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Development of a method based on on-line reversed phase liquid chromatography and gas chromatography coupled by means of an adsorption–desorption interface for the analysis of selected chiral volatile compounds in methyl jasmonate treated strawberries

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ABSTRACT

A method based on the use of the through oven transfer adsorption–desorption (TOTAD) interface in on-line coupling between reversed phase liquid chromatography and gas chromatography (RPLC–GC) for the determination of chiral volatile compounds was developed. In particular, the method was applied to the study of the influence of methyl jasmonate (MJ) treatment on the production and enantiomeric composition of selected aroma compounds in strawberry. The compounds studied were ethyl 2-methylbutanoate, linalool and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (i.e. furaneol), which were examined on days 3, 6 and 9 after treatment. The method developed resulted in relative standard deviations (RSDs) of 21.6%, 8.1% and 9.8% and limits of detection (LD) of 0.04, 0.07 and 0.02 mg/l for ethyl 2-methylbutanoate, linalool and furaneol, respectively. The application of the RPLC–TOTAD–GC method allowed higher levels of ethyl 2-methylbutanoate, linalool and furaneol to be detected, particularly after 9 days of treatment. Besides, MJ demonstrated to affect the enantiomeric distribution of ethyl 2-methylbutanoate. On the contrary, the enantiomeric composition of linalool and furaneol kept constant in both control and MJ-treated strawberries throughout the study. These results are discussed.

1. Introduction

The use of multidimensional systems may be of great usefulness in improving the resolution between compounds in complex matrices by transferring cuts of limited broadness from the first to the second dimension. Specifically, on-line coupling liquid chromatography–gas chromatography (LC–GC) enables an effective sample fractionation in the LC-step, even working in a reversed phase (RP) mode, as well as an efficient and rapid gas chromatographic (GC) separation. Considering the incompatibility between both analytical techniques, i.e. LC and GC, interfaces allowing the direct coupling are essential. Although different interfaces have been described in the past to perform on-line coupling RPLC–GC [1,2], all of them have been currently replaced by interfacing techniques based on vaporization [3–5]. These techniques are based on the use of a programmable temperature vaporizer (PTV) injector, which contains inside a polymeric material in which the analytes are retained. We have earlier reported the application of RPLC–GC via PTV to chemically different analytes present in complex matrices by transferring large volume fractions [6–8].

Nevertheless, despite this coupled technique offers many advantages, the full automation of the chromatographic system is particularly complicated as the detachment of the GC-column is necessary during the transfer of the target compounds. This is the reason why this approach has not been implemented in routine analysis as yet. To overcome this limitation a new interface for on-line RPLC–GC named TOTAD (through oven transfer adsorption desorption) has been proposed in the literature in the past few years [9]. This interfacing technique allows the full automation of the LC–GC system, minimizing thus the sample handling and shortening the analysis time. Applications of the TOTAD interface in the literature are mainly focused on the determination of residues of pesticides in foods [10–12]. More recently, we have also described its usefulness in the analysis of volatile compounds, specifically methyl jasmonate (MJ), the impact aroma compound in jasmine [13,14]. In the present study we intend to extend the application area of the TOTAD interface to the RPLC–GC analysis of chiral compounds. In this respect, although the enantiomeric composition has already been determined by different LC–GC systems [15,16], the consideration of the stereochemistry of the chiral compounds by using RPLC–TOTAD–GC has not been to our knowledge carried out

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to date. Enantiomeric determinations are necessary since many of the aromas, additives and preservatives used in the industry are chiral compounds characterized by a specific enantiomeric composition. Actually, the separation of enantiomers occurring at low concentration is particularly helpful in controlling industrial processes, evaluating food nutritional aspects, assessing treatment and storage effects, etc. [17,18].

On the other hand, the influence of MJ on the bioformation of a number of components of vegetables and fruits has already been widely demonstrated [19–21]. In particular, the modification of the volatile fraction as a consequence of MJ treatment has been reported in mango [22], apple [23] and strawberry [24–26]. However, the impact of MJ treatment on the enantiomeric composition of chiral compounds has not been accomplished so far.

This research was conducted with the aim of developing a method based on the fully automated on-line RPLC–GC via TOTAD for the study of the effect of the exogenous application of MJ on the production and enantiomeric distribution of selected chiral volatile compounds in strawberries. Our further intention was, on the one hand, to get an insight into the effect of MJ on distinct biochemical pathways, and, on the other, to consider chiral compounds whose contribution to strawberry aroma is regarded as relevant.

2. Experimental

2.1. Samples and materials

(±)-MJ, ethyl 2-methylbutanoate, linalool and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol) were purchased from Sigma–Aldrich (Steinheim, Germany). MJ is commercialized as a stereoisomeric mixture made up of 90% racemic MJ and 10% racemic epiMJ. A standard solution of ethyl 2-methylbutanoate, linalool and furaneol in methanol of 50 mg/l was used to select the RPLC fraction to be transferred to GC as well as estimate the repeatability and limit of detection (LD) of the method. Methanol (HPLC grade) and water were obtained from Labscan Ltd. (Dublin, Ireland) and from a Milli-Q purification system (Millipore, Milford, MA, USA), respectively. Tenax TA (80-100 mesh, Varian Inc., Middelburg, NL) was used as the packing material in the glass liner of the interface. Prior to use, Tenax TA was conditioned at 250 °C for 120 min under a stream of helium. Dichloromethane (HPLC grade) was purchased from Lab Scan (Dublin, Ireland).

2.2. Treatment of strawberry fruits with MJ

The exogenous application of MJ to the berries was accomplished as detailed elsewhere [25]. In brief, 15 berries (i.e. approximately 300 g each) were put in one 600 ml container to be treated. MJ standard (80 μl) was placed into one vial, which was placed inside the container whose lid was covered. MJ was allowed to spontaneously vaporize during 24 h at 25 °C. Subsequently, the vial containing MJ was withdrawn and the container was kept at 10 °C for 9 days. This procedure was applied exactly in the same way to untreated samples which were used as a control except for the use of the vial containing MJ, which was replaced by an empty vial. The volatile compounds of the treated fruits were examined on days 3, 6 and 9 after treatment as explained below.

2.3. Steam distillation-solvent extraction (SDE)

The analysis of the volatiles from the untreated and MJ-treated strawberries was performed by SDE using the high-density solvent configuration of the commercial version (Varian Inc., Middelburg, NL) of the microdistillation-extraction device. The extracts were obtained from a 100-g weight of berries and 200 ml of Milli-Q water. The sample was heated in a silicone bath at 140 °C whilst dichloromethane, which was used as the extraction solvent, was distilled by utilizing a water bath at 60 °C. A coldfinger at 2 °C (±1 °C) was employed to condense the vapours of both sample and solvent. The reflux was continuously maintained during the whole extraction (i.e. 1 h). Once the extraction was finished, the volatile compounds were collected in 2 ml of distilled dichloromethane. Between consecutive extractions the SDE device was cleaned with acetone and Milli-Q water. The extracts were then sampled into the RPLC–TOTAD–GC system as below specified.

2.4. RPLC–TOTAD–GC analysis

2.4.1. Instrumentation

The system used to carry out the analyses consisted of a liquid chromatograph and a gas chromatograph linked through an automated TOTAD interface, US Patent 6,402,947 B1 (exclusive rights assigned to KONIK-Tech, Sant Cugat del Vallés, Barcelona, Spain). This interface is based on a modification of the PTV injector and its design has been carried out by Pérez et al. as described in [9]. LC-pre-separation was performed with a Hewlett-Packard model 1050 (Wilmington, DE, USA) chromatograph fitted with a manual injection valve (model 7125, Rheodyne, Cotati, CA, USA) having a 20-μl sample loop and an ultraviolet (UV) detector operated at 210 nm. The GC analyses were performed with a Konik model HRGC 4000B chromatograph equipped with a flame ionization detector (FID) which was set at 250 °C throughout the experimentation. LC data were acquired by employing HP Chemstation software (Hewlett-Packard, Wilmington, DE, USA) and GC data acquisition was carried out using Ezchrom (Konik, Sant Cugat del Vallés, Barcelona, Spain).

2.4.2. RPLC conditions

The LC-pre-separation of the SDE extract of untreated and MJ-treated strawberries was accomplished on a 150 mm × 4.6 mm i.d., 10 μm-C_6 column (Waters, Madrid, Spain). All analyses were performed using methanol/water as the mobile phase. The initial eluent composition (methanol/water, 25:75, v/v) was maintained for 3 min and subsequently a linear gradient was applied within 7 min up to 100% methanol. The initial flow was 1.0 ml/min, which was kept up to 1.2 min, after that the flow was programmed at 0.2 ml/min at 1.5 min and maintained until the transfer was finished. The LC equipment was properly washed by passing methanol through the whole system after every single run.

2.4.3. RPLC–GC transfer via TOTAD

The TOTAD interface connecting the GC to the LC is fully automated through the employment of electrovalves, which control electronically helium flow and pressure. The transfer of the fraction of interest from the LC-pre-separation step to the GC system was performed by switching, from the waste position to the transfer position, a six-port valve, which is joined to the GC by silica capillary tubing (50 cm length × 0.25 mm i.d.). During transfer a 600 μl-volume is transferred from LC to GC at a flow rate of 0.2 ml/min. To facilitate the transfer a helium flow of 300 ml/min was applied and maintained an additional time (3 min) once the transfer step was finished in order to complete the remaining solvent removal. During the transfer step the volatile compounds transferred are retained on the packing material (1 cm of Tenax TA), which is placed inside the glass liner that is in turn located in the TOTAD interface. The temperature of the interface was set at 40 °C during the transfer step. Once finished the transfer step, the retaining compounds were desorbed from Tenax TA and moved to the GC-column by increasing the TOTAD temperature at 10 °C/s from the initial value (i.e. 40 °C) up to 200 °C. Finally, volatile compounds were analyzed by GC under the experimental conditions detailed below.

2.4.4. GC analysis

Chromatographic separations were performed on a 25-m × 0.25-mm i.d. fused-silica column coated with a 0.25-μm layer of permethylated β-cyclodextrin (Chirasil-β-Dex, Varian Inc., Middelburg, NL) [27]. The oven temperature was programmed at 4 °C/min from 40 °C up to 180 °C, which was kept for 5 min. In all analyses, FID was set at 250 °C and helium was used as the carrier gas at an initial pressure of 35 psi throughout the analysis. Split mode was used in all analyses (split ratio, 30:1). Identification of the investigated compounds in the samples was carried out by comparison of the retention time with that obtained from the RPLC–TOTAD–GC analysis of the standard under identical experimental conditions. Additionally, RPLC–GC analyses of spiked samples were in all instances accomplished to verify the identification. Each analysis was performed in duplicate.

3. Results and discussion

The compounds included in this study, i.e. ethyl 2-methylbutanoate, linalool and furaneol, were selected on the basis of their different chemical structure, their chiral nature and their importance in strawberry aroma. As previously mentioned in the introduction, we meant to study how MJ can affect distinct biochemical pathways of important contributors to strawberry aroma [28–30]. Two different RPLC fractions were transferred to GC via TOTAD to determine the three target chiral compounds. Fraction 1, which comprised from 3.2 to 6.2 min (flow rate 0.2 ml/min, transferred volume 600 μl) in the LC-pre-separation chromatogram, corresponded to the LC elution of linalool and furaneol and Fraction 2, which comprised from 5.0 to 8.0 min (flow rate 0.2 ml/min, transferred volume 600 μl), corresponded to the LC elution of ethyl 2-methylbutanoate.

The LD and repeatability, estimated as relative standard deviation (RSD), were calculated from three replicates of the SDE extraction of a standard solution containing ethyl 2-methylbutanoate, linalool and furaneol (1.5 ml in 200 ml of methanol) followed by the consecutive RPLC–TOTAD–GC analysis of fraction 1 and 2. As a result, LD values of 0.04, 0.07 and 0.02 mg/l for ethyl 2-methylbutanoate, linalool and furaneol were obtained. Likewise, RSD values, given as the sum of both enantiomers, were 21.6%, 8.1% and 9.8% for ethyl 2-methylbutanoate, linalool and furaneol, respectively. From these values, it can be stated that the developed method was satisfactory in terms of sensitivity and repeatability (<10%) for linalool and furaneol. In contrast, it was not so precise for ethyl 2-methylbutanoate. However, the repeatability was considered acceptable taking into account the complexity of the method proposed.

By injecting a 0.6 μl volume of the SDE extract of an untreated strawberry sample into GC in the split mode (ratio, 30:1) and under the experimental conditions described in Section 2, an apparent lack of chromatographic resolution was observed for peaks eluting below 30 min. This can probably be explained by the polarity of the compounds together with their co-elution with other matrix components. Specifically, peaks corresponding to ethyl 2-methylbutanoate, linalool and furaneol interfered with other chromatographic signals in such a way that the chromatogram was considered unacceptable. In this respect, it is necessary to keep in mind that over 360 compounds have been reported in strawberry aroma [28]. The analysis of such complex matrices usually demands for the selectivity provided by a multidimensional technique. For this reason, we believed convenient to set up a RPLC–GC method to accomplish this study.

Fig. 1 illustrates the relative areas of (a) ethyl 2-methylbutanoate and (b) linalool and furaneol, in control and MJ-treated strawberries on days 0, 3, 6 and 9 after treatment

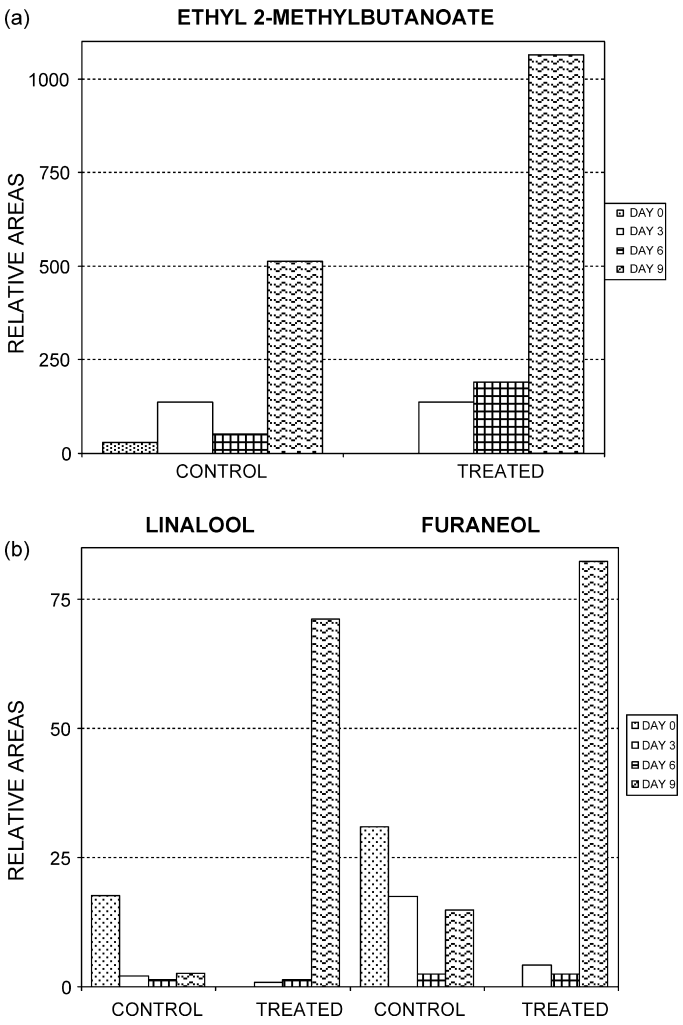

Fig. 1. Relative areas of (a) ethyl 2-methylbutanoate and (b) linalool and furaneol, in control and MJ-treated strawberries on days 0, 3, 6 and 9 after treatment obtained by SDE followed by RPLC–TOTAD–GC.

obtained by SDE followed by RPLC–TOTAD–GC. The relative areas were estimated from the ratio between a matrix component used as a reference and the target compounds. The reference compound was elected on the basis of its satisfactory resolution and constancy in all analyses. The design of Fig. 1 was calculated in all cases from the sum of both enantiomers and the mean values of duplicates obtained from the same sample. It is important to note that the data obtained from day 0 correspond to the same sample for both control and treated strawberries.

As seen in Fig. 1, the selected compounds were considerably affected by MJ treatment. Regarding ethyl 2-methylbutanoate, although its concentration did not exhibit apparent change with the treatment on day 3 (in both untreated and treated samples it increased with respect to day 0), it underwent a considerable enhancement in treated samples on days 6 and 9. This can be attributed to the effect of MJ on the biochemical pathways involved in the formation of esters in strawberry. Data concerning MJ effect on ethyl 2-methylbutanoate level cannot be found in the literature. Nevertheless reports on other esters in this regard suggest similar results encountered for ethyl butanoate, methyl acetate, butyl acetate and 3-hexenyl acetate in strawberry [24,25]. As far as linalool is concerned, it showed no striking alteration with MJ treatment on days 3 and 6 since its concentration decreased in both untreated and treated berries with respect to day 0. However, similarly to ethyl 2-methylbutanoate, its level was noticeably

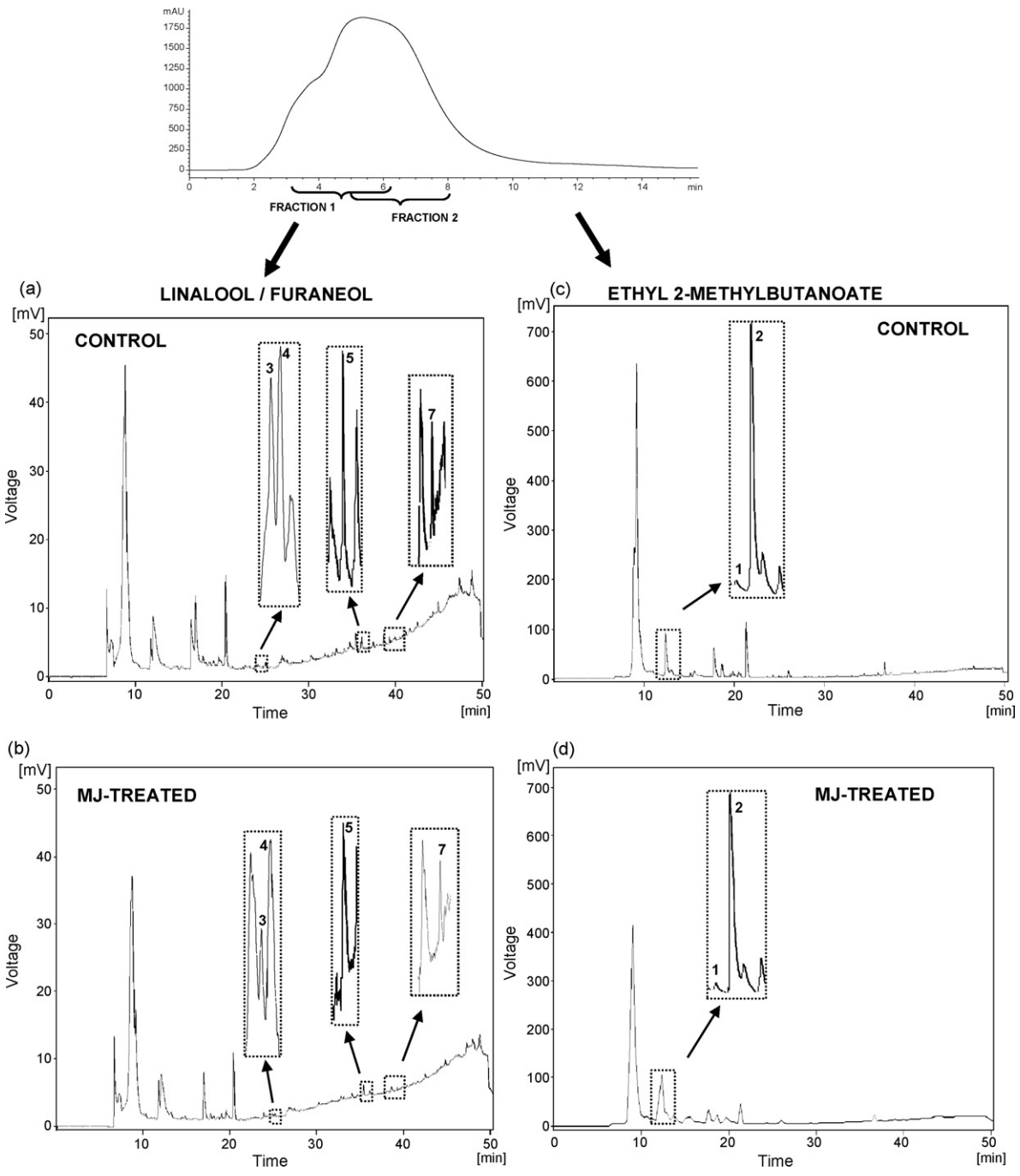

Fig. 2. Chromatograms provided by the RPLC–TOTAD–GC analysis of a SDE extract of strawberries corresponding to fraction 1 (control (a) and MJ-treated (b) samples) and fraction 2 (control (c) and MJ-treated (d) samples) after 6 days of treatment. Peak identification: (1) (−)-ethyl 2-methylbutanoate, (2) (+)-ethyl 2-methylbutanoate, (3) (−)-linalool, (4) (+)-linalool, (5) (−)-furaneol, (6) (+)-furaneol, and (7) reference compound.

improved on day 9. We have previously reported the increase of linalool in strawberry after 5 days of MJ treatment as well as a very slight decline after that in strawberries treated with MJ [25]. The discrepancy between the previous and the present studies might be due to the different amount of MJ used for the treatment in both works (40 μl *vs.* 80 μl). Delay in strawberry decay as a consequence of MJ treatment has already been described [24,25]. Consequently, it is possible that the higher amount of MJ used for the treatment in the present study results in a delay in the bioformation of linalool, which would mainly take place on day 9 instead of day 5. In this respect, concentrations of terpenes have been reported to increase with the stage of ripeness, reaching the maximum in the overripe fruit [30]. With regard to furaneol, in line with ethyl 2-methylbutanoate and linalool, the influence of MJ was more appreciable on day 9, which was the only day on that furaneol concentration was higher than that on day 0. In fact, furaneol amount was lower in MJ-treated berries than that of the control sample on day 3 and kept invariable in both samples on day 6. This agrees with the conclusion earlier reached [25], where an increase was obtained on days 5, 7 and, particularly on day 9 after MJ treatment.

All in all, it can be stated that MJ affects the biochemical pathways forming ethyl 2-methylbutanoate, linalool and furaneol resulting in an increase on day 9 after treatment. The affection

Table 1
Enantiomeric composition[a] (ee, %) of the selected chiral volatile compounds in strawberry samples untreated (control) and treated with MJ on days 3, 6 and 9 after treatment by on-line RPLC–GC via TOTAD.

Compounds selected	Treatment days					
	Day 3		Day 6		Day 9	
	Control	Treated	Control	Treated	Control	Treated
(+)-Ethyl 2-methylbutanoate[b]	90.9	68.6	99.7	82.5	98.2	85.3
(+)-Linalool	13.6	17.0	24.7	37.9	20.1	23.5
(−)-Furaneol	100	100	100	100	100	100

[a] ((Predominant enantiomer − minor enantiomer)/(predominant enantiomer + minor)] × 100.
[b] Predominant enantiomer in brackets.

of the three target compounds can reflect a lack of specificity of MJ. Besides, the higher amount of MJ used in the present study with respect to the previous work does not seem to affect the bioformation of esters and furanones. On the contrary, linalool was influenced by MJ differently according to the amount utilized. It is also worthy to emphasize that a 3 day period is not long enough to appreciate the effect of MJ on the strawberry volatile composition.

Fig. 2 depicts the chromatograms obtained from the transfer from RPLC to GC by using the TOTAD interface of the fraction 1 corresponding to the SDE extract of control (a) and MJ-treated (b) strawberries as well as of the fraction 2 corresponding to the SDE extract of control (c) and MJ-treated (b) strawberries on day 6 after treatment. The elution order of the target enantiomers was assigned on the basis of our previous experience as well as on bibliographic information [31–33].

Table 1 indicates the enantiomeric composition of ethyl 2-methylbutanoate, linalool and furaneol obtained from the SDE–RPLC–TOTAD–GC analysis of untreated and MJ-treated strawberries on days 3, 6 and 9 after treatment. As observed, whereas ethyl 2-methylbutanoate and linalool occurred as enantiomeric mixtures with a characteristic proportion in each case, furaneol was present as the pure (−)-enantiomer in all samples. Enantiomeric purities around 98–99% has been reported for (+)-ethyl 2-methylbutanoate in strawberry products [33]. These results are in accordance with the values found in the present study for control samples. However, a decrease in the enantiomeric composition of ethyl 2-methylbutanoate was observed after treatment. This might be due to the influence of MJ on the enzymes regulating the biosynthesis of esters in strawberry. In this respect, lipases, whose asymmetric character is already known [34], are usually the enzymes responsible for the hydrolysis of esters and esterification of fatty acids and alcohols [35,36]. Further experimentation would be in any case required to support the hypothesis about MJ effect on lipase activity since, although data in this regard have been reported on other enzymes [37,38], the specific effect on lipases has not, to our knowledge, been studied so far.

In contrast to ethyl 2-methylbutanoate, previous reports have demonstrated that linalool exhibits a wide range of enantiomeric excess values in nature. Although (+)-linalool has been described to prevail in strawberry flavour [39,40], varying enantiomeric excesses have also been described in other matrices. The predominance of the (−)-enantiomer and the occurrence of the racemic mixture in fruits other than strawberry have been published [39]. Similarly, the enantiomeric purity of the (+)-enantiomer in bergamot essential oil is also known [41]. Besides, it is also worthy to bear in mind that linalool is at times fraudulently added to natural samples to enhance their fragrance. On the other hand, no effect of MJ on the enantiomeric composition of linalool was observed, which implies that MJ does not influence the stereochemical enzymes involved in the formation of linalool enantiomers. Regarding furaneol, the data encountered in the present study on the enantiomeric purity of the (−)-enantiomer in strawberry support results published by other authors [35]. Besides, MJ did not have any effect on the prevalence of (−)-furaneol during the whole study.

In conclusion, the employment of SDE followed by on-line coupling RPLC–GC via TOTAD is a method suitable for the determination of the levels and enantiomeric composition of chiral volatile compounds in strawberry. Besides, the exogenous application of MJ modifies the concentrations of ethyl 2-methylbutanoate, linalool and furaneol in strawberry particularly after nine days of treatment, which involves an influence on the bioformation of these compounds. Considering the relevance of these compounds to strawberry aroma, the significant increase in their concentrations might result in an enhancement in strawberry aroma. On the other hand, MJ appears to affect the enzymes regulating the stereochemical bioformation of ethyl 2-methylbutanoate, whereas no influence in this respect was observed for linalool and furaneol.

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