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A survey of ethanol content in virgin olive oil

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Abstract

Ethanol is a substrate for the chemical synthesis of fatty acid ethyl esters (FAEE) during storage of virgin olive oil whose contents are officially regulated. Given the impact that the ethanol content might have on the olive oil commercialization, the level of this metabolite has been studied in an array of olive genotypes representing the diversity available in olive (*Olea europaea* L.). Substantial levels of ethanol have been found in the oils of all genotypes under study. Moreover, increasing levels of alcohol dehydrogenase activity have been found during olive fruit ripening in good correspondence with the accumulation of ethanol in advanced stages of fruit maturation. The results suggest that ethanol has a ubiquitous character in the fruits of *Olea europaea* and, therefore, in all the oils obtained from them. Besides, their concentration seems to depend on the cultivar, ripening stage and climatology, not discarding the influence of the growing conditions. Data suggest that the application of olive oil regulation for FAEE levels should consider the presence of basal levels of ethanol in the oils, which are quite high in many cultivars.

Keywords: *Olea europaea* L.; virgin olive oil; ethanol

1. **Introduction**

Ethanol is a component that is naturally present in the olive fruit issues and, consequently, could pass into virgin olive oil (VOO) during the extraction process as it has been observed in a number of cultivars (Luna et al., 2006; Beltrán et al., 2015). The occurrence of ethanol in plants is generally associated to an adaptation to oxygen deprivation (Davies, 1980). It is produced from pyruvate by the consecutive action of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzymes (Fig. 1). However, the presence of this compound in plants is not necessarily related to a decrease in the availability of oxygen in the tissues as this pathway may work under aerobic conditions (*i.e.* aerobic fermentation) playing different key roles in the plant physiology. In adverse environmental conditions such as cold, drought or high CO_2 concentrations, an increase of ethanol content in the plant tissues has been observed due to an induction of PDC and ADH gene expression (Tadege et al., 1999; Moyano et al., 2004). The aerobic fermentation pathway also participates actively in the strategies used by the different plants for the preservation of the species through seed dispersal. Thus, both PDC and ADH activities have been observed to increase during the strawberry fruit ripening parallel to the accumulation of sugars and the increase in the synthesis of aroma compounds, which corresponds largely to esters derived from ethanol (Pérez et al., 1992; Moyano et al., 2004). Unlike most of the fruits, the olive fruit uses a different strategy for seed dispersal. Olive fruit accumulates large amounts of triacylglycerol (TAG) along ripening, whose production in plants is carried out from the building block acetyl-CoA (Fig. 1). However, while acetyl-CoA is synthesized in seeds from pyruvate by the action of the enzymatic complex pyruvate dehydrogenase (PDH), the synthesis in olive fruit seems to be carried out from acetate through the so-called PDH bypass (Fig. 1), which is mediated by the acetyl-CoA synthetase (ACS) activity (Salas et al., 2000). In this pathway, pyruvate is previously transformed into acetate by the sequential action of PDC and aldehyde dehydrogenase (AlDH)(Fig. 1), as demonstrated to occur in photosynthetic tissues (Roughan & Ohlrogge, 1996) and microalgae (Ramanan et al., 2013; Avidan & Pick, 2015). According to this model, the ADH activity would function as a safety valve to protect the cell from the accumulation of the transient excess of the cytotoxic acetaldehyde through conversion into ethanol.

The presence of ethanol in the oil may represent a problem from the perspective of the VOO legislation as this compound is substrate for the chemical synthesis of fatty acid ethyl esters (FAEE) during VOO storage. FAEE is a quality parameter used for virgin olive oil (VOO) according to the European Commission and the International Olive Oil Council (European Commission Regulation, 2013; International Olive Oil Council, 2013). These compounds are produced during storage whenever there are free fatty acids and ethanol in the oil and its chemical synthesis is dependent on the content s of those substrates, temperature and time (Di Loreto et al., 2014; Gómez-Coca et al., 2016). FAEE was originally proposed as a fraud indicator for the identification of blends with low quality oils such as those subjected to deodorization as this industrial process causes an increase in the content of FAEE. Later, it was proven that FAEE

do not originate exclusively in this process as it is also found in VOO obtained from non-sound fruits. In this sense, the relationship between the FAEE content in VOO and its sensory classification have been described in different studies (Gomez-Coca et al., 2012; Perez-Camino et al., 2002). More recently, Di Serio et al. (2017) demonstrated the relationship that exists between the FAEE level in the oil and the sensory defects caused by fermentative processes in the olive fruits. Thus, this parameter is today used as an indicator of the health status of the fruit from which the oil was extracted (Bendini et al., 2009; Mariani & Bellan, 2008) based on the assumption that the presence of its substrate ethanol in the oil can solely be due to its generation in fermentative processes occurring in the fruit or in the oil (Bierdermann et al., 2008; Conte et al., 2014). This is the reason why EU Commission Regulation 1348/2013 (2013) substituted alkyl esters with ethyl esters. The former term also included originally the methyl esters (FAME), whose production in the oil uses as substrate the methanol present in the fruit. Unlike ethanol, the level of methanol in the fruit is considered to be physiological because methanol is liberated during the pectin degradation of the cell wall as olive fruit ripens.

It has been demonstrated that ethanol is not just a fermentation derivative as it accumulates in sound fruits during ripening (Beltran et al., 2015). The question is how much of the ethanol present in the oil is coming from the physiological levels in the olive fruit and how much is due to fermentative processes. Given the impact that the ethanol content might have, as substrate for the synthesis of FAEE, in the classification of olive oil as Extra-VOO and in line with the needs for research that Conte et al. (2014) suggested regarding the limits of the ethanol content in the oils, the aim of this work was to investigate the natural occurrence of ethanol in virgin olive oil and its genetic variability. For this purpose, a wide range of VOOs representing the available diversity of the olive species was screened. Moreover, the enzymatic activity responsible for the synthesis of ethanol in the olive fruit tissues was studied to verify its metabolic origin.

2. Materials and methods

2.1. Plant material.

Two sets of olive samples representative of the *Olea europaea* species were studied. On one hand, a collection of 97 commercial cultivars that gathered the genetic variability of the World Olive Germplasm Collection (WOGC) located at IFAPA Centre "Alameda del Obispo", (CAP-UCO-IFAPA) in Cordoba Spain. Trees, two per accession, were cultivated in the same conditions at the WOGC using standard culture practices. On the other, 136 genotypes featuring the same genetic background, from the cross of olive cultivars 'Picual' and 'Arbequina', were also included in the study. The harvest was carried out by hand during four consecutive years (2009-2012) at turning stage except for the studies related to fruit ripening. Additionally, for 'Picual', 'Arbequina' and five genotypes from their cross (characterized by having quite different levels of volatile compounds in their oils), fruits were harvested at three ripening stages [ripening index (RI) = 1, 2.5 and 5] in order to test the influence of RI on ADH activity.

2.2. Preparation of ADH crude extracts and enzymatic assay.

Preparation of crude extracts and measure of ADH activity were carried out according to Sánchez-Ortiz et al. (2012) with minor modifications. Acetone powders were prepared from the mesocarp of fresh harvested olive fruits by grinding with acetone at -20 °C (1:15, w/v) using a blender. After filtration, the residue was re-extracted twice with 20 mL of cold acetone. The whitish powder obtained was finally rinsed with diethylether, dried and stored at -20 ºC. ADH enzyme extracts were prepared from 125 mg of acetone powder in 3 mL of a buffer consisting of 50 mM sodium phosphate (pH 7.2), 14 mM β-mercaptoethanol, 2 mM ditiothreitol and 10% glycerol using an Ultraturrax homogenizer (3 x 1 min). The resulting homogenate was centrifuged at 27000*g* for 20 min at 4 ºC and filtered through Miracloth®. The clear supernatant was used as the crude extract.

ADH activity was assayed by mixing 1.5 ml of 100 mM sodium phosphate (pH 6.0), 30 µl of 10 mM reduced nicotinamide adenine dinucleotide (NADPH), 10 µl of 1 M hexanal and 20 µl of the crude extract. ADH activity was measured by monitoring the decrease in absorbance at 340 nm over time due to the oxidation of NADPH. One unit of ADH activity is defined as the amount of enzyme required to oxidize 1 µmol of NADPH per min at 25 °C, taking into account a molar extinction coefficient of 6160 $M^{-1} \cdot cm^{-1}$.

2.3. Olive oil extraction.

Olive oil was extracted from batches of 2–3 kg of olive fruits using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that mimics the industrial process of VOO production on a laboratory scale (Martínez et al., 1975). Milling of the olive fruit was performed using a stainless steel hammer mill operating at 3000 rpm and with a 5 mm sieve. The Abencor thermo-beater was used for the malaxation step for 30 min at 28 °C. Finally, centrifugation of the kneaded paste was carried out in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oils were decanted, paper filtered, and stored under nitrogen at -20 °C until they were analyzed.

2.4. Analysis of ethanol in the oil.

The content of ethanol in the oils was analyzed by means of HS-SPME/GC-MS-FID according to Pérez et al. (2016). Olive oil samples (0.5 g) were prepared in 10-mL vials and were conditioned to room temperature and then placed in a vial heater at 40 °C for a 10 min equilibration time. Volatile compounds from the headspace were adsorbed onto SPME fiber DVB/CAR/PDMS 50/30 µm (Supelco Co., Bellefonte, PA). Sampling time was carried out at 40 °C for at least 50 min, which is the time needed to reach the equilibrium. Desorption of volatile compounds was completed directly into the GC injector. Volatile compounds were identified out on a 7820A/GC-5975/MSD system (Agilent Technologies), equipped with a DB-Wax capillary column (60 m × 0.25 mm i.d., film thickness, 0.25 µm: J&W Scientific, Folsom, CA) and under the following conditions: the injection port was operated in splitless mode at 250 °C; He was

used as the carrier gas and the flow rate was 1 mL/min; column was held for 6 min at 40 °C and then programmed at 2 °C min^{-1} to 168 °C; the mass detector was operated in the electronic impact mode at 70 eV, the source temperature was set at 230 °C and the mass spectra were scanned at 2.86 scans/s in the m/z 40-550 amu range. Compound identification was performed by matching against the Wiley/NBS Library, and by GC retention time against absolute ethanol (Sigma-Aldrich, St. Louis, MO). For quantitative purposes, the volatile fraction was analyzed three times on a HP-6890 GC equipped with a FID detector (Agilent Technologies) using also a DB-Wax capillary column operated under the following conditions to reproduce the same retention timea than those of the 7820A/GC-5975/MSD system: N_2 was used as carrier gas at 17 psi constant pressure; injector and detector were held at 250 °C; column was held for 6 min at 40 °C and then ramped up at 2 °C/min. Calibration curves were made in re-deodorized high-oleic sunflower oil and a linear regression curve was obtained (R^2= 0.996).

3. Results and discussion

The ethanol content in oils from a large sample of genotypes representative of the *Olea europaea* species was studied. The sample covers around a quarter of the WOGC cultivars from IFAPA (Cordoba, Spain), including the Core-36 cultivars of the WOGC (Belaj et al., 2012) for guaranteeing a high genetic diversity, and a segregating progeny of the 'Picual' and 'Arbequina' cultivars having a similar genetic background. As described in Materials and Methods, the trees of these cultivars and the segregating progeny were grown under the same agronomic conditions and the fruits were hand-picked, transported at low temperature, and carefully washed prior to the immediate extraction of their oil. Thus, it was guaranteed that the oils were produced from apparently clean and healthy fruits. As shown in Figure 2, all the oils analyzed in these samples have been shown to contain ethanol. This ubiquity of ethanol in VOO would confirm the hypothesis made by Beltrán et al. (2015) that the ethanol, which is naturally found in the fruit, would later pass into the oil during the extraction process. The presence of ethanol in fruits is a common occurrence in many plant species, especially in those with fragrant fruits where large amounts of ethyl esters are synthesized during ripening and postharvest storage (Pérez et al., 1992; Moyano et al., 2004; Pérez & Sanz, 2008, Bangerth et al., 2012). Actually, the results of the analyses have also shown that all the oils examined contained ethanol-related compounds such as its metabolic precursor, acetaldehyde, and the esterified product ethyl acetate (data not shown). However, contrary to the latter, ethanol has no impact on the aroma of olive oil, even in the case of higher contents, due to the extremely high odour threshold of this compound (30 µg/g oil) (Romero et al., 2015).

A content range of 0.12-12.93 mg/kg oil was found for ethanol among the WOGC cultivar subset under study, with an average value of 2.10 mg/kg oil. These levels are in agreement with those found by Romero et al. (2015) for oils of cultivars Arbequina, Hojiblanca and Manzanilla (0.17-4.24 mg/kg oil) in a recent validation work of the HS-SPME/GC-MS method for the quantification of VOO volatile compounds. However, the same research group found higher

contents of ethanol (20-24 mg/kg oil) in the oils of the Chetoui and Chemlali cultivars produced in Tunisia (Tena et al., 2007). Even greater contents were found by Tura et al. (2008) for oil samples obtained from 18 Italian olive cultivars grown in the western coast of the Garda lake (northern Italy). The mean content (30.6 mg/kg oil) and content range (3.47-217.7 mg/kg oil) were an order of magnitude higher than what was found in this study.

As stated by Gómez-Coca et al. (2016), the FAEE content in the oil is dynamic, proving that it increases with time under certain storage conditions and reaching the limits established by the European Commission Regulation (2016) in a few months. It has been estimated that 11% of the cultivars under study would have given rise to oils with ethanol contents which, considering a complete conversion in the most favorable storage conditions, lead to FAEE levels above 35 mg/kg oil. These hypothetical FAEE levels would make those oils not to be considered as Extra-VOO according to the European Commission Regulation (2016) despite having been obtained with the best sanitary guarantees.

As in the WOGC cultivars, all the oils analyzed from the 'Picual' x 'Arbequina' progeny contained ethanol, which would support the hypothesis that it is a ubiquitous component in VOO (Fig. 2-B). A high level of segregation was found among the genotypes of the progeny for the ethanol content, displaying even higher ethanol contents (0.04-48.06 mg/kg oil) than those observed in the WOGC cultivars, clearly transgressing the levels found in the parents 'Arbequina' and 'Picual'. The average ethanol content in the cross progeny was also clearly higher than that found for the WOGC cultivars (3.58 mg/kg oil *versus* 2.10 mg/kg oil). However, in both olive samples a median value of 0.8 mg/kg oil was found. It has been estimated that 18% of the 'Picual' x 'Arbequina' progeny produced oils containing ethanol levels that could potentially lead to levels of FAEE above the VOO limit (FAEE > 35 mg/kg oil) established by the European Commission Regulation (2016), despite the fact that the oils were obtained in adequate sanitary conditions.

The content of ethanol in the oils must be contextualized in its harvest year, considering that this study has been developed over four years. In this sense, it has been investigated how the climatology of the year affects the level of ethanol in the oils from the two main cultivars grown in Spain, 'Picual' and 'Arbequina', and parents of the studied cross progeny. The content of ethanol in the oils varied depending on the season climatic conditions as displays in Figure 3-A. Interestingly, these cultivars do not follow the same pattern in the different seasons, as shown by the change in the proportions of the ethanol contents in the 2011 and 2012 seasons compared to those observed in the 2009 and 2010 seasons.

Another important aspect to be considered in this study is that the oils were obtained from olives at the turning stage (roughly RI = 2.5). As shown in Figure 3-B, the ethanol content follows different evolution patterns during the ripening process depending on the cultivar. Thus, while the oils of the 'Arbequina' cultivar show a slight increase in the ethanol content in the year of study, a strong increase was found for oils of the 'Picual' cultivar in advanced stages of ripening (RI = 5), approximately quadrupling the levels found in less advanced stages (RI = 1 and

2.5) to reach contents of 4.55 mg/kg oil. These levels coincide with those found by Beltrán et al. (2016) for this cultivar at the same stage of ripening. It should be noted that this ethanol content in the oil may potentially be converted, after storage under appropriate conditions, into FAEE contents dangerously close to the limit value marked by the VOO regulation for this parameter (European Commission Regulation, 2016).

Data demonstrated a substantial influence of the year climatology and the fruit ripening stage on the ethanol content in each cultivar, not ruling out the interaction between both variables and that derived from the growing conditions. The influence of fruit ripening stage on the ethanol content could be explained from a biochemical point of view. As shown in Figure 4-A, there is a good relationship between the level of ADH activity that synthesizes ethanol and the ethanol content in the oils (Fig. 3-B), experiencing an increase as ripening advances. The level of ADH activity was also studied during the ripening process of five genotypes from the 'Picual' x 'Arbequina' cross (Figure 4-B). As it happens to the two parents ('Picual' and 'Arbequina'), an increase in the level of this enzymatic activity was observed during fruit ripening for all the genotypes under study in good agreement with what found by Iaria et al. (2012) for the expression level of an olive ADH gene (*OeADH*). The confirmation of the natural presence of ethanol in VOO, and consequently in olive fruits, and of ADH activity would indirectly support the hypothesis of the PDH bypass working in the generation of TAG in olive fruit; this ADH activity acting as a safety valve against the synthesis of high levels of acetaldehyde, which produces ethanol.

4. Conclusions

It has been investigated the presence of ethanol in the oils produced by individuals from two olive samples, commercial cultivars from WOGC and genotypes from a segregating population of the 'Picual' x 'Arbequina' cross, representative of the *Olea europaea* species. Data suggest that ethanol is naturally found in the fruits of the *Olea europaea* species and, therefore, in all oils obtained from them, and that its concentration is a function of the cultivar, ripening stage and year climatology, not ruling out the dependence of the growing conditions. The levels of ADH activity found in the olive fruit might explain its accumulation during fruit ripening. Despite the fact that the presence of ethanol in the oil may be related to fermentation processes, the application of olive oil regulation for FAEE levels should take into account the presence of basal levels of ethanol in the oils as it is quite high in many olive cultivars.

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Figure captions

Figure 1. Biochemical mechanisms for generating acetyl-CoA, precursor of the *novo* fatty acid biosynthesis.

Figure 2. Content (mg/kg oil) of ethanol in the oils produced from cultivars from the World Olive Germplasm Collection (A) and from genotypes of the 'Picual' x Arbequinal' cross (B). The arrows mark individuals from which the ethanol content can potentially be converted into FAEE levels higher than the limit established by the European Commission Regulation (2016).

Figure 3. Content (mg/kg oil) of ethanol in oils from the cultivars 'Picual' and Arbequinal' throughout the seasons 2009-2012 (A), and during the fruit ripening (B).

Figure 4. Levels of ADH activity (U/g fruit) during ripening of 'Picual' and Arbequinal' fruits (A) and of fruits from selected genotypes of the 'Picual' x Arbequinal' cross (B).

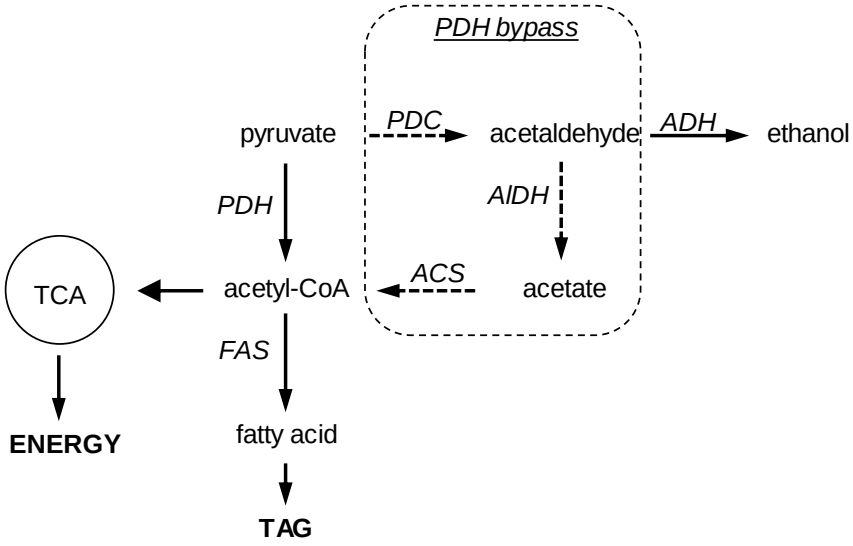
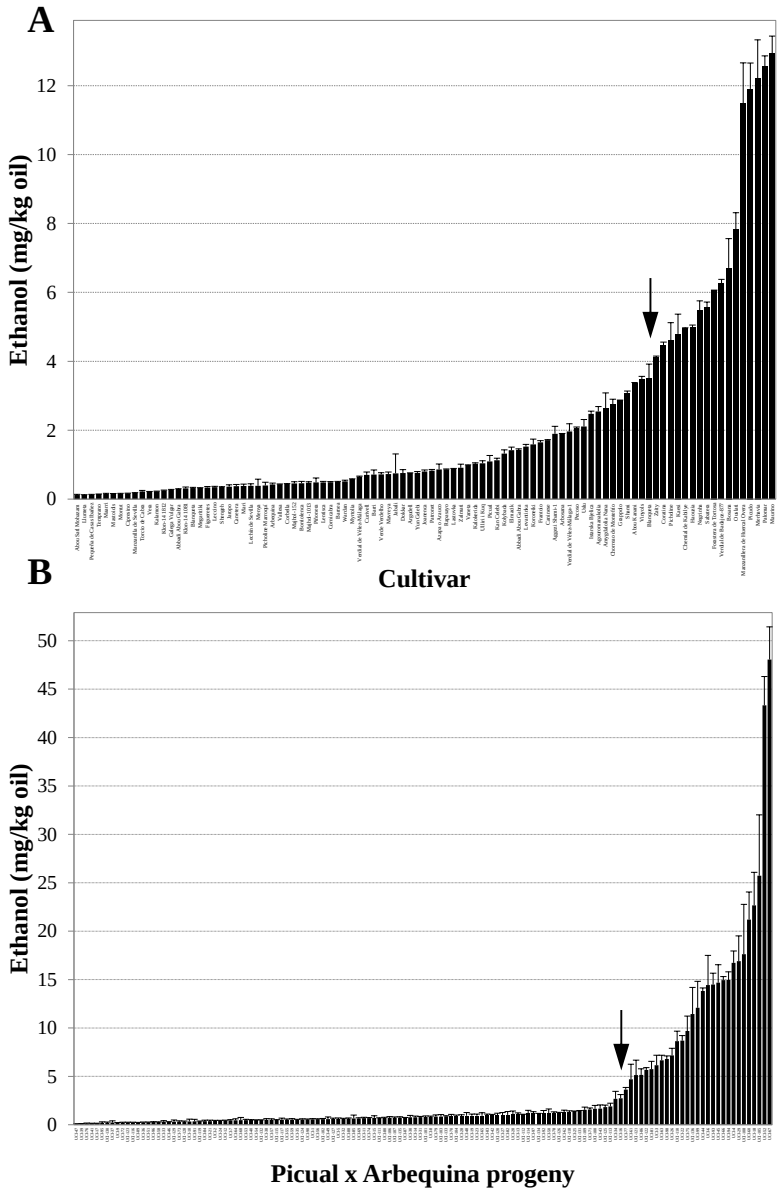

PDH bypass
pyruvate
PDC
acetaldehyde
ADH
ethanol
PDH
AlDH
TCA
acetyl-CoA
ACS
acetate
FAS
ENERGY
fatty acid
TAG

Figure 1


A
Ethanol (mg/kg oil)
0
2
4
6
8
10
12
Cultivar
B
Ethanol (mg/kg oil)
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10
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20
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30
35
40
45
50
Picual x Arbequina progeny

Figure 2

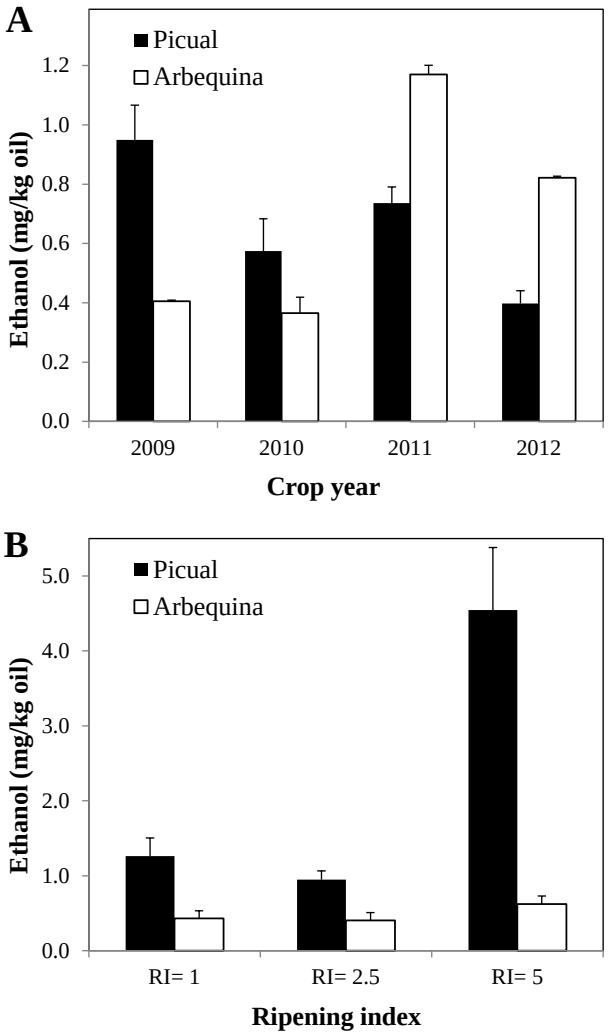
A
Picual
Arbequina
Ethanol (mg/kg oil)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
2009
2010
2011
2012
Crop year
B
Picual
Arbequina
Ethanol (mg/kg oil)
0.0
1.0
2.0
3.0
4.0
5.0
RI= 1
RI= 2.5
RI= 5
Ripening index

Figure 3

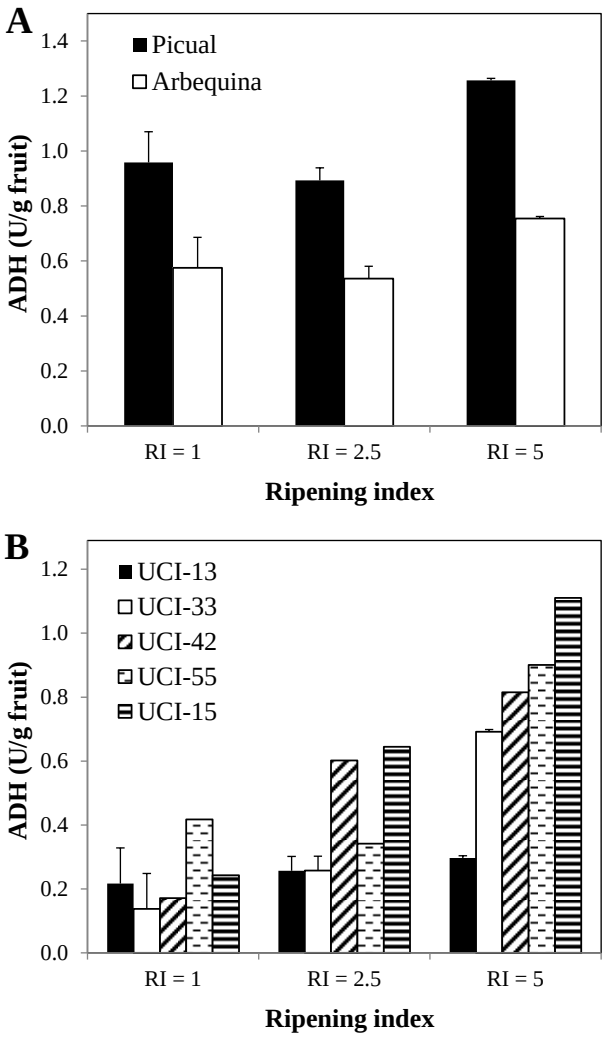
A
Picual
Arbequina
ADH (U/g fruit)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RI = 1
RI = 2.5
RI = 5
Ripening index
B
UCI-13
UCI-33
UCI-42
UCI-55
UCI-15
ADH (U/g fruit)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RI = 1
RI = 2.5
RI = 5
Ripening index

Figure 4