

RESEARCH ARTICLE

Fatty acid ethyl esters in virgin olive oils: A correlation study with the volatile profile

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ABSTRACT

Fatty acid ethyl esters are the newest mandatory quality parameters introduced for extra virgin olive oil classification linked to fermentative processes occurring in low-quality olives. Although it is clear the strong linkage of ethyl esters with sensory fermentative defects there is a lack of knowledge about their relationship with specific volatile compounds. Hence, a study was carried out to evaluate the correlation among extra virgin olive oil quality indices and its respective volatile profile with fatty acid ethyl esters. Thirty-five virgin olive oils were collected from local oily industry and analysed for the fatty acid ethyl esters content, the basic quality parameters, the total phenolic content and the volatile profile. The obtained results showed that the fatty acid ethyl esters were negatively correlated to total phenolic compounds ($p < 0.05$) and *trans*-2-hexenal content ($p < 0.001$) while positively correlated ($p < 0.001$) to ethyl acetate and ethyl acetate/*trans*-2-hexenal and ethyl acetate/hexanal ratios. Interestingly, ethanol was not correlated at all with the ethyl esters content in oils.

Keywords: Extra virgin olive oil; Fatty acid ethyl esters; Phenolic compounds; Volatile compounds

INTRODUCTION

Virgin olive oils are obtained directly from the fruits only by mechanical extraction (Official Journal of the European Communities, 2001). Only those oils that fulfil all the requirements established by the different regulations issued by the European Community can be classified in a specific category, such as extra virgin olive oil (EVOO) (Official Journal of the European Union, 2016). Good fruits and extraction practices are necessary conditions, but sometimes not sufficient, to obtain high quality oils. In fact, the final characteristics of a specific oil are dependent on pre-harvest as well as post-harvest factors (Mele et al., 2018). Among the former, genotype and ripeness degree strongly influence the phenolic and volatile amount and profile of the oil, as well as the basic chemical parameters such as free acidity which tended to rise, especially in damaged fruits (Conte et al., 2019; Giuffrè, 2018; Rotondi et al., 2010). Moreover, even soil, canopy position, and growing conditions/treatments could have a significant effect, as reported elsewhere (Caruso et al., 2017; Rached et al., 2017).

Among the analytical parameters of EVOO, the EU Regulation 61/2011 (Official Journal of the European Union, 2011) introduced for the first time the determination of the fatty acid alkyl esters (FAAEs), constituted by the sum of the fatty acids methyl esters (FAMEs) and ethyl esters (FAEEs), as quality parameter for the EVOO. Successively, the EU Regulation 1348/2013 (Official Journal of the European Union, 2013) focused only on the sum of FAEEs, which must not exceed 40 mg kg^{-1} in the 2013/14 crop year, $\leq 35 \text{ mg kg}^{-1}$ in the 2014/15 harvest year and $\leq 30 \text{ mg kg}^{-1}$ from the 2015/16 crop year. Finally, the EU Regulation 2016/2095 (Official Journal of the European Union, 2016) set the maximum value of FAEEs for EVOO at 35 mg kg^{-1} of oil.

The presence of FAMEs and FAEEs in olive oils has been known since the end of the 60s when were identified in stripping products (Bendini et al., 2009). Cert (2006) related the presence of such compounds to the fermentation phenomena that may occur in the olive fruit with formation of methyl and ethyl alcohols that react with free fatty acids (FFAs). Furthermore, the author studied the behaviour

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of these esters to deodorization by applying two types of technology, under steam and under nitrogen, and found that up to 150 °C there is no loss of these products. Based on these results, Pérez-Camino et al. (2008) and Jabeur et al. (2015) used the alkyl esters of fatty acids to detect the addition of deodorized oils in EVOO. Alongside, Mariani and Bellan (2008) suggested these esters as quality markers, useful in detecting the EVOO produced from poor quality olives. The authors analysed more than 150 olive oils of various classes. It was found that alkyl esters in *lampante* oils were significantly abundant than in EVOOs, so that they could be eligible as an analytical tool to detect illegal mixture of *lampante* oil in EVOO, even those subjected to mild treatments in order to remove negative volatile compounds and bring them closer to the virgin. In good quality EVOO the presence of these esters was very low and, often, the FAMEs were higher than ethyl ones (Pérez-Camino et al., 2002). Other studies have also highlighted as ethyl and methyl esters of fatty acids in EVOO could be affected by the technological operations during the extraction process, while they were not affected by oil storage (Alcalá et al., 2017; Squeo et al., 2017a; Caponio et al., 2018a). As a conclusion, when appreciable amounts of alkyl esters of fatty acids in EVOO were found there was a high probability of illegal mixture of refined oils with analytical characteristics very similar to those of EVOO. Furthermore, other authors (Biedermann et al., 2008; Gómez-Coca et al., 2012) have shown a close correlation between chemical parameters and sensory evaluation; in particular, EVOOs, characterized by a low content of alkyl esters and a low content of waxes, were recognized as high quality oils from the panel test, while those samples having high levels of FAAEs have not been judged as EVOOs. The FAAEs, in fact, have been reported to be strongly related with fermentative sensory defects such as winey, moldy and fusty-muddy (Gómez-Coca et al., 2012). As reported in several studies, both the sensory defect than the positive sensory attribute of the oils was strongly linked to the volatile profile of the oils (Cayuela et al., 2015; Kalua et al., 2007).

As far as we know, no investigations are available in literature in which the fatty acid ethyl esters in VOOs were correlated with the volatile profile of the same. In this framework, our aim was at studying the relationship between the total content of FAEE in virgin olive oils with the volatile profile and basic quality parameters.

MATERIALS AND METHODS

Sampling

One litre of thirty-five VOOs, packaged in green glass having about 3-mL of headspace, were collected by local oily

industries in Apulia region in the months of November and December 2017. All the oils, produced by a two- or three-phase centrifuge extraction system from drupes freshly harvested, upon arrival in the laboratory were subjected to analytical determinations and stored until completion of the analyses, about one month, in the dark at 4 °C. Each sample was representative of a large batch of oil, extracted from different cultivars and ripeness degree of the fruits, sampled from tanks having a capacity of about 10 tons.

Chemicals

n-hexane, Folin-Ciocalteu reactive, gallic acid, methyl heptadecanoate and Sudan I dye were purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC, St. Louis, USA); diethyl ether and acetic acid were purchased from Honeywell (Honeywell International, Inc., Morristown, NJ, USA); ethanol, potassium iodide, sodium thiosulfate, chloroform, isooctane and methanol were purchased from Carlo Erba (CARLO ERBA Reagents S.r.l., Cornaredo, Milan, Italy); phenolphthalein solution was purchased from Fluka (Honeywell International, Inc., Morristown, NJ, USA); silica gel was purchased from Merck (Merck KGaA, Darmstadt, Germany).

Analytical determinations

Routine analyses (free acidity, peroxide value and UV spectrophotometric constants) were carried out according to EC Regulations (Official Journal of the European Communities, 1991).

The total phenolic compounds (TPCs) of the oils were extracted by liquid-liquid extraction using 5 mL of methanol/water (70:30, v/v) mixture and 1 g of sample and analysed as reported in Tamborrino et al. (2019) using the Folin-Ciocalteu reagent. The results were expressed as mg of gallic acid equivalent per kg of oil.

The FAEEs were determined as prescribed by the official analytical methods of EU Regulations (Official Journal of the European Union, 2011). Briefly, alkyl esters were firstly recovered by silica gel column chromatography and then measured by capillary gas chromatography. Each sample was analysed twice.

The volatile compounds analysis was carried out as reported in Caponio et al. (2018b) by SPME-GC-MS. Volatiles were extracted by means of a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA) at 40 °C for 20 min and then desorbed in the GC injector. The identification was carried out by comparison of their mass spectra and retention times with those of authentic reference compounds and the NIST and Wiley libraries. The results were expressed as total ion count.

Statistical analysis

Minitab 17 software (Minitab Inc., State College, PA, USA) was used to carry out both the Principal Component Analysis (PCA) and the correlation analysis among variables and to calculate the respective significance p -values.

RESULTS AND DISCUSSION

Chemical characteristics of the analysed VOOs are showed in Fig. 1. In particular, i) FFAs mean value was $0.42 \text{ g } 100 \text{ g}^{-1}$ (range $0.19\text{-}2.28 \text{ g } 100 \text{ g}^{-1}$) with one sample with values higher than the limit provided for EVOO; ii) PV mean value was $8.6 \text{ meq O}_2 \text{ kg}^{-1}$ (range $1.8\text{-}23.1 \text{ meq O}_2 \text{ kg}^{-1}$) with one sample with values higher than the limit provided for EVOO; iii) K_{232} mean value was 1.95 (range $1.18\text{-}3.54$) with three samples exceeding the limit for EVOO; iv) K_{270} mean value was 0.22 (range $0.06\text{-}0.37$) with eleven samples exceeding the limit for EVOO, among which were included those out of the limit for the other chemical parameters; v) FAEEs showed mean value of 8.01 mg kg^{-1} (range $2.51\text{-}20.13 \text{ mg kg}^{-1}$) without samples with values exceeding the

limit for EVOO. Moreover, TPCs showed mean values of 226 mg kg^{-1} (range $21\text{-}537 \text{ mg kg}^{-1}$).

The oils were also characterized by means of the analysis of volatile compounds and, as showed in Fig. 2, the aldehydes were the most abundant compounds.

More in detail, *trans*-2-hexenal – arising from linolenic acid oxidation (Zunin et al., 2004), and accountable for bitter and cut grass notes (Angerosa et al., 2004) – was the most abundant, in agreement with other authors (Aparicio et al., 1996; Angerosa et al., 2001; Caponio et al., 2014), representing on average more than 70% of the volatile fraction. It was followed by (a) *trans*-2-hexen-1-ol, having green and pungent notes, deriving from the reduction *trans*-2-hexenal by alcohol dehydrogenase (ADH) (Bedoukian, 1971; Morales et al., 1997); (b) hexanal, resulting from both enzymatic oxidation and autoxidation of linoleic acid (Zunin et al., 2004) and responsible for the sensory notes of green, apple, cut grass (Morales et al., 1997); (c) hexan-1-ol, originating from hexanal through the activity of ADH and responsible of fruity, aromatic, soft, cut grass notes (Angerosa et al., 2004); (d) ethanol, arising from fermentation and responsible for sensory notes of alcoholic, ripe apple, floral (Reiners and Grosch, 1998); (e) *cis*-3-hexen-1-ol and *trans-trans*-2,4-hexadienal, responsible for banana, leaf-like, green-fruity, pungent and cut grass notes, respectively (Angerosa et al., 2004); (f) ethyl-acetate, that may be synthesised through a different pathway (Salas, 2004; Kalua et al., 2007), responsible for sweet and aromatic notes (Morales et al., 1997). Both nonanal, deriving from autoxidation of oleic acid (Paradiso et al. 2016, Paradiso et al., 2018a) and considered a marker of olive oil oxidation, and hexanoic acid, formed by oxidation of the corresponding aldehyde (Paradiso et al., 2018b), were present in very small amount, indicating a low incidence of the phenomenon of auto-oxidation in the examined samples.

PCA is a powerful multivariate exploratory analysis able to highlight underlying structure in the dataset and variables correlations. Fig. 3 shows the loading plot from PCA considering, as variables, the basic quality parameters, the TPC and, among the volatiles, *trans*-2-hexenal, hexanal, ethanol, and ethyl-acetate, in turn responsible for the characteristic positive flavour of EVOOs and for fermentative defects (Angerosa et al., 2004; Kalua et al., 2007). Moreover, also the relative amount of fermentation markers (ethanol (E) and ethyl-acetate (EA)) respect to the positive C6 aldehydes (*trans*-2-hexenal (t2H) and hexanal (H)) have been considered. The first two principal components, PC1 and PC2, explained together about 55% of total variability. The plot evidenced that FAEEs were quite strongly and positively correlated with ethyl-acetate

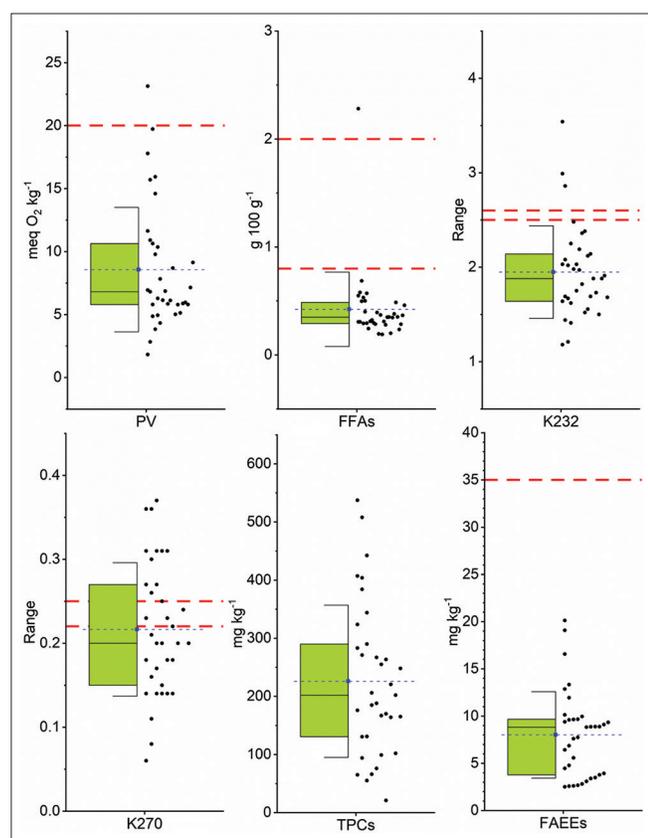


Fig 1. Chemical characteristics of the analysed VOOs. PV, peroxide value; FFAs, free fatty acids; K_{232} and K_{270} , specific absorption at 232 and 270 nm; TPCs, total polyphenol compounds; FAEEs, fatty acid ethyl esters. The dotted bars in red indicated the threshold for extra virgin (lower), virgin (higher) or both the oils, according to the Regulation EEC 2568/91.

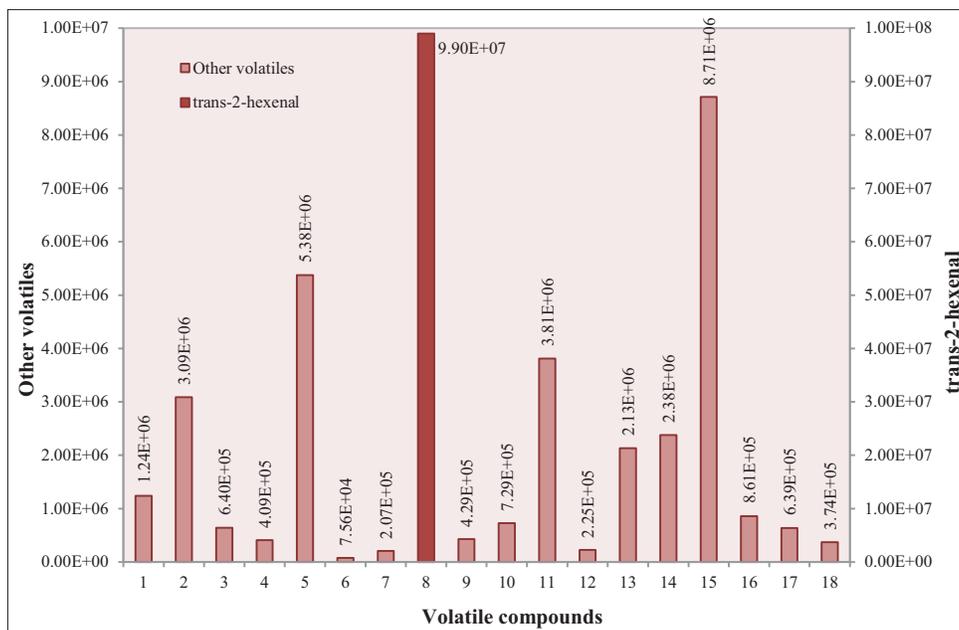


Fig 2. Mean values (total ion count of the integrated area) of the volatiles detected in the analysed VOOs. 1, ethyl acetate; 2, ethanol; 3, pentan-3-one; 4, 1-penten-3-one, 5, hexanal; 6, 1-penten-3-ol; 7, limonene; 8, *trans*-2-hexenal; 9, 3-hexen-1-ol, acetate; 10, hexan-1-ol; 11, nonanal; 12, *trans,trans*-2,4-hexadienal; 13, *cis*-3-hexen-1-ol; 14, *trans*-2-hexen-1-ol; acetic acid; 17, benzaldehyde; 18, hexanoic acid.

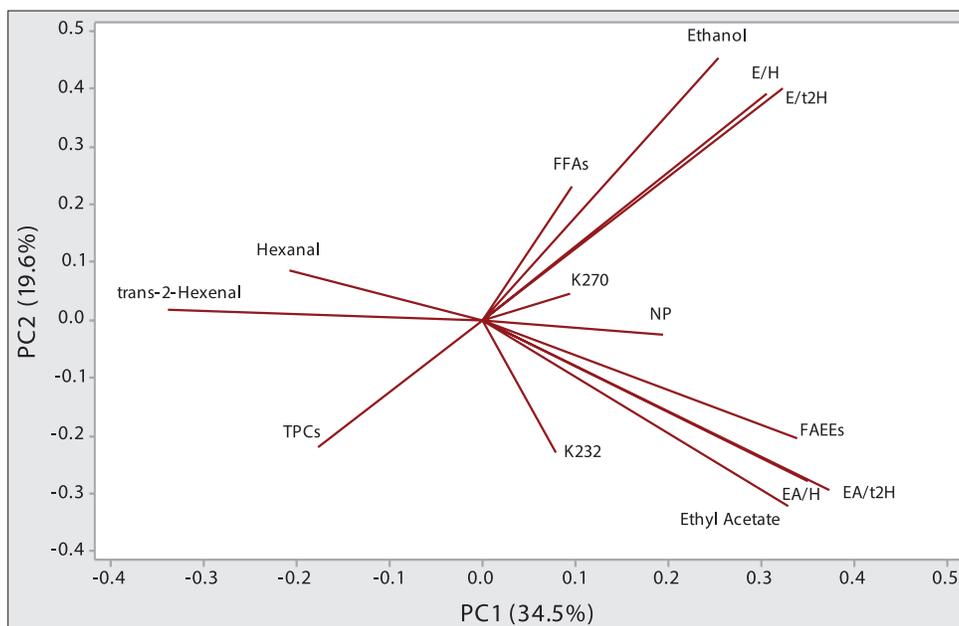


Fig 3. Loading plot from the principal component analysis (PCA) carried out on the selected data. PV, peroxide value; FFAs, free fatty acids; K_{232} and K_{270} , specific absorption at 232 and 270 nm; TPCs, total polyphenol compounds; FAEs, fatty acid ethyl esters; EA, ethyl acetate; E, ethanol; t2H, *trans*-2-hexenal; H, hexanal.

rather than ethanol which, instead, seemed to be not correlated at all.

The result is noteworthy considering that ethanol is one of the precursors of fatty acids ethyl esters and thus a positive correlation would be reasonably expected. On the other hand, a negative correlation with the positive fruity compounds, *trans*-2-hexenal and hexanal, was observed.

The ratios EA/H, EA/t2H, E/H, and E/t2H behaved very similar to the pure compounds being the EA/H and EA/t2H strongly correlated with FAEs. The Pearson's correlation coefficients were calculated along with the significance p -values. The results showed that a significant negative correlation existed between FAEs and *trans*-2-hexenal ($p < 0.001$) while, without statistical significance, among FAEs and hexanal ($p > 0.05$). Most important, a

very significant negative correlation was found between ethyl esters and ethyl-acetate ($p < 0.001$) while the former were totally not correlated with ethanol, confirming what already observed through the PCA. Considering the indices EA/H, EA/t2H, E/H, and E/t2H, a strong correlation was found among FAEEs and EA/H and EA/t2H, being the former the most strongly correlated ($r = 0.746$, $p < 0.001$). Neither E/H nor E/t2H were correlated with FAEEs. As previously deduced from PCA, and confirmed by the Pearson's correlation, ethyl-acetate has been proven to be the most sensitive marker of the presence of fatty acids ethyl esters in the virgin olive oils under study. With regard to the TPC, a significant negative correlation with FAEEs was reported ($p < 0.05$). Phenolic content in oils depends on the ripeness degree and quality of the drupes as well as on technological procedures of olive paste preparation and oil extraction (Gómez-Caravaca et al., 2008; Alagna et al., 2012; Squeo et al., 2017b; Caponio et al., 2018b). Considering that ethyl esters are formed mainly as a consequence of fermentative processes occurring during prolonged and improper drupes storage, and, in turn, the same events cause the depletion of phenolic compounds, the correlation observed is reasonable. The same conclusions can be drawn considering the negative correlation of FAEEs with *trans*-2-hexenal and hexanal. These findings confirmed that the processes responsible for the rising of ethyl esters in oils are linked together with a depletion of positive volatile and phenolic compounds as already known.

CONCLUSION

Our finding highlighted that FAEEs are strongly correlated with other analytical parameters of the EVOOs. In particular, FAEEs were significantly correlated in negative manner with TPCs and *trans*-2-hexenal and in positive manner with ethyl acetate and ethyl acetate/*trans*-2-hexenal and ethyl acetate/hexanal ratios.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Author contributions

Authors contributed equally to this study.

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