GC/IRMS Analysis of Mandarin Essential Oils. 1. $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ Values of Methyl *N*-Methylanthranilate

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The $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values of methyl *N*-methylanthranilate from mandarin essential oils have been measured by capillary gas chromatography, on-line coupled with isotope ratio mass spectrometry (GC/IRMS). Cold-pressed and distilled mandarin peel oils of different origins and petitgrain oils mandarinier as well as reference substances of methyl *N*-methylanthranilate and methyl anthranilate were investigated. The combination of the $\delta^{13}C$ and $\delta^{15}N$ values is useful in the authenticity control of mandarin oils.

Keywords: *GC/IRMS; $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values; methyl N-methylanthranilate; mandarin essential oil; Citrus reticulata Blanco*

INTRODUCTION

The determination of isotopic values is gaining increasing importance, especially in view of the desire for authenticity control and origin determination of essential oils and foods (Braunsdorf et al., 1993; Hener et al., 1995; Frank et al., 1995; Faber et al., 1995; Juchelka and Mosandl, 1996). Considering the well-known biochemical background of carbon isotope fractionation and the well-established methods for $\delta^{13}C$ analysis, most of the investigations deal with the ^{13}C isotope abundance.

Compared with the $\delta^{13}C$ measurement, the determination of nitrogen stable isotope ratio analysis ($\delta^{15}N$) has not yet found the same wide application.

Most studies of nitrogen stable isotope ratio analysis are related to plant nutrition and fertilization and the composition of soil nitrogen (Yoneyama et al., 1991; Delwhiche and Steyn, 1970).

The only carbon source of plants is atmospheric CO_2, whereas nitrogen sources mainly are the nitrate or ammonium ions of the soil, except for some plants which form N_2-fixing associations utilizing atmospheric N_2. Atmospheric nitrogen contains 0.366 atom % ^{15}N, defined as $\delta^{15}N_{AIR} = 0‰$ and considered as universally constant (Mariotti, 1983).

A possible explanation for the differences in ^{15}N content of atmospheric nitrogen and that of the biosphere and soil may be isotope discrimination in the reactions involved in the biological nitrogen cycle. Nitrogen isotope fractionation is based on kinetic isotope effects, which tend to favor the lighter isotope ^{14}N, which enriches the unreacted starting material in the heavier isotope. Isotope effects that occur during biological processes (nitrification and denitrification) account for the $\delta^{15}N$ values of soils that mostly show a ^{15}N abundance higher than atmospheric nitrogen (Delwhiche and Steyn, 1970; Shearer et al., 1974).

The uptake and assimilation of nitrogen by plants are accompanied by isotope effects, too. Normally the $\delta^{15}N$ value of plant nitrogen is slightly depleted in ^{15}N compared with the nitrogen source (Yoneyama et al., 1991).

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The $\delta^{15}N$ values of plant material are determined by the ^{15}N content of their nitrogen sources, mostly the nitrogen of the soil. Therefore, fertilization influences the isotopic composition of nitrogen in the soil and thus also reflects on the $\delta^{15}N$ value of plant material. Fertilizers have lower $\delta^{15}N$ values than most natural nitrogen sources available to plants, and one might expect that the $\delta^{15}N$ value of soil organic matter would show a shift toward lower values (Shearer et al., 1974; Létolle, 1980, and literature cited therein).

However, as reported in the literature (Létolle, 1980), the total organic material from soils cultivated with or without fertilizers of any type showed no differences in $\delta^{15}N$ values.

Other authors report that fertilizers will have an influence upon $\delta^{15}N$ values in the pedosphere, and it did appear that the $\delta^{15}N$ value of plant material reflected changes in the isotopic composition of the soil nitrogen (Hübner, 1986; Amberger and Schmidt, 1987). Soil characteristics as well as climatic factors also have an influence on the $\delta^{15}N$ value of plants (Hübner, 1986; Delwhiche and Steyn, 1970; Fry, 1991). As a result of these factors, which influence the $\delta^{15}N$ value of plants, the ^{15}N abundance could depend on the location of plants.

The $\delta^{13}C$ values are likewise influenced by isotope effects caused by exogenous factors such as differences in location, climate, or harvest time (Winkler and Schmidt, 1980; O'Leary, 1981).

So the connection between the $\delta^{13}C$ and $\delta^{15}N$ values could give more information than one value alone (Kornexl et al., 1996; van der Merwe and Lee-Thorp, 1990).

This paper describes the application of gas chromatography/isotope ratio mass spectrometry (GC/IRMS) measurements of methyl *N*-methylanthranilate in mandarin essential oils, evaluating $\delta^{13}C$ as well as $\delta^{15}N$ values.

Methyl *N*-methylanthranilate is a characteristic flavor compound of mandarin essential oil. The mandarin oil is the only citrus peel oil that contains this ester.

MATERIALS AND METHODS

Essence Oil Sources. *Commercially Available.* Cold-pressed mandarin oils were from Italy, Greece, Brazil, and Argentina and of unknown origin. Distilled mandarin oils

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were from Italy. Distilled petitgrain oils mandarinier were from Italy and of unknown origin (Misitano & Stracuzzi, Messina, Italy; Ziegler, Aufsess, Germany; Kaders, Hamburg, Germany; Liebich, Nürnberg, Germany; Dufti, Melle, Germany).

Self-Prepared. Mandarin oils from Italy were prepared by solvent extraction. The peel from 15 fruits (*Citrus reticulata* Blanco) was rubbed off using a grater and extracted with 700 mL of pentane/diethyl ether 1:1 (v/v) overnight (room temperature). The organic layer was filtered, dried with Na_2SO_4, and concentrated using a Vigreux column. Mandarin oil from Italy was also distilled. The essential oil was obtained from the rubbed peel by steam distillation according to the DAB 10 method (*Deutsches Arzneibuch*, 1991). The oil was separated from the water phase by liquid−liquid extraction, three times with 50 mL portions of *n*-pentane/diethyl ether 1:1 (v/v). The organic phase was dried with Na_2SO_4 and concentrated using a Vigreux column. Petitgrain oil mandarinier from Italy was prepared by distillation. The air-dried leaves were ground under liquid N_2. For further preparation details, see Distilled Mandarin Oil.

Preparative Layer Chromatography (PLC). Hydrocarbons are the major compounds of mandarin oil. Therefore, minor compounds such as methyl *N*-methylanthranilate must be enriched by PLC. For isolation, 300 μL of the oil sample was separated using silica gel plates (Merck, Darmstadt, Germany, silica gel 60 F_{254}, 2 mm layer thickness) as the sorbent and *n*-pentane/diethyl ether 97:3 (v/v) as the eluent. Detection was by UV at 254 nm. Methyl *N*-methylanthranilate $R_f = 0.25$; methyl anthranilate $R_f = 0.10$. The compounds were removed from the silica gel plate and sorbent extracted with 10 mL of diethyl ether. After concentration (Vigreux column) to about 0.5 mL, the solution was ready to use for GC and GC/IRMS. To exclude isotopic fractionation during sample cleanup (Braunsdorf et al., 1992), the PLC preparation was checked with methyl *N*-methylanthranilate and methyl anthranilate reference substances.

Gas Chromatography. For quantification and control of PLC fraction, a Fisons GC 8000 series equipped with a flame ionization detector connected to a Shimadzu Chromatopac C-R3A integrator was used. An OV-1701 fused silica capillary column (60 m × 0.32 mm i.d., 0.6 μm film thickness, homemade) was used for the analysis. Conditions were as follows: 1 μL split injection; split ratio, 1:18; flow rate of carrier gas (helium), 1.2 mL/min; injector temperature, 220 °C. The temperature program was as follows: the column was held at 50 °C for 5 min and then programmed at 3 °C/min to 240 °C, which was held for 20 min.

Gas Chromatography/Mass Spectrometry. The GC/MS analysis were performed with a Fisons GC 8000 series equipped with a mass selective detector (Fisons MD 800). A DB-5 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness, Durabond, J&W Scientific, Folsom, CA) was used as stationary phase. Conditions were as follows: 0.3 μL split injection: split ratio, 1:30; flow rate of carrier gas (helium), 1.4 mL/min; injector temperature, 220 °C; interface temperature, 250 °C; ion source temperature, 220 °C. The temperature program was as follows: the column was held at 40 °C for 5 min and then programmed at 2.5 °C/min to 280 °C, which was held for 20 min.

Gas Chromatography/Isotope Ratio Mass Spectrometry. Isotope ratio measurements were performed with a Finnigan MAT delta S isotope mass spectrometer (Finnigan MAT, Bremen, Germany) (Brand et al., 1994), on-line coupled to a Varian 3400 GC via a combustion interface (Figure 1). The GC was equipped with an OV-1701 fused silica capillary column (60 m × 0.32 mm i.d., 0.6 μm film thickness, homemade). Conditions were as follows for carbon isotope measurements: 1 μL split injection (split ratio 1:15); flow rate of carrier gas (helium), 1.9 mL; injector temperature, 220 °C; the column was held at 100 °C for 5 min and then programmed at 3 °C/min to 150 °C and held for 5 min followed by a 3 °C/min increase to 200 °C, held 5 min, followed by a 3 °C/min increase to 240 °C, which was held for 20 min; to prevent the combustion furnace from deactivation, the backflush mode was held for 600 s. Conditions for nitrogen isotope measurements

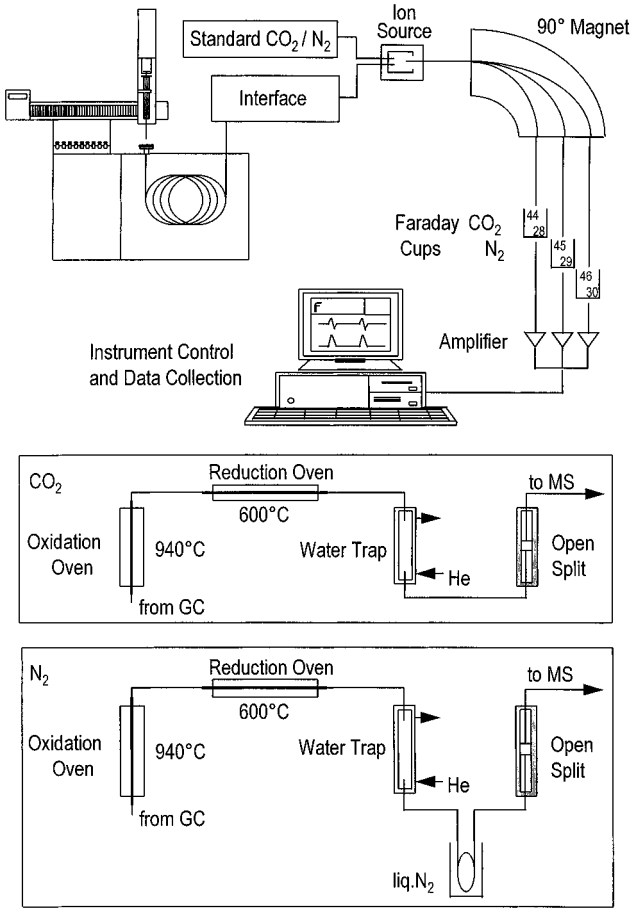

Figure 1. GC/IRMS schematic diagram.

were as follows: 1 μL split injection (split ratio 1:10); other parameters see above.

Isotope ratios are expressed as δ values (‰) versus a standard:

$$\delta^{13}C_{PDB} = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{PDB}}{(^{13}C/^{12}C)_{PDB}} \times 1000 \quad (1)$$

$$\delta^{15}N_{AIR} = \frac{(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{AIR}}{(^{15}N/^{14}N)_{AIR}} \times 1000 \quad (2)$$

In the case of carbon isotope measurement the system performance was checked by introducing a home-made mixture of references with well-known $\delta^{13}C_{PDB}$ values (references: 5-nonanone, −28.05‰; menthol, −26.55‰; γ-octalactone, −23.17‰; γ-decalactone, −30.12‰).

RESULTS AND DISCUSSION

Cold-pressed mandarin essential oils, widely used in the food industry and in perfumery, are well-appreciated olfactory quality products with high commercial value.

The oils are obtained by cold-pressing the peel of the fruits of *Citrus reticulata* Blanco. The less valuable distilled oils are obtained by distillation from the liquid of the screw-pressed residues of the cold extract of mandarin oil. Besides these products, reconstituted mandarin oils are available on the market.

These oils were generally obtained by mixing orange terpenes, γ-terpinene, natural and nature-identical methyl *N*-methylanthranilate, thymol, and other compounds to adapt the reconstituted oils to the mandarin oils (Dugo et al., 1992b). A natural source of methyl

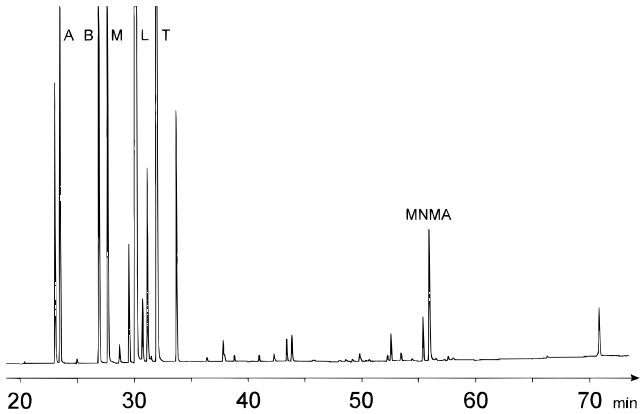

Figure 2. GC analysis of mandarin oil (OV-1701; for further conditions, see Materials and Methods): (A) α-pinene; (B) β-pinene/sabinene; (M) myrcene; (L) limonene; (T) γ-terpinene; (MNMA) methyl *N*-methylanthranilate.

N-methylanthranilate is the petitgrain oil mandarinier, obtained by steam distillation of the leaves of mandarin trees.

The composition of the peel oil is well reported as a mixture of monoterpenes (98.0%), oxygenated compounds (1.6%), and sesquiterpenes (0.3%). Among the hydrocarbons, which represent >98% of the oil content, limonene (65−75%) is the predominant compound of this essential oil, followed by γ-terpinene, α-pinene, and myrcene (Dugo, 1994).

Besides these main compounds, Kugler and Kovats (1963) noted methyl *N*-methylanthranilate as an important compound of mandarin flavor. Potential precursors of this compound are methyl anthranilate and anthranilic acid as intermediates in the aromatic amino acid pathway (Polak, 1973; Bell and Charlwoods, 1980). This ester occurs in an average amount of 0.4% in the oils (Figure 2).

To avoid coelution with other oil constituents and to increase the concentration, methyl *N*-methylanthranilate was necessarily isolated by preparative layer chromatography (PLC).

The applied sample cleanup allows the separation of methyl *N*-methylanthranilate without any isotopic fractionation, proven by comparing the $\delta^{13}C_{PDB}$ values of methyl *N*-methylanthranilate reference substances with and without PLC preparation.

The $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values of methyl *N*-methylanthranilate from different cold-pressed and solvent-extracted mandarin oils have been investigated. The results are outlined in Table 1.

The isotopic values of the authentic and self-prepared samples from Italy are rather similar, not only the carbon but also the nitrogen values. This is indicated by the standard deviation of the $\delta^{13}C_{PDB}$ and the $\delta^{15}N_{AIR}$ values of the 10 samples. Compared with the authentic oils, the commercial oil Co 3 from Italy is similar, whereas the authenticity of the commercial samples Co 1 and Co 2 is suspicious. They show lower δ values, and particularly the commercial oil Co 1 is extremely depleted in its ^{13}C content.

The carbon and nitrogen values of the essential oils from Greece, Brazil, and Argentina are not in accordance with those of the Italian oils. The samples from Greece are enriched in the ^{15}N content (7.4‰), whereas the $\delta^{13}C$ values are similar compared with the Italian oils. In the case of Brazilian and Argentinian oils the $\delta^{15}N_{AIR}$ values are also enriched in ^{15}N, but the

Table 1. $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ Values[a] of Methyl *N*-Methylanthranilate from Cold-Pressed and Solvent-Extracted Mandarin Essential Oils of Different Origins

sample	$\delta^{13}C_{PDB}$ [σ]	$\delta^{15}N_{AIR}$ [σ]	concn (area %)
Authentic Samples from Italy			
Au 1	−31.07 [0.03]	4.42 [0.25]	0.53
Au 2	−30.05 [0.04]	4.26 [0.12]	0.64
Au 3	−29.99 [0.04]	4.18 [0.24]	0.57
Au 4	−30.94 [0.23]	3.62 [0.19]	0.47
Au 5	−30.73 [0.18]	3.70 [0.24]	0.40
Au 6	−30.18 [0.21]	4.47 [0.22]	0.56
Au 7[b]	−29.96 [0.17]	3.82 [0.23]	0.74
Au 8[b]	−29.90 [0.14]	4.56 [0.04]	0.51
Au 9[b]	−29.54 [0.12]	4.66 [0.36]	0.47
Au 10[b]	−30.07 [0.17]	4.02 [0.15]	0.26
av	−30.24 [0.50]	4.17 [0.37]	0.52
Commercial Samples from Italy			
Co 1	−32.42 [0.06]	3.26 [0.22]	0.45
Co 2	−31.42 [0.15]	3.16 [0.08]	0.45
Co 3	−29.97 [0.10]	4.78 [0.20]	0.45
Commercial Samples from Greece			
Co 4	−29.11 [0.11]	7.40 [0.31]	0.38
Co 5	−30.37 [0.21]	7.44 [0.05]	0.35
Commercial Sample from Brazil			
Co 6	−32.66 [0.14]	6.04 [0.25]	0.45
Commercial Sample from Argentina			
Co 7	−32.66 [0.13]	8.32 [0.26]	0.15
Commercial Samples of Unknown Origin			
Co 8	−30.22 [0.11]	4.56 [0.27]	0.52
Co 9	−30.42 [0.10]	5.20 [0.22]	0.30
Co 10	−32.05 [0.07]	6.03 [0.21]	0.66

[a] $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values [‰]; standard deviation $n = 4$ [σ]; authentic sample (Au); commercial sample (Co). [b] Self-prepared by solvent extraction.

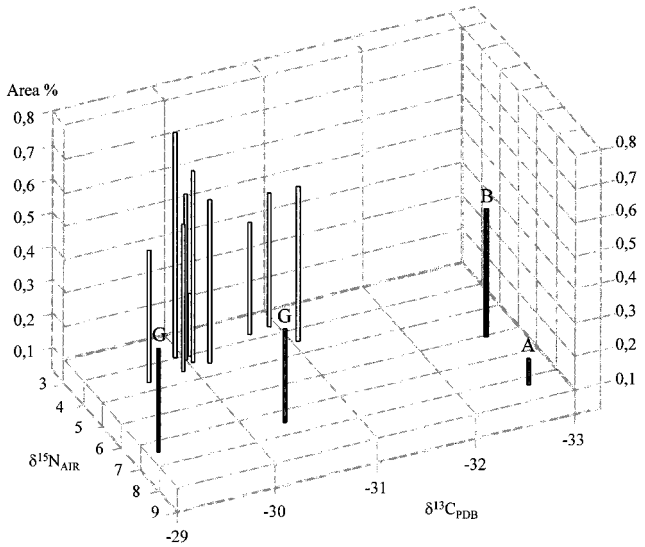

Figure 3. $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values of methyl *N*-methylanthranilate from cold-pressed and solvent-extracted mandarin essential oils of different origins: white columns, authentic samples from Italy (Au 1−Au 10); black columns, commercial samples from Greece = G (Co 4, 5), Brazil = B (Co 6), and Argentina = A (Co 7).

$\delta^{13}C_{PDB}$values are depleted in ^{13}C compared with the Italian oils (Figure 3).

A previous study concerning total carbon and nitrogen δ values of orange juice pulps reports the same tendency, lower $\delta^{13}C_{PDB}$ and higher $\delta^{15}N_{AIR}$ values of samples from Argentina and Brazil compared with those from Italy (Kornexl et al., 1995).

Isotope effects caused by growing conditions, for example, differences in location, climate, and fertiliza-

Table 2. $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ Values[a] of Methyl *N*-Methylanthranilate from Distilled Mandarin Peel Oils from Italy

sample	$\delta^{13}C_{PDB}$ [σ]	$\delta^{15}N_{AIR}$ [σ]	concn (area %)
Co 11	−32.48 [0.16]	3.20 [0.17]	0.30
Co 12	−33.42 [0.13]	2.98 [0.37]	0.31
Au 13[b]	−30.30 [0.10]	4.21 [0.25]	0.32

[a] $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values [‰]; standard deviation $n = 4$ [σ]; authentic sample (Au); commercial sample (Co). [b] Self-prepared by steam distillation according to the DAB 10 method.

tion, influence the δ values of plant material. Therefore, the $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values can be characteristic for a location. These observations must be confirmed with other samples from Argentina and Brazil.

Comparing the commercial samples of defined and undefined origins, it appears that sample Co 8 is an Italian oil (Table 1). The δ values of samples Co 9 and Co 10 are suspicious. Whereas Co 10 could be assigned to the Brazilian oil, sample Co 9 has a carbon value comparable to those of the Italian oils but a higher nitrogen value. In contrast to all other samples, this oil contains methyl anthranilate (0.03%).

Methyl *N*-methylanthranilate is present in the investigated oils between 0.26 and 0.74% (Table 1) with an average amount of 0.5%. Nearly the same natural range (0.26−0.66%, with an average amount of 0.4%) is described in the literature (Dugo, 1994). The content of methyl *N*-methylanthranilate depends on the state of ripeness. Green unripe fruits have high amounts, which decrease during the ripening process (Dugo, 1994).

The oil Co 7 from Argentina contains only 0.15% of methyl *N*-methylanthranilate. Moreover, a remarkable quantitative composition is detected, especially a high amount of δ-3-carene, which is normally a trace compound (≤0.005%) (Dugo et al., 1992a). Investigations show that the oil is diluted with orange peel oil (S. Faulhaber, dissertation in preparation, 1997).

In Table 2 the δ values of methyl *N*-methylanthranilate from distilled mandarin peel oils from Italy are presented. These products are less valuable than the cold-pressed oils. For economic reasons, cold-pressed mandarin oils are blended with the less valuable distilled oils.

The δ values of the commercially distilled oils are depleted in the ^{13}C and ^{15}N contents related to the authentic cold pressed oils (Table 1). In contrast to the commercially distilled samples the isotope ratios of the self-prepared distilled oil (Au 13) are similar to those of the cold-pressed oils. The depletion of the commercial oils reflects a possible effect of oil production.

Commercially distilled oils are obtained by distillation from the liquids of the screw-pressed residues of cold extraction.

The distillation parameters could effect the differences between the commercial and self-prepared samples. The differences of the δ values of cold-pressed and commercially distilled oils from Italy allow a differentiation of both products.

At the present state of knowledge partial blending with 10−20% distilled mandarin oils seems to be difficult to detect by this method.

The δ values of methyl *N*-methylanthranilate from petitgrain oils mandarinier and from commercial chemicals are outlined in Table 3.

Petitgrain oils mandarinier, as a source of natural methyl *N*-methylanthranilate, contain this compound in concentrations >50%. Genuine mandarin oils may

Table 3. $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ Values[a] of Methyl *N*-Methylanthranilate from Petitgrain Oils Mandarinier and Commercially Available Compounds

sample	$\delta^{13}C_{PDB}$ [σ]	$\delta^{15}N_{AIR}$ [σ]
Petitgrain Oils Mandarinier		
Au Italy 14[b]	−33.07 [0.17]	3.96 [0.16]
Co Italy 15	−32.59 [0.27]	4.00 [0.22]
Co Italy 16	−32.78 [0.11]	4.01 [0.11]
Co 17	−32.08 [0.20]	3.58 [0.03]
Co 18	−31.80 [0.02]	4.21 [0.27]
Methyl *N*-Methylanthranilate		
MNMA na 1	−32.20 [0.22]	3.06 [0.25]
MNMA na 2	−34.02 [0.20]	2.02 [0.18]
MNMA ni	−37.13 [0.26]	−5.04 [022]
Methyl Anthranilate		
MA na	−31.53 [0.12]	2.53 [0.28]
MA ni	−31.58 [0.30]	−2.71 [0.23]
Commercial Sample (Co 9) Containing 0.03% Methyl Anthranilate		
Co 9	−30.71 [0.49]	−7.45 [0.23]

[a] $\delta^{13}C_{PDB}$ and $\delta^{15}N_{AIR}$ values [‰]; standard deviation $n = 4$ [σ]; authentic sample (Au); commercial sample (Co); natural (na); nature-identical (ni). [b] Self-prepared by steam distillation according to DAB 10 method.

be partially blended with less valuable reconstituted oils, and the mixture is sold as genuine mandarin oil.

Compared to the cold-pressed fruit oils, the δ values of the petitgrain oils show a depletion in the ^{13}C contents, whereas the δ values of the commercial chemicals of natural origin are depleted in the ^{13}C and ^{15}N contents.

As can be seen from Tables 1 and 3 the $\delta^{13}C$ (^{15}N) values are not significant enough to prove the blending of small amounts of methyl *N*-methylanthranilate from strange natural sources to genuine cold-pressed mandarine oils.

On the other hand, the compounds methyl *N*-methylanthranilate and methyl anthranilate declared as nature-identical are extremely depleted in the ^{15}N contents (negative $\delta^{15}N_{AIR}$ values). The only mandarin peel oil that contains methyl anthranilate in detectable amounts is the sample Co 9. The $\delta^{15}N_{AIR}$ value of this compound is also extremely depleted in its ^{15}N content. This indicates an addition of nature-identical methyl anthranilate.

The δ values of carbon and nitrogen permit an origin assignment, as shown on the samples from Brazil, Argentina, Greece, and Italy. To confirm these results, further investigations with samples from the different locations are necessary. For further authenticity assignment of the suspicious mandarin oils, continued isotopic investigations of other oil compounds are in preparation (S. Faulhaber, dissertation in preparation, 1997).

The isotopic values of methyl *N*-methylanthranilate, an important aroma compound of mandarin peel oil, were determined using GC/IRMS. The $\delta^{13}C_{PDB}$ as well as the $\delta^{15}N_{AIR}$ values were measured. $\delta^{15}N$ and $\delta^{13}C$ values seem to be characteristic for the country of origin, and the combination of carbon and nitrogen isotopic values is useful in the authenticity control of mandarin essential oils.

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