TITLE: DETERMINATION OF MAJOR VOLATILE COMPOUNDS DURING THE PRODUCTION OF FRUIT VINEGARS BY STATIC HEADSPACE GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD

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ABSTRACT

A static headspace gas chromatography coupled to mass spectrometry (SHS-GC-MS) method was validated to determine several major volatile components during the production process of fruit vinegars. The method is simple, fast, linear in the working range, suitably sensitive, repeatable and reproducible, and has a good degree of accuracy for most of the compounds studied. Different conditions were tested in the production process of vinegars by means of double fermentation. The addition of SO_2 and pectolytic enzymes produced a considerable increase in methanol and acetaldehyde, especially in strawberry purees, whereas pressing led to a loss of these volatile compounds. In the alcoholic fermentation of persimmon and strawberry purees, the *Saccharomyces cerevisiae* strain used had a great influence on the production of acetaldehyde and higher alcohols in wines. Considering the influence of these studied compounds in the final profile of the vinegars, our results showed that the

Saccharomyces cerevisiae strain isolated in this study produced the most suitable wine substrates for the production of vinegars. Moreover, semisolid fruit substrate provides better results than liquid substrate. Inoculated acetification in wood recipients yielded vinegars with a better volatile profile, as these contained higher levels of most compounds except acetaldehyde.

KEYWORDS: Volatile compounds; persimmon; strawberry; vinegar; wine; SHS-GC-MS

1. Introduction

Vinegar is one of the most widespread and common products in the world because it is available in every country in several different varieties (Mazza & Murooka, 2009). The traditional use and integration of vinegars in numerous cultures can be traced back to ancient times. Today, the most widely marketed vinegar is wine vinegar, although vinegar can be produced from a variety of very different raw materials.

In today's market, there is a growing demand for fruit vinegar sold as a health food product (Ou & Chang, 2009). This consumer trend has led to the development of new products with the aim of expanding the range of vinegars available on the market. Furthermore, the production of these vinegars provides a use for surpluses of second quality fruit.

Different quality parameters should be studied in selecting the best production procedure for new fruit vinegars. Such parameters should include volatile compounds responsible for aroma and close attention should be paid to which of these compounds might be influenced by the production process.

Aroma is certainly one of the most important determinants of food quality and acceptance. The particular aroma of vinegar is the result of high quantities of volatile compounds. These compounds may come from the raw material or may be formed

during the production process. Different authors have pointed out the importance of the production process in the final aroma of vinegars and therefore in their organoleptic qualities (Morales et al., 2001; Callejón et al., 2009). Moreover, the content of several major volatile compounds found in vinegar such as methanol is restricted by Spanish legislation (<1g/L) (Presidencia del Gobierno, 1993).

Gas chromatography coupled to a mass spectrometry detector is widely used in the study of volatile compounds. To analyse these constituents in a liquid sample, the sample is introduced into a gas chromatograph, the volatile components are evaporated, and their vapour is carried through the column by the mobile phase (Ettre, 2002). However, the non-volatile matrix remains in the injector, thereby contaminating it. Researching volatile components present in a solid sample is even more complicated. This type of sample obviously cannot be introduced into an instrument; it requires an elaborate sample preparation procedure that includes extracting the volatile components, among other steps (Ettre, 2002).

Headspace is a fast, simple, efficient and environmentally friendly sampling method used with capillary GC for the analysis of volatile fractions in many food samples. Headspace (HS) is essentially a sampling method that permits analysts to take an aliquot of the gas phase in equilibrium with a liquid or solid phase (Ettre, 2002). During static HS analysis, equilibrium between the sample and the headspace above is achieved, and a fraction of this headspace gas phase is withdrawn for GC analysis (Bylaite & Meyer, 2006). In equilibrium, the distribution of the analytes between the two phases depends on their partition coefficients. The composition of the original sample can therefore be established from the analytical results of this aliquot (Ettre, 2002).

Static HS-GC works well with high precision and accuracy for liquid samples since calibration can be performed easily by either external or standard addition without any

serious problems (Li et al., 2009). With static headspace sampling, sample headspace volatiles are automatically brought directly to the GC, thus offering good validation as well as the possibility for a high number of samples to be processed (Sriseadka et al., 2006). The main disadvantage of static HS-GC compared to dynamic HS-GC is its relatively low sensitivity (Snow & Slack, 2002). However, sensitivity can be increased by salting-out, pH control or increasing the equilibration temperature during sample heating (B'Hymer, 2003). Static headspace GC is mostly useful for applications in the high-ppb to percent concentration ranges (Wang at al., 2008). In the headspace analysis, parameters such as temperature and equilibrium time, headspace volume and instrumental conditions must be carefully standardized (Arisseto and Toledo, 2008).

The overall goal of this work was to develop and to optimize a simple and fast method based on GC-MS to monitor the evolution of major volatile compounds in the production process of fruit vinegars. Firstly, to monitor changes in these compounds a sampling method had to be selected that was suitable for all three products studied: raw material (fruit puree), fruit wine and fruit vinegar[1], which all have very different consistencies. We decided to test headspace sampling. Next, we optimized the static headspace sampling and injection conditions. Finally, the method was successfully applied to determine the major volatile compounds in these kinds of matrices.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used were analytical-reagent grade and provided from the following sources: acetaldehyde, methyl acetate, methanol, ethyl acetate, 1-propanol, isobutanol, isoamyl acetate, 2-methyl-1-butanol, 3-methyl-1-butanol, ethanol, acetic acid and 4-methyl-2-pentanol (IS) from Merck (Darmstadt, Germany); sodium chloride from

100 Sigma–Aldrich (Madrid, Spain); and water from a Milli-Q purification system
101 (Millipore, Bedford, USA).

102 **2.2. Standards and sample preparation**

103 6 g of sample saturated in sodium chloride (2 g) and 10 μL of internal standard (391 ug
104 kg^{-1}) were placed into a 20 mL HS vial and sealed immediately with a white
105 silica/PETF lined septum and aluminium crimp cap (VWR International Eurolab S.L.,
106 Barcelona, Spain) and then placed in the autosampler tray for HS sampling.
107 A standard mix was used to establish the best injection volume. A dearomatised fruit
108 puree spiked with standards was used to select sample incubation temperature and time.
109 Fruit was dearomatised as follows: 5 mL of dichloromethane were added to 20 g of fruit
110 puree. This mixture was stirred with a stir bar over night, and then was centrifuged at
111 4,000 rpm for 10 min and the dichloromethane was withdrawn. This procedure was
112 repeated. To eliminate remains of dichloromethane, the puree was submitted to a
113 nitrogen stream for 20 min. After this, 5 mL of acetone were added and the mixture was
114 stirred for three hours, followed by centrifugation (4,000 rpm for 10 min), the solvent
115 was withdrawn and a nitrogen stream was subsequently applied for 20 min. We spiked a
116 commercial fruit puree and vinegar with the analytes for repeatability, intermediate
117 precision and recovery assays.

118 **2.3. Vinegars production and samples studied**

119 Fruit processing and pre-treatment was performed as follows: fruit was crushed with a
120 beater; 60 mg L^{-1} of sulphur dioxide were added to prevent the growth of undesirable
121 micro-organisms; 15 mg L^{-1} of each of two kinds of pectolytic enzymes (Depectil extra-
122 garde FCE® and Depectil clarification® from Martin Vialatte Oenologie, Epernay,
123 France), were then added to the puree. 50 g L^{-1} and 75 g L^{-1} of sucrose were also added
124 to 2008 and 2009 strawberry puree respectively to ensure an appropriate final acidity in

125 the resulting vinegar. Samples of fruit puree were taken before and after the addition.
126 One portion of 2008 strawberry fruit puree was pressed to study the effect of two types
127 of starting substrates (semisolid and liquid) (Table 1).
128 The alcoholic fermentation of the fruit substrate was similar in persimmons and 2008
129 strawberries and slight modifications were made in the case of 2009 strawberries. 6 L of
130 fruit puree was distributed into various glass recipients: six for persimmons, eight for
131 2008 strawberries (four of purees and two of liquid substrate) and eight for 2009
132 strawberries. These recipients were then divided into two groups: half of them were
133 inoculated with the oenological yeast *Saccharomyces cerevisiae* QA23 at a
134 concentration of $2x10^6$cells mL^{-1}, and spontaneous alcoholic fermentation was allowed
135 to take place in the other half. The inoculated fermentation in the 2009 strawberries was
136 performed with the yeast strain *Saccharomyces cerevisiae* RP1, isolated during the
137 spontaneous alcoholic fermentation of the 2008 strawberry puree.
138 Acetification was carried out in glass vessels by spontaneous processes except for
139 strawberry wines from the 2009 harvest. These wines were acetified in three different
140 containers: a glass vessel, and oak and cherry wood barrels. Each of them was filled
141 with 5.5 L of wine. All the wine obtained from inoculated alcoholic fermentation was
142 mixed and dispensed in the abovementioned recipients and inoculated with acetic acid
143 bacteria. The wines from spontaneous alcoholic fermentation were processed in the
144 same way and acetified spontaneously.
145 All vinegars obtained in 2007 and 2008 were pressed. Additionally two different final
146 treatments were applied to strawberry vinegars from the 2008 harvest: some were
147 centrifuged and others pasteurized. Strawberry vinegars from 2009 were only
148 pasteurized. The 2007 persimmon vinegars presented an average acetic degree between
149 4.4 (from inoculated wines) and 4.5 (from spontaneous wines). The acetic acid contents

150 average in 2008 strawberry vinegars were 4.8 (from spontaneous wines) and 4.9 (from
151 inoculated wines). Finally, inoculated vinegars from 2009 harvest reached an acetic
152 degree of 5.5 (glass vessel), 6.6 (oak barrel) and 6.3 (cherry barrel).
153 Furthermore, part of the puree from the 2009 strawberries was concentrated by heating
154 to test another form of increasing the sugar content and prevent having to add it in; the
155 resulting product was a cooked must (Table 1). One litter of this substrate was
156 fermented by a spontaneous process and 1 L by inoculating it with RP1 strain yeast.
157 Finally, the inoculated wines were acetified by adding the selected acetic acid bacteria
158 and the spontaneous wines were left to acetify spontaneously.
159 Different samples were taken throughout these production processes and a total of 53
160 samples were analysed: 6 fruit purees and 1 liquid substrate, 22 wines and 24 vinegars.
161 All the samples were stored in 30 mL amber glass flasks at -20°C until the analysis. The
162 codes and characteristics of the samples are shown in Table 1.

163 **2.4. Optimization of static headspace conditions and method validation**

164 Several headspace conditions were optimized: spit ratio, injection volume, time and
165 temperature of incubation. Different split ratios (2, 5, 10, 15, 20 and 40) and injection
166 volumes (250 and 350 uL) were tested.
167 We studied different incubation times (10, 20, 30 and 40 min) and temperatures (55, 65,
168 75 and 85ºC). A sample of commercial fruit puree was spiked with all the compounds
169 studied for these trials. The quantities added were roughly 25 mg kg^{-1} except for ethyl
170 acetate, which was 150 mg kg^{-1}.
171 The method was validated with respect to linearity, sensitivity (LOQ), precision
172 (repeatability and intermediate precision) and accuracy.

The quantification limits were obtained injecting successive dilutions of standards and were calculated as the concentration which would result in a signal-to-noise ratio higher than or equal to 10. These values were determined for liquid and semisolid matrices.

Repeatability and intermediate precision were checked using a dearomatized commercial fruit puree and vinegar spiked with the analytes. These spiked samples were injected six times in a single day for the repeatability assay and three times a day on six different days for the intermediate precision assay. The results, expressed as relative standard deviation (%RSD).

The accuracy of the method was evaluated only in the case of vinegar since the calibration lines were built using hydroacetic solutions instead of a real matrix. A commercial vinegar was spiked with standards at three levels of concentration.

2.5. Static headspace GC-MS instrumentation and conditions

Analyses were conducted using an Agilent 6890 GC system coupled to an Agilent 5975inert quadrupole mass spectrometer and equipped with a Gerstel MP2 headspace autosampler (Müllheim an der Ruhr, Germany).

Static headspace equilibration was performed at 65ºC for 20 min, while a low shaking at 250 rpm was applied during sample heating. 350 μL of headspace gas were injected using a heated (85ºC) gastight syringe (1 mL) in split mode 10:1. The split/splitless inlet temperature was 200ºC. Syringe injection speed was 50 μL s^{-1}.

Separation was performed on a CPWax-57CB column (50m×0.25mm, 0.20μm film thickness, Varian, Middelburg, The Netherlands). The carrier gas was He at a constant flow rate of 1mL/min. The column oven temperature was initially set at 35ºC for 5 min, and then was increased to135ºC at 4ºC min^{-1} and then at 10ºC min^{-1} to 200ºC and held for 5 min.

The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 250ºC, respectively. Electron ionization mass spectra in SIM mode were recorded at 70 eV electron energy. A solvent delay of 3.0 min was used and the following ions were monitored: 31, 43, 44, 45, 55, 57, 61 and 74. All data were recorded using an MS ChemStation. The samples were analyzed in triplicate and blank runs were done before and after each analysis.

2.6. Qualitative and quantitative analyses

Compounds were identified based on the comparison of the retention times of individual standard and computer matching with the reference mass spectra from the NIST 98 library. Acquisition was performed in selected ion monitoring mode (SIM). Initially, standard solutions and several samples were analysed in full scan mode (mass range: 29-350 amu). These data were acquired to identify the compounds and determine appropriate ions for the later acquisition in SIM mode.

The quantitative determination of volatile compounds was performed by using the relative area calculated as the ratio between the target ion of each compound and the internal standard (4-methyl-2-pentanol). Calibration curves at seven levels and three replicates per level were built by adding a standard mixture of all compounds in both matrices: a commercial dearomatised fruit puree enriched with ethanol and hydroacetic solution. This procedure was performed in keeping with that described in Mestre et al. (2002) in order to obtain a matrix that was as representative as possible and to ensure that the calibration graphs were applicable to the majority of the real sample. The range of the calibration curves was chosen to cover the possible concentrations in real samples (Table 2-3).

2.7. Statistical analysis

All statistical analyses were performed using Statistica software (StatSoft, 2001). One-way ANOVA was used to evaluate significant differences (significance levels $p<0.05$).

A principal component analysis (PCA) was carried out as an unsupervised method in order to ascertain the degree of differentiation between samples and which compounds were involved. Data were auto-scaled before PCA.

3. Results and discussion

The main aim of this work was to explore the possibility of using the headspace sampling method in major volatile GC-MS analysis. Headspace gas chromatography (HS-GC) is a powerful technique for the analysis of volatile compounds in food and non-food products (Linssen et al., 1995). There are many instrumental parameters of the headspace autosampler that can affect the sensitivity, precision and accuracy of static headspace analysis. We therefore optimized this sampling technique by evaluating the effect of the following parameters: injection volume, temperature and equilibrium time. The addition of salt into the aqueous extract determined an increment of the ionic strength for the analytes resulting in an increase of their diffusion into the headspace and of the sensitivity (Pawliszyn, 1997). Although the effect of salting-out may play a key role in headspace sampling, taking into account our previous work (Callejon et al., 2008) in which the saturation of samples with salt gave the best results, it was not considered among parameters to optimize and we decided to use an enough amount of sodium chloride to saturate the samples. Good chromatographic data, maximum recovery, sensitivity, and time saving were selected as criteria for optimization. The method was then validated and, finally, applied to the analysis of real samples.

3.1. Optimization of static headspace conditions: the effect of injection volume, equilibrium temperature and time

247 Among the different split ratios tested, the lowest (2:1 or 5:1) provided poorly defined
248 peaks and the highest resulted in small peaks. The best results were obtained with 350
249 μL injection volume and a 10:1 split ratio.
250 After the injection conditions were selected, we studied the incubation parameters. As
251 shown in Figure 1, we found that the higher the extraction time, the lower all relative
252 areas of chromatographic peaks. However, no significant differences were found among
253 relative areas obtained between 10 and 20 min of extraction. Between 10 and 30 min we
254 found significant differences for isoamyl acetate, and between 10 and 40 min for ethyl
255 acetate and isoamyl acetate. Therefore, we considered 20 min to be an appropriate
256 extraction time. On the other hand, incubation temperature showed different trends
257 depending on the compound. Relative areas of 1-propanol and 2-methyl-1-butanol
258 clearly increase as temperature rises. However, the values of relative areas for ethyl
259 acetate, isoamyl acetate and acetaldehyde decrease as temperature increases. These
260 decreases begin to be statistically significant for isoamyl acetate when the temperature
261 rises from 65º to 75ºC.
262 An increase in temperature entailed a loss of sensitivity in some of the compounds
263 studied; because no significant losses were observed at 65ºC, this is the incubation
264 temperature we chose. In summary, the best incubation conditions were established at
265 20 min at 65ºC.
266 **3.2. Method validation**
267 The method was evaluated with respect to linearity, sensitivity (LOQ), precision
268 (repeatability and intermediate precision) and accuracy. The relationship between
269 detector response measured in terms of relative area and amount of standard was linear
270 as suggested by the correlation coefficient obtained (0.996 -1.000). The linearity ranges,
271 the equation of linear regression and the correlation coefficient are shown in Tables 2-3.

The quantification limits obtained were low enough to quantify the different kinds of samples of this study.

Repeatability and intermediate precision results are in agreement with the values proposed by AOAC (1993) for both kinds of matrices (fruit puree and vinegar).

The recovery percentage obtained in the accuracy assays ranged between 68.0 and 108.2. In general, a good degree of accuracy was achieved for most of the compounds, except for acetaldehyde and ethyl acetate.

3.3. Sample analysis

The optimized method was applied to study the changes in nine major volatile compounds throughout the production process of fruit vinegars. These products were obtained through a double fermentation process (alcoholic and acetic). Different conditions were tested at each stage of production. We will discuss the results considering the effect of each stage on the concentration of these compounds. They are involved directly in the aroma of products because they either provide particular aromatic notes such as ethyl acetate or isoamyl acetate or contribute to the overall aromatic profile. Moreover, some of them are also precursors of other volatile compounds present in vinegars. For example, acetaldehyde undergoes condensation reactions to produce acetoin, a volatile compound characteristic of vinegar. On the other hand, vinegars have a considerable content of volatile acids formed from higher alcohols, especially isovaleric acid from 3-methyl-1-butanol. This alcohol is also a precursor of isoamyl acetate.

Pre-treatments of fruit puree

Methanol was the most abundant compound in the initial fruit puree, especially in the persimmon puree (Tables 4-6). The addition of SO_2 and pectolytic enzymes gave rise to a notable increase in this compound (about 100 mg kg^{-1}) in the strawberry samples.

297 Added pectolytic enzymes act as hydrolysing pectins releasing methoxyl groups and
298 producing an increase in methanol, as Ribéreau-Gayon et al. (2006) described for red
299 wines. The second compound that underwent a considerable change in concentration
300 was acetaldehyde. This aldehyde is a natural aroma component in almost all fruits. This
301 compound appears as a result of fruit metabolism during ripening (Pesis, 2005). In our
302 case, the fruit puree (persimmon and strawberry) presented values between 5.4-10.4 mg
303 kg^{-1}. These amounts increased after the addition of SO_2 and pectolytic enzymes,
304 especially in the strawberry samples. In grape must, SO_2 combines with acetaldehyde to
305 form a stable compound (Ribereau-Gayon et al., 2006). Therefore, the addition of this
306 substance may cause a loss of acetaldehyde. However, we observed an increase, leading
307 us to deduce that pectolytic enzyme may favour the release of acetaldehyde. This effect
308 seems to be stronger than the loss caused by combination with SO_2.
309 The remaining compounds increased in most cases, the highest changes were found in
310 the strawberry samples except for methyl acetate, which mainly increased in persimmon
311 puree.
312 One portion of strawberry puree from the 2008 harvest was pressed to obtain a liquid
313 substrate. The pressing process resulted in a decrease in all the compounds (Table 5),
314 especially ethyl acetate and acetaldehyde, which diminished by up to 80%.
315 **Alcoholic fermentation**
316 Two types of alcoholic fermentations were performed. One part of the fruit puree was
317 spontaneously fermented and the other part was inoculated with a selected strain of
318 *Saccharomyces cerevisiae* yeast.
319 In general, as can be seen in Tables 4-6, the higher alcohols increased in all cases as
320 expected; in some cases, reaching concentrations close to the lowest values of the
321 content range found in grape wine (Ribereau-Gayon et al., 2006). During alcoholic

322 fermentation, yeast can synthesize these compounds through two metabolic pathways,
323 one of which is amino acid metabolism (Ribereau-Gayon et al., 2006; Bayonove, et al.,
324 2000). Just as occurs in grape wines, the higher alcohol that reached the largest amounts
325 was 3-methyl-1-butanol (Romano et al., 2003; Garde-Cerdán & Ancín-Azpilicueta,
326 2007).
327 If we compare the two kinds of fermentations, the inoculated alcoholic fermentation of
328 persimmon puree produced higher alcohol contents than spontaneous fermentation,
329 except for isobutanol, which reached a similar concentration in both types of
330 fermentations. However, in 2008 strawberry wines produced by spontaneous
331 fermentation were richer in isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol than
332 inoculated wines, with the latter containing higher levels of 1-propanol than those
333 produced with spontaneous fermentation. Persimmon and strawberry purees were
334 inoculated with the same yeast strain, but the only common trend found was the
335 production of 1-propanol in greater proportion than any other higher alcohol. This
336 alcohol is synthesized by yeast in relation to the metabolism of amino acid sulphur
337 (Bayonove et al., 2000). Otherwise, the observed increases in 2-methyl-1-butanol and 3-
338 methyl-butanol in the inoculated processes were similar in both substrates. These results
339 suggest that the production of 1-propanol could be further conditioned by the type of
340 substrate and the production of the other two alcohols by the yeast strain. Ibarz et al., in
341 (2005), pointed out that the production of higher alcohols in grape wines depends on
342 both factors: the yeast and must used.
343 Interestingly, the results of the 2009 wines showed opposite changes in higher alcohols
344 to those observed in 2008 wines, being these changes for the inoculated 2009 wines
345 similar to the 2008 spontaneous wines and vice versa (Tables 5-6). As explained in
346 section 2.3., the yeast strain used in the production of 2009 inoculated strawberry wines

was isolated from 2008 spontaneous wines. Therefore, the strain involved in the fermentation process has a strong influence on the end levels of these compounds in wines (Torrea et al., 2003; Ribereau-Gayon et al., 2006).

Methanol levels increased in persimmon and 2008 strawberry during alcoholic fermentation, although these differences were only statistically significant in the case of persimmon. Methanol is a non-fermentative alcohol; therefore, the only source of this compound during alcohol fermentation is the hydrolysis of pectins. In these reactions, ester bonds between galacturonic acid and methanol are cleaved, releasing this alcohol into the medium, which is carried out by pectin esterases (Fernandez-Gonzalez et al., 2005). Several authors have shown that some *Saccharomyces cerevisiae* strains have pectin-esterase activity (Pretrorius and Van der Westhuisen, 1991; Gainvors et al., 1994; Fernandez-Gonzalez et al., 2005). Thus, the increase in methanol in this fermentative stage may have come from two possible hydrolytic pathways: due to the pectin esterase activity of the yeast and/or to the pectolytic enzymes added to the substrate that continued to act.

Acetaldehyde is a secondary product of yeast alcoholic fermentation; it is produced during the first days of fermentation (Bosso and Guaita, 2008). This aldehyde increased in persimmon case, being slightly higher in inoculated fermentations than in spontaneous fermentations, although the changes were not statistically significant. Meanwhile, in strawberry alcoholic fermentation acetaldehyde values decreased, especially in spontaneous fermentation. Strawberries are rich in anthocyanins, which are responsible for the berry's red colour. In the production of red wines, these compounds undergo condensation reactions in which different molecules are linked by acetaldehyde bridges (Bosso and Guaita, 2008). These reactions involve a loss of this aldehyde. These types of reactions could explain the diminution of acetaldehyde in strawberry

372 wine production. Opposing trends were found in terms of the final amount of this
373 compound in strawberry wines depending on the year of harvest. In 2008, strawberry
374 wines from inoculated fermentation, "inoculated wines", were found to have higher
375 values than "spontaneous wines". However in 2009 strawberry wines, the highest
376 results for acetaldehyde were found in spontaneous wines. As mentioned above, the
377 yeast strain employed for the production of 2009 inoculated wines was the same as that
378 used for 2008 spontaneous wines. Furthermore, these 2008 spontaneous wines and 2009
379 inoculated wines presented similar values for this compound. The influence of the
380 *Saccharomyces cerevisiae* strain on the differing production of acetaldehyde has been
381 reported by several authors (Antonelli et al., 1999; Regodon et al. 2006).
382 Among the esters studied, the most abundant in our fruit wines was ethyl acetate
383 followed by methyl acetate and isoamyl acetate, this last related to a fruity aroma.
384 Ethyl acetate is the most prevalent ester in grape wines (Ribereau-Gayon et al., 2006).
385 In persimmon puree, the concentration of this ester was below the quantification limit;
386 however the wines presented extremely high levels compared to the normal values in
387 grape wines (30-110 mg kg^{-1}, Regodon et al., 2006). In 2008 strawberry, ethyl acetate
388 underwent a slight increase only during alcoholic fermentation in the inoculated wines.
389 Although the starting concentrations in 2009 wines were very low (below the
390 quantification limit), the wines obtained through spontaneous fermentation presented
391 high concentrations (633-761 mg kg^{-1}) while in those obtained through inoculated
392 fermentation this compound was not detected. Several authors have shown that the
393 formation of esters during alcoholic fermentation is closely related to the enzymatic
394 activity of the yeast strain (Barre et al., 2000). In keeping with this, we observed that
395 this compound was not produced in the 2009 inoculated process and it was only

produced in one case in 2008 spontaneous wines (Table 5-6). The ester isoamyl acetate increased in all cases studied.

Methyl acetate is formed by the condensation of methanol and acetic acid. We found that during the alcoholic fermentation of persimmon the amount of this ester doubled. This is consistent with the high levels of methanol found in persimmon substrate.

This compound remained practically unchanged in strawberry wine production except in the case of the 2009 spontaneous process, in which the levels of methyl acetate concentration increased. Finally, all compounds were found to have increased in the alcoholic fermentation of strawberry liquid substrate. Figuring among the most outstanding changes, we might mention a considerable increase (up to 70%) in acetaldehyde, higher alcohols and isoamyl acetate. The liquid substrate was fermented in the absence of solid colorants so the binding reaction between acetaldehyde and monomeric anthocyanins did not frequently occur. This is a likely explanation for why levels of this aldehyde were found to increase in wines from this substrate. Furthermore, the largest increase in acetaldehyde occurred in inoculated alcoholic fermentation. We observed the same behaviour for higher alcohols as in the fermentation of semisolid substrate, showing the highest contents of 1-propanol in inoculated wines and the other three higher alcohols in spontaneous wines. These results again indicate the relevance of the yeast strain in the production of higher alcohols.

Comparing the final content of the volatile compounds analysed in wines from different substrates (liquid and semisolid), it is clear that methanol and 1-propanol reached higher values in liquid wines than in wines from semisolid substrate. Wines from liquid resulted in lower values of methyl and ethyl acetate than wines from the other type of substrate.

Acetic fermentation

In the acetic fermentation of persimmon wine, levels of acetaldehyde increased in most cases. In 2008 strawberry vinegar, concentrations of this compound increased in all cases. The transformation of ethanol to acetic acid takes place in two steps, with acetaldehyde being the intermediary product. These reactions can be performed by acetic acid bacteria as well as by chemical oxidation. When performed by a micro-organism, each step is catalyzed by different enzymes (alcohol deshidrogenase and aldehyde deshydrogenase, respectively). In chemical oxidation, the step from acetaldehyde to acetic acid depends on the presence of oxygen (Ribereau-Gayon et al., 2006).

The acetification process in samples from the 2009 harvest was carried out in different containers (glass vessels, cherry and oak wood barrels). In the vinegar from glass vessels, we noticed a remarkable amount of acetaldehyde together with lower levels of ethanol and acetic acid than in vinegar produced in wood barrels. The main difference between these kinds of recipients is the better oxygen transference that occurs through wood pores. This might suggest that ethanol is being transformed into acetaldehyde while the second reaction is not taking place at a similar rate, probably due to the lower proportion of oxygen in the glass vessel. This result coincides with that reported by other authors on the accumulation of this aldehyde during acetification due to oxygen impoverishment (Polo and Sanchez-Luengo, 1991). Acetaldehyde tends to accumulate under low oxygen conditions instead of being oxidized to acetic acid (Zoecklein et al., 1995). Furthermore, we have observed increases in acetaldehyde in previous studies during glass bottle aging of red vinegars in which acetification and aging processes took place simultaneously (Callejon et al., 2010). And during accelerated aging in glass vessels with wood chips we observed an increase in acetaldehyde due to the chemical oxidation of ethanol (Tesfaye et al., 2004). Although these studies prove that the

446 accumulation of acetaldehyde in vinegars can take place by means of the two
447 abovementioned pathways (microbiological or chemical oxidation), in our case,
448 microbiological transformation is the most likely cause of the accumulation of this
449 compound.
450 The samples from cherry wood barrels had higher concentrations of acetaldehyde than
451 those from oak barrels, regardless of the type of acetification. This compound may be
452 released into the liquid medium from this type of wood, as this phenomenon has been
453 observed in white wine vinegars aged in different kinds of wood (oak, cherry, chestnut
454 and acacia) (Callejon et al., 2010).
455 A loss of higher alcohols occurred during the acetification stage. Callejon et al. (2009)
456 showed that acetic acid bacteria consume other alcohols apart from ethanol, with 3-
457 methyl-1-butanol being the most frequently consumed followed by isobutanol and 2-
458 methyl-1-butanol, in keeping with the abundance order in the substrate. In our case, a
459 similar behaviour was observed, and in agreement with these authors, the pattern of
460 higher alcohols consumption varied depending on the abundance of these alcohols in
461 the starting wines. In other words, the higher the concentration of the alcohol, the more
462 it was consumed.
463 The 2009 strawberry wines were divided into two groups: one underwent spontaneous
464 fermentation and the other was inoculated with acetic acid bacteria. In the inoculated
465 processes the vinegars reached 6ºAc while spontaneous processes they only reached
466 4ºAc as a consequence of the unexpected halt of the acetification process. Therefore, in
467 terms of the changes in higher alcohols, the consumption of these compounds was more
468 pronounced in vinegars produced using selected acetic acid bacteria.
469 Although the consumption of methanol by acetic acid bacteria has not been previously
470 reported, the acetification process implied a decrease in this alcohol. Generally, these

471 micro-organisms have a defence mechanism that transforms alcohols into less toxic
472 products such esters. Persimmon vinegars showed a reduction in the concentration, with
473 about 150 mg kg^{-1}, and a similar diminution was observed for 2008 strawberry samples.
474 In the 2009 acetification processes, spontaneous fermentation produced a larger
475 decrease in methanol than did inoculated fermentation and this difference was more
476 pronounced in samples produced in glass vessels. The concentration of methanol in all
477 final products was below the legal level allowed for vinegars (Presidencia del Gobierno,
478 1993).
479 On the other hand, methanol is involved in the synthesis of methyl esters, in this case,
480 especially of methyl acetate. We observed higher levels of methyl acetate in persimmon
481 vinegars, and as in alcoholic fermentation, during the production process the content of
482 this ester doubled. In 2008 strawberry samples, acetic fermentation produced significant
483 increases in this compound. However, these condensation reactions alone are not
484 sufficient to explain the diminution of methanol mentioned above.
485 In samples from the 2009 harvest, both strawberry vinegars produced in glass vessels
486 experienced a similar decrease in methyl acetate. However, an increase in methyl
487 acetate was found in the vinegar produced in wood barrels, with slightly higher levels
488 recorded in the case of oak barrels, which may be due to concentration phenomena.
489 Furthermore, we might point out a considerable increase in inoculated processes in
490 barrels. In general, despite the different evolutions observed, the final concentrations of
491 methyl acetate in vinegars were correlated with initial concentrations of methanol
492 ($r=0.7$).
493 Different trends were found in levels of ethyl acetate, a characteristic compound of
494 vinegar, which were especially conditioned by the fruit substrate used. In persimmon,
495 the concentrations of this ester in the resulting vinegars were similar to those in wines

and no clear tendency was observed (Table 4). In 2008 strawberry vinegars, ethyl acetate reached more than twice the concentration of that in wines. From the 2009 harvest, the vinegars obtained through inoculated acetification showed values between 83 for glass vessels and 663-682 for the others. This indicates a considerable formation along with a slight concentration of this compound in wood recipients. The results of the spontaneous acetifications in the 2009 samples were the opposite because a hydrolysis of ethyl acetate was taking place. This behaviour has been observed by several authors who have shown that the active consumption of ethanol by acetic acid bacteria induces the hydrolysis of most ethyl esters (Callejon et al., 2009).

Isoamyl acetate usually increases during surface acetification processes, however, in our vinegars in most cases it was found to diminish. This might be explained again by a hydrolysis reaction due to the consumption of alcohol 3-methyl-1-butanol by acetic acid bacteria.

Comparing the two final treatments applied to the 2008 strawberry vinegars, pasteurization and centrifugation, no statistically significant differences in the volatile compounds studied between them were found (Table 5).

Special vinegars were also produced for this study which used cooked strawberry must (Table 6). Only inoculated acetifications we obtained final products. The main difference in these heated strawberry vinegars was the high levels of acetaldehyde compared to vinegars obtained from uncooked strawberry fruit puree. These high levels would adversely affect the organoleptic properties of the end product.

3.4. Principal component analysis

The compounds studied underwent a series of changes during the production of the vinegars. Several principal component analyses were performed to evaluate whether these changes were great enough to distinguish the different samples obtained

521 throughout the production process based on substrate, production stage or production
522 method. In the case of persimmons, the PCA allowed us to separate the samples into
523 three groups: the substrate, wines and vinegars, with the first three components
524 accounting for 93.9% of the variance. Similar results were obtained when the PCA was
525 applied to the 2008 strawberry sample data. However, in the products obtained from the
526 2009 harvest the separation was not so clear.
527 Moreover, this analysis was applied to the data of the strawberry puree substrates to
528 study the influence of the addition of enzymes and SO_2. Each sample appears in a
529 different quadrant in the plan of the two principal components. The PC1 is able to
530 separate the substrates depending on the harvest and the PC2 separates the samples with
531 and without treatment.
532 PCA of strawberry wines from 2008 harvest reveals that substrate pressing affects more
533 than the inoculation. This is deducted from the samples separation into the plan of two
534 first PC. The liquid wines inoculated and spontaneous appear in the same quadrant
535 whist the group of wines from inoculated semisolid substrate are separated in different
536 quadrant from the spontaneous group.
537 On the other hand, the result of this analysis on the data obtained from all the wine
538 samples showed that the principal three components explained 92.6% of the variance.
539 Data scores and variable loadings are plotted simultaneously into the plan made up of
540 the first two principal components in Figure 3. This figure shows that the samples are
541 distributed into three groups. The figure shows that PC2 successfully separates the 2008
542 strawberry spontaneous and 2009 inoculated wines from the other strawberry wines.
543 Thus, the wines obtained through the use of the same yeast strain appear together in the
544 same quadrant. This reinforces the theory that the yeast strain has a strong influence on
545 these compounds of the aromatic profile. We confirmed a high degree of association

between strawberry wines inoculated with the RP1 strain and the production of higher alcohols such as 2-methyl and 3-methyl-1-butanol and isobutanol. Moreover, if we consider only the persimmon and 2008 strawberry wines, the PCA revealed that PC1 allows us differentiates between persimmon wines and strawberry wines and PC2 distinguishes between inoculated and spontaneous wines. PC1 was positively correlated with acetaldehyde, the three acetates and methanol, and PC2 was positively correlated with acetaldehyde, isoamyl acetate and propanol. In the analysis of the final vinegars, the score plot obtained by selecting the first two PCs as axes showed that the samples were distributed in three groups, one formed by persimmon vinegars, another which included 2008 strawberry vinegars and 2009 strawberry vinegars produced in a glass vessel, and a third group, very far from the previous ones, comprised of the 2009 strawberry vinegars produced in barrels. This shows the importance of the type of recipient in which the acetification is carried out on the final content of these compounds.

Conclusions

The headspace sampling method proposed has proved to be a valuable methodology for the determination of major volatile compounds during the production process of fruit vinegars. From a practical point of view, this method does not require any complicated sample preparation. The validation of the method was satisfactory, recovery values and limits detection are acceptable for most of the compounds studied, and the method was successfully applied to real samples.

The addition of SO_2 and pectolytic enzymes produced a considerable increase in methanol and acetaldehyde, especially in the strawberry samples. However, pressing led to a loss of these volatile compounds. In alcoholic fermentation, the *Saccharomyces cerevisiae* strain used had a great influence on the production of acetaldehyde and

higher alcohols in wines. Taking into account the influence of these compounds studied in the final profile of vinegar, the results show that the *Saccharomyces cerevisiae* strain isolated in this study produces the most suitable wine substrates for the production of vinegars. Moreover, the use of semisolid fruit substrate provides better results than the use of a liquid substrate.

In terms of acetic fermentation, inoculated acetifications in wood recipients resulted in vinegars with better volatile profiles as these presented higher levels of most compounds except acetaldehyde.

Acknowledgments

This research was made possible thanks to financial support from the Spanish Government by means of a predoctoral grant and the research project AGL2007-66417-C02-01 funded by the Ministry of Science and Innovation. Moreover, the researchers are grateful to the enterprises Hudisa S.A., Agromedina and Grupo Alconeras for providing the fruit used in vinegar production.

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684
685 **Figure Captions**
686 Figure 1. Optimization of headspace conditions. Effect of incubation time on relative
687 areas of volatiles compounds.
688 Figure 2. Optimization of headspace conditions. Effect of incubation temperature on
689 relative areas of volatiles compounds.
690 Figure 3. Data scores and variable loadings plot on the plan made up of the first two
691 principal components (PC1 against PC2) of wine samples.
692 **Footnotes Table**

[1]Given the acidic nature of these products and the lack of a suitable alternative term, we have decided to refer to these products as vinegars throughout the text, despite the fact that according to Spanish regulations, some of these products are not sufficiently acidic to be classified as vinegars.

Figure 1

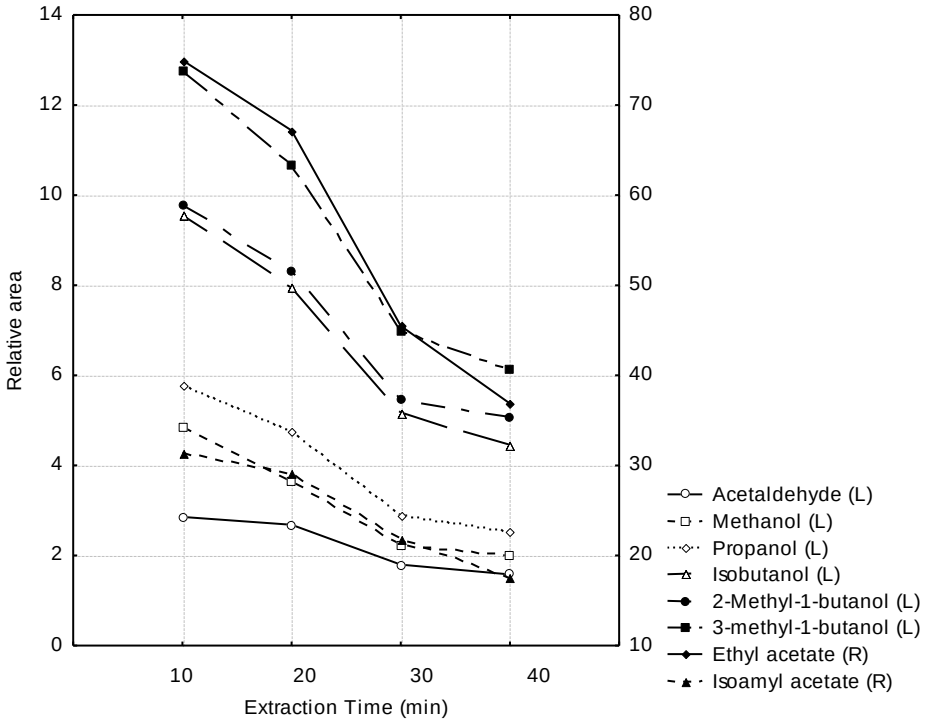


Figure 2

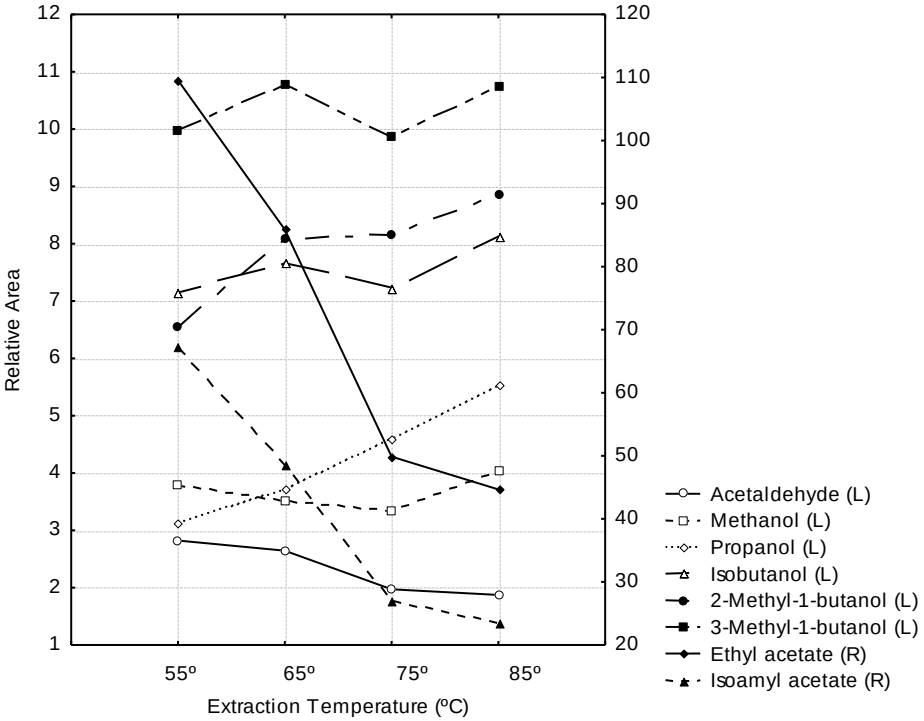
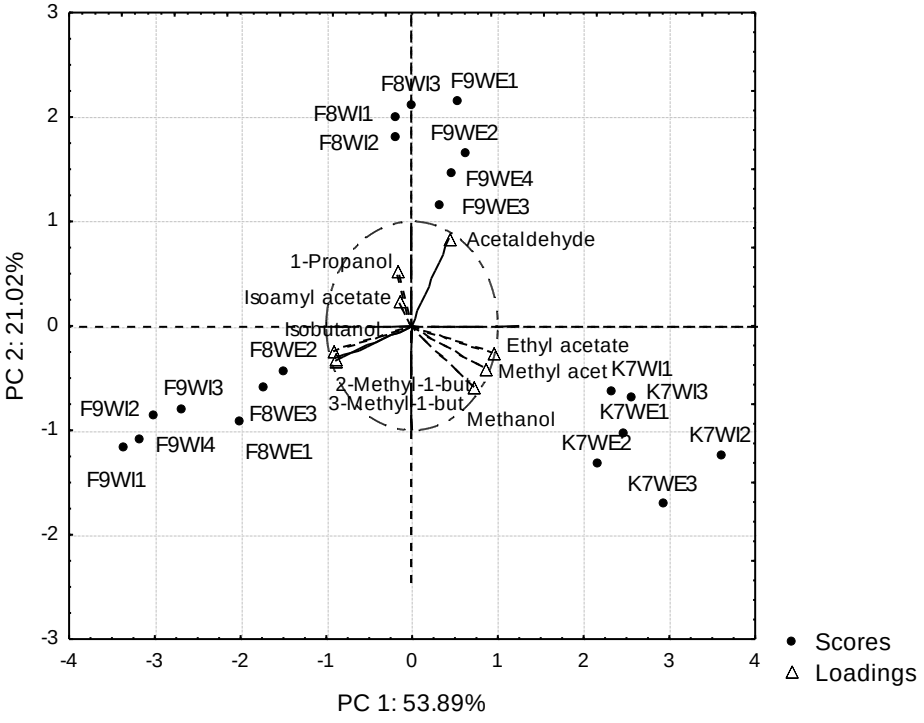

Relative Area
Extraction Temperature (ºC)
55º
65º
75º
85º
Acetaldehyde (L)
Methanol (L)
Propanol (L)
Isobutanol (L)
2-Methyl-1-butanol (L)
3-Methyl-1-butanol (L)
Ethyl acetate (R)
Isoamyl acetate (R)

Figure 3


PC 2: 21.02%
PC 1: 53.89%
F8WI3
F9WE1
F8WI1
F8WI2
F9WE2
F9WE4
F9WE3
Acetaldehyde
1-Propanol
Isoamyl acetate
Isobutanol
F8WE2
Ethyl acetate
Methyl acet
2-Methyl-1-but
3-Methyl-1-but
Methanol
K7WI1
K7WI3
K7WE1
K7WI2
K7WE2
K7WE3
F9WI3
F9WI2
F8WE3
F9WI4
F8WE1
F9WI1
Scores
Loadings

727 Table 1. Treatment and codex of samples.
728

Fruit and harvest	Treatment	Puree Sample	Treatment	Substrate sample	Alcoholic fermentation	Wine sample	Acetification	Treatment o Recipient	Vinegar sample
Persimmon 2007	Crushed	K7Z1	SO2 Pectolytic enzymes	K7Z2	Inoculated	K7WI1-K7WI3	Spontaneous	Pressing Centrifugation	K7VE1- K7VE3
					Spontaneous	K7WE1-K7WE3	Spontaneous	Pressing Centrifugation	K7VI1- K7VI3
Strawberry 2008	Crushed	F8P1	SO2 Pectolytic enzymes sucrose	F8P2	Inoculated	F8WI1- F8WI3	Spontaneous	Centrifugation	F8SVI1C-F8SVI2C
								Pasteurization	F8SVI1P-F8SVI2P
					Spontaneous	F8WE1- F8WE3		Centrifugation	F8SVE1C-F8SVE2C
								Pasteurization	F8SVE1P-F8SVE2P
	-	F8P2	Pressing	F8L	Inoculated	F8LWI	-	-	-
					Spontaneous	F8LWE			
Strawberry 2009	Crushed	F9P1	SO2 Pectolytic enzymes sucrose	F9P2	Inoculated	F9WI1- F9WI4	Inoculated	glass vessel	F9SVIG
								oak barrel	F9SVIO
								cherry barrel	F9SVIX
					Spontaneous	F9WE1- F9WE4	Spontaneous	glass vessel	F9SVEG
								oak barrel	F9SVEO
								cherry barrel	F9SVEX
		-	Heating Concentrated	F9MC	Inoculated	-	Inoculated	glass vessel	F9MCVI1-F9MCVI2

					Spontaneous	-	Spontaneous	glass vessel	-

729
730 Table 2. Analytical characteristics of the method for vinegar.
731

Compound	**Retention Time (min)**	**m/z**	**Linear range (mg kg^{-1})**	**r^2**	**LOQ (ug kg^{-1})**	**Added (mg kg^{-1})**	**Recovery (%)**	**Mean Recovery (%)**	**Repeatability (%RSD)**	**Intermediate Precision (%RSD)**
Acetaldehyde	3.95	44	1-200	0.998	0.30	37 50 62	65.1 67.0 72.0	68.0 ± 3.6	1.88	3.80
Methyl acetate	5.01	74	2-500	0.998	0.15	15 20 25	103.5 100.0 103.5	102.3 ± 2.0	2.64	4.10
Ethyl acetate	6.03	61	74-2002	0.9995	0.095	450 600 750	82.0 70.8 68.3	73.7 ± 7.3	3.90	1.60
Methanol	6.54	31	10-700	0.9992	4.0	150 200 250	90.0 90.0 85.6	88.5 ± 2.5	1.65	2.22
Propanol	10.8	31	1-75	0.9999	0.24	3.37 4.50 5.62	90.3 91.0 85.0	88.8 ± 3.3	2.09	3.00
Isobutanol	12.7	43	1-124	0.9998	0.21	9 12 15	96.0 109.7 102.0	102.6 ± 6.9	1.54	1.97
Isoamyl acetate	13.3	55	0.57-20.5	0.9999	0.015	0.375 0.500 0.625	84.1 89.4 77.0	83.5 ± 6.2	4.92	5.20
2-Methyl-1-butanol	16.9	57	1-75	1.000	0.11	2.62 3.50 4.37	102.7 95.0 96.5	98.1 ± 4.1	0.87	2.52

3-Methyl-1-butanol	17.0	55	1-76	0.9993	0.13	10 14 17	99.0 108.7 117.0	108.2 ± 9.0	2.54	3.97

732
733
734 Table 3. Analytical characteristics of the method for wine and puree of fruit.
735

Compound	**Linear range (mg kg^{-1})**	**r^2**	**LOQ (mg kg^{-1})**	**Repeatability (%RSD)**	**Intermediate Precision (%RSD)**
Acetaldehyde	1-200	0.9986	4.63	4.85	5.75
Methyl acetate	0.9-170	0.9982	2.77	3.12	4.19
Ethyl acetate	61-4500	0.9960	3.1	4.24	4.30
Methanol	51-3000	0.9991	38.1	4.26	5.19
Propanol	1-200	0.9989	2.40	4.96	6.00
Isobutanol	1-200	0.9991	1.54	4.70	6.88
Isoamyl acetate	0.05-10.4	0.9989	0.17	2.86	7.08

2-Methyl-1-butanol	1-200	0.9989	0.27	6.93	8.15
3-Methyl-1-butanol	1-202	0.9967	0.30	0.83	5.73

736
737
738
739 Table 4. Changes in volatile compounds during the elaboration of persimmon vinegars.
740

Samples	**Mean concentration of compounds (mg kg^{-1}) ± SD**								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
K7Z1	10.4 ± 0.3	9.5 ± 0.9	n.q.	343 ± 9	2.27 ± 0.01	1.340 ± 0.003	0.1177 ± 0.0004	0.140 ± 0.004	n.q.
K7Z2	28.2 ± 1.9^{a}	18.1 ± 1.7^{a}	n.q.	376 ± 39	2.97 ± 0.08^{a}	1.99 ± 0.05^{a}	0.140 ± 0.004	0.31 ± 0.02^{a}	n.q.
K7WE1	32.1 ± 1.9	36.1 ± 1.3^{b}	1221 ± 45^{b}	551 ± 17^{b}	8.9 ± 0.6^{b}	15.8 ± 1.3^{b}	0.94 ± 0.03^{b}	7.69 ± 0.25^{b}	27.98 ± 1.04^{b}
K7WE2	25.1 ± 3.2	34 ± 3^{b}	1046 ± 107^{b}	554 ± 41^{b}	8.5 ± 0.5^{b}	15.6 ± 1.3^{b}	0.82 ± 0.06^{b}	8.5 ± 0.4^{b}	33 ± 3^{b}
K7WE3	30.8 ± 1.9	42.2 ± 0.7^{b}	1459 ± 17^{b}	758 ± 18^{b}	11.10 ± 0.05^{b}	20.5 ± 0.5^{b}	1.33 ± 0.08^{b}	8 ± 1^{b}	38 ± 4^{b}
K7WI1	39.2 ± 0.7^{b,c}	38.8 ± 0.9	1094 ± 58^{b}	581 ± 40^{b}	14.8 ± 1.2^{b,c}	15.3 ± 0.9^{b}	1.31 ± 0.15^{b}	10.466 ± 0.024^{b,c}	40.6 ± 0.5^{b,c}
K7WI2	40.47 ± 0.14^{b,c}	67 ± 5	1942 ± 90^{b}	695 ± 6^{b}	15.46 ± 0.15^{b,c}	16.67 ± 0.03^{b}	2.87 ± 0.19^{b}	10.93 ± 0.03^{b,c}	42.1 ± 0.3^{b,c}
K7WI3	36.8 ± 1.6^{b,c}	47.8 ± 0.9	1354 ± 140^{b}	539 ± 74^{b}	16 ± 2^{b,c}	16.3 ± 1.7^{b}	1.86 ± 0.07^{b}	9.3 ± 0.9^{b,c}	41 ± 3^{b,c}
K7VE1	37 ± 3	103 ± 7^{b}	1447 ± 152^{b}	471 ± 42	3.07 ± 0.07^{b}	7.01 ± 0.03^{b}	1.25 ± 0.16	5.19 ± 0.11^{b}	16.0 ± 0.6^{b}
K7VE2	32.81 ± 0.19	79.89 ± 0.17^{b}	1203 ± 24^{b}	444 ± 31	3.42 ± 0.14^{b}	7.59 ± 0.15^{b}	0.89 ± 0.04	5.3 ± 0.4^{b}	17.9 ± 0.3^{b}

K7VE3	47 ± 3	86 ± 6[b]	1278 ± 100[b]	464 ± 12	3.37 ± 0.07[b]	8.17 ± 0.13[b]	0.90 ± 0.07	5.91 ± 0.16[b]	17.5 ± 0.3[b]
K7VI1	61 ± 4	86.10 ± 5.03[b]	1094 ± 59[d]	374 ± 19[b,d]	4.8 ± 0.1[b,d]	5.83 ± 0.04[b,d]	0.9980 ± 0.0001	4.9 ± 0.4[b]	17.55 ± 0.24[b]
K7VI2	33.4 ± 2.1	67 ± 4[b]	921 ± 70[d]	326 ± 22[b,d]	4.47 ± 0.15[b,d]	5.38 ± 0.13[b,d]	0.89 ± 0.03	5.14 ± 0.07[b]	17.1 ± 0.3[b]
K7VI3	38.1 ± 2.4	87 ± 6[b]	1024 ± 84[d]	385 ± 8[b,d]	4.169 ± 0.002[b,d]	5.11 ± 0.12[b,d]	0.95 ± 0.12	4.6 ± 0.1[b]	15.3 ± 0.3[b]

n.q.: concentration under quantification limit.
[a]: significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA)
[b]: significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[c]: significant differences ($p<0.05$) with respect to spontaneous process (ANOVA)
[d]: significant differences ($p<0.05$) with respect to the vinegars obtained from spontaneous wines (ANOVA)

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743 Table 5. Changes in volatile compounds during the elaboration of strawberry vinegars in harvest 2008.
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Samples	**Mean concentration of compounds (mg kg^{-1}) ± SD**								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
F8P1	9.9 ± 0.5	6.9 ± 0.6	96 ± 10	190 ± 14	4.46 ± 0.11	11.6 ± 0.5	0.249 ± 0.004	3.4 ± 0.1	15.5 ± 0.9
F8P2	52.9 ± 2.2[a]	9.30 ± 0.11[a]	140 ± 7[a]	292. ± 9[a]	5.97 ± 0.14[a]	16.9 ± 0.7[a]	0.360 ± 0.007[a]	5.3 ± 0.4[a]	22.5 ± 1.2[a]
F8L	7.2 ± 0.3[e]	1.93 ± 0.08[e]	53 ± 2[e]	244 ± 7[e]	3.51 ± 0.09[e]	10.3 ± 0.2[e]	0.107 ± 0.015[e]	3.7 ± 0.4[e]	12 ± 9[e]
F8LWE1	9.2 ± 0.6	2.1 ± 0.4[f]	59 ± 9[f]	489 ± 33	49.9 ± 2.1	65.0 ± 2.4[f]	0.96 ± 0.07[f]	16 ± 2[f]	80.9 ± 1.8[f]
F8LWI1	27.4 ± 2.4[c, f]	2.9 ± 0.4[f]	86 ± 6[c, f]	530 ± 14[f]	90.3 ± 0.4[c]	40.44 ± 0.08[c, f]	0.678 ± 0.024[c, f]	11.0 ± 0.3[c]	72.0 ± 1.1[c, f]
F8WE1	21.8 ± 0.9[b]	9.2 ± 0.7	170 ± 11	462 ± 9	37 ± 2[b]	83 ± 3[b]	0.95 ± 0.06[b]	34.0 ± 0.3[b]	108.3 ± 0.6[b]

F8WE2	19.0 ± 0.6[b]	7.62 ± 0.22	127 ± 5	290.1 ± 2.3	24.9 ± 0.9[b]	61.2 ± 2.3[b]	1.14 ± 0.08[b]	24.3 ± 1.3[b]	80.1 ± 1.9[b]
F8WE3	19.8 ± 1.4[b]	8.1 ± 0.4	129 ± 11	303 ± 22	26.0 ± 1.7[b]	69 ± 7[b]	0.969 ± 0.021[b]	26.2 ± 1.9[b]	92 ± 7[b]
F8WI1	51 ± 5[c]	8.47 ± 0.09	173 ± 6[b]	305 ± 3[b]	43.9 ± 0.8[b,c]	33.9 ± 0.4[b,c]	1.382 ± 0.007[b,c]	14.4 ± 0.3[b,c]	62 ± 5[b,c]
F8WI2	46 ± 4[c]	8.9 ± 0.5	184 ± 11[b]	317 ± 16[b]	44.8 ± 1.7[b,c]	35 ± 1[b,c]	1.50 ± 0.12[b,c]	12.1 ± 0.7[b,c]	64 ± 3[b,c]
F8WI3	52.5 ± 1.4[c]	9.07 ± 0.25	207 ± 17[b]	327 ± 28[b]	45 ± 4[b,c]	34.3 ± 2.4[b,c]	1.52 ± 0.15[b,c]	12.3 ± 1.6[b,c]	58.0 ± 1.9[b,c]
F8SVE1C	34.3 ± 0.4[b]	17.7 ± 0.5[b]	439 ± 31[b]	259 ± 13	4.40 ± 0.09[b]	11.93 ± 0.04[b]	0.610 ± 0.023[b]	5.74 ± 0.21[b]	13.7 ± 0.4[b]
F8SVE1P	40 ± 4[b]	19.4 ± 1.9[b]	483 ± 47[b]	246 ± 33	4.3 ± 0.3[b]	11.7 ± 0.5[b]	0.61 ± 0.08[b]	5.1 ± 0.3[b]	13.7 ± 0.3[b]
F8SVE2C	75.9 ± 3.0[b]	13.00 ± 0.11[b]	368 ± 15[b]	195 ± 12	4.16 ± 0.23[b]	11.6 ± 0.6[b]	0.46 ± 0.07[b]	5.47 ± 0.16[b]	14.2 ± 0.6[b]
F8SVE2P	79 ± 4[b]	14.2 ± 0.9[b]	374 ± 34[b]	181 ± 14	4.05 ± 0.13[b]	11.4 ± 0.3[b]	0.31 ± 0.03[b]	5.4 ± 0.3[b]	14.4 ± 0.3[b]
F8SVI1C	67.9 ± 2.3[b]	14.0 ± 0.5[b]	374 ± 11[b]	174 ± 10[b]	6.78 ± 0.24[b,c]	5.32 ± 0.22[b,c]	0.271 ± 0.003[b,c]	3.0 ± 0.4[b,c]	9.7 ± 0.4[b,c]
F8SVI1P	77.4 ± 2.3[b]	16.5 ± 0.9[b]	446 ± 24[b]	180 ± 5[b]	7.3 ± 0.4[b,c]	5.8 ± 0.3[b,c]	0.251 ± 0.013[b,c]	2.98 ± 0.19[b,c]	10.5 ± 0.8[b,c]
F8SVI2C	89.2 ± 1.6[b]	15.50 ± 0.19[b]	424.11 ± 2.23[b]	179 ± 5[b]	7.72 ± 0.25[b,c]	5.87 ± 0.19[b,c]	0.260 ± 0.016[b,c]	2.93 ± 0.03[b,c]	10.85 ± 0.15[b,c]
F8SVI2P	98 ± 4[b]	18.2 ± 0.8[b]	498 ± 34[b]	181 ± 15[b]	8.24 ± 0.13[b,c]	6.3 ± 0.3[b,c]	0.248 ± 0.014[b,c]	2.9 ± 0.3[b,c]	11.6 ± 0.3[b,c]

[a]: significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA)
[b]: significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[c]: significant differences ($p<0.05$) with respect to spontaneous process (ANOVA)
[d]: significant differences ($p<0.05$) with respect to the vinegars obtained from spontaneous wines (ANOVA)
[e]: significant differences ($p<0.05$) with respect to F8P2 sample (ANOVA)
[f]: significant differences ($p<0.05$) with respect to semisolid wines obtained with similar alcoholic process (spontaneous or inoculated) (ANOVA)

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Table 6. Changes in volatile compounds during the elaboration of strawberry vinegars in harvest 2009.

Samples	Mean concentration of compounds (mg kg^{-1}) ± SD								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
F9P1	5.4 ± 0.1	3.21 ± 0.05	n.q.	159 ± 12	2.34 ± 0.02	1.371 ± 0.011	0.118 ± 0.001	0.211 ± 0.002	n.q.
F9P2	95 ± 8[a]	4.6 ± 0.4[a]	n.q.	293 ± 31[a]	3.47 ± 0.12[a]	2.60 ± 0.08[a]	0.133 ± 0.001	1.37 ± 0.07[a]	3.9 ± 0.3[a]
F9WE1	65.1 ± 0.7[b]	11.5 ± 0.3[b]	639 ± 11[b]	237 ± 10[b]	14.4 ± 0.5[b]	25.0 ± 0.7[b]	2.94 ± 0.04[b]	16.5 ± 0.5[b]	48.8 ± 0.9[b]
F9WE2	55.1 ± 0.6[b]	12.4 ± 0.1[b]	761 ± 8[b]	254 ± 11[b]	15.0 ± 0.5[b]	26.0 ± 0.8[b]	2.85 ± 0.04[b]	13.4 ± 0.4[b]	48.6 ± 1.3[b]
F9WE3	44 ± 1[b]	11.3 ± 0.4[b]	667 ± 31[b]	239 ± 8[b]	14.4 ± 0.4[b]	25.0 ± 0.4[b]	2.66 ± 0.18[b]	15.19 ± 0.11[b]	47.6 ± 0.7[b]

F9WE4	49 ± 4[b]	11.1 ± 0.7[b]	633 ± 47[b]	222 ± 3[b]	13.6 ± 0.3[b]	23.9 ± 0.3[b]	2.55 ± 0.11[b]	12.69 ± 0.15[b]	46.0 ± 0.5[b]
F9WI1	23.6 ± 1.3[b,c]	4.72 ± 0.07[c]	n.q.	303 ± 4	12.81 ± 0.22[b,c]	69.7 ± 0.5[b,c]	2.64 ± 0.06[b]	52.7 ± 1.3[b,c]	171 ± 7[b,c]
F9WI2	25.1 ± 1.9[b,c]	4.45 ± 0.15[c]	n.q.	279 ± 16	12.05 ± 0.22[b,c]	67.6 ± 1.1[b,c]	2.60 ± 0.17[b]	42.4 ± 0.8[b,c]	167 ± 10[b,c]
F9WI3	23.2 ± 1.3[b,c]	4.02 ± 0.12[c]	n.q.	235 ± 5	11.1 ± 0.1[b,c]	59.4 ± 0.7[b,c]	1.98 ± 0.06[b]	39 ± 3[b,c]	152 ± 5[b,c]
F9WI4	20.0 ± 0.6[b,c]	4.52 ± 0.03[c]	n.q.	277 ± 12	11.9 ± 0.5[b,c]	67.2 ± 2.4[b,c]	2.72 ± 0.08[b]	44 ± 3[b,c]	173 ± 11[b,c]
F9SVEG	1.43 ± 0.07	7.0 ± 0.5	45 ± 5	120 ± 1	0.71 ± 0.01	1.569 ± 0.022	n.q.	2.111 ± 0.003	2.739 ± 0.004
F9SVEO	23.6 ± 0.6	16.2 ± 0.5	148 ± 5	165.6 ± 0.4	1.16 ± 0.01	3.036 ± 0.012	0.065 ± 0.007	2.914 ± 0.008	5.64 ± 0.07
F9SVEX	63.15 ± 0.11	14.22 ± 0.02	439 ± 17	198.2 ± 1.1	2.001 ± 0.003	5.176 ± 0.014	0.158 ± 0.014	4.67 ± 0.07	9.5 ± 0.3
F9SVIG	129 ± 5	3.4 ± 0.3	83 ± 5	146.7 ± 0.9	1.493 ± 0.024	11.5 ± 0.3	0.27 ± 0.04	8.81 ± 0.22	27.0 ± 0.7
F9SVIO	42 ± 3	20.4 ± 1.4	682 ± 41	276 ± 5	2.364 ± 0.012	24.7 ± 0.9	1.4 ± 0.1	21.2 ± 0.6	47.5 ± 0.06
F9SVIX	64.4 ± 1.0	17.3 ± 1.1	663 ± 5	278 ± 16	2.82 ± 0.09	26.2 ± 0.8	1.282 ± 0.023	23.3 ± 1.0	52.1 ± 0.4
F9MCVI1	719 ± 58	22.8 ± 2.1	341 ± 17	318 ± 15	11.3 ± 0.4	9.9 ± 0.6	0.57 ± 0.03	9.1 ± 0.6	43 ± 3
F9MCVI2	410 ± 17	25.4 ± 2.1	452 ± 39	370 ± 27	15.1 ± 0.8	11.3 ± 0.4	0.65 ± 0.05	11.3 ± 1.0	48.9 ± 1.9

n.q.: concentration under quantification limits
[a]: significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA)
[b]: significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[c]: significant differences ($p<0.05$) with respect to spontaneous process (ANOVA)
[d]: significant differences ($p<0.05$) with respect to the vinegars obtained from spontaneous wines (ANOVA)

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