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Identification of Monomenthyl Succinate in Natural Mint Extracts by LC−ESI-MS−MS and GC−MS

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Fresh and dried mint leaves *Mentha piperita* (peppermint) and *Mentha spicata* (spearmint) were extracted in two different ways and the extracts investigated by high performance liquid chromatography−tandem mass spectrometry. All the ethanolic extracts prepared with Soxhlet apparatus were used in the identification of monomenthyl succinate as previously reported. The highest level was found in fresh spearmint leaves. The analysis of the extractions, prepared under mild conditions using a fluorinated solvent (HFC 134-a), confirmed the natural occurrence of monomenthyl succinate in the leaves, ruling out the hypothesis that this constituent could be an artifact of the Soxhlet extraction process. A method for identifying this compound in such a fluorinated solvent extract of mint leaf using preliminary esterification with diazomethane and then GC−MS is described.

KEYWORDS: ***Mentha piperita*; *Mentha spicata*; monomenthyl succinate; cooling agent; methyl menthyl succinate; HFC 134-a; fluorinated solvent**

INTRODUCTION

(−)-Menthol is widely used in food products because of its cooling effect. This sensation of freshness also finds many applications in oral health care products, cosmetics, pharmaceuticals, and the tobacco industry. The mechanism of the effect of menthol on receptors has been studied (*1*). This molecule exerts its effect on cold receptors, causes receptor depolarizations and increases nervous discharge by inhibiting the efflux of calcium from these cold receptors (*2*, *3*).

Menthol (*4*) is available from both natural and synthetic sources and is relatively inexpensive, but certain organoleptic characteristics of the molecule, namely a burning sensation and a mint flavor, are not suitable for some applications, hence the determination to develop near odorless physiological cooling substitutes. Many studies (*5*, *6*) have been carried out on the synthesis of molecules and correlating their structure and their physiological cooling activity. It was found that a wide range of different chemical classes impart this effect, e.g. menthyl esters (*7*−*9*), carboxamides (*10*), and menthane glycol ketals (*11*, *12*). Nowadays many synthetic cooling agents are commercially available, and a recent publication (*13*) analyzes this subject and compares their cooling strengths. Only three of these molecules have as yet been identified in nature, L-monomenthyl succinate, L-monomenthyl glutarate, and L-dimenthyl glutarate, which were recently reported by Hiserodt and co-workers (*14*) in natural extracts using high performance liquid chromatography−tandem mass spectrometry. L-monomenthyl succinate was found in *Lycium barbarum* and *Mentha piperita*, while the glutarates were identified in *Litchi chinesis*. A European patent (*15*) from the same research team claims the use of plant extracts containing monomenthyl succinate as a coolant in food and nonfood products as well as the methods for isolating these plant extracts.

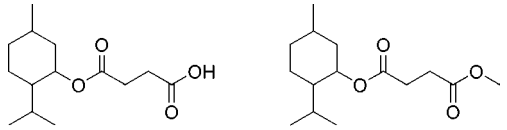

Figure 1. Structures of compounds.

Monomenthyl succinate, **1** (**Figure 1**), also known as Physcool is recognized as safe for use in foodstuff, and it has FEMA (Flavor and Extract Manufacturer's Association) GRAS (generally recognized as safe) status (FEMA 3810). The purpose of the present study was to confirm the natural occurrence of **1** in mint extracts in order to rule out the hypothesis that this ester was an artifact resulting from the extraction stage. Ethanolic Soxhlet extracts and 1,1,1,2-tetrafluoroethane (HFC 134a) extracts were prepared from dried and fresh leaves of *M. piperita* and *Mentha spicata* and investigated using LC−MS−MS. Since monomenthyl succinate had not been identified directly in mint leaf extract using GC−MS, due to its relatively low level abundance in nature and the lack of sensitivity of this analytical technique for such a molecule, diazomethane was used to derive this monoester and hence increase its volatility.

MATERIALS AND METHODS

Reagents. Monomenthylsuccinate was prepared in our laboratory using classical methods. The GC purity of this ester was higher than 99%. The melting point was 63 °C.

Methyl monomenthyl succinate, **2**, was synthesized from **1** as a starting material using a straightforward esterification method. This

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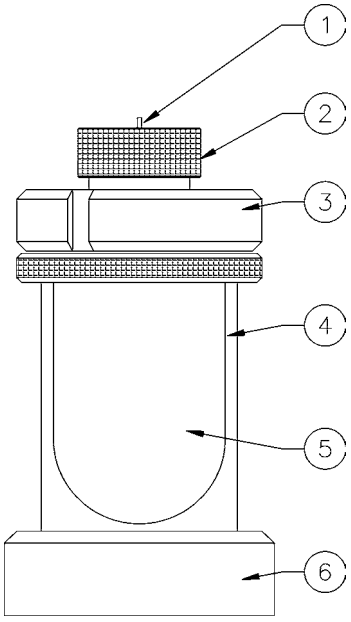

Figure 2. Extractor to carry out an extraction with HFC 134-a: (1) aerosol valve; (2) knurled finished nut; (3) castellated nut; (4) polycarbonate tube; (5) glass vessel; (6) stainless steel base.

standard substance was obtained with a purity higher than 99% in GC. Analytical characterizations were carried out by NMR, IR, MS.

Fresh and dried mint leaves (*M. piperita* and *M. spicata*) were purchased from the producer (R. C. B. International Ltd, Albany, OR). The leaves were grown in the northwestern state of Oregon in 2003 for dried leaves and 2004 for fresh leaves.

Ethanol and HFC 134-a were food-grade quality. HFC 134-a was obtained from Dupont De Nemours (Le Grand Saconnex, Genève, France; Dymel HFC 134-a/P).

Methanol (HPLC grade, Riedel De Haën) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Water used for LC mobile phase was purified with a Millipore Super Q apparatus (Millipore, Bedford, MA).

Diazomethane was prepared from Diazald purchased from Sigma-Aldrich. All solvents for synthesis procedures were commercially available.

Extraction Procedures. Two different kinds of extraction were carried out: a Soxhlet extraction with 96% ethanol and a soft extraction process using a fluorinated solvent, HFC 134-a. HFC 134-a has a boiling point of −26 °C, which facilitates handling and avoids energy consumption.

The vessel used to perform extraction with HFC 134-a is shown **Figure 2**. The leaves were placed in a small Pyrex extractor with an O-ring seal and a valve equipped with filter. A metallic adapter was screwed down by hand to close the system. An aerosol filled with HFC 134-a was inverted and attached to the valve. After the required quantity of liquefied gas was allowed to enter into the vessel, the aerosol was removed. Extraction was done at ambient temperature with vibrating, using a rotary shaker. The solvent was removed by simple vaporization and evaporation resulting in a concentrated solvent-free extract. The use of HFC-134-a improves the olfactory quality of the extract called Jungle Essence (Mane USA, Wayne, NJ).

Sample preparation was as follows: Four Soxhlet extractions were carried out using exactly the same method. Small pieces of fresh or dried leaves, *M. pipperita* or *M. spicata* (15.5 g), were extracted for 10 h with 96% ethanol (500 mL). The solvent was then filtered and concentrated under vacuum using a rotatory evaporator to obtain 2 mL of extract. The samples obtained are referred to as A (dried leaves of *M. piperita)*, B (dried leaves of *M. spicata*), C (fresh leaves of *M. piperita*), and D (fresh leaves of *M. spicata*).

Small pieces of dried leaves of *M. piperita* (28.0 g) were extracted for 3.5 h with HFC 134-a (192 g) and hence the extract E (0.23 g) was obtained**.** This method was repeated using small pieces of dried *M. spicata* leaves (24.2 g) and HFC 134-a. (222 g). The mixture was stirred for 5 h to obtain extract F (0.13 g). These samples were prepared in a 300-mL vessel.

For GC−MS investigation, a larger quantity of extract was prepared from dried leaves of *M. piperita* using HFC 134-a in a 3000-mL Jungle Essence extractor.

Small pieces of dried leaves (250 g) were extracted with solvent (1802 g) for 7 h to produce sample G (1.11 g). The extraction yield was 0.44%.

Synthesis Procedures. Preparation of the Authentic Sample of Succinic Acid Menthyl Ester Methyl Ester. Physcool (succinic acid monomenthyl ester) was treated with an excess of methanol in the presence of catalytic H_2SO_4. The mixture was heated under reflux until completion of the reaction, as monitored by thin-layer chromatography. After cooling, most of the methanol was evaporated and the residue was diluted with *tert*-butyl methyl ether and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and filtered, and the solvents were evaporated. The crude product was then purified by distillation under reduced pressure (bp 100−102 °C/ 0.12 mbar).

The derivatization of **1** to **2** was easily characterized by analytical data: ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.72 (d, 3, J = 6.9 Hz, $-CH_2-CH(CH_3)CH_2-$), 0.8−1.15 (m, 3, $-CH_2CH(CH_3)CH_2-$, CH_2), 0.86 (d, 3, J = 7 Hz, $CH(CH_3)_2$), 0.87 (d, 3, J = 6.4 Hz, $CH(CH_3)_2$), 1.25−1.55 (m, 2, $-CH_2-$), 1.25−1.72 (m, 2, $-CH_2-$), 1.75−1.9 (m, 1, $CHCHO$), 1.9−2.05 (m, 1, $CH(CH_3)_2$), 2.6 (s, 4, $CO(CH_2)_2CO$), 3.7 (s, 3, OCH_3), 4.67 (td, 1, J = 4.4 Hz, J = 10.9 Hz, $CHCHO$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 16.7 ($-CH_2CH(CH_3)CH_2-$), 21.1 and 22.4 ($CH(CH_3)_2$), 23.8 ($-CH_2-$), 26.6 ($-CH_2CH(CH_3)CH_2-$), 29.4 ($COCH_2CH_2COOMe$), 29.9 ($COCH_2CH_2COOMe$), 31.7 ($CH(CH_3)_2$), 34.6 ($-CH_2-$), 41.2 (CH_2CHO), 47.4 ($CHCHO$), 52.1 (OCH_3), 74.9 ($CHCHO$), 172.1 (CO), 173.1 (CO); IR (neat) 2955 (s), 2932 (s), 2871 (m), 1735 (vs), 1456 (m), 1438 (m), 1368 (m), 1264 (m), 1215 (s), 1165 (s), 990 (m), 845 (w) cm^{-1}; MS [m/z (EI)] 270 (M+), 155 (7), 139 (10), 138 (32), 133 (10), 123 (19), 115 (100), 96 (11), 95 (48), 83 (14), 82 (12), 81 (32), 67 (10), 59 (13), 55 (39), 41 (11).

Treatment of the Peppermint Leaves Jungle Essence Extract with Diazomethane. The Jungle Essence extract (G) was diluted with *tert*-butyl methyl ether and treated twice with a cold 5% aqueous sodium carbonate solution. The combined aqueous basic phases were then acidified with a cold 10% aqueous HCl solution and extracted twice with *tert*-butyl methyl ether. An excess of diazomethane generated from Diazald (*16*, *17*) was then bubbled directly into the above extract of the Jungle Essence. Excess dissolved diazomethane was removed using acetic acid before analysis. After treatment, the solvent was evaporated using a flow of nitrogen until 0.5 mL remained.

Diazald was placed in a flask with ethanol and *tert*-butyl methyl ether as the solvent, a solution of 37% aqueous KOH was added dropwise, and a stream of nitrogen was applied to force the generated diazomethane into the extract solution.

Analytical Conditions (LC−MS−MS). Chromatographic Conditions. The LC system was an Agilent Technologies model 1100 equipped with an autosampler model G1313A.

The analytes were separated on a 150 × 2.1 mm i.d., 5 μm, Zorbax SB-C18 column obtained from Agilent Technologies (Palo Alto, CA). The mobile phase was similar to those used by Hiserodt et al. (*14*): a binary mixture of A (water with 10 mM NH_4OAc) and B (methanol with 10 mM NH_4OAc). The flow rate was 0.2 mL/min.

For the analysis of the first four samples (A, B, C, and D), the gradient was programmed as follows: linear from 90% to 0% A in 10 min, remaining at 100% B for 15 min, and then returning to 90% A in 2 min. This composition was kept constant for a further 8min. The analytical column was kept at a constant temperature of 30 °C.

This gradient was slightly modified for the two last samples (E and F), which were investigated some months later: linear from 90% to 0% A in 10 min, remaining at 100% B for 30 min, and then returning to 90% A in 5 min. In this case, the column was kept at 20 °C. The system was allowed to equilibrate for 20 min before each injection. The sample injection volume was 20 μL.

Mass Spectrometry Conditions. The LC system was coupled to a triple quadrupole mass spectrometer API III Plus Sciex (PE Sciex,

Concorde, Ontario, Canada) equipped with an electrospray ionization (ESI) interface operated both in positive and negative ionization mode.

Full-scan MS spectra and MS−MS spectra for **1** were obtained by infusion of the standard in methanol with 3 mM of NH_4OAc. The analyte was detected by selected reaction monitoring (SRM) and the analytical conditions were optimized to achieve the highest sensitivity. The precursor and product ion for the analyte, together with the applied collision energy are

ionization mode	ISV injection source voltage, V	OR orifice, V	transition SRM	collision energy, eV
ESI+	5000	60	257 → 119	10
ESI–	–4800	–65	255 → 99	14

Preparation of Extracts for Quantitative Analysis. The extracts were diluted in methanol. For each sample, the best dilution rate and the more sensitive ionization mode was chosen.

The quantification is done by external calibration. In these extracts, the matrix is complex and reliable quantification requires spiking samples. The different extracts were analyzed in triplicate with monomenthyl succinate standard addition at two different concentrations.

To summarize this standard addition procedure, samples A and B were analyzed, in negative ion mode, after 50% dilution without spiking and at the same dilution after spiking with 0.52 and 1.30 μg/mL of **1**. Sample C was analyzed in negative ion mode after 25% dilution without spiking and at the same dilution after spiking with 0.52 and 1.04 μg/mL of **1**. Sample D was analyzed in positive ion mode after 10% dilution without spiking and at the same dilution after spiking with 0.52 μg/mL of **1**. Samples E and F were analyzed in positive ion mode after 50% dilution without spiking and at 25% dilution after spiking with 0.52 and 1.30 μg/mL of **1**.

Analytical Conditions (GC−MS). GC−MS analyses were performed using an Agilent model 6890 equipped with a 60m × 0.25 mm i.d., 0.25 μm, HP1 fused silica column and interfaced with an quadrupole MS model 5973 (Agilent, Palo Alto, CA). The column was programmed from 150 to 280 °C at an increase rate of 2 °C/min. The injection temperature was 250 °C. The injection mode was splitless and the injection volume was 1 μL. Helium was used as the carrier gas at a constant flow rate of 1.1 mL/min. The mass spectrometer was operated at 70 eV. Data were acquired in scan mode over the range 35−500 amu and in SIM mode (*m*/*z* 95, 115, and 138).

The identification of methyl menthyl succinate, **2**, was based on comparing retention data and mass spectra with those of the reference.

NMR spectra were obtained on a 200 MHz Brücker spectrometer and IR on a FTS Biorad 155.

RESULTS AND DISCUSSION

The monomenthyl succinate quantification by LC−MS−MS was validated for the following parameters: limit of detection (LD), limit of quantification (LQ), linearity, exactitude, and repeatability. LD and LQ were calculated according to ref *18*, referring to the variations of a "blank" signal obtained by injection of 20 μL of methanol.

For samples A−D in ESI+ mode, LD = 0.03 μg/mL, LQ = 0.06 μg/mL, the linearity of the response was established in the concentration range 0.10−1.04 μg/mL with $R^2 = 0.9992$, the repeatability was 2.6−7.9%, and the exactitude was 2.6−11.6% (minimum and maximum values for the calibration range).

For samples A−D in ESI− mode, LD = 0.02 μg/mL, LQ = 0.05 μg/mL, the linearity of the response was established in the concentration range 0.05−1.04 μg/mL with $R^2 = 0.9993$, the repeatability was 2.0−6.6%, and the exactitude was 2.4−11.1%.

Table 1. Analysis of Extract C

experiment	determined concn of the spiked samples, 0 μg/mL	0.52 μg/mL	1.04 μg/mL	t	*T*, %	$C_{initial}$, μg/mL
1	<0.05	0.44	0.62	2.889	65	0.75
2	<0.05	0.48	0.66	2.889	65	0.87
3	<0.05	0.48	0.65	3.059	67	0.95

Table 2. Concentrations of Monomenthyl Succinate in the Investigated Extracts

sample	% dilution	ESI ionization	[**1**], μg/mL
A	50	–	1.2
B	50	–	1.5
C	25	–	3.4
D	10	+	11.7
E	25	+	0.8
F	25	+	0.8

For the second series of measurements (samples E and F), in ESI+ mode, the LD and QD were respectively 0.01 and 0.02 μg/mL. The linearity of the method was investigated for **1** in the range 0.105−1.05 μg/mL with $R^2 = 0.9997$.

The monomenthyl ester **1** was identified in mint extracts based on retention time and fragmentation correlations with authentic external reference.

An example of the chromatogram is given in **Figure 3**.

The peak eluted at 20.4 min in the upper chromatogram (**Figure 3A**) is **1**, as confirmed by the increase in the signal of the spiked samples (**Figure 3B,C**). The concentration of **1** in the nonspiked sample could not be directly quantified, as it was less than the lowest limit of the linearity range. The analysis of the spiked samples allowed the determination, from the experimental concentrations, of the percentage signal suppression (*T*,%) and the initial concentration of monomenthyl succinate in the different samples

$$C_{\text{initial}} + \text{addition} = C_{\text{experimental}}t$$

where *t* is the correction factor of suppression phenomenon and $T = (1 - 1/t) \times 100$.

The quantitative results for sample C are detailed in **Table 1**. The experimental concentrations of **1** in each solution are given. Among all the samples investigated, only the concentration of **1** in extract D without spiking was quantifiable; hence, in this case, only one standard addition was carried out.

Concentrations of **1**, corrected from recoveries and dilution in the different extracts, are detailed in **Table 2**. Monomenthyl succinate was detected in all samples. Extract D contains the highest level of **1**: the evaluated (100% recovery in extraction step) quantity in the fresh leaves (*M. spicata*) was approximately 1500 ppb. The results obtained for samples C and A relating to *M. piperita* were in the same range as those obtained by Hiserodt et al. (*14*): 150−450 ppb in the leaves and 600 ppb previously reported.

The extracts prepared with HFC 134-a were less rich in monomenthyl succinate due to the low polarity of this solvent compared to ethyl alcohol. Quantitative data showed the level of **1** in sample E to be approximately 800 ppb, which corresponds to 7 ppb in the dried leaf. HFC 134-a is known for its high selectivity and low boiling point, which facilitates its

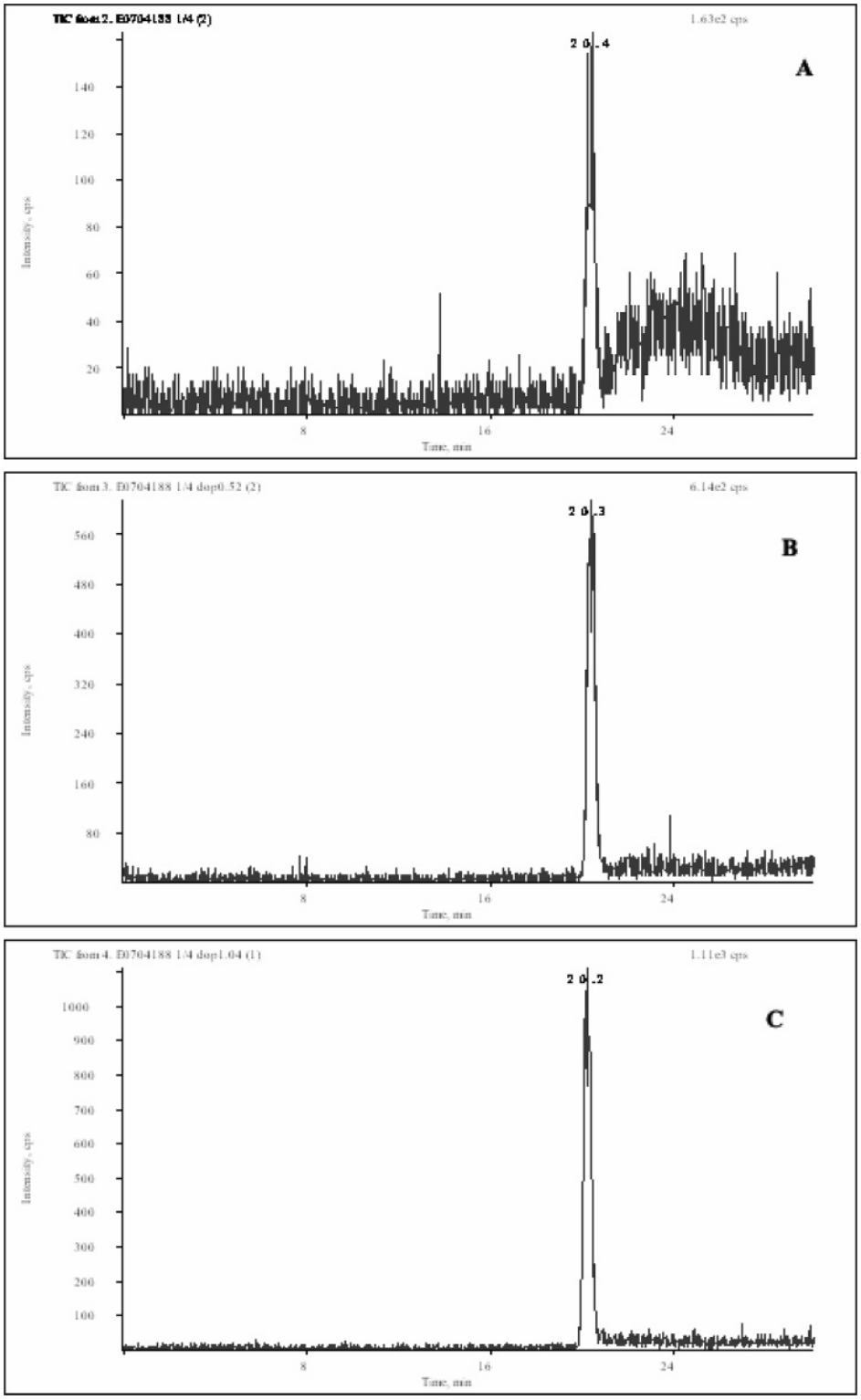

Figure 3. Chromatograms for sample C: (**A**) 25% dilution; (**B**) 25% dilution and spiked with 0.52 μg/mL **1**; (**C**) 25% dilution and spiked with 1.04 μg/mL **1**.

use at low pressure. This solvent is extremely suitable for the extraction of natural products, as it produces high-quality extracts that cover an olfactory spectrum similar to that of the raw material. No artifact is produced because the extraction is carried out at low-temperature, avoiding hydrolysis, transesterifications, and thermolysis of some molecules: during the extraction process, the temperature is about 15 °C and never exceeds 18 °C. This solvent, which is also used in automobile car conditioners, is accepted by the laws and regulations currently in force to prepare extracts for food products (88/344/CEE).

The GC−MS analysis of peppermint dried leaves HFC 134-a extract (sample G) through direct injection did not indicate the presence of **1**, despite a very dense fingerprint area. This could be explained by the low concentration of the component, the presence of a free acid function, and the possible interaction

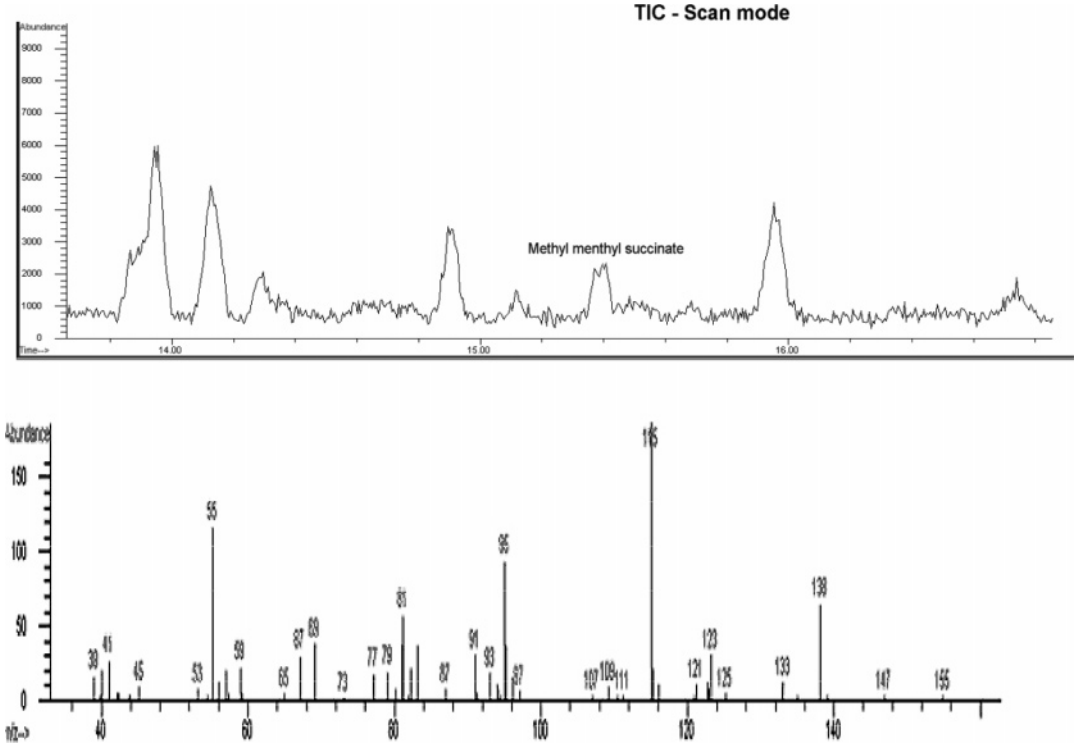

Figure 4. Partial chromatogram (HP1) showing the elution of methyl menthyl succinate and mass spectra of this compound.

with other compounds. This extract contains large amounts of aliphatic monomenthyl esters.

The GC−MS injection of the Jungle Essence extract after treatment with diazomethane permitted the observation of the target compound both in scan and SIM modes. **Figure 4** shows a part of the total ion chromatogram in scan mode with the elution of the diester. Its mass spectra is very slightly contaminated by another compound. The SIM approach, confirms the occurrence of the molecule.

Identification was based on comparison between the mass spectra and the retention time under the same experimental conditions. The retention time of the methyl menthyl ester was 15.4 min; the retention indices were t_R= 1742 on HP1 and t_R = 2317 on polar INNOWAX.

Quantitative data was obtained by an external standard method. A value of 10 ppm in the extract was found which, in relation to the quantity of raw material used, equates to a result of 20 ppb in the dried leaf. This value should be compared with 7 ppb found in sample E because the same leaves were used. For sample E, quantification was done by LC and for sample G treated, quantification was carried out using GC−MS. The levels were found to be in the same range. Any differences could be explained by the various size and shape of the extractors, leading to a change in the efficiency of the extraction.

This work confirms the previous publication of Hiserodt et al. (*14*) on the identification of **1** in natural plant extracts. The use of a solvent such as HFC 134-a demonstrates the fact that monomenthyl succinate is not an artifact of the extraction process. This result is supported by a personal communication of Hirerodt and co-workers with the International Organization of Flavor Industry, in that they detected **1** in a spearmint/peppermint residue diluted in methanol.

The transformation of **1** into its more volatile methyl ester **2** allows detection by GC−MS.

Monomenthyl succinate is a flavor compound known for its cooling effect. It is widely used as Physcool in oral care products and chewing gum and it was important to confirm the natural occurrence of this molecule.

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LITERATURE CITED

(1) Hensel, H.; Zotterman, Y. The effect of menthol on the thermoreceptors. *Acta Physiol. Scand.* **1951**, *24*, 27−34.
(2) Schafer, K.; Braun, H. A.; Hensel, H. Static and dynamic activity of cold receptors at various calcium levels. *J. Neurophysiol.* **1982**, *47*, 1017−1027.
(3) Schafer, K.; Braun, H. A.; Isenberg C. Effect of menthol on cold receptor activity. *J. Gen. Physiol.* **1986**, *88*, 757−776.
(4) Clark, G. S. *Menthol. Perfumer Flavorist* **1998**, *23*, 33−46.
(5) Watson, H. R.; Hems, R.; Rowsell, D. G.; Spring D. J. New compounds with the menthol cooling effect. *J. Soc. Cosmet. Chem.* **1978**, *29*, 185−200.
(6) Ottinger, H.; Soldo, T.; Hofmann, T. *J. Agric. Food Chem.* **2001**, *49*, 5383−5390.
(7) Mane, J. M.; Ponge, J. L. Coolant compositions. US Patent 5,725,865, 5,843,466, and 5,752,529 to V. Mane Fils S. A. 1998.
(8) Pelan, B. M. C.; Vermeulen, C. J.; Wemmenhoven, B. WO 2003/043431 to Unilever N. V.
(9) Grainger, B. T.; Kleinhenz R. J.; Christensen G. W. US Patent 6,365,215 to International Flavors and Fragrances Inc., 2002.
(10) Watson, H. R.; Roswell D. G.; Spring, D. J. US Patent 4,150,052 to WilkinsonSword Ldt, 1979.
(11) Grub, H.; Pelzer, R.; Hopp, R.; Emberger, R.; Bertram, H. J. US Patent 5,266,592 to Haarmer and Reimer GmbH, 1993.

(12) Greenberg, M. J. Preparation of menthone ketals for use as flavour enhancers. EP 485170 to Wrigley, 1991.
(13) Erman, M. Progress in physiological cooling agents. *Perfumer Flavorist* **2004**, *29*, 34−50.
(14) Hiserodt, R. D.; Adedeji, J.; John, T. V.; Dewis, M. L. *J. Agric. Food Chem*. **2004**, *52*, 3536−3541.
(15) Hiserodt, R. D.; Adedeji, J.; John, T. V., Eckert, M. EP 1 518 466 A1, to International Flavors and Fragrances Inc. 2004.
(16) De Boer, Th. J.; Backer, H. J. Diazomethane. *Organic Syntheses;* Wiley: New York, 1963; Collect. Vol. 4, pp 250−253.
(17) Lombardi, P. A rapid, safe and convenient procedure for the preparation and use of diazomethane. *Chem. Ind.* **1990**, 708.
(18) Caporal-Gautier, J.; Nivet, J. M.; Algranti, P. Guide de validation analytique. S. T. P. *Pharma Pratiques* **1992**, *2*, 205−226.

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