JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Online RPLC-GC via TOTAD Method To Isolate (+)-Methyl Epijasmonate from Lemon (*Citrus limon* Burm.)

MARIA DEL MAR CAJA, GRACIA PATRICIA BLANCH, AND MARIA LUISA RUIZ DEL CASTILLO*

Instituto de Fermentaciones Industriales CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

Pure (+)-methyl epijasmonate was isolated from lemon (*Citrus limon* Burm.) for the first time. To that aim, two commercial essential oils and one homemade extract were included in the present paper. First, a study on the appropriate chromatographic conditions to avoid the epimerization from methyl epijasmonate to the more stable methyl jasmonate was accomplished. The results obtained are discussed. The presence of (+)-methyl epijasmonate in the three samples studied was initially established through the direct injection into GC-MS. However, the overlapping of (+)-methyl epijasmonate with other matrix components made it necessary to employ a multidimensional technique. RPLC-GC analysis via through-oven transfer adsorption−desorption (TOTAD) provided the selectivity and sensitivity required, reflecting that the homemade lemon extract was an adequate natural source to obtain pure (+)-methyl epijasmonate by means of the collection of the corresponding RPLC fraction.

KEYWORDS: Methyl epijasmonate; RPLC; lemon; essential oil

INTRODUCTION

Methyl jasmonate (MJ) is an endogenous plant growth regulator that is involved in the control of developmental processes and stress-related responses (*1*). It possesses two stereochemical centers at C-3 and C-7, which means that it can exist in four different stereoisomeric configurations. These forms are known as (−)- and (+)-MJ [(3*R*,7*R*)- and (3*S*,7*S*)-isomers, respectively] and (−)- and (+)-methyl epijasmonate [epiMJ, (3*S*,7*R*)- and (3*R*,7*S*)-isomers, respectively].

The exogenous application of low MJ concentrations can influence the biosynthesis of important plant and fruit components such as carotenes and chlorophylls (*2, 3*) and vitamins (*4*). Similarly, the modification of the biochemical pathways of aromatic compounds by the application of MJ has also been demonstrated (*5*). In this respect, reports in the literature have already described the impact of MJ on the biosynthesis of volatile compounds in apple (*6*) and strawberry (*7*) as well as the alteration of the volatile emission in plants as a consequence of the exposure to MJ (*8*). Nevertheless, all of these reports consider the exogenous application of standard MJ, which is commercialized as a mixture of the four stereoisomers in such a way that, to our knowledge, the effect of the individual stereoisomers of MJ on the biosynthesis of volatile compounds has not been studied as yet.

In this regard, it is known that the biological properties of a chiral compound depend to a great extent on the absolute configuration of its stereochemical centers. Particularly, for MJ the relationship between its structural requirements and its bioactivity has been previously studied (*9–11*). For instance, it has been pointed out that the odor of (+)-epiMJ is far stronger than that of the other three stereoisomers (*12*). Actually, certain investigations have proved that the odor of (+)-epiMJ is especially strong in ripe lemons (*13*) and that this epimer is in addition the major form present in lemon peels and the only biologically active form in the hair pencils of the oriental fruit moth (*14, 15*). As a result of these studies, we could state that MJ stereoisomers, including (±)-epiMJ, have different mechanisms of action for various biological activities and that, therefore, the effect of the exogenous application of MJ on the biochemical pathways must be studied for each individual stereoisomer. To that end, it is first essential to separately obtain each stereoisomer with sufficiently high purity. This is particularly complicated for MJ because individual enantiomers are not commercially available. As a consequence, pure enantiomers have to necessarily be obtained either from preparative-scale chiral chromatographic separation of the commercial stereoisomeric mixture, from enantioselective synthesis, or from natural sources.

In this paper we report the development of a method to isolate (+)-epiMJ from a natural source, lemon peel (*Citrus limon* Burm.), with a view to studying its effect on the biosynthesis of food components, mainly volatile compounds. For that purpose, the search for lemon natural products that can be used as a source of pure (+)-epiMJ was required in the first place.

MATERIALS AND METHODS

Samples. (±)-Methyl jasmonate standard was obtained from Sigma-Aldrich (Steinheim, Germany). This standard is a stereoisomeric mixture

* Corresponding author (telephone 91-5622900; fax 91-5644853).

10.1021/jf800425p CCC: $40.75
Published on Web 06/28/2008

formed by 95% of (±)-MJ and 5% of (±)-epiMJ. A standard solution of approximately 1.0 mg of the standard in 10 mL of methanol was employed in the identification of the target compound, in the selection in RPLC of the fraction to be transferred to GC, and in the study of the epimerization from epiMJ to MJ. Methanol used to prepare this solution was purchased from Labscan Ltd. (Dublin, Ireland).

Two commercial essential oils from lemon (*C. limon* Burm.) (samples 1 and 2) were acquired from the local market. Sample 1, used as a fragrance, was obtained by cold pressing, whereas sample 2, used for therapeutic purposes, was extracted by steam distillation from fresh plants and flowers from ecological crops. A third sample (sample 3), which was obtained by ourselves in our laboratory, was also included in this study. The extraction procedure followed is detailed below.

Experimental Procedure To Obtain Lemon Peel Extract. Peels from five lemons (Fino variety, 150 g) obtained from a local grocer were soaked in 400 mL of acetone/ether (1:1) at room temperature with constant stirring for 96 h. The extract was then concentrated at 40 °C by using a rotary evaporator to an oily residue. Subsequently, the obtained oil was shaken with 100 mL of ether and extracted with 50 mL of a saturated solution of NaCl. The ether extract was twice dried over sodium sulfate and finally concentrated to a yellow oil (0.43 g).

All samples studied (samples 1−3) were diluted in hexane (1:5) prior to their introduction in the gas chromatography−mass spectrometry (GC-MS) system.

GC-MS Analysis. The chromatographic analyses of standard MJ as well as of the three lemon samples were carried out by using a Hewlett-Packard model 6890 gas chromatograph coupled to an Agilent 5989A quadrupole instrument (Palo Alto, CA). This equipment is fitted with a split/splitless injector, which operated at the splitless mode at all times. The injector temperature was studied by testing different values (150, 170, 200, 250, and 300 °C), selecting 250 °C to accomplish the analyses. The GC separations were performed on a 25 m × 0.25 mm i.d. capillary column coated with a 0.25 μm layer of polyethylene glycol modified with acid (007-FFAP, Quadrex). The GC column was initially programmed at 5 °C/min from 40 °C (5 min) to 90 °C, subsequently at 1 °C/min to 120 °C, and finally at 4 °C/min to 190 °C. Helium was used as the carrier gas in the constant flow mode (1 mL/min). The source and the quadrupole temperatures were set at 230 and 100 °C, respectively. Data acquisition from the MS was accomplished with the HP-ChemStation system. In all instances (+)-epiMJ in the samples was identified by using its mass spectrum in combination with its linear retention index (LRI). Specifically, the obtained mass spectrum was initially matched with that contained in the Wiley library for epiMJ. Additionally, (+)-epiMJ identity was confirmed by comparison with the mass spectrum and retention time data provided by MJ standard, which, as already mentioned, contains 5% of epiMJ. LRI for (+)-epiMJ and (−)-MJ was measured by co-injection of the samples with a solution containing the homologous series of C_8−C_{24} *n*-alkanes, as described in the literature (*16*). As later explained under Results and Discussion, the certainty of the identity of epiMJ as just the (+)-enantiomer was based on various bibliographic references (*9*–*12*). The effectiveness of this approach was finally verified by running the spiked samples in the same experimental conditions.

RPLC-GC System. The multidimensional system used to analyze the lemon peel essential oils (samples 1 and 2) and the extract (sample 3) was composed of a liquid chromatograph and a gas chromatograph linked through an automated through-oven transfer adsorption−desorption (TOTAD) interface, U.S. Patent 6,402,947 B1 (exclusive rights assigned to KONIK-Tech, Sant Cugat del Vallés, Barcelona, Spain). LC preseparation was carried out by using a Hewlett-Packard model 1050 (Wilmington, DE) chromatograph fitted with a manual injection valve (model 7125, Rheodyne, Cotati, CA), a 20 μL sample loop, and an ultraviolet (UV) detector, which operated at 205 nm throughout the experimentation. The GC runs were accomplished with a Konik model HRGC 4000B chromatograph equipped with a flame ionization detector (FID) set at 250 °C. Data acquisition from LC was carried out by using HP Chemstation software (Hewlett-Packard), whereas an Ezchrom (Konik, Sant Cugat del Vallés, Barcelona, Spain) was utilized to acquire data from the GC analysis.

RPLC Conditions. The LC preseparation of samples 1−3 was accomplished on a 150 mm × 4.6 mm i.d., 10 μm C_6 column (Waters, Madrid, Spain) kept at room temperature. All analyses were performed using methanol/water as the mobile phase. The initial eluent composition (methanol/water, 70:30, v/v) was maintained for 3 min; subsequently a linear gradient was applied within 1 min to 78% methanol, which was kept during 6 min. After that, a linear gradient was once more applied within 2 min to 100% methanol. The flow rate was modified in such a way that the transfer of the RPLC fraction corresponding to MJ stereoisomers was carried out at 0.3 mL/min and the rest of the matrix components were eluted at faster flow rate (i.e., 1.0 and 2.0 mL/min before and after the transfer time, respectively). The transferred fraction volume corresponded to 750 μL (fraction width = 2.5 min). The LC equipment was properly washed by passing methanol through the whole system after every run.

RPLC-GC Transfer. The GC was connected to the LC system through the TOTAD interface. The system was automated by means of electrovalves and an electronic pressure control, which is used to control helium flow through the interface. The transfer of the RPLC fraction containing MJ stereoisomers was performed by switching, from the waste position to the transfer position, a six-port valve, which is connected to the GC by silica capillary tubing (62.15 cm length × 0.32 mm i.d., 50 μL internal volume). A helium flow was set during the transfer, and once the transfer step was finished, an additional time (so-called purge time = 2 min) was set with the intention of achieving the complete elimination of the eluent. During the transfer step and purge time, MJ stereoisomers are retained in the glass liner placed inside the TOTAD interface and packed with 1 cm length of Tenax TA, which was kept in place with two glass wool plugs. The temperature of the interface during the retention of the investigated compounds was 40 °C. Subsequently, MJ was desorbed from Tenax TA and finally transferred to the GC column by increasing the TOTAD temperature at 10 °C/s from 40 to 200 °C. Finally, MJ stereoisomers were analyzed by GC under the experimental conditions specified below.

Gas Chromatographic Analysis. GC analysis of the RPLC fraction transferred was performed on a 30 m × 0.25 mm i.d. fused-silica column coated with a 0.25 μm layer of (5% phenyl)-methyl polysiloxane (DB-5 ms, J&W, Folsom, CA) using helium as the carrier gas at an initial pressure of 15 psi and constant flow of 1.2 mL/min. The oven temperature was increased from 40 °C (10 min) to 170 °C at 5 °C/min. Splitless mode was used in all cases (splitless time = 30 s), and FID was set at 250 °C.

RESULTS AND DISCUSSION

Selection of the GC conditions. Taking into account that epiMJ has already been reported to be isomerized to MJ above 180 °C (*13, 17*), a study on the conditions to be used during the chromatographic analysis was considered to be mandatory. The study was faced in such a way that a standard solution of MJ, made up of 95% (±)-MJ and 5% (±)-epiMJ, in methanol was injected into GC at different injection temperatures, as previously specified under Materials and Methods, the oven temperature being 170 °C. All measurements were carried out in triplicate. The relative standard deviation (RSD) calculated from three replicates ranged between 2.2 and 11.9% for epiMJ and between 0.4 and 10.1% for MJ according to the temperature tested. The ratio of both compounds was estimated from the peak areas. As a result, the (±)-MJ to (±)-epiMJ ratio remained constant (i.e., around 17) at all values tested. We concluded therefore that the injection temperature used during the GC analysis did not appear to bring about the epimerization from (±)-epiMJ to (±)-MJ and, in short, any value might be equally set during the experimentation. These results are in disagreement with those reported by other authors, who have described the conversion of (±)-epiMJ to (±)-MJ when working at column temperatures >180 °C and injection temperatures >170 °C (*13, 17*). This discrepancy might be due to the distinct chromatographic conditions other than the temperatures used in these works with respect to the present study (i.e., column length, carrier gas, etc.), which result in a longer permanence

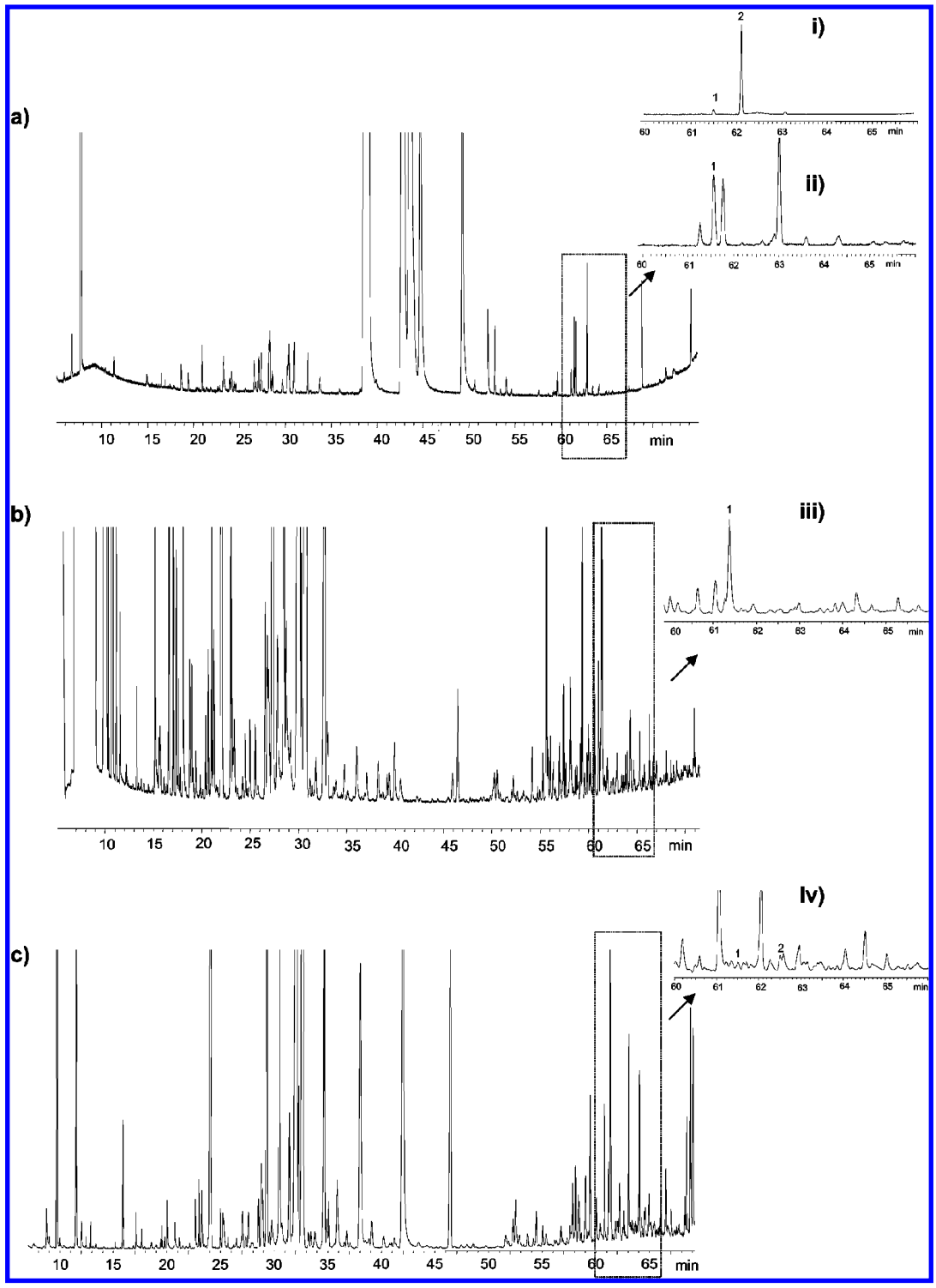

Figure 1. Chromatograms obtained from the GC-MS analysis of sample 1 (**a**), sample 2 (**b**), and sample 3 (**c**). All chromatograms were recorded at the same full range. Plot i represents the GC-MS chromatogram corresponding to the standard run under identical conditions. See text for further details. Peak identification: 1, (+)-methyl epijasmonate; 2, (−)-methyl jasmonate.

of (±)-epiMJ inside the chromatographic column. In any case, a more extensive study including the determination of the epimerization barriers from (±)-epiMJ to (±)-MJ is necessary to ensure the experimental conditions to be applied during the chromatographic analysis. This aspect will be the aim of a further study.

Determination of (+)-epiMJ in Lemon Samples. The occurrence of (+)-epiMJ and (−)-MJ as pure enantiomers in nature (*18, 19*) has already been demonstrated. Equally, it has also been reported that epiMJ is clearly the major form in lemon peel (*13*). On the basis of these studies, we chose to obtain pure (+)-epiMJ from lemon samples. For that purpose, two commercial lemon essential oils and one homemade lemon extract were considered in the present study.

Figure 1 represents the chromatograms obtained from the direct injection into GC-MS of sample 1 (**a**), sample 2 (**b**), and sample 3 (**c**). All chromatograms were recorded at the same full range. For comparison, the GC-MS chromatogram corresponding to the standard run under identical conditions is also shown in **Figure 1** as plot i. Similarly, the chromatogram area

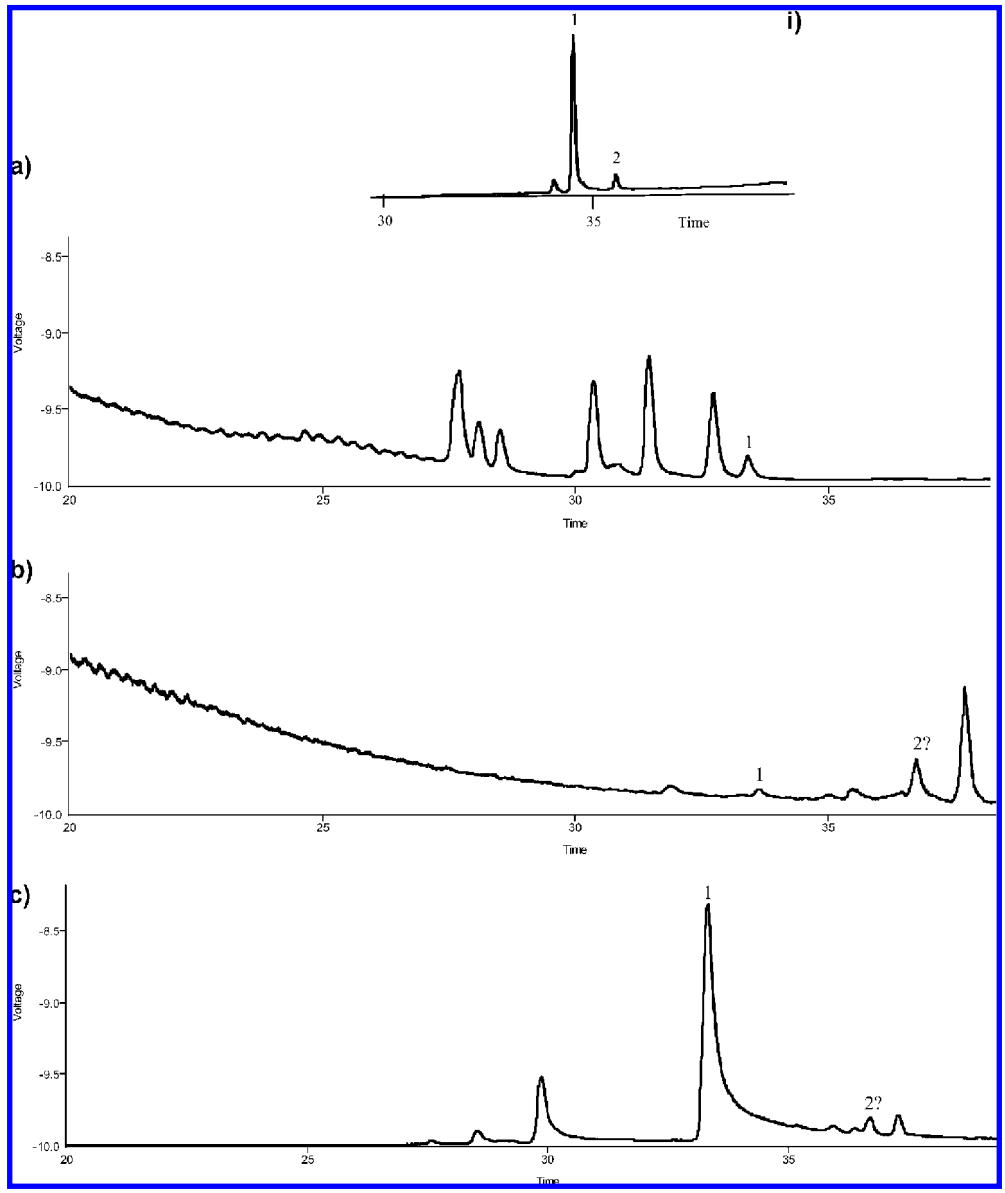

Figure 2. Chromatograms obtained from the RPLC-GC analysis via TOTAD of sample 1 (**a**), sample 2 (**b**), and sample 3 (**c**). Transfer time, 2.5 min; transfer speed, 0.3 mL/min; transfer volume, 750 μL. All chromatograms were recorded at the same full range. Plot i illustrates the RPLC-GC via TOTAD chromatogram corresponding to the standard analyzed under identical conditions. See text for further details. Peak identification: 1, (+)-methyl epijasmonate; 2, (−)-methyl jasmonate.

corresponding to the elution of (+)-epiMJ has been enlarged and included as plots ii, iii, and iv for samples 1, 2, and 3, respectively. First, it is important to highlight that, as can be seen in **Figure 1**, (−)-MJ (retention time = 62.5 min) was not detected in samples 1 and 2. This confirms that the experimental conditions used do not cause the epimerization from epiMJ to MJ. Its detection in sample 3 is most likely due to the actual occurrence of small amounts of (−)-MJ in the sample rather than to the epimerization from (+)-epiMJ. On the other hand, (+)-epiMJ was identified in the three samples considered by using its retention time (61.5 min), its linear retention index (2284) on the 007-FFAP gas chromatographic column, and its mass spectrum. In fact, the mass spectrum provided by the Wiley library showed the typical fragmentation pattern of epiMJ: *m/z* (real intensity) 67 (34), 77 (34), 79 (45), 81 (30), 82 (27), 83 (100), 91 (32), 93 (42), 95 (50), 109 (45), 133 (32), 151 (72), 156 (38), 193 (21), molecular ion 224 (50). However, the mass spectra corresponding to other components occurring in the matrix were also observed in the epiMJA signal in samples 1 and 2. As a matter of fact, the utilized software verified the coelution of several components together with (+)-epiMJ in these samples. Therefore, an additional cleanup before chromatographic determination was considered to be crucial to isolate (+)-epiMJ with sufficiently high purity. For this reason, we additionally analyzed samples 1−3 by online coupling RPLC-GC. The RPLC step enables the fraction containing the target compound to selectively be transferred from RPLC to GC and, as a consequence, to separate (+)-epiMJ from the other matrix components (*20*). Besides, the possibility of preconcentrating the analyte inside the packing material placed in the RPLC-GC interface, especially when the TOTAD interface is used, provides the sensitivity required when for the determination of minor compounds, such as (+)-epiMJ (*21*).

Figure 2 illustrates the chromatograms resulting from the RPLC-GC analysis via TOTAD of sample 1 (**a**), sample 2 (**b**), and sample 3 (**c**). All chromatograms were recorded at the same full range. For comparison, the RPLC-GC via TOTAD chromatogram of the standard analyzed in the same conditions is

also shown in **Figure 2** as plot i. As observed in **Figure 2**, the presence of (+)-epiMJ (retention time = 33.4 min) could be established in all of them. From these analyses, the absence of (−)-MJ (retention time = 35.2 min) seemed to be apparent in sample 1. In contrast, its detection in samples 2 and 3 appeared to be uncertain. These results agree with those encountered by GC-MS concerning samples 1 and 3. In contrast, this did not happen with sample 2, in which a very minor compound was detected at (−)-MJ retention time. This disagrees with the clear lack of (−)-MJ observed by GC-MS (**Figure 1b**, plot iii), which suggests the possible elution of some kind of interference other than (−)-MJ along with (+)-epiMJ in **Figure 2b**. From **Figure 2** it is evident that the amount of (+)-epiMJ was far higher in sample 3 than in samples 1 and 2. An approximate estimation of the concentration of the (+)-epiMJ in the tested three lemon samples was performed by comparing the absolute areas provided from the RPLC-TOTAD-GC analyses of the studied samples and the model solution (1 mg/10 mL) under the same experimental conditions. The concentrations of (+)-epiMJ obtained were 13.0, 13.5, and 65.0 mg/L in samples 1, 2, and 3, respectively. This fact can be explained by the different extraction procedure used in each case to obtain the essential oils and the extract from lemon peel. With regard to the RPLC fraction purity, the elution of other components together with (+)-epiMJ was found in sample 1, whereas the RPLC fractions provided by samples 2 and 3 were far purer. Actually, as also can be seen in **Figure 2**, (+)-epiMJ occurred practically pure in sample 3.

The repeatability (expressed as RSD) for (+)-epiMJ estimated from three replicates of the RPLC-TOTAD-GC analysis of samples 1−3 varied from 3.4 to 4.5% depending on the sample. The detection limit of the method was calculated from the RPLC-TOTAD-GC analysis of the model solution (1 mg/10 mL) by considering a signal/noise ratio equal to 5, 0.01 mg/L being the value obtained. The recovery was also estimated from the model solution and the direct injection into GC of 0.4 μL of the same model solution in the splitless mode, which was used as a reference. The recovery obtained for (+)-epiMJ by the proposed method was 71%.

As a conclusion, the extract obtained in our laboratory from natural lemon peel (Sample 3) appeared to be the most appropriate sample to be used as a natural source of pure (+)-epiMJ. The collection of the RPLC fraction corresponding to the elution of (+)-epiMJ instead of its transfer to GC via TOTAD may enable pure (+)-epiMJ to be obtained from a natural source. Either removal or modification of the RPLC eluent in which the analyte is collected can be necessary according to the specific study to be carried out.

LITERATURE CITED

(1) Sembdner, G.; Parthier, B. The biochemistry and the physiological and molecular actions of jasmonates. *Annu. Rev. Plant Physiol.* **1993**, *44*, 569–589.

(2) Saniewski, M.; Czapski, J. The effect of methyl jasmonate on lycopene and β-carotene accumulation in ripening red tomatoes. *Experientia* **1983**, *39*, 1373–1374.

(3) Pérez, A. G.; Sanz, C.; Richardson, D. G.; Olías, J. M. Methyl jasmonate vapor promotes β-carotene synthesis and chlorophyll degradation in Golden Delicious apple peel. *J. Plant Growth Regul.* **1993**, *12*, 163–167.

(4) Wolucka, B. A.; Goosens, A.; Dirk, I. Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. *J. Exp. Bot.* **2005**, *56*, 2527–2538.

(5) Lalel, H. J. D.; Singh, Z.; Tan, S. C. The role of methyl jasmonate in mango ripening and biosynthesis of aroma volatile compounds. *J. Hortic. Sci. Biotechnol.* **2003**, *78*, 470–484.

(6) Kondo, S.; Setha, S.; Rudell, D. R.; Buchanan, D. A.; Mattheis, J. P. Aroma volatile biosynthesis in apples affected by 1-MCP and methyl jasmonate. *Postharvest Biol. Technol.* **2005**, *36*, 61–68.

(7) Fernando, J.; Wang, S. Y.; Wang, C. Y.; González-Aguilar, G. A. Methyl jasmonate in conjuction with etanol treatment increases antioxidant capacity, volatile compounds and postharvest life of strawberry fruit. *Eur. Food Res. Technol.* **2005**, *221*, 731–738.

(8) Degenhardt, D. C.; Lincoln, D. E. Volatile emissions from an odorous plant in response to herbivory and methyl jasmonate exposure. *J. Chem. Ecol.* **2006**, *32*, 725–743.

(9) Yamane, H.; Sugawara, J.; Suzuki, Y.; Shimamura, E.; Takahashi, N. Synthesis of jasmonic acid related compounds and their structure−activity relationships on the growth of rice seedlings. *Agric. Biol. Chem.* **1980**, *44*, 2857–2864.

(10) Koda, Y.; Kikuta, Y.; Tazaki, H.; Tsujino, Y.; Sakamura, S.; Yoshihara, T. Potato tuber-inducing activities of jasmonic acid and related compounds. *Phytochemistry* **1991**, *30*, 1435–1438.

(11) Koda, Y.; Kikuta, Y.; Kitahara, T.; Nishi, T.; Mori, K. Comparisons of various biological activities of stereoisomers of methyl jasmonate. *Phytochemistry* **1992**, *31*, 1111–1114.

(12) Acree, T. E.; Nishida, R.; Fukami, H. Odor thresholds of the stereoisomers of methyl jasmonate. *J. Agric. Food Chem.* **1985**, *33*, 425–427.

(13) Nishida, R.; Acree, T. E. Isolation and characterization of methyl jasmonate from lemon (*Citrus limon* Burm.). *J. Agric. Food Chem.* **1984**, *32*, 1001–1003.

(14) Baker, T. C.; Nishida, R.; Roelofs, W. L. Close-range attraction of female oriental fruit moths to herbal scent of male hairpencils. *Science* **1981**, *214*, 1359–1361.

(15) Nishida, R.; Baker, T. C.; Roelofs, W. L. Hair pencil pheromone components of male oriental fruit moths, *Grapholitha molesta*. *J. Chem. Ecol.* **1982**, *8*, 947–959.

(16) Van Den Vool, H.; Kratz, P. D. A generalization of the retention index system including linear temperature programmed gas−liquid partition chromatography. *J. Chromatogr.* **1963**, *11*, 463–471.

(17) Kobayashi, A.; Kawamura, M.; Yakamoto, Y.; Shimizu, K.; Kubota, K.; Yamanishi, T. Methyl jasmonate in the essential oil of tea. *Agric. Biol. Chem.* **1988**, *52*, 2299–2303.

(18) Vick, B. A.; Zimmerman, D. C. Biosynthesis of jasmonic acid by several plant species. *Plant Physiol.* **1984**, *75*, 458–461.

(19) Tamogami, S.; Awano, K.; Kitahara, T. Analysis of the enantiomeric ratios of chiral components in absolute jasmine. *Flavour Fragrance J.* **2001**, *16*, 161–163.

(20) Blanch, G. P.; Ruiz del Castillo, M. L.; Herraiz, M. Evaluation of a transfer technique for direct coupling of reversed phase liquid chromatography and gas chromatography (RPLC-GC). *J. Chromatogr. A* **1998**, *818*, 77–83.

(21) Flores, G.; Blanch, G. P.; Ruiz del Castillo, M. L. Through oven transfer adsorption-desorption (TOTAD) interface for the analysis of aromatic samples by on-line reversed phase liquid chromatography−gas chromatography (RPLC-GC). *J. Sep. Sci.* **2008**, *31*, 1207–1214.

Received for review February 12, 2008. Revised manuscript received May 8, 2008. Accepted May 9, 2008. Financial assistance from the Ministerio de Educación y Cultura, Project AGL2007-65772, is gratefully acknowledged.

JF800425P