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Note

Even carbon number predominance of plant wax *n*-alkanes: a correction

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Abstract

The distributions of *n*-alkanes of four species of *Micromeria* have the conventional higher plant pattern of high carbon preference index (CPI) and odd-numbered carbon dominance (maxima at *n*-C_{31} or *n*-C_{33}), rather than the even-numbered predominance previously reported (Palić, R., Ristić, N., Simić, N., Kitić, D., Kapetanović, R., 1997. The alkanes from some plants of *Micromeria* genus. Journal of the Serbian Chemical Society 62, 619–622). The stable carbon isotope ratio values ($\delta^{13}C$) of the individual *n*-alkanes (−38 to −34‰) are typical of C_3 plants. Homologous series of odd-numbered predominant *iso*-alkanes (*i*-C_{27} to *i*-C_{35}) and even-numbered predominant *anteiso*-alkanes (*a*-C_{27} to *a*-C_{35}) are also present (8 to 18% of the total identified alkanes) and have similar carbon isotopic ratios (−36.8 to−35.1‰).

Keywords: *Micromeria*; *anteiso*-Alkanes; *iso*-Alkanes; Carbon isotope ratios; *n*-Alkanes; Leaf waxes

1. Introduction

Odd carbon-numbered dominant distributions of long chain *n*-alkanes, typically in the range of C_{25} to C_{35}, are characteristic components of the epicuticular leaf waxes of higher plants (Douglas and Eglinton, 1966; Eglinton and Hamilton, 1967; Kolattukudy, 1969). Such carbon number patterns have proved of great value in environmental and paleo-environmental biomarker-based research. For example, they have been used in qualitatively and semi-quantitatively apportioning sources of hydrocarbons found in recent aquatic sediments polluted with fossil fuels (Volkman et al., 1992). The validity of this basic paradigm needs to be rigorously re-examined whenever a contrary indication occurs. For example, recently an exception was reported (Palić et al., 1997), where the extracted *n*-alkanes from *Micromeria albanica* and *M. thymifolia* had an even carbon-number dominated distribution (even/odd ratio ~7), maximizing at *n*-C_{30} and *n*-C_{32}.

We have now examined four species of *Micromeria* (the two previously investigated and two additional species: *M. cristata* and *M. juliana*) by dual column, high-resolution GC, together with coinjection of authentic hydrocarbon standards and independent confirmation by GC–MS, and we find that this anomalous result (Palić et al., 1997) was incorrect. Thus, in all four extracts the *n*-alkanes form an entirely typical, high carbon preference index (CPI) (5.6 to 7.2) and odd carbon number dominated distribution maximized at either *n*-C_{31} or *n*-C_{33}. These higher plants are part of the *Labiatae* (mint family) and are endemic to the Balkan peninsula (Palić et al., 1997).

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2. Experimental

2.1. Samples

M. albanica, *M. cristata*, *M. juliana* and *M. thymifolia* were collected in the blooming growth phase in the country of Serbia. Leaves, flowers and green parts of stems were air-dried for 10 days at room temperature and kept in a cold and dark place until extracted.

2.2. Extraction and isolation

The dried plant material (60 to 200 g) was extracted with distilled petroleum ether (40–70°C) in a Soxhlet apparatus for 24 h. The extracts were evaporated to a small volume and dried for 2 h with anhydrous $MgSO_4$. After removal of $MgSO_4$, the extracts were evaporated under vacuum to constant weight. The extraction of *M. albanica* gave 10.05 g (5.02%), *M. cristata* 5.0 g (7.9%), *M. juliana* 5.8 g (8.3%) and *M. thymifolia* 6.06 g (3.06%) of extractable material. The alkanes were then isolated with silica-gel column chromatography and SiO_2 thin layer chromatography. The yields (based on the original extract) were 160 mg (1.59%, *M. albanica*), 46 mg (0.9%, *M. cristata*), 105 mg (1.8%, *M. juliana*) and 50 mg (0.82%, *M. thymifolia*), respectively.

2.3. Identification and abundance of alkanes by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

Alkane fractions were completely dissolved in hexane with the aid of mild sonication. A small aliquot (1 μl) was injected into a single inlet, dual-column gas chromatograph with two flame ionization detectors (FID). The compounds were separated on J&W DB-1 and DB-5 columns (both 60 m×0.32 mm; film thickness, 0.25 μm) (Palić et al., 1999).

The *n*-alkanes were identified by direct comparison of retention times of known standards and samples on both capillary columns. In addition, two *n*-alkanes (*n*-C_{24} and *n*-C_{32}) were spiked into one extract after the original GC analysis and then re-analyzed. The *anteiso*- and *iso*-alkanes were identified by comparing their retention pattern to published chromatograms (Kavouras et al., 1998), and by comparison of mass spectra (see below).

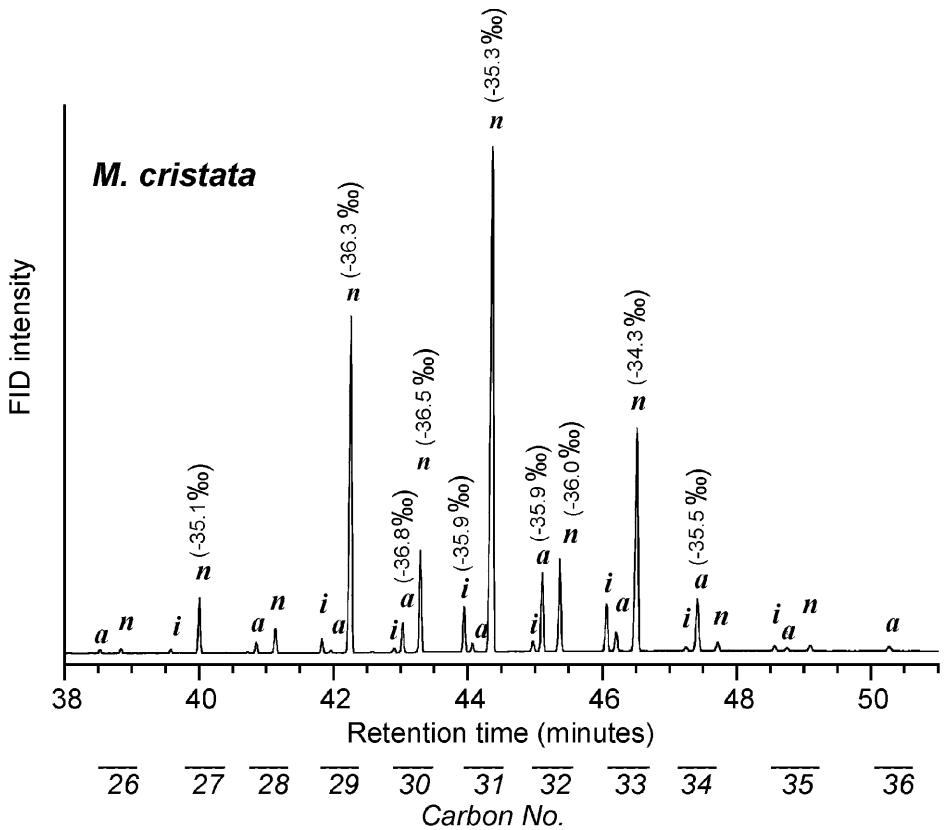

Fig. 1. Gas chromatogram of the *M. cristata* extract. *Anteiso*-, *iso*- and *n*-alkanes are labeled with *a*, *i* and *n*, respectively. The mean $\delta^{13}C$ values for each compound are listed in parentheses.

GC–MS was performed using a HP 6890 gas chromatograph interfaced to a Hewlett Packard 5973 mass selective detector. A DB-5 capillary column was used, and mass spectra were acquired at ~70 eV across a scan range of 50–800 amu at a rate of ~1 scan/s. The *anteiso*- and *iso*-alkanes were tentatively identified by their fragmentation patterns, which generally show prominent ions for ($M^{\cdot+}$–$C_2H_5^-$) and ($M^{\cdot+}$–$C_3H_7^-$), respectively (Kavouras et al., 1998).

2.4. Compound-specific stable carbon isotope ratio analysis by isotope ratio monitoring gas chromatography–mass spectrometry (irmGC–MS)

The stable carbon isotope analyses were performed on a Hewlett Packard 6890 GC interfaced to a modified Finnigan GC Combustion III unit followed by a Finnigan Delta Plus isotope mass spectrometer. Compounds were separated on a J&W DB-5 fused silica capillary

Table 1
The distribution and abundance of *n*-alkanes, *anteiso*-alkanes and *iso*-alkanes in *Micromeria*[a]

		M. albanica	*M. cristata*	*M. juliana*	*M. thymifolia*
% *n*-Alkanes		92.3	81.9	87.2	84.5
% *anteiso*-Alkanes		1.4	11.1	6.2	2.3
% *iso*-Alkanes		6.3	7.0	6.7	13.2
n-Alkanes					
	n-C_{22}	0.0	0.1	0.1	0.0
	n-C_{23}	0.0	0.1	0.1	0.0
	n-C_{24}	0.0	0.2	0.1	0.0
	n-C_{25}	0.1	0.6	0.2	0.1
	n-C_{26}	0.0	0.3	0.1	0.1
	n-C_{27}	0.3	3.1 (−35.1)	2.2 (−35.2)	2.7
	n-C_{28}	0.1	1.4	0.7	0.8
	n-C_{29}	3.1	22.7 (−36.3)	12.4 (−35.2)	15.9 (−36.4)
	n-C_{30}	1.2	5.9 (−36.5)	5.3 (−35.9)	3.1 (−38.1)
	n-C_{31}	20.5 (−34.1)	40.9 (−35.3)	45.0 (−34.7)	34.9 (−37.1)
	n-C_{32}	7.6 (−34.5)	5.8 (−36.0)	8.0 (−35.8)	6.9 (−38.0)
	n-C_{33}	60.2 (−34.2)	17.7 (−34.3)	24.5 (−34.5)	33.6 (−38.0)
	n-C_{34}	4.1 (−34.8)	0.7	1.0	1.2
	n-C_{35}	2.8 (−34.8)	0.5	0.4	0.6
anteiso-Alkanes					
	a-C_{27}	0.0	0.6	0.4	0.0
	a-C_{28}	2.2	4.5	3.6	2.4
	a-C_{29}	0.0	0.7	0.6	0.0
	a-C_{30}	3.8	12.3 (−36.8)	8.6	6.7
	a-C_{31}	1.4	4.1	3.0	3.9
	a-C_{32}	16.0	36.1 (−35.9)	35.5 (−35.1)	28.0
	a-C_{33}	13.9	9.8	15.6	18.6
	a-C_{34}	52.8	29.9 (−35.5)	30.2	37.3
	a-C_{35}	9.9	2.1	2.5	3.1
iso-Alkanes					
	i-C_{27}	0.4	2.8	2.8	0.3
	i-C_{28}	0.1	0.8	0.8	0.0
	i-C_{29}	1.1	9.2	6.6	2.8
	i-C_{30}	0.4	3.0	2.4	1.2
	i-C_{31}	9.7	30.6 (−35.9)	30.0	25.1 (−35.2)
	i-C_{32}	4.1	7.9	11.2	8.0
	i-C_{33}	54.9	36.3	37.7	52.1
	i-C_{34}	15.1	4.3	5.6	6.6
	i-C_{35}	14.1	5.2	2.9	3.8

[a] For each class of alkanes, the data are the percent relative abundance of each compound. The mean $\delta^{13}C$ values (in ‰) are listed in parentheses.

column (60-m length; 0.32-mm diameter; 0.25 μm film thickness). During each analysis, pulses of reference CO_2 were bled into the mass spectrometer and were used to calibrate it relative to Vienna Pee Dee belemnite (VPDB). Alkane fractions were injected three times and the values reported here are the mean. The precision (expressed as the standard deviation of the three injections) was no greater than 0.66‰ and averaged 0.28‰. The $\delta^{13}C$ values for coinjected perdeuterated n-C_{32} were within 0.5‰ of the actual value (as determined with standard off-line techniques).

3. Results and discussion

3.1. Distribution and abundance of alkanes

Fig. 1 shows a partial GC trace (DB-5 stationary phase) of the alkane fraction for *M. cristata*. The results for individual alkanes from all four samples are presented in Table 1 and parameterized in Table 2. The *n*-alkane distributions in each case reflect the conventional higher plant pattern of high CPI (~6 to ~7), average chain length (ACL) (31 to 32) and odd-numbered carbon dominance (maxima at n-C_{31} or n-C_{33}), rather than the even-numbered predominance previously reported (Palić et al., 1997). The *anteiso*- (3-methyl) and *iso*- (2-methyl) alkanes also have similar homolog distributions (Table 1), which dominate the same region of the chromatogram as the *n*-alkanes (C_{29} to C_{35}) (Table 1 and Fig. 1). The *anteiso*-alkanes have a predominant even-numbered distribution, whereas the *iso*-alkanes are mainly odd-numbered. Similar patterns have been reported previously and reflect the biosynthetic pathways of these compounds (Kolattukudy, 1969). The *anteiso*-alkanes originate mainly with a 2-methylbutanoyl (C_5)–CoA "starter", which is elongated by C_2 units and then decarboxylated, resulting in an even-numbered dominance. The *iso*-alkanes have an odd-numbered dominance because they are mostly biosynthesized with a 2-methylpropanoyl (C_4)–CoA "starter".

The relative abundance of *anteiso*- and *iso*-alkanes compared to the total alkanes varied from 1.4 to 11.1% and 6.3 to 13.2%, respectively (Table 2). Generally, most higher plants contain lower amounts of branched alkanes than reported here (Eglinton and Hamilton,

Table 2
Summary of molecular and isotopic data in *Micromeria*

		M. albanica	*M. cristata*	*M. juliana*	*M.thymifolia*
n-Alkanes					
	Odd max.	33	31	31	31
	Even max.	32	30	32	32
	CPI (22 to 35)[a]	6.6	6.0	5.6	7.2
	ACL (22 to 35)[b]	32.4	30.7	31.2	31.3
	Mean $\delta^{13}C$ (‰)	−34.5 ($n=5$)	−35.6 ($n=6$)	−35.2 ($n=6$)	−37.5 ($n=5$)
	Mean $\Delta\delta^{13}C$ (‰)[c](even–odd)	−0.3	−0.9	−1.0	−0.9
anteiso-Alkanes					
	Odd max.	33	33	33	33
	Even max.	34	32	32	34
	CPI (28 to 35)	0.34	0.20	0.28	0.34
	ACL (27 to 35)	33.3	32.2	32.5	32.8
	Mean $\delta^{13}C$ (‰)	NA	−36.0 ($n=3$)	−35.1 ($n=1$)	NA
iso-Alkanes					
	Odd max.	33	33	33	33
	Even max.	34	32	32	32
	CPI (28 to 35)	4.0	5.1	3.9	5.3
	ACL (27 to 35)	33.1	31.8	31.9	32.4
	mean $\delta^{13}C$ (‰)	NA[d]	−35.9 ($n=1$)	NA	−35.2 ($n=1$)

[a] CPI (carbon preference index) = (∑odd C from the range of carbon numbers listed in parentheses)/(∑even C from the range of carbon numbers listed in parentheses).

[b] ACL (average chain length) = (∑[Cj]×j)/ (∑[Cj]) where j is the range of carbon numbers listed in parentheses and Cj is the relative concentration of the alkane containing j carbon atoms.

[c] $\Delta\delta^{13}C$, the difference between the average $\delta^{13}C$ values of the even and odd numbered *n*-alkanes for n-C_{29} to n-C_{33}, except for *M. albancia* where the range was n-C_{31} to n-C_{35}.

[d] NA = not available.

1967; Rogge et al., 1994). One notable exception is the tobacco plant, *Nicotiana tabacum*, which can have approximately equivalent amounts of *anteiso*-, *iso*-, and *n*-alkanes (Rogge et al., 1994; Kavouras et al., 1998). These species of *Micromeria* fall between most higher plants and tobacco in terms of their content of *anteiso*- and *iso*-alkanes. Recent environmental studies have relied on the large abundance of *anteiso*- and *iso*-alkanes in tobacco to use these compounds as biomarkers of cigarette smoke pollution in indoor and urban aerosols (Rogge et al., 1994; Kavouras et al., 1998). Combustion of vegetation in some regions might complicate such use due to a natural abundance of these branched alkanes.

3.2. Stable carbon isotopes

The $\delta^{13}C$ values of individual alkanes are illustrated in Fig. 1, plotted in Fig. 2 and summarized in Table 2. Only the most abundant alkanes were measured, which limited the analysis to 5 to 6 *n*-alkanes and a few *anteiso*- and *iso*-alkanes in each extract. The $\delta^{13}C$ values of the *n*-alkanes ranged from −38 to −34‰ and are similar to values measured for *n*-alkanes in other C_3 plants (Collister et al., 1994; Lockheart et al., 1997). *M. thymifolia* was the most depleted in ^{13}C, followed by *M. juliana* and *M. cristata*. The most enriched was *M. albanica*. These intra-species differences may be genetic or due to climatic and temporal effects such as light irradiance, temperature and growing period (Collister et al., 1994; Lockheart et al., 1997). A slight trend towards more negative $\delta^{13}C$ values with a longer ACL emerges when compared with those from Collister et al. (1994) for 10 different C_3 plants. There is no correlation between $\delta^{13}C$ values and CPI for the same suite of data. All four *Micromeria* species exhibit a tendency for the even-numbered *n*-alkanes to be depleted in ^{13}C by ~−1‰ relative to the neighboring odd-numbered *n*-alkanes, except for *M. albanica*, which shows a smaller difference (−0.3‰) (Fig. 2 and Table 2).

There were no obvious differences between the $\delta^{13}C$ values of the *n*-alkanes and those of the branched alkanes. While it is likely that the different "starters" used to biosynthesize these compounds may have differing isotopic abundances, these differences are attenuated by the presumably rather constant carbon isotopic abundance of the C_2 units used in chain elongation. No data are available for the $\delta^{13}C$ values of *anteiso*- and *iso*-alkanes in other higher plants.

3.3. Cautionary warning

Long chain *n*-alkanes distributions dominated by odd carbon-numbered homologs are reliable indicators of terrigenous inputs in environmental and paleo-environmental studies. However, the integrity of the basic paradigm that these *n*-alkane distributions are characteristic of a specific source (in this case, higher plant epicuticular leaf waxes) needs to be tested constantly. Necessary protocols include the usage of high resolution glass capillary columns, high purity solvents, clean sample and handling techniques, co-injection of authentic standards, and analysis by GC–MS. In addition, external factors such as contamination of the leaf waxes by petroleum-derived alkanes (derived from local pollution or solvents used in applying pesticides) need to be considered. In this study, we have corrected a previous misidentification of an even-numbered dominance in *Micromeria* species (Palić et al., 1997).

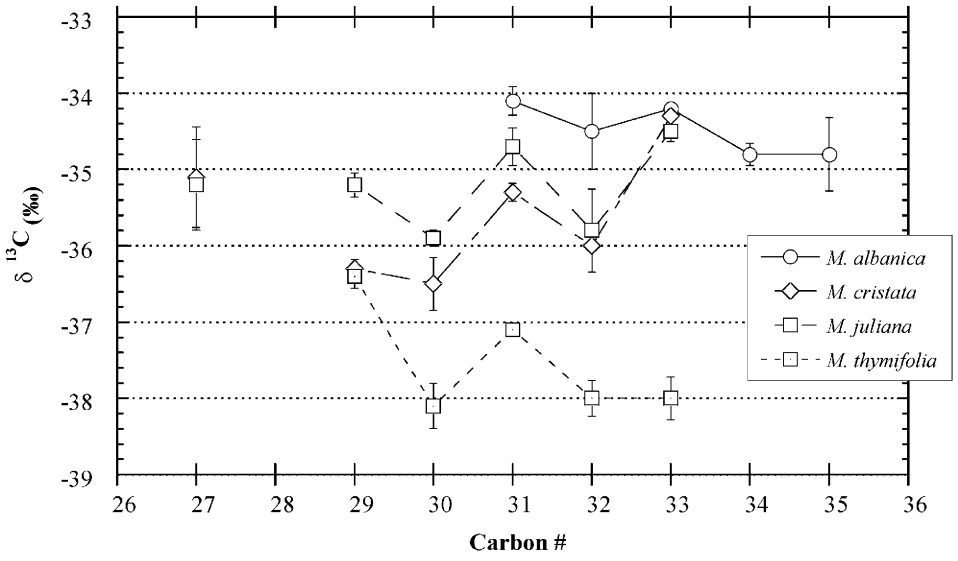

Fig. 2. Average $\delta^{13}C$ values of *n*-alkanes for each *Micromeria* species. Error bars are the standard deviations from triplicate analyses.

The literature contains a number of other reports of even-numbered dominance or no odd/even preference in epicuticular leaf wax *n*-alkanes. Those instances from the 1960s may possibly be the result of human or instrumental error, extraction and analysis of the whole leaf, or petroleum contamination, especially when low amounts of *n*-alkanes were involved (Herbin and Robins, 1969). However, sporadic reports of even-numbered dominance have continued into the 1990s. Osborne et al. (1989, 1993), using a short capillary column, did not find an odd/even preference in the epicuticular waxes of several species of *Encephalartos*, *Ceratozamia*, *Dioon* and *Zamia*. The authors suggested that in these *Cycadales* an α-oxidation process leads to *n*-alkanes in the range of *n*-C_{19} to *n*-C_{22}. Using packed-column GC, Bondada et al (1996) investigated epicuticular leaf waxes of cotton (*Gossypium hirsutum*) and found a majority of even-numbered *n*-alkanes ranging from *n*-C_{24} to *n*-C_{32}. No biosynthetic explanation was presented. We recommend that these and other unusual results be reexamined. Overall, we believe that the usefulness of odd-numbered long chain *n*-alkanes as powerful indicators of terrigenous inputs persists.

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