Identification of Monomenthyl Succinate, Monomenthyl Glutarate, and Dimenthyl Glutarate in Nature by High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Menthol, menthone, and other natural compounds provide a cooling effect and a minty flavor and have found wide application in chewing gum and oral care products. Monomenthyl succinate, monomenthyl glutarate, and dimenthyl glutarate provide a cooling effect without the burning sensation associated with menthol. Additionally, because they do not have a distinct flavor, they can be used in applications other than mint flavors. Because these menthyl esters have not been reported in nature, we undertook to identify a natural source for these cooling compounds. Using high performance liquid chromatography-tandem mass spectrometry, monomenthyl succinate was identified in *Lycium barbarum* and *Mentha piperita*, and monomenthyl glutarate and dimenthyl glutarate were identified in *Litchi chinesis*. The identifications were based on the correlation of mass spectrometric and chromatographic retention time data for the menthyl esters in the extracts with authentic standards which resulted in a 99.980% confidence in the identifications.

KEYWORDS: *Lycium barbarum*; *Mentha piperita*; *Litchi chinesis*; monomenthyl succinate; monomenthyl glutarate; dimenthyl glutarate; high performance liquid chromatography-tandem mass spectrometry (LC/MS/MS); Nature Identical (NI)

INTRODUCTION

The most widely known cooling compound in flavor chemistry is menthol. Menthol is of botanical origin, occurring in mint, specifically in mint oils from *Mentha piperita* (peppermint) and *M. arvensis* (cornmint). Natural cooling compounds such as menthol, menthone, menthyl acetate, and peppermint oil have been used for many years to provide a cooling effect, a minty flavor, and the sensation of nasal clearing. These cooling compounds act on the common chemical sense (*1*), which has been termed chemesthesis (*2*), and are responsible for chemoreception (*3*). The value of the synthetic cooling compounds discussed here is that they give enhanced cooling without the burning sensation associated with menthol. Additionally, because they possess few properties associated with olfaction, they impart little or no flavor and can be used in applications other than mint flavors.

Historically, chewing gum, mint, and oral care products were the main end application for cooling compounds. In these historical applications, the noncooling effects exhibited by the compounds such as olfaction and irritation are acceptable, and they remain the primary use. However, the desire to use cooling compounds in other applications, along with the desire to make

new and traditional products with flavors other than mint, has resulted in the commercialization of a number of synthetic cooling compounds.

Since the original work of Watson and co-workers (*4*, *5*), synthetic compounds providing the physiological sensation of cooling have been used in the flavor industry. The current view regarding the physical properties of cooling compounds required for activity was reported by Watson et al. (*5*). Additionally, Eccles (*6*) has published a review article on menthol and related cooling compounds.

A selection of synthetic cooling compounds with commercial significance include L-menthyl lactate, **1**; 3-(menthoxy) propane-1,2-diol, **2**; *N*-ethyl-*p*-menthane-3-carboxamide, **3**; 2-isopropyl-*N*,2,3-trimethylbutanamide, **4**; L-monomenthyl succinate, **5**; L-monomenthyl glutarate, **6**; and L-dimenthyl glutarate, **7** (**Figure 1**). The use of monomenthyl succinate in cooling compositions has been disclosed by Mane and Ponge (*7*), and there are several subsequent patents, for example, Grainger et al. (*8*), disclosing the use of several cooling compounds in combination in both flavor and fragrance applications.

In the European Union and in some other countries around the world, flavor chemicals fall into three classifications: Natural, flavor chemicals obtained using a natural process or of natural extraction or distillation from material of vegetable or animal origin; Nature Identical (NI), flavor chemicals of

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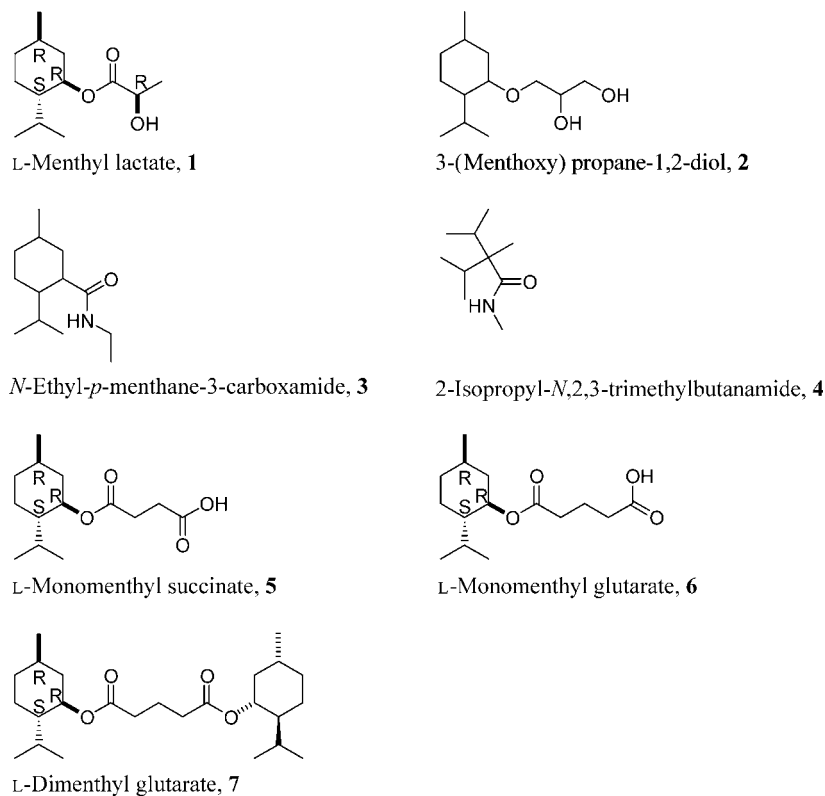
L-Menthyl lactate, **1**   3-(Menthoxy) propane-1,2-diol, **2**

N-Ethyl-*p*-menthane-3-carboxamide, **3**   2-Isopropyl-*N*,2,3-trimethylbutanamide, **4**

L-Monomenthyl succinate, **5**   L-Monomenthyl glutarate, **6**

L-Dimenthyl glutarate, **7**

Figure 1. Structures of compounds.

synthetic origin but indistinguishable from a substance naturally present in material of vegetable or animal origin; and Artificial, flavor chemicals of synthetic origin with no known natural occurrence.

NI is of key importance because NI labeling status is greatly preferred over artificial labeling in the European Union. This is particularly true in some product categories, of which beverages are a leading example. Because cooling compounds, in general, deliver sensory perception best in polar systems, their use in beverages is an attractive final application.

The purpose of this investigation was to identify a natural source of **5**, **6**, and **7**. An exhaustive review of the literature showed that none of these menthyl esters were reported as occurring in nature. However, Kim et al. (*9*) reported that menthol and succinic acid, precursors to **5**, were identified in *Lycium chinesis* P. Mill. (Chinese desert-thorn) leaves. Menthol was identified in *Litchi chinesis* Sonn. (lychee) by Johnston et al. (*10*) and glutaric acid in the same plant by Chan et al. (*11*). On the basis of this information, the possibility was considered that **6** and **7** might be present in *L. chinesis* and that **5** might be present in *Lycium barbarum* L. (wolfberry) leaves, a close relative of *L. chinense*. This could be through a metabolic pathway or as a result of natural drying. *L. barbarum* dried fruit and *M. piperita* L. (peppermint) dried leaves were also analyzed for **5** because they are readily available and are a food source.

L. chinesis (lychee) is a subtropical fruit native to China and belongs to the family Sapindaceae. Lychee is a delicacy in many Asian countries. However, its commercial importance and popularity among consumers has continued to expand to markets outside Asia. In North America, trade in fresh lychee has shown a steady increase. The availability of the fresh fruit is limited to the summer months, consequently, there is a global demand for processed fruit (*12*). *L. barbarum* (wolfberry) is also a native of China and belongs to the family Solanaceae. The fruits are red when ripe and are commonly used in Asia along with licorice to maintain good health (*13*).

M. piperita (peppermint) is a native of Europe and belongs to the mint family, Lamiaceae. Peppermint is a usually sterile hybrid from water mint (*M. aquatica*) and spearmint (*M. spicata*). In Britain, as in the rest of Europe, true peppermint is used almost exclusively for confectioneries and sweet liquors, where its cooling and fresh pungency balances the sweetness of the sugar. For all such purposes, the usage of pure essential oil is preferred to avoid the astringent to bitter notes of the peppermint leaves (*14*).

MATERIALS AND METHODS

Reagents. Compound **5** (CAS 77341−67−4) was obtained from International Flavors & Fragrances Inc. (South Brunswick, NJ) and was ≥98% pure by GC. Compound **6** (CAS 220621-22-7) was also obtained from International Flavors & Fragrances Inc. (South Brunswick, NJ) and consisted of 60−70% **6**, 30−40% **7**, and ≥97% **6** and **7** by GC. *L. barbarum* dried fruit and *L. chinesis* dried fruit were purchased from a local Asian food store. *M. piperita* dried leaves were obtained from Plant It Herbs (Athens, Ohio).

Extraction Procedures. *L. barbarum* dried fruit was frozen overnight at −25 °C and then powdered in a blender. The powdered fruit (205 g) was extracted in a glass Soxhlet extractor using 800 mL of 95% ethyl alcohol. The extraction continued for 13.5 h over a period of 2 days. The extract was filtered and the filtrate concentrated to 50 g in a rotary evaporator under vacuum at 55 °C. The filtrate, on keeping overnight, developed a sediment that was filtered and discarded. The remaining filtrate was concentrated to yield 25.5 g of extract.

M. piperita dried leaves were extracted using the same procedure as for *L. barbarum* dried fruit. However, because of the difference in the bulk density compared to the *L. barbarum* dried fruit and equipment size limitations, a smaller sample was extracted. The powdered leaves (115 g) were extracted to produce 15 g of extract.

Unbleached all purpose flour (200 g) was spiked with 3.9 mg of menthol and 5.7 mg succinic acid, thoroughly blended, and extracted using the same procedure as that for *L. barbarum* dried fruit. This procedure yielded 7 g of extract. The extract was analyzed as-is.

The rough, woody husks of *L. chinesis* dried fruit were removed and discarded and the sticky fruit cut away from the nut. The dried
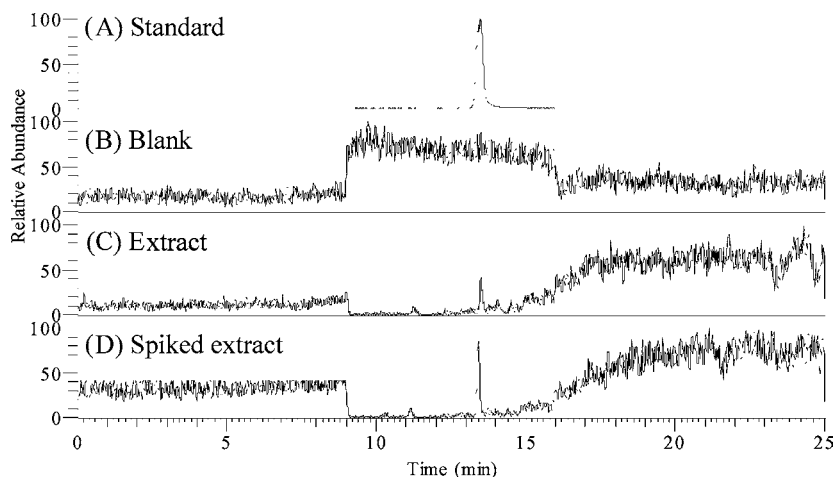
Figure 2. LC/MS/MS chromatograms of (**A**) 1.78 μg/mL monomenthyl succinate standard, (**B**) Blank, (**C**) 0.54 g/mL *M. piperita* dried leaf extract, and (**D**) 250 μL of *M. piperita* dried leaf extract (0.54 g/mL) spiked with 2 μL of monomenthyl succinate (178 μg/mL).
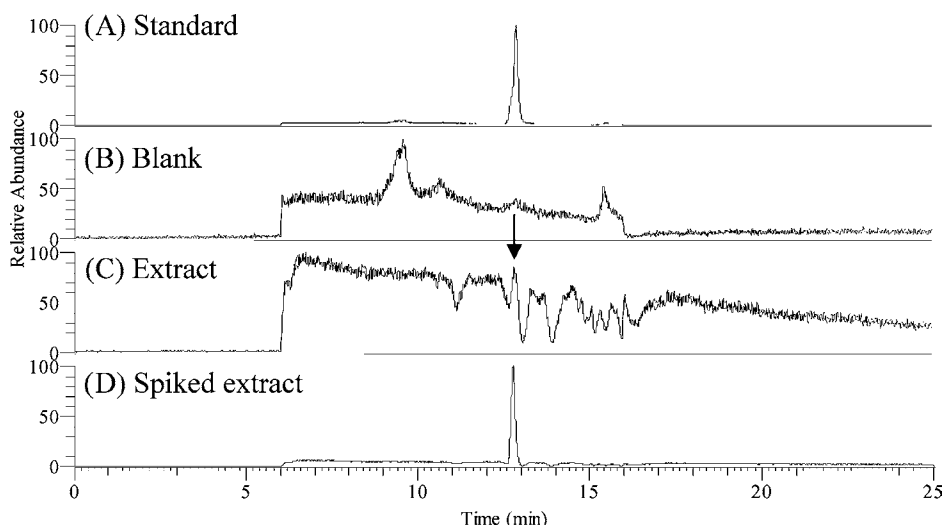

Figure 3. LC/MS/MS chromatograms of (**A**) 0.68 μg/mL monomenthyl succinate standard, (**B**) blank, (**C**) flour extract analyzed as-is (**D**) 250 μL of flour extract spiked with 1 μL of monomenthyl succinate (68 μg/mL).

fruit (600 g) was ground under liquid nitrogen in a precooled 2 M100 mill (Brinkmann, Westbury, NY). To keep the ground fruit from sticking and clumping when it thawed, Celite was added to the still frozen powdered fruit and mixed thoroughly. The fruit-Celite mixture was extracted with 2 L of methylene chloride using a Soxhlet extractor for 3 h at 40 °C. The extract was distilled to 150 mL, transferred to a Zymark concentrator tube, and concentrated to 50 mL on a TurboVap concentrator (Zymark Corporation, Hopkinton, MA). The methylene chloride layer was dried over 10 g of anhydrous sodium sulfate and then concentrated to 0.5 mL. The extract was analyzed as-is.

Instrument Conditions. A TSQ 7000 triple quadrupole mass spectrometer with an API2 source was interfaced to a SpectraSystem P4000 gradient pump (Thermo Electron Corporation, San Jose, CA). The sample components were separated on a 250- × 2.1-mm, 5-μm, Zorbax SB−C18 analytical column (Agilent Technologies, Palo Alto, CA) using gradient elution with a mobile phase consisting of H_2O (10 mM NH$_4$OAc) with CH$_3$OH (10 mM NH$_4$OAc) as the organic modifier. The gradient for the analysis of **6** and **7** was 40−100% CH$_3$OH (10 mM NH$_4$OAc) in 15 min with a 15 min hold at 100% CH$_3$OH (10 mM NH$_4$OAc). The gradient for the analysis of **5** was 10−100% CH$_3$-OH (10 mM NH$_4$OAc) in 10 min with a 15 min hold at 100% CH$_3$OH (10 mM NH$_4$OAc). The flow rate was 0.2 mL/min, the column temperature was ambient, and a 20-μL sample injection was made using a SpectraSystem AS3000 autosampler (Thermo Electron Corporation, San Jose, CA).

The mass spectrometer was operated in the SRM mode using APCI in both the positive and negative ion mode. The following transitions were monitored: **5**, *m/z* 255/99 at +20 V, −APCI; **6**, *m/z* 269/113 at +20 V, −APCI; **6**, *m/z* 271/133 at −10 V, +APCI; and **7**, *m/z* 409/271 and 409/133 at −13 V, +APCI. The collision cell (Q2) was pressurized to 2 mT with argon (Ar). The sheath gas (N$_2$) and auxiliary gas (N$_2$) were 60 psi and 20 units, respectively. Q1 and Q3 were calibrated and tuned using MRFA/myoglobin.

Preparation of Extracts for Quantitation by Standard Addition. *M. piperita* dried leaf extract (2.37 g) was diluted with 2.0 mL CH$_3$-OH (spl. concentration = 0.54 g/mL assuming d_{extract} = 1 g/mL). A 400-μL aliquot of this solution was added to each of four vials, which were subsequently spiked with 0, 3.9, 7.8, and 11.7 ppm **5**.

L. barbarum dried fruit extract (3.05 g) was diluted with 3.0 mL CH$_3$OH (spl. concentration = 0.50 g/mL assuming d_{extract} = 1 g/mL). A 200-μL aliquot of this solution was added to each of four vials, which were subsequently spiked with 0, 1.1, 2.2, and 3.2 ppm **5**.

A 200-μL aliquot of flour extract was spiked as-is with 0, 0.054, 0.11, and 0.16 ppm **5**.

RESULTS AND DISCUSSION

Compound **5** was identified in extracts of *M. piperita* dried leaves and *L. barbarum* dried fruit, and **6** and **7** were identified in an extract of *L. chinesis* dried fruit by high performance liquid chromatography-tandem mass spectrometry (LC/MS/MS). The menthyl esters were identified in the extracts based on retention time correlation with an authentic external standard and their
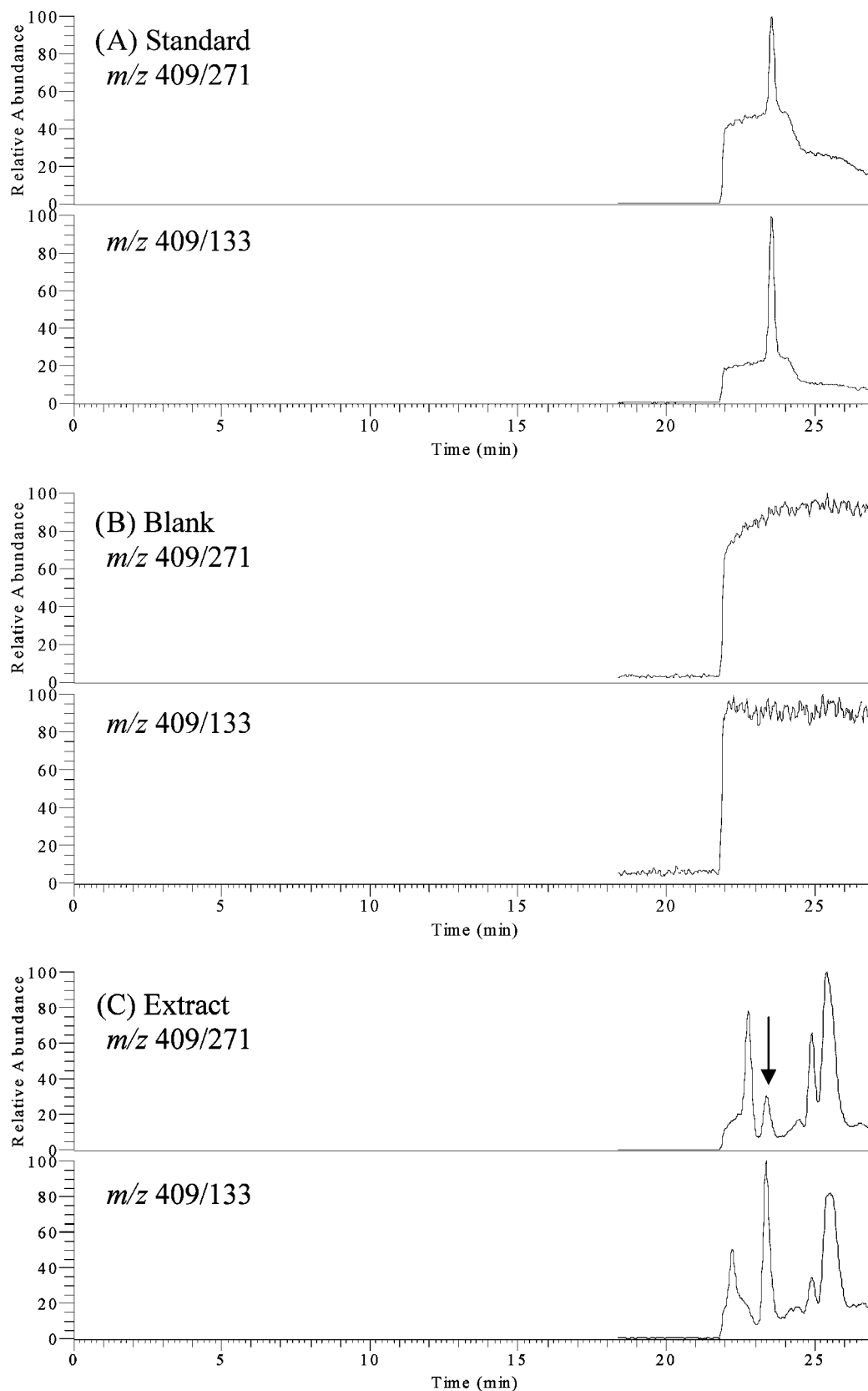
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Figure 4. LC/MS/MS chromatograms of (**A**) 0.13 μg/mL dimenthyl glutarate standard, (**B**) Blank, and (**C**) *L. chinesis* dried fruit extract analyzed as-is.

spectrometric properties. Components in the plant extracts were first separated by reverse phase LC based on partitioning between the eluent and the hydrophobic stationary phase, followed by mass spectrometric analysis, so that only components meeting specific criteria, based on their mass spectra, were detected. Compounds **5** and **6** were identified using APCI in

the negative ion mode and single reaction monitoring (SRM). Compound **7** was identified in the positive ion mode, using the same technique, because **7** did not respond in the negative ion mode. SRM is similar to acquiring a full scan product ion spectrum, but the full spectrum is not obtained. Rather, the deprotonated precursor ions for **5** and **6** and the protonated

precursor ion for **7** were selected in the first quadrupole region (Q1) of a triple quadrupole mass spectrometer. They were fragmented in the collision cell (Q2) that was pressurized with 2 mT Ar. The third quadrupole region (Q3) was set up to focus the principal fragment ion(s) for **5**, **6**, or **7** for detection. This resulted in the high degree of sensitivity and selectivity that was necessary for the analysis of trace compounds in complex matrixes and is often the only analytical approach where the confidence level for a positive identification is very high. Porter et al. (*15*) evaluated European Commission criteria for LC/MS operated in the SRM mode (LC/MS/MS). They concluded that SRM with a single precursor/product ion pair yielded a unequivocal identification of the analyte in question (99.980% confidence) when supported by retention time data from an external standard.

For instance, in the example of **5**, for a plant compound other than **5** to be misidentified as **5**, it must be acidic, have a molecular weight equal to 256, fragment to produce a product ion at m/z 99 and have the same retention time as an authentic standard of **5** using the same LC conditions described earlier. The same general considerations apply to **6** and **7**. In addition to this, *L. barbarum* and *M. piperita* extracts were spiked with **5** at an appropriate level, so that if, by chance, another compound with identical mass spectrometric properties but with a slightly different retention time was detected, it would appear as a shoulder. Additionally, all purpose white flour was spiked with menthol and succinic acid and carried through the extraction process to verify that **5** was not an artifact of the extraction process. The identification of **6** in *L. chinesis* was further verified by analyzing the extract in the positive mode of APCI in addition to the negative ion mode. This significantly increased the probability of a correct identification. Specificity was increased for **7** by monitoring two product ions in the positive ion mode.

Identification of Monomenthyl Succinate and Monomenthyl Glutarate in the Plant Extracts. The identification of **5** and **6** in the plant extracts was accomplished by setting Q1 to m/z 255 or 269 for the deprotonated precursor ion for **5** or **6** respectively. Q3 was set to m/z 99 or 113 for the respective product ion. A divert valve was used so that only components eluting in the retention time window of **5** or **6** would enter the mass spectrometer. Compound **6** was also identified, in the positive ion mode, by monitoring the m/z 271/133 transition. **Figure 2C** shows a chromatogram of **5** in *M. piperita* dried leaf extract (0.54 g/mL). **Figure 2A** shows a chromatogram of a standard (1.78 μg/mL) and **Figure 2D** a chromatogram of *M. piperita* dried leaf extract spiked with **5**. The blank (**Figure 2B**) was an injection of sample solvent that was made immediately before sample analysis to demonstrate that **5** detected in the extract was not the result of carry over from the previous injection of the standard.

All purpose white flour was spiked with menthol and succinic acid equivalent to four times the level of **5** detected in the *M. piperita* dried leaf extract and 25 times the level in the *L. barbarum* dried fruit extract. The purpose of this experiment was to show that **5** detected in *M. piperita* and *L. barbarum* was not an artifact of the extraction process. The spiked flour was extracted and analyzed using the same procedures as that for the plant extracts. The results are shown in **Figure 3**. **Figure 3C** shows the possible detection of **5**, but quantitative analysis showed this to be significantly lower than the level detected in the *M. piperita* or *L. barbarum* extracts.

Quantitative data obtained by the standard addition method showed the level of **5** in the *M. piperita* dried leaf extract to be 5 ppm ($\equiv$600 ppb in the dried leaf) and the level in the *L.*

barbarum dried fruit extract to be 0.8 ppm ($\equiv$100 ppb in the dried fruit). The level of **5** in the flour extract was 0.003 ppm. This is 1700 times less than the level found in the *M. piperita* extract and 270 times less than the level found in the *L. barbarum* extract. Although it is possible that some of **5** detected in the *M. piperita* and *L. barbarum* extracts resulted from the extraction process, clearly the majority detected was from the plant extracts themselves. Linear regression analysis of the standard addition data resulted in $y = 0.35x + 1.63$ ($R^2 = 0.992$) and $y = 268x + 212$ ($R^2 = 0.985$) for **5** in the *M. piperita* and *L. barbarum* extracts, respectively, and $y = 441306x - 1316$ ($R^2 = 0.982$) for **5** in the spiked flour extract. The level of **6** in the extract of *L. chinesis* dried fruit was 0.9 ppm ($\equiv$0.7 ppb in the dried fruit) and was determined by external standard quantitation.

Identification of Dimenthyl Glutarate in *L. chinesis*. As mentioned above, **7** was analyzed in the positive ion mode only because it does not form a negative ion. However, because **7** had two significant product ions, the specificity of this analysis was further increased by monitoring both ions. Q1 was set to m/z 409 for the protonated precursor ion and Q3 was set to m/z 271 and 133 for the product ions. **Figure 4** shows the respective data for the identification of **7** in *L. chinesis* dried fruit extract. **Figure 4A** shows a chromatogram of a standard (0.13 μg/mL). The blank (**Figure 4B**) was an injection of sample solvent that was made immediately before sample analysis to demonstrate that **7** detected in the extract (**Figure 4C**) was not the result of carry over from the previous standard injection. The level of **7** in the extract was estimated to be 0.2 ppm ($\equiv$0.1 ppb in the dried fruit) based on external standard quantitation.

Conclusions. Compound **5** was identified in *L. barbarum* dried fruit and *M. piperita* dried leaf extracts, and **6** and **7** were identified in *L. chinesis* dried fruit extract using LC/MS/MS in the SRM mode. These identifications were based on the correlation of mass spectrometric and chromatographic retention time data of the menthyl esters in the extracts with authentic standards. Identification was further verified by spiking the *L. barbarum* and *M. piperita* extracts with **5** and analyzing the *L. chinesis* extract for **6** in both the positive and negative ion mode. For the analysis of **7**, two precursor/product ion transitions were monitored to increase selectivity. Because menthol, succinic acid, and glutaric acid occur in nature, flour was spiked with menthol and succinic acid and the extract analyzed to show that **5** is not an artifact of the extraction process. Flour was not spiked with glutaric acid, but the same principle applies. Using these techniques resulted in a 99.980% confidence in the identities being correct.

ABBREVIATIONS USED

NI, Nature Identical; SRM, single reaction monitoring; MRFA/myoglobin, L-methionyl-arginyl-phenylalanyl-alanine acetate/horse skeletal apomyoglobin.

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