PAPER

GC-C-IRMS CHARACTERIZATION OF SYNTHETIC BIS(METHYL-THIO)METHANE IN TRUFFLE FLAVORINGS

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ABSTRACT

Bis(methyl-thio)methane (BMTM), the molecule which provides "white truffle-like" flavor was characterized by physico-chemical methods. Analysis by GC-C-IRMS of eight samples of synthetic BMTM from various raw material suppliers allowed the investigation of the $\delta^{13}C$ values. More, ten samples purchased on the Italian flavoring market, declared as synthetic BMTM principal component diluted in olive oil were analyzed by GC-C-IRMS. The results of both sample groups allowed us to define the range of $\delta^{13}C$ values of synthetic BMTM.

We verified if the simple proposed extraction method allows to measure the $\delta^{13}C$ value of BMTM also identified in seasonings of the Italian market declared on label as "white truffle flavored olive oil". In all twenty samples purchased on the market, the data strictly corresponded with synthetic BMTM as the principal component.

Measurements by ^{1}H NMR made on synthetic BMTM and BMTM extracted from "white truffle-like flavor" confirmed that the adopted extraction method using methanol-d4 determined the isotopic distribution of $^{13}C/^{12}C$ ratio in two characteristic sites of this molecule.

Keywords: white truffle flavoring, BMTM, GC-C-IRMS, ^{1}H NMR

1. INTRODUCTION

Tuber magnatum (white truffle), *Tuber aestivum* (summer truffle), and *Tuber melanosporum* (black truffle) are the most well-known species belonging to the genus *Tuber F.H.* These species have been the object of numerous studies using various analytical systems for the identification of the compounds that provide the distinctive aroma of these fungi (BELLESIA *et al.*, 1996; DÍAZ *et al.*, 2002; DÍAZ *et al.*, 2003; TALOU *et al.*, 1989).
Several reports have considered the constituents responsible for the typical aroma and have also studied the quantitative and qualitative fluctuations of these compounds, depending upon truffle type and geographical origin (COSTA *et al.*, 2015; FIECCHI *et al.*, 1967; GIOACCHINI *et al.*, 2008; MAURIELLO *et al.*, 2004; PELUSIO *et al.*, 1995). Presently, pure natural BMTM derived from truffles is not available on the raw material flavor market because the levels of BMTM in truffles are too low for satisfactory isolation of a significant quantity of this molecule (SCHMIDBERGER and SCHIEBERLE, 2017). Also, the commercial cost of the natural BMTM from the white truffle would be unsustainable because the raw material is very expensive (BORSINO DEL TARTUFO, 2018).
Therefore, due to the non-feasibility of BMTM isolation from the natural matrix, no measurements using Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) have been previously carried on.
The European Union (EU) Regulation 1334/2008 allows the citation of the flavoring source (in our case "white truffle") only if the flavoring components are obtained exclusively or by at least 95% (w/w) from the source material referred to and the other maximum 5% can derive only from other natural sources. Therefore, the citation in the label "natural flavoring", without the citation of a defined natural source, may only be used if all the flavoring components derive from natural sources. If one or more synthetic compounds are present in a flavoring formulation, the label must report only the term "flavor" without any reference to a food, food category or a vegetable or animal flavoring source (REG. EU 1334, 2008).
The corresponding naturally-occurring identical compound, easily synthesized by the oil industry and supported in olive oil (PACIONI et al., 2014), is used as a flavoring agent for truffle flavored food products. Among the "white truffle-like" flavored foods, extra virgin olive oil, flavored with bis(methyl-thio)methane (BMTM), used as a seasoning, occupies the most important position in the market.
Using a simple extraction method and the GC-C-IRMS analytical technique, we aimed to ascertain the reliability of characterizations of the synthetic BMTM molecule present in "white truffle-like" flavors and seasonings. Following the EU Regulation 1334/2008 before cited, the identification of synthetic BMTM in the formulation of flavors and seasonings does not allow the use on the label the term "natural white truffle flavor", or "natural flavor" or "white truffle flavor", but only the term "flavor".
Synthetic BMTM characterization by GC-C-IRMS, confirmed by ^{1}H-NMR, allowed us to demonstrate the identity of the BMTM molecule extracted from "white truffle-like" flavors purchased on the Italian flavoring market and from olive oil seasonings available on the Italian consumer market.

2. MATERIALS AND METHODS

2.1. Solvents

Methanol (anhydrous) (99.8) and methanol d-4 were purchased from Sigma Aldrich (Milan, Italy).

2.2. Samples analyzed by GC-C-IRMS

- Eight samples of BMTM ($C_3H_8S_2$) declared to be synthetically derived were obtained. The first purchased was a standard (> 99%) from Sigma Aldrich (Italy) and the others were purchased on the flavor raw material market (FCI, Frutarom, Moellhausen, Penta, Treatt, Sterling, Sigma Aldrich).
- Ten samples of "white truffle-like" flavors purchased on the Italian flavorings market and declared as synthetic BMTM diluted in olive oil.
- Twenty "white truffle-like" seasonings purchased on the Italian market and consisting of flavored extra virgin olive oil, or olive oil, or sunflower oil. All the seasonings were declared to contain olive oil or extra-virgin olive oil except the seasoning n. 4, which contains sunflower oil. The product n. 8 reported on the label "natural flavor". The product n. 13 reported on the label "white truffle natural flavor". The products n. 6, 11, 16, and 19 reported on the label "white truffle flavor". The other products reported on the label "flavor".

2.3. Samples analysis by ^{1}H NMR

Two samples were analyzed, one corresponding to the standard BMTM (sample n.1 in Table 1) and another corresponding to a "white truffle-like" flavoring (sample n.1 in Table 2).

2.4. Samples preparation

For GC-C-IRMS analysis, samples of synthetic BMTM (Table 1) were dissolved in anhydrous methanol (99.8) at a concentration of 60 μL mL^{-1}. The mix was vortexed for 1 min.
For samples of "white truffle-like" flavoring (Table 2) an aliquot of 3 mL was vortexed with 1 mL of methanol for 5 min and then centrifuged at 5000 rpm for 5 min. The methanol layer was isolated by a Pasteur pipette and utilized for the analysis.
For samples of seasonings, an aliquot of 5-10 mL was vortexed with 1 mL of methanol for 5 min and then centrifuged at 5000 rpm for 5 min. The methanol layer was isolated by a Pasteur pipette and utilized for the analysis.
For NMR analysis, a sample of standard BMTM (sample n.1 in Table 1) was dissolved in methanol d-4 at a concentration of 60 μL mL^{-1}. The mix was vortexed for 1 min. For sample of a "white truffle-like" flavoring (sample n.1 in Table 2), an aliquot of 3 mL was vortexed with 1 mL of methanol d-4 for 5 min and then centrifuged at 5000 rpm for 5 min. The methanol layer, isolated by a Pasteur pipette, was utilized for the analysis.

2.5. GC-C-IRMS analysis

The system consisted of an Agilent Technology 7890A gas chromatograph equipped with a G4513A autosampler (Agilent Technology, Germany) and coupled to an IsoPrime stable IRMS GC5 (Isoprime, Cheadle, UK) via a combustion interface under a continuous flow of helium. The combustion interface consisted of a ceramic furnace with a copper oxide and platinum catalyst at 850°C. The carbon stable isotope ratio was determined by referring to the international standard Vienna PeeDee Belemnite ($\delta^{13}C_{VPDB}$) with a defined ^{13}C content. The CRM used for the GC-C-IRMS multipoint calibration were *n*-undecane (δ^{13}C: -26.11‰, Chiron C0414.11-150-CY), *n*-pentadecane (δ^{13}C: -30.22‰, Chiron C0418.15-150-CY) and *n*-hexadecane (δ^{13}C: -34.87‰). The hexadecane delta value was obtained by EA-IRMS using the following primary standards: glucose (δ^{13}C: -10.76‰, Sigma Aldrich BCR657), polyethylene ($\delta^{13}C$: -32.15‰, IAEA IAEA-CH-7) and lithium carbonate ($\delta^{13}C$: -46.60‰, NIST RM 8545).

Isotope ratios were expressed as values (‰) and calculated on the basis of the following equation:

$$\delta i\ E = \frac{(i\ R_{SA} - i\ R_{REF})}{i\ R_{REF}}$$

where "i" is the mass number of the heavier isotope of element E (in this case ^{13}C), R_{SA} is the respective isotope ratio of the sample and R_{REF} is the relevant internationally recognized reference material. The delta values were multiplied by 1000 and expressed in units "per mill" (‰) (COPLEN, 2011).

The GC was operated using a HP-5 capillary column, 30 m x 0.32 mm i.d., 0.25 µm film thickness (Agilent – Italy). Helium was used as the carrier gas at a flow rate of 1.2 mL. The oven temperature program was initially 50°C (held for 1 min), then increased to 150°C at a rate of 5°C min^{-1}, then increased to 250°C at a rate of 20°C min^{-1} (held for 1 min). The injector temperature was 220°C and the injection volume was 1 mL (split 1:10). Data were collected in triplicate.

2.6. NMR analysis

The ^{1}H NMR spectra were measured on a Bruker spectrometer AVIII400 equipped with a SampleXpress sample changer and using a BBIz probe. The spectra were acquired using a 30° excitation pulse width of 2.66 µs, relaxation delay of 40 sec, and acquisition time of 5 s at 298 K. T1 relaxation times of the quantified peaks were measured with inversion-recovery experiments to assure that no bias from a short D1 would arise. The longest T1 measured for the standard BMTM (sample n.1 in Table 1) was 6.96 s, found for the CH_2 peak, while for the "white truffle-like" flavoring (sample n.1 in Table 2) we observed 6.02 sec for the same peak as the longest T1. Thus, the chosen D1 was sufficient to ensure a complete relaxation of the magnetization and to give fully quantitative results. The spectral width was 40 ppm centered at 6.5 ppm to ensure that the baseline was perfectly flat, as this is a precondition to correct integration and quantification of the peaks. For each spectrum, 64 scans were summed. The acquisitions were performed in a fully automated way. The spectra were processed with 260 K points and an exponential multiplication of 0.3 Hz. The data were acquired, processed, and analyzed using the software program Topspin 3.5 from Bruker Biospin.

Phasing and baseline correction were performed manually. In the case of the standard BMTM (sample n.1 in Table 1), the two main peaks of the H_1-C_{12} were considered together with both satellites for each peak. However, in the case of the "white truffle-like" flavoring (sample n.1 in Table 2), only one satellite per peak was considered due to the superposition with other signals in the matrix. The intensity of the peak was doubled for the calculation of the isotopic ratio.

3. RESULTS

3.1. GC-C-IRMS

The samples of commercially available synthetic BMTM could be readily prepared for GC-C-IRMS analysis by simple dilution in anhydrous methanol (Table 1).

Similarly, the extraction method adopted for samples reported in Tables 2 and 3 allowed us to isolate BMTM as a methanolic extract for use in GC-C-IRMS measurements.
For samples reported in Tables 1 and 2, corresponding to BMTM declared from synthesis and "white truffle-like" flavors purchased on the Italian flavorings market, respectively, the $\delta^{13}C$ values varied in the tight range of -42.24 and -43.40‰.
The above cited range, deduced from analysis of a sufficiently large number of samples (standards and flavorings) produced with "BMTM" certified from synthesis, was very useful for characterization of the "synthetic" authenticity of the molecule subjected to $\delta^{13}C$ measure. In addition, the same data, as expected, was not consistent with the carbon isotope ratio natural abundance ranges, as documented in literature data (VAN LEEUWEN *et al*., 2014).
Data obtained for seasonings of Table 3 are also included in the range between -42.34 and -43.26‰, and was not significantly different from the values corresponding to the synthetic samples cited above.

Table 1. GC-C-IRMS data produced by eight samples declared as BMTM from synthesis and available on the flavoring market.

Sample	$\delta^{13}C$ ‰ (mean)	s
1	-43.35	0.43
2	-42.30	0.35
3	-43.05	0.34
4	-42.47	0.32
5	-42.24	0.35
6	-43.40	0.34
7	-42.26	0.34
8	-43.14	0.41

Table 2. GC-C-IRMS data produced by ten samples, all declared as "white truffle-like" flavors, purchased on the Italian flavorings market and consisting of synthetic BMTM as the principal component diluted in olive oil.

Sample	$d^{13}C$ ‰ (mean)	s
1	-42.50	0.58
2	-42.43	0.53
3	-43.12	0.41
4	-42.40	0.34
5	-42.55	0.38
6	-42.64	0.47
7	-43.18	0.52
8	-42.38	0.45
9	-42.46	0.37
10	-42.52	0.46

Table 3. GC-C-IRMS data produced by twenty samples of white truffle flavored oils purchased on the Italian market (glass pack 40-250 mL).

Seasoning	$\delta^{13}C$ ‰ (mean)	s
1	-42.34	0.42
2	- 43.08	0.34
3	-42.81	0.52
4	-42.63	0.36
5	-43.16	0.38
6	-42.86	0.48
7	-43.02	0.35
8	n.d.	----
9	-42.53	0.46
10	-43.12	0.43
11	-43.06	0.37
12	-43.18	0.51
13	-42.68	0.45
14	-42.48	0.39
15	-43.04	0.37
16	-43.10	0.41
17	-42.66	0.56
18	-42.74	0.46
19	-43.26	0.38
20	-42.45	0.42

3.2. ^1H NMR characterization of BMTM

The aim of our study was to develop an approach using NMR methodology to characterize the BMTM synthetic molecule of a standard sample and to verify the possibility of using the extraction method referred in "2.4 Sample preparation" to characterize the synthetic BMTM present in a "white truffle-like" flavor. We measured the values $^{13}C/^{12}C$ for the two sites corresponding to CH_2 and CH_3 in the BMTM molecule extracted from the two samples. Our data showed the structural isotopic distribution of $^{13}C/^{12}C$ in the characteristic sites of these two molecules.

3.2.1 BMTM from synthesis (sample 1, Table 1)

The ^1H NMR spectra of the synthetic standard BMTM were acquired using two aliquots of the same sample diluted in methanol-d4 and in replicates to evaluate the experimental CV% (coefficient of variation %) from 8 trials.
The BMTM ^1H NMR spectrum in methanol-d4 displayed two singlets, resonating at 3.68 and at 2.15 ppm. The signals are attributed to the CH_2 (3.68 ppm) and the CH_3 (2.15 ppm). The ^{13}C satellites of the two peaks were easily recognizable and they appeared as doublets with a heteronuclear J coupling of 149.9 Hz for the CH_2 and of 138.5 Hz for the CH_3. The doublets were centered at the isotropic resonances of the peaks. As expected, the intensities of the satellites were significantly lower than those of the main peaks, which were attributed to the ^1H attached to the ^{12}C.

The ratio between the intensities of satellites due to the ^{1}H - ^{13}C heteronuclear coupling and that of the main peak allowed us to extract the integral ratio between ^{13}C and ^{12}C in a site specific manner.
The ^{1}H NMR spectrum (Fig. 1) and the results (Table 4) indicated that the ratio of $^{13}C/^{12}C$ gave the same value for both sites (CH_2 and CH_3). This value is in agreement with the expected $^{13}C/^{12}C$ global ratio.

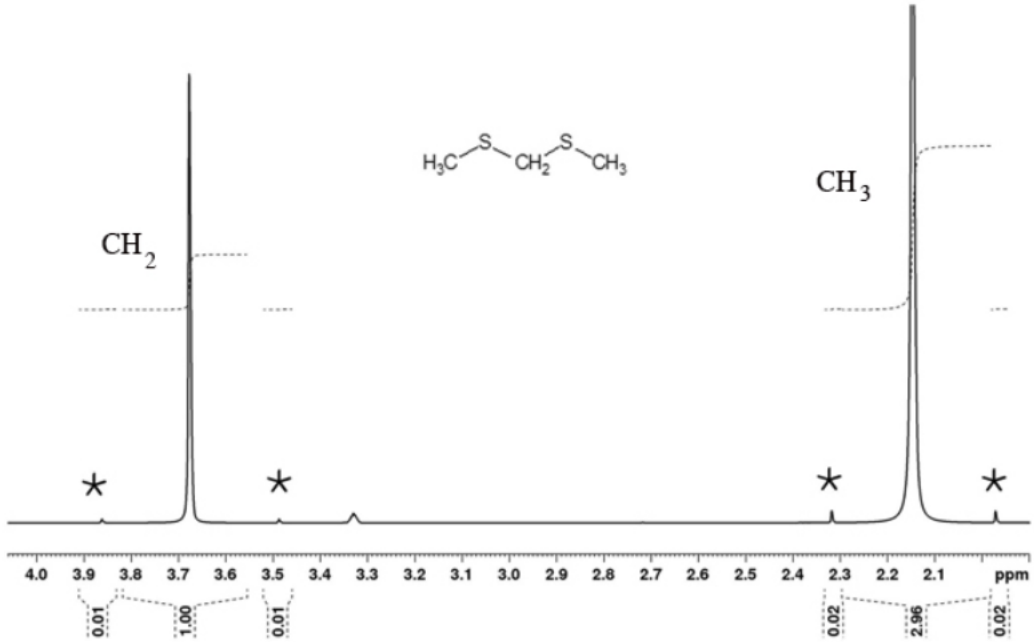

Figure 1. ^{1}H spectrum of the BMTM from synthesis, available on the flavor raw material market (sample 1, Table 1). The asterisk indicates the ^{13}C satellites. The dotted lines indicate the integral regions.

Table 4. Data %^{13}C measured by ^{1}H NMR spectra in replicates of two aliquots for each sample: synthetic BMTM standard (sample 1 in Table 1), synthetic BMTM from "white truffle-like" flavor (sample 1 in Table 2).

		% ^{13}CH			
		BMTM synth. std.		**BMTM synth. flav.**	
replicates		$-CH_2-$	$-CH_3$	$-CH_2-$	$-CH_3$
aliquot 1	1	1.02	1.06	1.01	0.99
	2	1.03	1.02	1.07	0.99
	3	1.05	1.05	1.03	1.06
aliquot 2	1	1.05	1.05	0.99	1.02
	2	1.02	1.00	0.99	0.99
	3	1.00	1.01	0.99	1.04
	4	1.01	1.00	0.99	1.02
	5	1.03	0.99	1.03	1.05
mean		1.03	1.02	1.01	1.02
CV%		0.02	0.03	0.03	0.03

3.2.2 BMTM extracted from synthetic white truffle-like flavoring (sample1, Table 2)

The same approach described for the synthetic BMTM standard was applied to the synthetic BMTM in "white truffle-like" flavoring. As in the case of the molecule BMTM from synthesis, the spectrum was acquired in 8 replicates derived from two extracts produced from two aliquots of synthetic BMTM from "white truffle-like" flavoring. The ^1H spectra of the two aliquots extracted with methanol-d4 showed similar features to that of the synthetic BMTM standard, with the two singlets in the same position (3.67 ppm for CH_2 and 2.14 ppm for CH_3) and the same heteronuclear J couplings (149.9 Hz for the CH_2 and of 138.5 Hz for the CH_3). The 0.01 ppm shift is attributable to a matrix effect and it is reproducible in all the replicates. The spectrum acquired is reported in Fig. 2 and also in this case, as reported in Table 4, the ratio of $^{13}C/^{12}C$ gave the same value for both sites (CH_2 and CH_3).
As shown in Fig. 2, the presence of a potentially interfering substance often found in "white truffle" flavor (identifiable by GC/MS as methylsulfinyl(methylthio)-methane) did not hinder the identification of ^{13}C satellites and the % ^{13}C values were strictly comparable to data obtained for synthetic BMTM standard.
To clarify, the ^1H NMR spectrum of the sulfoxide is easily recognizable by these features: one singlet at 2.71 ppm attributable to one terminal methyl; one singlet at 2.33 ppm attributable to the second terminal methyl; two doublets centered at 3.98 ppm and at 3.83 ppm with a J=13.8 Hz attributable to the CH_2 protons that became diastereotopic upon asymmetric oxidation.

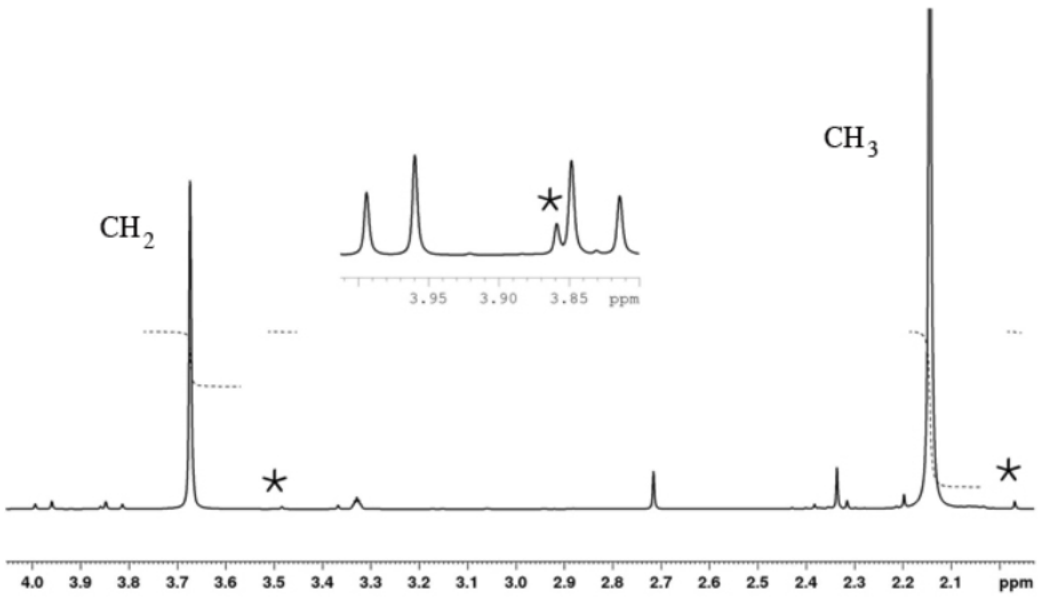

Figure 2. ^1H spectrum of the "white truffle" flavor purchased on the Italian flavor market and consisting of synthetic BMTM as the principal component diluted in olive oil. The asterisk indicates the ^{13}C satellites. The dotted lines indicate the integral regions. The inset shows the expansion of the 4.05-3.75. These peaks are attributed to the CH_2 protons in a molecule identified as methylsulfinyl(methylthio)-methane.

4. DISCUSSION

The simple extraction method, adopting methanol as the solvent, is useful for application to provide data by GC-C-IRMS of a synthetic BMTM standard, in flavoring from the raw material market (see Table 1), and in Italian flavoring from the commercial market (see Table 2). The $\delta^{13}C$‰ data collected for all these matrices ranged between -42.24 (σ 0.35) and -43.40‰ (σ 0.34). Clearly, the range is external to the natural abundance of the carbon isotope ratio, and allows one to identify the synthetic origin of BMTM if this molecule is used to produce seasonings made by the addition to olive oil or other vegetable oils.

The $\delta^{13}C$‰ data obtained with the same simple extraction method on twenty seasonings purchased on the Italian market allowed us to deduce that the synthetic BMTM molecule is clearly identifiable. In fact, the $\delta^{13}C$‰ values reported in Table 3, except for the sample n. 8, which does not contain BMTM, exhibited data in the range -42.34 and -43.26‰, identified as characteristic of synthetic BMTM.

Most seasoning samples – representing about 75% of the total seasoning samples examined (namely, n. 1-5, 7, 9, 10, 12, 14-15, 17-18 and 20 in Table 3) are compliant with the EU Regulation 1334/2008 because they contain synthetic BMTM and on the label only the term "flavor" is correctly used.

Samples n. 6, 11, 13, 16, and 19 in Table 3 are not compliant with the EU Regulation 1334/2008 because they contain synthetic BMTM and report on label the reference to the term "truffle". Specifically, the term "white truffle flavor" was used for samples 6, 11, 16, 19, while "natural white truffle flavor" for sample 13.

For the sample n. 8 in Table 3, the only one resulting as not containing BMTM, and reported on label as produced with "natural flavor", the judgment of compliance or otherwise with the EU Regulation 1334/2008 does not depend from the BMTM identification (natural or synthetic), but from the origin of all the molecules constituting the flavor. In fact, a natural flavor used for a seasoning can be realized totally with natural molecules. In this case, the final judgment is not linked to the BMTM identification, but from the naturalness of all the components of the flavor used.

There was no evidence of data that did not fall within the range characteristic of synthetic BMTM, and it is justified by being anyhow unavoidable due to the lack of commercial availability of natural BMTM in the raw material flavor market.

Adopting the same extraction method, but using methanol d-4 as the solvent, we demonstrated the feasibility of ^{1}H NMR measures to calculate % $^{13}C/^{12}C$ in two characteristic sites -CH_2- and -CH_3. This investigation, applied to a declared synthetic BMTM standard (sample 1 in Table 1) characterizes the integral ratio between ^{13}C and ^{12}C in a site specific manner. In fact, the ^{1}H NMR spectrum (Fig. 1) and the results (Table 4) indicated that the ratio of $^{13}C/^{12}C$ gave the same value for both sites (CH_2 and CH_3), in agreement with the expected $^{13}C/^{12}C$ global ratio.

Data produced from a synthetic BMTM standard were not statistically different from the corresponding data produced for a "white truffle-like" flavor, as shown in Table 4.

5. CONCLUSIONS

The data reported in this paper are the first GC-C-IRMS and ^{1}H NMR contributions to the characterization of synthetic BMTM available on the flavoring market and in some "white truffle-like" flavors. All the analyzed seasoning (except one sample not containing BMTM) produced GC-C-IRMS data of BMTM in agreement with the values of the synthetic molecule.

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Paper Received April 30, 2018 Accepted July 25, 2018