Quantitation of endogenous amount of ethanol, methanol and acetaldehyde in ripe fruits of different Spanish olive varieties

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ABSTRACT

BACKGROUND

The use of healthy olives and their good management along the production process are necessary to obtain the best quality virgin olive oils. One of the parameters related to the health of the olives is the content of fatty acid alkyl esters. Since these come from the esterification of C16 and C18 free fatty acids with short chain alcohols, the control of methanol, ethanol and acetaldehyde (precursor of ethanol) and their origin (endogenous or from fermentation) is essential.

This paper reports the endogenous amount of these compounds in some of the main Spanish olive varieties. For their analyses, headspace solid phase micro-extraction was applied and, to ensure the quantitation reliability, the matrix-matched technique was used to build the calibration lines.

RESULTS

For healthy and mature olives, the contents of ethanol and methanol are much higher and vary within a wider range than those corresponding to acetaldehyde. Since olives were not directly analyzed but previously homogenized, there was no correlation between the olive size parameters and the contents of the studied compounds. However, these contents are characteristic of each variety. When comparing healthy and unhealthy olives, significant differences were observed only for ethanol contents.

CONCLUSION

Higher contents of short alcohols are not always due to an unhealthy or poor state of the fruits, but to the variety. Therefore, as these alcohols are precursors of fatty acid alkyl esters, the maximum permissible content of the latter should not be set at a single value for all olive varieties.

1. INTRODUCTION

Virgin olive oil is an important element in the Mediterranean diet; it is widely appreciated and consumed for its taste and aroma as well as for its nutritional and health benefits [1-3]. Extra virgin olive oil (EVOO) is the highest quality oil, and comes from fresh and healthy olives and only by the application of mechanical extraction procedures. Because of its high added value the International Olive Oil Council (IOOC) and the European Commission have issued an international standard that regulates its quality by evaluating not only their organoleptic characteristics but also some physicochemical parameters.

In recent years, to ensure the use of healthy olives and their good management along the whole oil production process a new criterion has also been introduced: the content in Fatty Acid Ethyl Esters (FAEEs) [4]. To classify an olive oil as EVOO the content of FAEEs has to be lower than 30 mg kg^{-1}, with a total amount of Fatty Acids Alkyl Esters (FAAEs) lower than 75 mg kg^{-1} [5-7].

FAAEs derive from the esterification of C16 and C18 free fatty acids with low molecular weight alcohols such as ethanol and methanol, producing methyl and ethyl esters, respectively. This is a very fast reaction, so the FAAEs level in olive oil depends upon

availability of precursors and enzymes [8-10]. Therefore, to get a low FAAEs amount it is necessary to control the production of fatty acids and alcohols.

Fatty acids are specific to both the oil and the cell membrane of the pulp or can be produced after the lipolysis of the triacylglycerols. Short-chain alcohols come from many different sources such as pectin degradation, inadequate practices during olive harvest and post-harvest that cause the rupture of the drupe, fermentation of fruit sugars during the extraction and the storage process of olive oil or reduction of acetaldehyde to ethanol through the alcohol-dehydrogenase (ADH) activity during the glycolysis process. Therefore, it is obvious that the more damaged an olive is, the higher amounts of FAAEs the final oil will contain. However, Beltran et al., [11] claimed that part of the ethanol comes from the own metabolism of the olive and it is accumulated during fruit maturation on the olive tree, which makes the situation more complex. In fact, this implies that ripening stage and variety may have an effect on the amounts of ethanol and methanol, which may increase the content of FAAEs not related to the health status or quality of the olive, but will have legal implications on the limits of the alkyl esters.

Although there are many studies in the literature related to the origin of low molecular weight alcohols in plants and fruits, there are very few that quantify them in olive fruits and, so far, only one has focused on the effect of the maturity of olive varieties over ethanol concentration [11]. Therefore, a deeper knowledge on this topic is necessary for a better control of the natural content of FAAEs due to the lack of joint results about these compounds. This involves the analysis of more olive varieties and the determination not only of ethanol contents but also of methanol and acetaldehyde (as a precursor of ethanol). From these premises, the objective of

the present study was to assess the effect of olive variety on the endogenous amount of ethanol, methanol and acetaldehyde in healthy ripe olive fruits, including a great range of the main Spanish varieties.

2. MATERIAL AND METHODS

2.1. Fruit sampling

The experiment was carried out by analysing 13 different Spanish varieties typical of different Spanish regions (specified in parentheses): 'Arbequina', 'Arbosana', 'Argudell', 'Fulla de Salze', 'Morrut' (Catalonia), 'Hojiblanca', 'Picual', 'Picudo' (Andalusia), 'Marfil', 'Llumet', 'Sevillenca' (Valencia), 'Cornicabra' (Castilla-La Mancha) and 'Empeltre' (Aragon). Olive samples were supplied by the Institute of Agrifood Research and Technology (IRTA) olive germplasm bank [12], located in an area of 0.6 ha at the Mas de Bover Center (Constantí, Tarragonès, Spain) with coordinates: 41º 10 "north latitude, 1º 10" east longitude, 15 km from the Tarraconense coast, and 120 m above sea level. The climate is typically Mediterranean, with high environmental humidity (60-70%) and an average rainfall of 450 mm per year, irregularly distributed. The potential evapotranspiration (PET) is 965 mm per year. The soil is narrow (40-50 cm) and has a loamy texture, a basic pH (8.1) and a 4% content of active limestone. The collection keeps three trees by variety with a plantation framework of 7 x 5 m. All the olive trees grow in the same edaphoclimatic conditions and all of them have the same age (planted in 1992).

Fruit samples were collected by hand at the end of December 2017 so, taking into account the Mediterranean climate where the plantation is located, we could consider that all the olive

varieties were mature and that there could be no further evolution since the cold would paralyze any type of plant metabolism. Between 3 kg and 5 kg of olive fruits were randomly taken around the olive canopy from each one of the two trees selected (the two ones with similar fruit load) of each variety and no distinction between healthy and unhealthy fruits was considered.

To determine the physical parameters of the olives, 100 olives were randomly taken from the sampling of each tree. Then, average values for fruit and pit weight, maturity index (M.I.) and visual diagnostic of health status (undamaged olives, olives with knocking marks, olives damaged by fly, olives damaged by fungus, other defects) were measured.

The M.I. values were calculated by using the equation proposed by Uceda & Frias [13], based on the number of fruits of each maturity category (category 0 -when the skin of the fruit is deep green- and category 7 -when the colour of the skin and also of the pulp is purple-black) divided by the total number of fruits considered (100). Regarding health status, olive fruits were considered unhealthy when more than 10 olives (> 10%) present any defect, regardless of which one it was.

Finally, the healthy olives coming from the sampling of each tree were split into two groups and were processed separately. As far as the damaged fruits are concerned, these were separated from the rest of olive samples and were processed separately.

2.2. Preparation of the olive homogenates

The method followed to obtain the homogenates from each one of the two samples of each tree was based on the one proposed by Beltran et al. [11]. Once the healthy olives (i.e. with a

percentage of defects <10%) arrived at the laboratory, they were processed as soon as possible (15-20 min) to avoid any sample alteration. Each sample of olives (coming from the split into two from each tree) was rinsed with deionized cold water (4ºC) and totally dried with absorbent tissues for laboratory use. Immediately after, the olives were placed in a hammer mill (from the ABENCOR System) and were ground at room temperature obtaining a paste (sieve of 6 mm). 15 g of this olive paste were placed into a 50 mL tube and mixed together with 15 g of cold milli-Q water, homogenized and left to stand for 5 minutes in such a way that the particles corresponding to the pits were deposited in the lower part of the tube. Then, 1.00 g (± 0.01 g) of the homogenate was weighed in a 20 mL glass vial, together with 1.00 g (± 0.01 g) of saturated $CaCl_2$ solution and stirred to prevent any biological activity that could alter the sample before its analysis. This procedure was performed in duplicate for each pressing and the two vials were tightly sealed with a septum cap. Finally, the vials were kept at -18 ˚C until their analysis by solid phase microextraction and gas chromatography coupled to mass spectrometry (SPME-GC-MS).

2.3. Chemicals and standards

The standards of ethanol absolute (gradient HPLC grade) and methanol (supragradient HPLC grade) were purchased from Scharlab (Barcelona, Spain). Acetaldehyde (99% for synthesis) and calcium chloride anhydrous (97%) were purchased from Panreac (Barcelona, Spain). Milli-Q quality water used was obtained from a purification system (Millipore, Bedford, USA).

2.4. Solid Phase Microextraction (SPME)

The SPME holder for manual sampling and the 75 µm/85 µm Carboxen/polydimethylsiloxane (Carboxen/PDMS), 100 µm polydimethylsiloxane (PDMS) and 50/30 µm divinylbenzene/Carboxen on polydimethylsiloxane (DVB/CAR/PDMS) fibres were purchased from SUPELCO (North Harrison Road -Bellefonte, PA - USA). All the fibres were conditioned before use and thermally cleaned between analyses by inserting them in the GC injector port at the temperature recommended by the manufacturer.

2.5. Analytical procedure: Headspace-Solid Phase Microextraction (HS-SPME)

Ethanol, methanol and acetaldehyde contents in the samples were determined using the solid phase microextraction technique applied to the headspace of the samples (HS-SPME) and followed by gas chromatography coupled to a quadrupole mass spectrometer (GC-MS). HS-SPME is a fast and clean technique that allows the extraction and the concentration of these compounds from the homogenate samples contained in the vials and with no extra sample handling.

The different parameters that affects the SPME yield were optimized: fibre coating, extraction time and temperature, and sample volume.

The best results were obtained by pouring 1.00 ± 0.01 g of sample homogenate with 1.00 ± 0.01 g of saturated $CaCl_2$ solution into a 20 mL glass vial and pre-equilibrating it in a thermostated water bath at 40 ºC for 5 minutes. Then, the SPME fibre DVB/CAR/ PDMS 50/30 µm, Stableflex, 2 cm fibre was inserted manually through the vial septum and exposed to the headspace over the sample for 50 min at 40 ºC under medium orbital agitation.

Afterwards, the fibre was pulled into the sheath, removed from the vial and immediately introduced into the GC-MS injector port for thermal desorption at 270 ºC for 1 minute in the splitless mode.

2.6. Chromatographic conditions

Chromatographic analyses were performed with an HP-6890 gas chromatograph (HP, Palo Alto, CA, USA) equipped with an HP-5973 mass selective detector (HP, Palo Alto, CA, USA). Chromatographic separations were carried out by using a fused silica capillary column Chromapack (Varian, Middelburg, The Netherlands) CP-WAX 57CB (50 m x 0.25 mm i.d., 0.2 μm film thickness) and the best ratio "peak resolution/retention time" was achieved with the following oven temperature program: 40 ºC (5 min), 5 ºC min^{-1} to 100 ºC, 10 ºC min^{-1} to 215 ºC (5 min). The carrier gas was helium (He), with a head pressure of 14.8 psi at a constant flow of 1.8 mL/min. The split-splitless injection port operated in the splitless mode at 270 ºC. The mass spectrometer operated in the electron impact ionization mode at 70 eV. Interface, ion source and mass quadrupole temperatures were 200 ºC, 230 ºC and 150 ºC, respectively. The mass-to-charge (m/z) ratio range used was 28-300 amu, and spectra matching were performed using the Wiley/NBS library.

2.7. Quantitation of the analytes

To get reliable calibration lines it was first necessary to evaluate the effect of the sample matrix over the SPME yield. Then, to avoid quantitation errors due to the matrix effect found, the calibration lines were built by using the matrix-matched calibration technique [14, 15]. To

obtain an olive fruit matrix without any of the studied compounds, a homogenate from unripe

olive fruits was prepared. The absence of ethanol, methanol and acetaldehyde in the

homogenate was checked by HS-SPME and GC-MS prior to its use.

Then, six different calibration standards were prepared by adding different known

concentrations of each analyte to the homogenate of unripe olive fruits (not containing the

analytes studied) by combining high, low and medium concentrations of the three analytes. In

this way, it was possible to avoid measurements biases due to a different extraction yield

when all the analyte concentrations were very low compared to when they were very high.

Each calibration standard was prepared in triplicate and the calibration ranges were: ethanol

(0-125 mg kg^{-1}), methanol (0-200 mg kg^{-1}) and acetaldehyde (0-15 mg kg^{-1}).

Finally, the concentration of ethanol, methanol and acetaldehyde in the olive fruits was

calculated from the chromatographic peak area and taking into account the exact dilution of

the sample performed when obtaining the homogenate by mixing water and also when adding

CaCl$_2$ solution to the vial (already explained in "2.2. Preparation of the homogenates olive

samples" section).

2.8. Statistical analysis

Statistical analysis was performed using the SAS-Stat Software (V9.3.Cary, SAS Institute

Inc.). The variety factor was analysed by ANOVA (Analysis of Variance) using the

Generalised Linear Model (GLM) procedure, and mean comparisons were performed by

using the Duncan´s multiple range test ($\alpha < 0.05$).

The slopes and intercepts of the calibration lines – calculated by ordinary least-squares regression and evaluated by the coefficient of determination (r^2) and the standard errors of the slope and intercept coefficients – were obtained by using the ULC (Univariate Linear Calibration) software [16]. This program was also used to carry out the comparison between the slopes of the calibration lines when it was required.

3. RESULTS AND DISCUSSION

3.5. HS-SPME optimization

Considering the low molecular weight and the high volatility of the compounds studied, the fibre coatings tested were 75 µm/85 µm Carboxen/polydimethylsiloxane, 100 µm polydimethylsiloxane and 50/30 µm divinylbenzene/Carboxen on polydimethylsiloxane. The results showed that the best overall extraction efficiency (20-40% higher) and the best reproducibility (5-10% better) was obtained when the last coating was used.

The sample weight (always maintaining the ratio 1:1 between the homogenate and the $CaCl_2$ saturated solution to ensure the biological inactivity) was optimized together with the vial volume. When using vials of 20 mL, more sample quantity resulted in more signal, until the maximum volume that prevented the immersion of part of the fiber into the sample was reached. The results showed that, when using vials of 40 mL, there was no significant signal increase.

Related to extraction time and temperature, ranges between 30-40ºC (to avoid thermal sample alteration) and 30-60 minutes were essayed and the best results were obtained when extracting at 40 ºC for 50 min, values similar to the ones obtained in previous studies [17].

3.2 Performance parameters

The performance parameters evaluated were: matrix effect, limit of quantitation, repeatability and intermediate precision.

Due to the complexity of the olive paste composition and to the high extraction efficiency of the SPME fibers, it was necessary to check the effect that other olive compounds might have on the extraction of the analytes studied. Thus, known amounts of ethanol, methanol and acetaldehyde were added to Milli-Q water and to an olive paste (which did not contain any of the analytes). In each case 1.00 ± 0.01 g of spiked water or spiked paste were poured together with 1.00 ± 0.01 g of saturated $CaCl_2$ solution in a 20 mL vial. The chromatographic responses obtained after HS-SPME were compared and, as expected, when working with water as matrix, the response was 25-35% higher than when working with olive paste. However, it was still necessary to determine the differences, if any, between the SPME responses provided by the different varieties. This effect was evaluated by studying four different olive pastes from four different varieties randomly selected: 'Arbequina', 'Picual', 'Panisello' and 'Hojiblanca'. Four different concentrations (in duplicate) of the compounds under study were added to each one of these olive matrices and they were analyzed following the procedure specified above. From the chromatographic peak areas obtained, four different calibration lines were built by plotting each response against the added concentration. Then, the slopes of the four lines were compared in order to detect a different behaviour related to the variety matrix effect. The results showed that the slope of the different matrices were comparable (significance level, $\alpha = 0.05$). This means that when working with the matrix-

matched technique, the use of a paste of any olive variety as calibration matrix to build the calibration lines and quantify the analytes provides reliable results. Finally, the calibration lines were built using an 'Arbequina' olive paste as it provided the cleanest chromatographic signal.

The limits of quantitation (LOQ) were calculated from the amount of each compound required to give a signal/noise ratio 10:1 when working with olive paste. The LOQ values were 0.4 mg kg^{-1} for ethanol, 0.9 mg kg^{-1} for methanol and 0.2 mg kg^{-1} for acetaldehyde. Within-day and between-day precision of the method were evaluated. In both cases, the results were calculated as a relative standard deviation (% rsd). Within-day precision was calculated by analyzing 5 times a matrix spiked with 25.0 mg kg^{-1} of ethanol, 50.0 mg kg^{-1} of methanol and 5.0 mg kg^{-1} of acetaldehyde. From their chromatographic response, the values obtained were rsd < 5.5%. The between-day precision was calculated from the results obtained when analyzing 10 times a matrix spiked with 5 mg kg^{-1} of each analyte, in alternate days. The precision values were rsd < 7.9 %.

Figure 1 shows the chromatogram obtained when analyzing a real sample, in this case from 'Arbequina' variety.

3.6. Physical analysis of samples.

Once samples were collected, 100 olives of each tree were randomly selected and their physical characteristics were immediately determined. Since all the olive samples were handpicked at the end of December and almost all the fruits looked black on the trees, the maturity index (M.I.) values were equal or greater than 4 for all samples. The most notable

values were those corresponding to the 'Arbequina', 'Empeltre', 'Sevillenca' and 'Hojiblanca' varieties that, when studying the flesh colour in the lab, resulted in M.I. from 5 to 7. **Table 1** shows the average M.I. values together with those corresponding to other parameters evaluated for each variety: fruit weight, pit weight and flesh/pit ratio. As expected and because of the great differences between the varieties studied, the statistical analysis showed significant differences in all cases (**Table 1**). These values are in agreement with those previously reported for the same varieties [12, 18]. Regarding the visual health status, no significant differences were found between varieties (data not shown). These results were the ones expected because all the trees under study were in good health and only 5-8% of the olives showed defects. It should be noted that these defective olives were studied separately, as explained below.

3.7. Determination of ethanol, methanol and acetaldehyde in samples

The chromatographic results after the HS-SPME application to the studied samples are summarized in **Table 2**. When considering the different varieties all together, we observed that the ethanol content varied over a very wide range (from around 6 mg kg^{-1} for 'Sevillenca' to around 111 mg kg^{-1} for 'Morrut'). These different content values could not be correlated to any of the physical parameters determined in the present study. This is because we did not analyze olives directly, instead we analyzed the homogenate prepared from each sample and all the olives were mature. In this way, we avoided the effect both of the olive size and the maturity status over the olive composition and, therefore, over ethanol concentration. However, each variety has a different average ethanol content with an uncertainty of less than

10% in all cases. This low data spread indicates that the ethanol content is characteristic of each variety. Thus, according to this content cultivars can be grouped in four main groups (**Figure 2**): 1) 'Morrut' and 'Hojiblanca', with values close to 100 mg kg^{-1}, 2) 'Picual', 'Llumet', 'Cornicabra', 'Picudo', 'Empeltre'and 'Arbequina', with values between 44 and 57 mg kg^{-1} of ethanol, 3) 'Fulla de Salze' and 'Marfil', with a quantity of ethanol between 25 and 35 mg kg^{-1}, and 4) 'Argudell', 'Sevillenca' and 'Arbosana', with less than 14 mg kg^{-1} of ethanol.

As it can be seen, the ethanol values found are noticeable higher than those presented by Beltrán et al. [11]. This behaviour was the one expected because ethanol concentration increases with the fruit ripening and our samples presented higher M.I. in all cases. Regarding the cultivars, our results agree and confirm that the 'Hojiblanca' variety is one of those with the highest content of endogenous ethanol. However, when comparing the results for 'Picual' and 'Arbequina', our results did not match those found by Beltrán et al. [11]. As said before, in our study, these varieties showed a similar ethanol content but the results found by Beltrán et al. [11] showed a lower amount for 'Picual'. By carefully reviewing the results of these authors, it can be seen that, in the case of the 'Picual' variety, the amount of ethanol found at the last sampling point was 5 times higher than at the penultimate point while, for 'Arbequina', this increase was only of 3 times. Therefore, taking into account that the increase in ethanol is faster at the end of ripening, we can assume that if the MI of those samples were similar to ours, the ethanol values would tend to get closer as happened in our study.

Concerning methanol, its levels also varied significantly among cultivars, ranging from 23.5 mg kg^{-1} for 'Empeltre' to 180.6 mg kg^{-1} for 'Argudell' with a low dispersion of the results obtained for each cultivar (less than 7%). In this case, the application of hierarchical cluster analysis showed three groups (**Figure 3**). The first group contained the varieties with the highest methanol content, above 140 mg kg^{-1} ('Picudo', 'Morrut', 'Hojiblanca', 'Cornicabra' and 'Argudell'), the second group included the varieties having medium methanol content, between 74 and 94 mg kg^{-1} ('Marfil' and 'Arbequina'), and the third group contained the rest of cultivars, with methanol levels below 60 mg kg^{-1}.

As it can be seen, in some cases such as 'Hojiblanca', 'Morrut', 'Picudo' or 'Cornicabra', a high amount of ethanol coincided with a high amount of methanol but, statistically speaking, there is no relationship between the concentrations of these two alcohols. The most obvious example of this lack of correspondence was observed in 'Argudell' behaviour since, for this variety, the higher methanol content corresponds to one of the lowest ethanol concentration values. In addition, as with ethanol, no correlation was found between methanol concentrations and physical parameters, so the different concentrations found are related to the variety.

Finally, for acetaldehyde, it can be seen that the amounts were much lower than the ones for ethanol and methanol. This finding is the expected one as acetaldehyde is reduced to ethanol by the enzyme ADH from 13 to 25 weeks after flowering and, considering the sampling data (December 2017), most of the acetaldehyde amount had been already consumed. No relationship between acetaldehyde and ethanol contents was observed, but the presence of low amounts of acetaldehyde in all samples suggests that the ethanol found might be derived from

acetaldehyde by a natural physiological process from the healthy fruits. Depending on their acetaldehyde content, the olive varieties studied can be grouped in three different groups (**Figure 4**).

All these results were also reviewed by considering the two trees of each cultivar separately, as it can be seen in **Figure 5**, where ethanol and methanol for the two trees of each cultivar were graphically represented. It can be observed that results are very consistent. In fact, both trees of each cultivar have similar behaviour, except 'Morrut' variety which, moreover, shows the highest ethanol concentrations. Acetaldehyde data are not shown in **Figure 5**, as the scale used is too large to see differences in such small concentrations.

Other values considered were the ratios between the analytes studied (**Table 2**). Since ethanol and methanol are emitted by ripening fruit [19], the EtOH/MeOH ratio in fruits, and specifically in olives, can be considered a quality index because its value will mainly increase when the ethanol is above its natural concentration mainly due to fermentative processes. However, as shown in Table 2, this is a parameter highly depending on the olive variety. For example, the 'Empeltre' variety, even with healthy olives, will always show high EtOH / MeOH ratio values because it is a variety that has a very low MeOH content. The opposite case would be the 'Argudell' variety, with such high amounts of MeOH that, even when analysing damaged olives, the value of the EtOH / MeOH ratio will be very low.

Since in the present study the olives analysed were healthy and the ethanol origin could not be due to any spoilage, the EtOH / MeOH ratio was studied from another point of view. Concretely, we attempted to use this ratio as an index to predict whether the esters that will predominate in the final olive oil will be fatty acid methyl esters (FAMEs) or fatty acid ethyl

esters (FAEEs). Thus, EtOH / MeOH > 1 implies a probable greater proportion of FAEEs in the final oil and EtOH /MeOH <1 implies a probable greater proportion of FAMEs. By looking at the results, only 'Empeltre' is clearly over 1, whereas the rest of varieties resulted in ratios closer or lower than 1.Therefore, we could conclude that the majority of olive varieties will present a higher content of FAMEs than of FAEEs, which coincides with studies already published [8, 20].

In the other hand, the ratio acetaldehyde/ethanol (AA/EtOH) was proposed as an indicator of fruit anaerobic respiration occurring in ripen and over-ripen fruits, due to a reduction of the mitochondrial activity that implies a decrease of ADH activity. **Table 2** shows the AA/EtOH ratios obtained; and it can be seen that this ratio is lower than 0.6 in all cases, value similar to the one reported by Beltran et al. [11] when dealing with ripe olives. For each individual variety we found that 'Arbosana', 'Argudell', 'Marfil' and 'Sevillenca' show a ratio higher than 0.2 (low metabolic activity) whereas the rest of varieties show a ratio lower than 0.2 (high metabolic activity).

In agreement with previous studies [21], from all these results it can be concluded that alcohols are clearly related to the olive cultivar when dealing with ripe healthy fruits. In fact, when looking at R-square values (%) (**Table 2**), the cultivar accounts for 90% of the total variability for ethanol and 78% for methanol and acetaldehyde. Furthermore, the olive cultivar explains more than 90% of the total variability for EtOH/MeOH ratio and total studied volatile compounds.

As discussed above, unhealthy fruits were processed separately and analyzed as described in the methodology to detect any possible abnormal behaviour. The chromatographic results

were compared to those provided only by completely healthy olives, i.e. olives showing zero defects (the previously so - called "healthy olives" with defects <10%, were not included). In this way, we intended to assess that the differences, if any, were due solely to the health state of the olives. When comparing the alcohol content values obtained, no conclusive results were obtained. This was because the varieties that provided completely healthy olives did not match the varieties that gave damaged olives. For example, within the "unhealthy group" there were 'Argudell' olives that, naturally, have very low amounts of EtOH so, even when these olives were damaged (and their content in EtOH increases[22]), they still provided low values of EtOH. Contrary, within the "completely healthy group" there were 'Hojiblanca' olives that naturally present very high values of EtOH. Thus, to make a comparison possible, we evaluated the results obtained from varieties of which we had both completely healthy and unhealthy olives. **Table 3** reports the average concentration values of ethanol, methanol and acetaldehyde in 'Morrut' and 'Empeltre' varieties. It can be seen that both varieties show significant differences for ethanol: there is an increase of 43% (39 mg kg^{-1}) and 23% (10 mg kg^{-1}) of its content in damaged fruits compared to healthy fruits of 'Morrut' and 'Empeltre', respectively. However, there are not significant differences regarding both methanol and acetaldehyde contents of healthy and unhealthy olives. It should be noted that the average content for these two compounds (Table 3) does not differ from the average values shown in Table 2 for these two varieties. Therefore, methanol and acetaldehyde contents in the so-called "healthy olives" (with less than 10% of defects) are at the same range level as the completely healthy and unhealthy ones.

Regarding the ratio EtOH/MeOH, it shows significant differences because of the significant differences of ethanol contents. As expected for individual varieties, there is a clear increase of the EtOH/MeOH value for the unhealthy fruits in both 'Morrut' and 'Empeltre', so in this case the ratio can be considered a good quality index.

Finally, when analysing the overall data, it can be concluded that some healthy olive varieties provide an alcohol concentration (Table 2) much higher than the values provided by unhealthy olives from other varieties (Table 3). This behaviour proves that a high content of short alcohols is not always due to an unhealthy or poor state of the fruits. Hence, as these alcohols are the precursors of FAEEs, a unique value of FAEEs for all olive varieties should not be used. The olive variety has to be considered as it is closely related to this parameter.

Conflict of interest

The authors declare to have no potential sources of conflict of interest.

Aknowledgements

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Table 1: Average values of fruit physical traits (fruit weight, pit weight, flesh/pit ratio and maturity index – M.I.)

Variety	Fruit weight (g)	Pit weight (g)	Flesh/Pit ratio	M.I.	Reference values [12, 18] Fruit weight	Flesh/pit ratio
ARBEQUINA	1.6 ed	0.3 f	5.2 ± 0.0 cb	6.4 ± 0.5 ab	1.9	5.6
ARBOSANA	2.4 ced	0.4 fe	5.9 ± 0.7 cb	4.2 ± 0.9 ef	1.4	4.9
ARGUDELL	2.9 cbd	0.5 fde	6.2 ± 0.2 b	3.8 ± 0.3 f	2.2	5.6
CORNICABRA	4.0 b	0.8 b	4.9 ± 0.3 cbd	4.3 ± 0.9 def	3.5	4.1
EMPELTRE	3.4 cb	0.6 cde	5.8 ± 0.8 cb	6.9 ± 0.0 a	2.8	5.2
FULLA DE SALZE	1.5 e	0.4 f	3.9 ± 0.4 ed	4.5 ± 0.4 def	1.8	4.1
HOJIBLANCA	3.9 b	1.2 a	3.4 ± 0.1 e	5.1 ± 0.0 dc	4.5	3.7
LLUMET	2.5 ced	0.5 fde	5.5 ± 0.3 cb	4.9 ± 0.0 de	1.9	3.8
MARFIL	2.3 ced	0.4 fe	5.6 ± 0.2 cb	3.8 ± 2.7 f	2.1	4.3
MORRUT	3.4 cb	0.9 b	3.9 ± 1.2 ed	4.5 ± 1.4 def	3.9	5.7
PICUAL	2.9 cbd	0.6 cd	4.7 ± 0.3 cd	4.7 ± 0.5 de	3.6	3.5
PICUDO	5.9 a	0.8 cb	7.8 ± 0.9 a	4.6 ± 0.2 def	5.5	4.1
SEVILLENCA	3.5 cb	0.7 cb	4.9 ± 0.1 bd	5.8 ± 0.4 bc	3.4	5.3
Data analysis R-square	0.949	0.974	0.924	0.860		
F value	11.52	23.14	8.11	15.69		
α <0.05	0.0009	<0.0001	<0.0031	<0.0001		

By column, means with the same latter are not significantly different according to Duncan's multiple range tests (p<0.05).

Table 2: Average of the amount of ethanol, methanol and acetaldehyde contents for the studied varieties

Variety	Ethanol (mg kg⁻¹)	Methanol (mg kg⁻¹)	Acetaldehyde (mg kg⁻¹)	EtOH/MeOH*	AA/EtOH*	∑EtOH+MeOH+AA (mg kg⁻¹)*
ARBEQUINA	48.3 ± 2.0 bc	93.4 ± 1.6 bc	4.4 ± 0.1 a	0.52 ± 0.02 cd	0.10 ± 0.01 ef	146.2 ± 2.8 c
ARBOSANA	7.6 ± 0.2 e	28.5 ± 0.5 cd	4.1 ± 0.1 ab	0.27 ± 0.01 de	0.54 ± 0.01 a	40.2 ± 0.5 f
ARGUDELL	13.4 ± 0.4 e	180.6 ± 8.2 a	3.7 ± 0.1 bcd	0.07 ± 0.00 e	0.27 ± 0.02 d	197.7 ± 8.0 b
CORNICABRA	47.3 ± 0.8 bc	152.7 ± 5.4 ab	3.8 ± 0.1 bc	0.30 ± 0.01 de	0.08 ± 0.00 ef	203.8 ± 6.1 b
EMPELTRE	48.6 ± 0.5 bc	23.5 ± 0.9 d	3.2 ± 0.1 ecd	2.07 ± 0.08 a	0.07 ± 0.00 ef	75.3 ± 1.1 e
FULLA DE SALZE	34.4 ± 3.4 cd	39.7 ± 1.0 cd	3.5 ± 0.1 bcd	0.87 ± 0.06 bc	0.10 ± 0.01 ef	77.6 ± 4.4 e
HOJIBLANCA	98.1 ± 1.5 a	140.8 ± 9.9 ab	3.7 ± 0.1 bcd	0.70 ± 0.01 c	0.04 ± 0.00 c	257.5 ± 6.9 a
LLUMET	44.4 ± 3.2 cbd	51.5 ± 0.3 cd	2.6 ± 0.1 e	0.86 ± 0.06 bc	0.06 ± 0.01 ef	98.4 ± 2.9 de
MARFIL	25.3 ± 2.1 de	74.3 ± 2.3 cd	3.3 ± 0.1 cd	0.34 ± 0.03 de	0.20 ± 0.03 e	102.9 ± 2.0 de
MORRUT	111.2 ± 5.5 a	140.6 ± 9.2 ab	3.8 ± 0.2 bc	0.84 ± 0.09 bc	0.03 ± 0.00 f	255.5 ± 8.3 a
PICUAL	57.1 ± 3.2 b	53.3 ± 6.0 cd	3.1 ± 0.2 efd	1.13 ± 0.09 b	0.05 ± 0.01 ef	113.5 ± 8.7 d
PICUDO	48.7 ± 5.1 bc	137.2 ± 6.0 ab	3.3 ± 0.2 cd	0.36 ± 0.04 de	0.06 ± 0.01 ef	189.2 ±10.7 b
SEVILLENCA	5.9 ± 0.1 e	31.8 ± 2.1 dc	2.6 ± 0.1 ef	0.19 ± 0.02 de	0.43 ± 0.02 b	40.3 ± 2.0 f
Data analysis R-square	0.9093	0.7869	0.7870	0.9193	0.9679	0.9589
Data analysis F value	20.90	8.31	8.31	23.76	62.80	48.69
Data analysis α <0.05	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

By column, means with the same letter are not significantly different according to Duncan's multiple range tests (P<0.05).
* EtOH: Ethanol; MeOH: Methanol; AA: Acetaldehyde.

Table 3: Comparative test on the mean values of ethanol, methanol and acetaldehyde contents for completely healthy and unhealthy fruits from 'Morrut' and 'Empeltre' varieties.

Variety		Ethanol (mg kg^{-1})	Methanol (mg kg^{-1})	Acetaldehyde (mg kg^{-1})	EtOH/MeOH*
MORRUT	completely healthy fruits	90.1 ± 5.7 a	124.6 ± 1.6 a	3.6± 0.4 a	0.72 ± 0.05 a
	unhealthy fruits	129.8 ± 3.7 b	132.6 ± 4.8 a	3.8 ± 0.3 a	0.98 ± 0.02 b
EMPELTRE	completely healthy fruits	43.8 ± 0.4 A	25.4 ± 1.1 A	3.2 ± 0.1 A	1.86 ± 0.05 A
	unhealthy fruits	53.9 ± 1.8 B	22.6 ± 1.6 A	3.1 ± 0.1 A	2.37 ± 0.19 B

By column, means with the same letter are not significantly different according to Tukey Grouping (P<0.05)

* EtOH: Ethanol; MeOH: Methanol

FIGURES

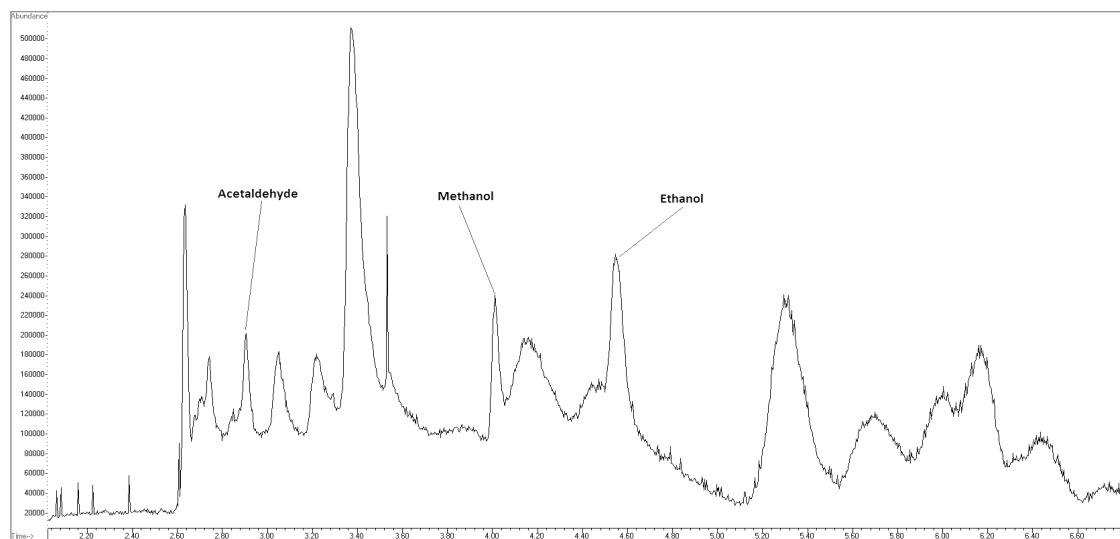**Figure 1.** Chromatogram of an 'Arbequina' sample analysed with the proposed HS-SPME procedure.

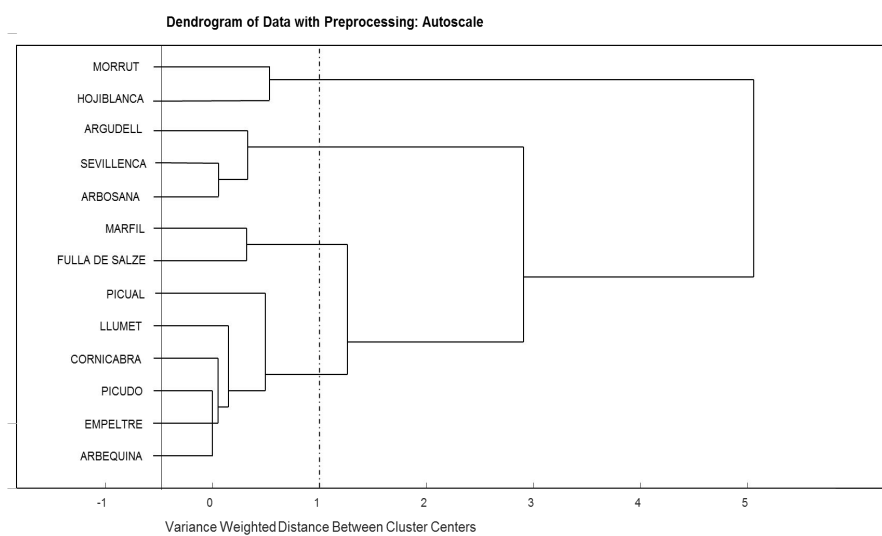**Figure 2.** Hierarchical cluster for ethanol

Dendrogram of Data with Preprocessing: Autoscale

Figure 3. Hierarchical cluster for methanol

Dendrogram of Data with Preprocessing: Autoscale
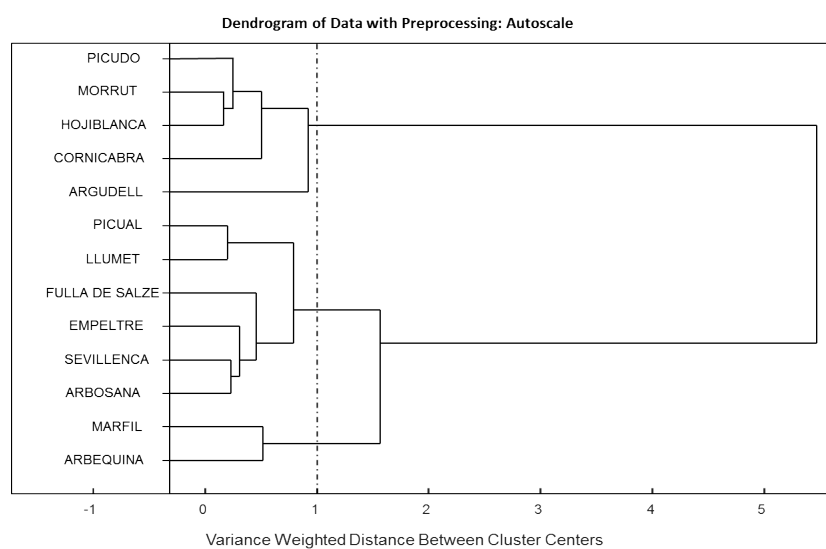
Figure 4. Hierarchical cluster for acetaldehyde

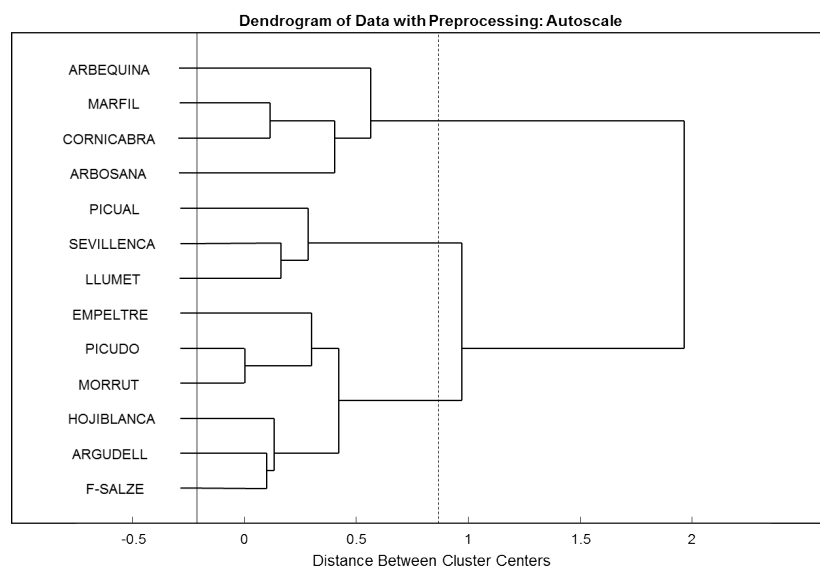Dendrogram of Data with Preprocessing: Autoscale

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Figure 5. Representation of the individual results of the amounts of ethanol, methanol and the ratio ethanol/methanol. EtOH: Ethanol, MeOH: Methanol