Isolation of the four methyl jasmonate stereoisomers and their effects on selected chiral volatile compounds in red raspberries

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ABSTRACT

The four stereoisomers present in a commercial sample of methyl jasmonate (MJ) were isolated at semi-preparative scale by HPLC, using a permethylated β-cyclodextrin column. This allowed the baseline resolution and collection of both major (methyl jasmonates) and minor (epi-methyl jasmonates) stereoisomers. When 1.5 mL of a 5 mg per mL MJ solution were injected, isolated amounts were 3.56 mg for (−) and (+)-methyl jasmonates, with respective purities of 96.1% and 99.9%, and 0.18 mg for (−)- and (+)-epi-methyl jasmonates, with 98.6% and 91.6% respective purities. The post-harvest treatment of red raspberry fruits with the pure methyl jasmonate stereoisomers isolated proved that (−)-epi-MJ either promotes the bioformation of (+)-limonene or inhibits that of (−)-limonene to a greater extent than the other three MJ stereoisomers, while the biosynthesis of the (+)-enantiomer of α-ionone is favoured equally, whichever MJ stereoisomer used. The results obtained in the present study might be used to obtain food products with improved sensory characteristics.

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1. Introduction

Methyl jasmonate (MJ) is a bioactive compound existing endogenously in higher plants that regulates a wide range of physiological processes. In the last few years, it the exogenous application of MJ to modify plant food composition has been reported. In this regard, MJ has been demonstrated to influence the biosynthesis of vitamins (Wolucka, Goosens, & Dirk, 2005), flavonoids (Wang, Bowman, & Ding, 2008), and aroma compounds (Blanch, Flores, & Ruiz del Castillo, 2011). Exposure to MJ may also induce anthocyanin accumulation in fruits (De la Peña Moreno, Monagas, Blanch, Bartolomé, & Ruiz del Castillo, 2010; Kondo & Mattheis, 2006), enhance the antioxidant activity (De la Peña Moreno, Monagas, et al., 2010) and improve food quality (Meng, Han, Wang, & Tian, 2009).

The relationship between the molecular shape and the response of biological receptors is already well-known. Stereoisomers of a chiral molecule can show different biological activities depending on the arrangements of the substituents around the stereocentres. Different mechanisms of action for various biological activities have been ascribed to endogenous MJ stereoisomers. It has been described that potato tuber-inducing activity of (+)-epi-MJ is higher than that of (−)-epi-MJ (Koda, Kikuta, Kitahara, Nishi, & Mori, 1992). The senescence-promoting activity of (+)-epi-MJ is far stronger than those of the other three stereoisomers, i.e. (−)-epi-MJ, (+)-M and (−)-M (Koda et al., 1992), (+)-epi-MJ has been reported to be the stereoisomer possessing strongest odour and, hence, is considered the jasmine aroma-impact compound (Acree, Nishida, & Fukami, 1985). In addition, (+)-epi-MJ is the only biologically active form in the hair pencils of the oriental fruit moth (Baker, Nishida, & Roelofs, 1981; Koda et al., 1992).

The chemical structure of MJ contains two chiral centres at C-3 and C-7, each of which can have either the R or S absolute configuration (see Fig. 1). Therefore MJ can exist in four different stereoisomeric configurations. The (+)-(3R,7S)-isomer and its mirror image, the (−)-(3S,7R)-isomer, which are a pair of enantiomers with the two side chains in the cis arrangement with respect to the plane of the cyclopentane ring, are known as (+)- and (−)-epi-MJ, respectively. The second pair of enantiomers, the (−)-(3R,7R)- and the (+)-(3S,7S)-forms, commonly called (−)- and (+)-MJ, respectively, have the two side-chains in the trans arrangement (Acree et al., 1985).

One of the most interesting applications of MJ is its use as an elicitor in plant treatments. The influence of exogenous MJ on the bioformation of food components has been extensively reported in the literature, with numerous articles describing the effect of MJ treatments on various fruits (Ghasemnezhad & Javaherhadi, 2008; Tzortzakis, 2007; Wang et al., 2008, 2009). Similar studies have also been carried out in our laboratory (Blanch & Ruiz del Castillo, 2012; Blanch et al., 2011; De la Peña Moreno, Blanch, Flores, & Ruiz del Castillo, 2010; Ruiz del Castillo, Flores, & Blanch, 2010).
All these studies have been accomplished by using commercial MJ, which is a stereoisomeric mixture. To date, the effect of the treatment of foodstuffs with pure MJ stereoisomers had not been studied. The reason is probably the analytical difficulty of separating the four MJ stereoisomers. Recently, we have reported the different influence of (−)- and (+)-MJ on the active-aroma esters in strawberry fruit (De la Peña Moreno, Blanch, & Ruiz del Castillo, 2010a) and on flavonols in red raspberries (De la Peña Moreno, Blanch, & Ruiz del Castillo, 2010b). However, in that work MJ enantiomers used in the treatment were isolated on an analytical scale, in such a way that the accumulation of HPLC fractions was necessary to reach the amount required for the treatments. Also, the used method did not allow the minor stereoisomers (i.e., (−)-epi-MJ and (+)-epi-MJ) to be isolated.

We here study the effect of the four MJ stereoisomers on the evolution of the enantiomeric composition of selected chiral volatile compounds in red raspberry fruits. To that end, a semi-preparative-scale high-performance liquid chromatography (HPLC) method allowing the isolation of pure (−)-epi-MJ, (+)-epi-MJ, (−)-MJ and (+)-MJ was developed.

2. Materials and methods

2.1. Standards and chemicals

Commercial MJ was obtained from Sigma–Aldrich (Steinheim, Germany). This compound is purchased as a stereoisomeric mixture formed by four stereoisomer, 45% of each (−)- and (+)-MJ and 5% of each (−) and (+)-epi-MJ, which is obtained by synthesis. Two model solutions were prepared: the first one (10 mg in 10 mL of methanol) was used to resolve the four stereoisomers of MJ by HPLC and the second one (50 mg in 10 mL of methanol) was meant to isolate the four MJ stereoisomers. Triethylammonium acetate (TEAA) was also supplied by Sigma–Aldrich. HPLC-grade methanol and water were both purchased from Labscan Ltd. (Dublin, Ireland). Ethanol was obtained from Prolabo (Fontenay, France). SPE cartridges were obtained from International Sorbent Technology (IST, Hengoed, UK).

2.2. Isolation of MJ stereoisomers by semi-preparative high-performance liquid chromatography

The isolation of the HPLC fractions corresponding to MJ stereoisomers was carried out by sampling a 1500-μL volume of the model solution of MJ into an Agilent model 1260 Infinity Series (Agilent, Wilmington, DE) chromatograph fitted with two preparative pumps, autosampler, a 5.0-mL sample loop and a diode array detector (DAD). The separation of MJ stereoisomers was carried out on a 30 m × 20.0 mm i.d. column packed with a 5-μm layer of permethylated β-cycloDEXtrin (Nucleodex β-PM, Macherey–Nagel, Düren, Germany). Since this column is not commercially available, it was specifically designed and prepared for this work. No temperature control was used for the column.

In the present study, different flow rates (10, 11, 12 and 13 mL/min), sampling volumes (1000, 1200 and 1500 μL) and TEAA concentrations in MeOH (0.1%, 0.2% and 0.3%) were tested to select the best experimental HPLC conditions. The isocratic elution was accomplished by applying methanol/water percentages of 55/45 at a flow rate of 13 mL/min. In all cases, the methanol used contained 0.1% TEAA at pH 7.0. The collection of the four fractions containing the (−)-MJ (Fraction 1), (−)-epi-MJ (Fraction 2), (+)-epi-MJ (Fraction 3), and (+)-MJ (Fraction 4) was carried out by means of a connection placed right after the DAD. The signal was registered by setting the DAD at 210 nm. The LC equipment was properly washed by passing methanol through the whole system for 15 min after every single run.

2.3. Preparation of the HPLC fractions containing MJ stereoisomers for treatments

The collected HPLC fractions containing the four stereoisomers of MJ were prepared and concentrated by solid-phase extraction (SPE) followed by the use of a rotary evaporator. Packed conventional 3-mL cartridges with a 500-mg weight of silica chemically modified by octadecyl (C18) were used. SPE procedure for each HPLC fraction was accomplished as follows: (i) SPE cartridge packing was conditioned by rinsing the tube with a 1-mL volume of 55/45 methanol/water, (ii) each HPLC fraction collected was slowly poured through the SPE cartridge, (iii) (−)-MJ, (−)-epi-MJ, (+)-epi-MJ, and (+)-MJ were respectively eluted with a 2-mL aliquot of ethanol at an approximate flow rate of 1 mL/min. Taking into account that MJ stereoisomers isolated were being used to treat raspberries, a harmless solvent had to be used. For this reason, we chose ethanol as the elution solvent. For the treatments, fractions were concentrated to about 1 μL in 300 μL ethanol. The MJ mixture was used as a GC reference. Finally the purity of each stereoisomer in the concentrated ethanol fractions was determined by GC as detailed in Section 2.7.

Fig. 1. Scheme representing the chemical structures of the four stereoisomers of MJ.
2.4. Plant material

Ripe red raspberry fruits (variety Glen Lyon, Huelva, Spain) were acquired from the local supermarket. We selected the fruits on the basis of regular size, colour and state of ripeness. Once chosen, the fruits were immediately treated with the isolated stereoisomers of MJ.

2.5. Post-harvest treatments of red raspberries

The raspberry fruits were treated with (−)-MJ, (+)-MJ, (−)-epi-MJ and (+)-epi-MJ. The treatments were accomplished by following the same procedure: one raspberry (2.5 g approximately) was placed in a 50-mL container. The concentrated ethanol fraction containing each MJ stereoisomer individually isolated and separated from the other three stereoisomers (i.e., 1 mL MJ stereisomer in 300 μL of ethanol) was put into a vial, which was, in turn, placed inside each container. The lids of the four containers were hermetically covered to avoid losses. MJ stereoisomers fractions were allowed to spontaneously vaporise for 24 h at 25 °C. For each treatment, one raspberry was at the same time placed at an additional container to be used as a control. The procedure followed for the control samples was exactly the same as that used for the treated fruit, with the exception of the use of an empty vial instead of a vial containing the ethanol fractions. Subsequently, the containers were kept at 5 °C for 5 days. Previous studies carried out in our laboratory have proven that storage times should comprise between 5 and 7 days. Longer storage times after treatment result in decay of the fruit (De la Peña Moreno, Monagas, et al., 2010). The enantiomeric compositions of limonene, β-pinene and α-ionone were then examined in all treated raspberries and compared with those in untreated fruits on day 5 after treatment by SPME-GC.

2.6. Solid phase microextraction of untreated and treated red raspberries

The extractions of the volatile compounds from untreated (i.e., control) and treated berries were performed by solid-phase microextraction (SPME). A fused silica fibre coated with a 65-μm layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB) installed in a holder for manual use (Supelco, Madrid, Spain) was utilised. As recommended by the supplier, the fibre was conditioned in the injector of the gas chromatograph at 250 °C for 30 min before use. A 10-g weight of raspberries was cut into pieces and placed in a 25-mL vial. The vial was then sealed with plastic film with characteristics suitable for the SPME extraction (i.e., inert and possessing low water permeability). Prior to the actual extraction, an incubation time of 10 min was applied to enrich the sample headspace in the volatile components. Experimentation was performed by exposing the fibre to the headspace of the sample for 30 min at 40 °C. The extraction conditions applied were selected as a result of the following optimisation process carried out in an earlier work (Blanch, Flores, Caja, & Ruiz del Castillo, 2009). Once the extraction was finished, the compounds retained on the SPME fibre were thermally desorbed into a GC injector and analysed by gas chromatography–mass spectrometry (GC–MS).

2.7. Gas chromatography–mass spectrometry

Chiral gas chromatography (CGC) was used to both determine the enantiomeric purity of the HPLC fractions collected and analyse the SPME extract obtained from the untreated and treated raspberries. In both cases, a Hewlett-Packard Model 6890 gas chromatograph fitted with a split/splitless injector and a flame ionisation detector (FID) was used. The GC analyses were accomplished on a 25-m × 0.25-mm i.d. capillary column coated with a 0.25-μm layer of permethylated β-cyclodextrin (Chirasil-β-Dex, Agilent). The injector was set at 250 °C, operating in splitless mode at all times. Helium was used as the carrier gas at a constant flow of 1 mL/min. The source and quadrupole temperatures were set at 230 and 100 °C, respectively. Data acquisition from the MS was accomplished with ChemStation software.

The enantiomeric purity of the HPLC fractions collected and concentrated by SPE was evaluated by programming the GC-column at 4 °C/min (5 min) from 40 to 160 °C and subsequently at 3 °C/min to 180 °C (10 min), as optimised elsewhere (Blushan & Kumar, 2009). The elution order of the four MJ stereoisomers on the GC column was established on the basis of optical rotation data carried out in a previous study (Ruíz del Castillo & Blanch, 2007). The identity of MJ stereoisomers was confirmed by matching their mass spectra with those provided for MJ by the Wiley library. The overall analytical procedure described, including collection of the HPLC fractions, SPE concentration and GC analysis, was performed in triplicate.

To determine the enantiomeric composition of selected chiral volatile compounds (i.e., limonene, β-pinene and α-ionone) in untreated-control and treated raspberries, the SPME fibre was desorbed at 250 °C for 10 min into the GC injector. The GC analysis was performed by programming the column at 5 °C/min to 70 °C, subsequently at 2 °C/min to 90 °C and finally at 5 °C/min to 180 °C. The mass spectrometer scanned from m/z 550 to 50. The identification of the volatile compounds analysed was made by matching their mass spectra with those provided by the Wiley library. Additionally, identities were verified by running the standards under the same chromatographic conditions. The SPME–GC–MS analysis of each sample was carried out in duplicate.

3. Results and discussion

3.1. Semi-preparative scale chromatographic resolution of the four MJ stereoisomers

Figure 2 indicates the HPLC chromatogram obtained from applying 13 mL/min as the flow rate, 1500 μL as the sampling volume and 0.1% as TEA percentage. Considering that the elution order of MJ stereoisomer on GC has already been reported (Caja, Blanch, & Ruiz del Castillo, 2008; Ruiz del Castillo & Blanch, 2007) the identification of each stereoisomer was established by injection of each HPLC fraction into the GC. This way, the elution order of the four MJ stereoisomers on the semi-preparative Nucleodex β-PM column was: (−)-MJ, (−)-epi-MJ, (+)-epi-MJ and (+)-MJ.

As seen in the figure, the four stereoisomers of MJ were baseline resolved on the Nucleodex β-PM column used. In fact, α-values were always higher than 1.0 and Rf factors higher than 1.5. The complete resolution of the four MJ stereoisomers is essential to guarantee the purity of each HPLC fraction collected.

Methods reported in the literature on the isolation of the four MJ stereoisomers are focused on asymmetric synthesis (Beale & Ward, 1998; Montforts, Gesing-Zibulak, & Grammenos, 1989), enzymatic resolution (Nishida, Acree, & Fukami 1985) and the use of high-performance liquid chromatography (HPLC) after previous formation of diastereoisomers (Kiyote, Higashi, Koike, & Oritani, 2001). The asymmetric synthesis and enzymatic resolution methods are only useful to obtain pure (−)-MJ. The other three MJ stereoisomers cannot be isolated using this method. The HPLC approach mentioned above enables both (−)- and (+)-MJ to be separated (Kiyote et al., 2001). However, the separation by this method requires the previous formation of diastereomeric ketais and does not allow (±)-epi-MJ to be resolved either. To our knowledge, only two procedures to obtain individually the four MJ stereoisomers have been thus far reported (Beale & Ward, 1998; Yamane,
Takahashi, Ueda, & Kato, 1981). However, these procedures are based on asymmetric synthesis and enantioselective resolution, in such a way that both procedures are time-consuming and laborious. This implies losses and sources of error. The HPLC method here proposed enables the four MJ stereoisomers to be separated with no need for prior derivatisation. It is therefore rapid and efficient. Once chromatographic separation of the four MJ stereoisomers was achieved, the time ranges most suitable for collection were selected.

### 3.2. Semi-preparative scale isolation of the four MJ stereoisomers

Table 1 indicates the HPLC fraction size (min), collection volume (mL) and collected amounts (mg) of each MJ stereoisomer obtained from each HPLC run.

<table>
<thead>
<tr>
<th>MJ stereoisomers</th>
<th>Collection times</th>
<th>Collected volumes</th>
<th>Collected amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-MJ</td>
<td>14.2–16.0</td>
<td>23.4</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>(1.8 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)-epi-MJ</td>
<td>16.2–17.5</td>
<td>16.9</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(1.3 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)-epi-MJ</td>
<td>17.6–18.8</td>
<td>15.6</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(1.2 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)-MJ</td>
<td>19.8–22.2</td>
<td>31.2</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>(2.4 min)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As seen in Figure 3, the elution order was (-)-MJ, (-)-epi-MJ, (+)-MJ, and (+)-epi-MJ. Specifically, the retention times obtained were 37.61, 37.82, 38.31 and 38.65 min for (-)-MJ, (-)-epi-MJ, (+)-MJ, and (+)-epi-MJ, respectively. As also appreciated in Figure 3, the four MJ stereoisomers were isolated with very high purity. In particular, the purities of the HPLC fractions collected were 96.1%, 98.6%, 91.6% and 99.9% for (-)-MJ, (-)-epi-MJ, (+)-epi-MJ and (+)-MJ, respectively.

The repeatability of the method here proposed to isolate the four individual MJ stereoisomers was estimated as relative standard deviation (RSD) from three replicates of the whole analytical procedure (i.e., semi-preparative HPLC separation, collection of the HPLC fractions, SPE concentration and GC verification). As a result, RSD values of 3.5%, 14.1%, 12.5% and 9.6% were obtained for (-)-MJ, (-)-epi-MJ, (+)-epi-MJ and (+)-MJ, respectively. It is not surprising that the HPLC fractions 1 and 4, which corresponded to the major MJ stereoisomers and (+)-MJ, exhibited lower RSD than the HPLC fractions 2 and 3 corresponding to the minor stereoisomers (-)- and (+)-epi-MJ. This is due to the greater easiness of fixing the collection times when the chromatographic signals are larger. In any case, the repeatability of the method was lower than 15% for the four stereoisomers and, therefore, the method proposed to isolate the four MJ stereoisomers was considered satisfactory. The recovery of the method was estimated by using a GC injection of 0.2 μL of a 1 μL/10 mL model solution as a reference. The recovery values of the method were 98.8, 94.5, 92.6 and 99.9% for (-)-MJ (HPLC fraction 1), (-)-epi-MJ (fraction 2), (+)-epi-MJ (fraction 3), and (+)-MJ (fraction 4), respectively.

### 3.3. Post-harvest treatment of red raspberries with the four MJ stereoisomers

Table 2 shows the enantiomeric excess (ee, %) of limonene, β-pinene and α-ionone in red raspberries untreated and treated with (-)-MJ-EtOH, (-)-epi-MJ-EtOH, (+)-epi-MJ-EtOH and (+)-MJ-EtOH. The measurements were made on Day 5 after treatment. As seen in the table, the (-)-enantiomers were predominant for limonene and β-pinene, whereas the (+)-enantiomer prevailed for α-ionone. These results agree with data previously obtained from raspberries untreated and treated with commercial MJ in combination with EtOH (Blanch et al., 2011). From the table it can also be observed that the effect of the different treatments varied according to the

![Figure 2](image-url) Chromatogram obtained from the direct semi-preparative scale HPLC separation of the four MJ stereoisomers. Experimental conditions: stationary phase: permethylated β-cyclodextrin (Nucleodex β-PM); mobile phase composition: MeOH (0.1% TEAA)/H2O (55/45); flow rate: 13 mL/min; sampling volume: 1500 μL; 0.1% TEAA percentage. HPLC elution order: (-)-MJ, (-)-epi-MJ, (+)-epi-MJ and (+)-MJ.
specific compounds considered. In particular, the enantiomeric composition of \((-\)-limonene) decreased slightly in all treated samples with respect to the control fruit, although all values varied within a reasonably narrow range. An exception was the samples treated with \((-\)/\((-\)-epi-MJ-EtOH, which showed values that decreased from 15.1% in the controls to 8.4% in treated red raspberries. This indicates that \((-\)-epi-MJ-EtOH might promote the bioformation of \((+\)-limonene) or inhibit the formation of \((-\)-enantiomer, probably because of the direct action of \((-\)-epi-MJ on the enantioselective enzymes regulating the biosynthesis of both

### Table 2

<table>
<thead>
<tr>
<th>Chiral compounds</th>
<th>Treatments</th>
<th>Control-Utreated</th>
<th>((-)-MJ-EtOH)</th>
<th>((-)-epi-MJ-EtOH)</th>
<th>((+)-epi-MJ-EtOH)</th>
<th>((+)-MJ-EtOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>((-)-Limonene</td>
<td></td>
<td>15.2</td>
<td>11.7</td>
<td>8.4</td>
<td>12.1</td>
<td>10.0</td>
</tr>
<tr>
<td>((-)-(\beta)-Pinene</td>
<td></td>
<td>80.8</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>((+)-(\alpha)-Ionone</td>
<td></td>
<td>83.1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>96.0</td>
</tr>
</tbody>
</table>

n.d.: not detected.
enantiomers of limonene in raspberries. On the contrary, the other three MJ stereoisomers did not appear to affect the formation of limonene enantiomers.

Regarding α-ionone, its enantiomeric excess exhibited in all cases an increase with the treatments, regardless of the treatment used. This means, on the one hand, that the post-harvest treatment of raspberries with MJ-EtOH promotes, equally to limonene, the bioformation of the (+)-enantiomer of α-ionone and, on the other, that the employment of one MJ enantiomer or another did not make any difference in this regard.


