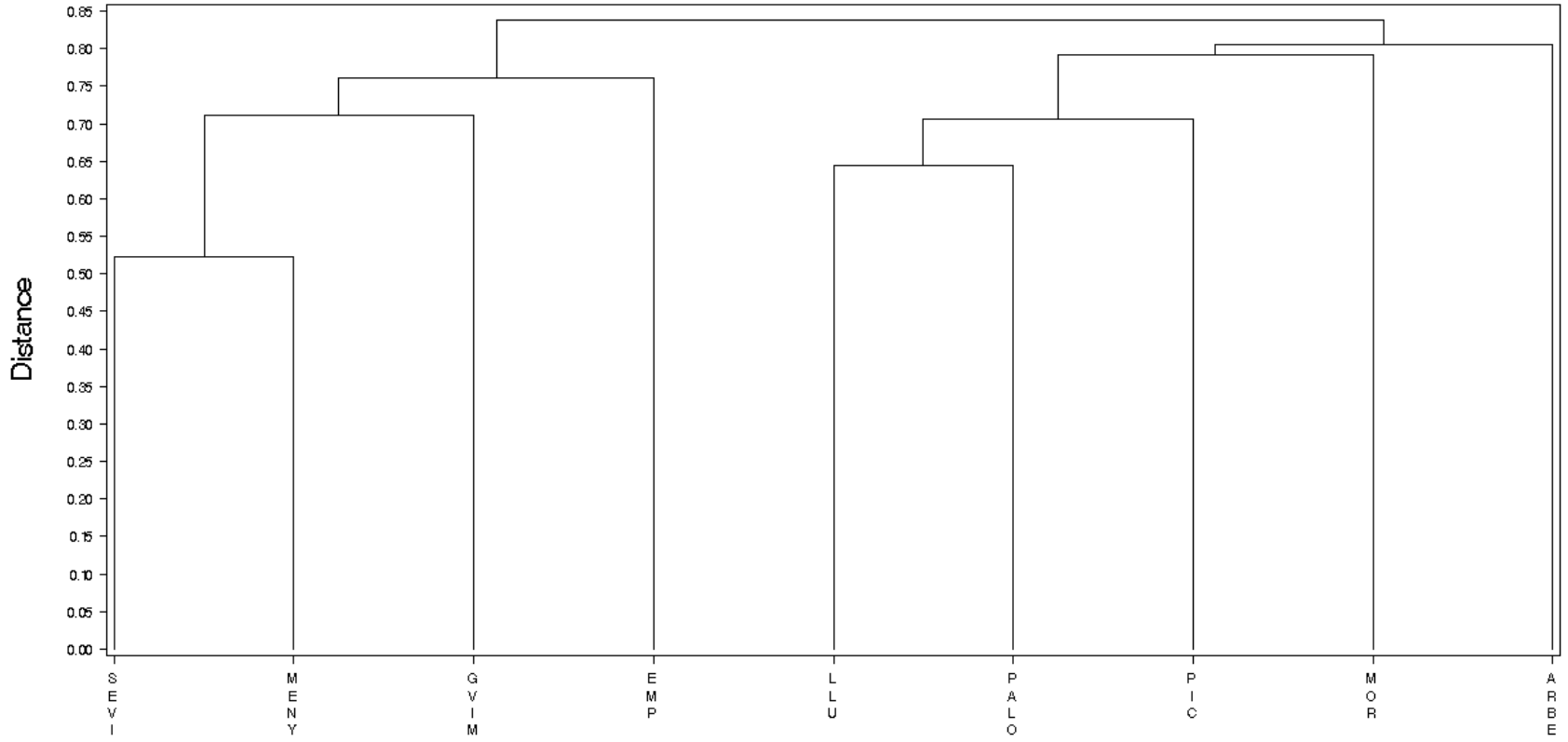
8.5 RDBE
-0.3087 ppm
Relative Abundance
m/z

Figure 1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0.00
SEVI
MENY
GVIM
EMP
LLU
PALO
PIC
MOR
ARBE

Figure 2