

1 **Zeolite reduces losses and minimizes fractionation of various flavor compounds during EA-**  
2 **IRMS analysis**

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12 **Key words:** EA-IRMS;  $\delta^{13}\text{C}$ , zeolite, flavor compounds

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14 **Abstract**

15 Among the methods for confirming the natural origin of flavour compounds,  $\delta^{13}\text{C}$  measurements  
16 using IRMS technique are becoming increasingly important. EA-IRMS measures can suffer from  
17 errors caused by the volatility of the analyzed compounds and the consequent isotope fractionation.  
18 This work suggests a method involving the adsorption of volatile molecules in zeolite before the EA-  
19 IRMS analysis, minimizing the possible error due to the volatility of the structures analyzed. The  
20 stabilizing effect on any fractionation was proven during the analysis of various molecules which  
21 cover a wide range of volatility. The work demonstrates that, by operating with the advised method,  
22 the repeatability of EA-IRMS data is very high not only for samples prepared before analysis, but  
23 also for samples prepared in a time span of up to 6 hours. The proposed method facilitates serial  
24 preparation and prevents erroneous results due to the natural volatility of the molecules under  
25 evaluation.

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27 **1. Introduction**

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29 The formulation of natural flavors, as classified according to the current European Community  
30 Directive, provides for the use of flavoring preparations derived by physical procedures of extraction  
31 from aromatic herbs and spices. These flavoring components may be single substances isolated by  
32 physical procedures of extraction from plant and animal sources or as flavoring substances produced  
33 or isolated by biotechnological processes (EC Reg. 1334/2008).

34 The flavor industry produces formulations of natural and synthetic flavouring substances  
35 using raw materials available on the industrial market. Therefore, quality control can be particularly  
36 problematic with respect to verification of the integrity of the chemicals declared “natural” at the  
37 point of purchase.

38 Currently, the analytical methods to confirm the natural origin of a molecule marketed as such are  
39 based on chiral analysis. The enantiomeric distribution of the molecule extracted from a natural  
40 source is compared to data in the literature. However, isotope ratio mass spectrometry (IRMS) affords  
41 a more certain characterization. The measure of the stable isotope ratio of carbon is an advanced  
42 method used for discrimination from natural and synthetic organic substances (Winkler & Schmidt,  
43 1980; Schmidt, 1986; Rossmann, 2001). With this methodology, the reference data that is  
44 characteristic of natural molecules may not be available as “official” data but, for many molecules,  
45 the data are traceable within the literature (Schmidt, Gatfield, Hilmer, Werkhoff, Krammer, Sommer,  
46 Herbrand, Meier & Bertamm, 2001; Strojnik, L., Hladnik, J., Weber, N.C., Koron, D., Stopar, M.,  
47 Zlatić, E., Kokalj, D., Strojnik, M., & Ogrinc, N., 2021). In some cases, the  $\delta^{13}\text{C}$  values are available  
48 even for flavor molecules stemming from different biotechnologies or physical procedures (Goupry,  
49 Rochut, Robins & Gentil, 2000). However,  $\delta^{13}\text{C}$  values available at the present time do not cover the  
50 complete range of molecules used in flavoring formulations. The isotopic characterization of organic  
51 compounds is possible by coupling classical elemental analysis (EA) with isotope ratio mass  
52 spectrometry (IRMS). Moreover, data acquired through conventional EA-IRMS analytical methods  
53 may suffer from errors caused by the extreme volatility of many flavor compounds. With respect to  
54 the reproducibility of  $\delta^{13}\text{C}$  measurements using EA-IRMS approaches, one must take into account  
55 spontaneous isotope fractionation, even though this event is difficult to evaluate. The standard tin  
56 capsules generally used for solid sample containment in EA-IRMS analysis do not restrict the  
57 volatility of the molecule under analysis, thus impairing the correct determination of the  $\delta^{13}\text{C}$  values  
58 associated with the chromatography peaks of  $\text{CO}_2$  arising from the complete combustion of the  
59 samples. In particular, the exact measurement of  $\delta^{13}\text{C}$  values is impeded by: i) the recording of the  
60  $\text{CO}_2$  signals, which often present insufficient intensity and extreme variability to be compared with  
61 those measured for the  $\text{CO}_2$  in the working gas reference; ii) the volatilization related isotope effects  
62 that lead to potential isotopic fractionation.

63 In recent decades, zeolites, a class of natural and synthetic microporous aluminosilicate  
64 minerals, have been investigated and used in a wide range of applications as adsorbents and catalysts  
65 (Davis, 2002; Kumar, Li, & Rimer, 2016; Li & Yu, 2014; Zones & Davis, 1996). The pore diameter  
66 in zeolites ranges from 0.5–2.0 nm and has been proven to be suitable for both adsorption and

67 retention of various volatile molecules that can freely access the pores (Veerapandian, De Geyter,  
68 Giraudon, Lamonier, & Morent, 2019).

69 In this study we present a method that combines the adsorption of volatile molecules in zeolitic  
70 materials with conventional EA-IRMS analysis to reliably measure the stable carbon isotope  
71 composition of volatile chemicals minimizing potential C isotope fractionation.

72 The use zeolite increases the residence time of the volatile molecules inside the tin cap,  
73 reducing losses and thus minimizing the fractionation effects due to volatilization. This report gives  
74 the results the  $\delta^{13}\text{C}$  values obtained by the analysis of 10 volatile compounds, sourced from the flavor  
75 market. These flavor components were chosen to cover a wide range of boiling points and volatilities.  
76 The results, in addition to contributing to the knowledge of the carbon stable isotope composition of  
77 the molecules analyzed, could aid in the development of novel reliable reference protocols for EA-  
78 IRMS analysis of volatile compounds.

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## 80 **2. Materials and methods**

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### 82 *2.1 Samples*

83 Natural samples (ethyl propionate  $\geq 97\%$ , ethyl butyrate  $\geq 98\%$ , isoamyl acetate  $\geq 97\%$ , ethyl  
84 octanoate  $\geq 98\%$  and ethyl heptanoate  $\geq 98\%$ ) were purchased by Merk (Milan, Italy); synthetic liquid  
85 samples and solid sample vanillin ex fer ac (produced from ferulic acid) were provided by Interflavors  
86 (Milan, Italy). All the synthetic compounds had a purity  $> 98\%$ .

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### 88 *2.2 $\delta^{13}\text{C}$ determination*

89 Liquid samples were prepared by adding 1  $\mu\text{L}$  of each material under analysis into a 5 x 9  
90 mm tin capsule containing 5 mg of zeolite (MS-13X - Molecular Sieve Adsorbent, 20304,  
91 Supelco; 1.3 nm pore diameter). For the solid sample vanillin, 0.1 mg were added to a tin capsule  
92 containing 2.5 mg of zeolite, and then covered with an additional 2.5 mg of zeolite. Capsules were  
93 carefully closed by folding them with cleaned tweezers and then transferred to the auto-sampler.

94 The  $\delta^{13}\text{C}$  values of samples were measured using a Flash 2000 HT elemental analyzer  
95 coupled, via a ConFLo IV Interface, with a Delta V Advantage Isotope Ratio Mass Spectrometer  
96 (IRMS), interconnected to the software Isodat 3.0 (Thermo). The combustion/reducing reactors,  
97 combined in a single quartz tube, were heated at 1020 °C. The He gas flow was 120 mL  $\text{min}^{-1}$  and  
98 100 mL  $\text{min}^{-1}$  for carrier and reference, respectively. The  $\text{O}_2$  purge for flash combustion was 3 s at a  
99 flow rate of 175 mL  $\text{min}^{-1}$  per sample. The GC separation column was tempered to 45 °C. The  $\text{CO}_2$

100 reference gas pulse was introduced three times (20 s each) at the beginning of each run. The run time  
101 of the analysis was 600 sec for a single run. The analysis of each sample was performed five times.  
102 Calibration was performed using three secondary reference materials provided by IAEA: NBS18  
103 ( $\delta^{13}\text{C} \text{‰} = -5.014 \pm 0.035$ ); IAEA-600 ( $\delta^{13}\text{C} \text{‰} = -27.771 \pm 0.043$ ); IAEA-612 ( $\delta^{13}\text{C} \text{‰} = -36.722 \pm$   
104  $0.006$ ). Two in-house solid nonvolatile standards – sulfanilamide ( $\delta^{13}\text{C} = -27.23 \pm 0.06 \text{‰}$ ) and  
105 methionine ( $\delta^{13}\text{C} = -30.01 \pm 0.05 \text{‰}$ ) standards were used for normalization and quality assurance.  
106 The carbon stable isotope ratio was determined by referring to the international standard Vienna  
107 PeeDee Belemnite ( $\delta^{13}\text{C}_{\text{VPDB}}$ ) with a defined  $^{13}\text{C}$  content.

108 The relative difference of isotopic ratio (isotope-delta value) was calculated on the basis of  
109 the following equation:

$$\delta^{i/j}\text{E} = \frac{{}^{i/j}\text{R}_{\text{P}} - {}^{i/j}\text{R}_{\text{Ref}}}{{}^{i/j}\text{R}_{\text{Ref}}}$$

114 where “i” is the higher and “j” is the lower atomic mass number of element E (in this case  $^{13}\text{C}$ ),  $\text{R}_{\text{P}}$   
115 is the respective isotope ratio of the sample and  $\text{R}_{\text{Ref}}$  is the respective isotope ratio of the relevant  
116 internationally recognized reference material. The delta values were multiplied by 1000 and  
117 expressed in units “per mil” (‰) (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014).

### 119 3. Results and Discussions

120  
121 To prove the efficiency of zeolitic materials in providing reliable measurement of the stable  
122 carbon isotope composition of flavor compounds, we analyzed 10 molecules of diversified structure  
123 and boiling point (b.p.). In particular, we used a synthetically produced zeolite (MS-13X) with pore  
124 size of 10 Å which was able to absorb molecules of large critical diameter, such as aromatics and  
125 branched-chain hydrocarbons (Veerapandian, De Geyter, Giraudon, Lamonier, & Morent, 2019; Yi,  
126 Yang, Tang, Zhao, Wang, Cui, Feng, & Ma, 2017).

127 Results, expressed using the standard  $\delta^{13}\text{C}$  notation (Table 1), showed that the measured values  
128 ranged from -37.01 to -17.32‰. Data analysis clearly indicated that the preventive adsorption of the  
129 flavor compounds on the zeolitic materials allowed highly repeatable measurements of the stable  
130 carbon isotope composition of the samples. The values of the standard deviations (s.d.) obtained in 5  
131 independent measurements ranged from 0.02 to 0.19, suggesting that the adsorption of volatile  
132 molecules in zeolitic materials may minimize potential isotope effects occurring during sample

133 preparation and analysis (Julien, Parinet, Nun, Bayle, Höhener, Robins, & Remaud, 2015) were  
134 solved using zeolite as adsorbent material.

135 To test the reproducibility of the methods and the stability of the adsorption over time, we  
136 performed  $\delta^{13}\text{C}$  measurements on a set samples at different times after the preparation. To this  
137 purpose the encapsulated samples were kept on the auto-sampler under continuous flux of He, and  
138 then analyzed at 0, 2, and 6 hours. Figure 1 shows the results of one representative experiment  
139 performed using the ethyl butyrate N as a test molecule. The signal amplitudes for the ethyl butyrate  
140 N were stable over the measurement time, indicating that no significant losses of sample occurred  
141 until at least 6 hours after sample preparation.

142 The  $\delta^{13}\text{C}$  measurement data at times of 0, 2 and 6 hours fell within a very narrow range with  
143 maximum variation within  $\pm 0.06$  ‰.

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#### 145 **4. Conclusions**

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147 This work took into consideration the problem of optimized determination of the IRMS values  
148 obtained for EA on a group of molecules that differed in boiling point and volatility. These  
149 representative compounds can be considered as prototypes for verifying the efficiency of the method  
150 suggested in this paper.

151 The experiments showed that the sample preparation as conducted in this work allowed us to  
152 obtain repeatable results not only on samples prepared immediately before the analysis, but also on  
153 prepared samples which underwent stationing in the environment for times up to 6 hours.

154 This study showed that the use of zeolite prevents diffusion or fractionation of the molecules  
155 being measured, contrary to what is commonly found by preparing the containment capsule without  
156 the use of any support. The suggested method facilitates the serial preparation of many samples for  
157 analysis without the results being distorted by the natural volatility of the molecules under  
158 examination.

159 This paper suggests a model to confirm the efficiency of this proposed method, useful for the  
160 EA-IRMS analysis of pure volatile organic compounds without the use of liquid autosampler.

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#### 162 **Author contribution**

163 Monica Bononi and Fernando Tateo designed the study. Fabio Francesco Nocito carried out the  
164 experiment. Monica Bononi, Fabio Francesco Nocito and Fernando Tateo wrote the manuscript.

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167 **Declaration of Competing Interest**

168 The authors declare that they have no known competing financial interest or personal relationship  
169 that could have appeared to influence the work reported in this paper.

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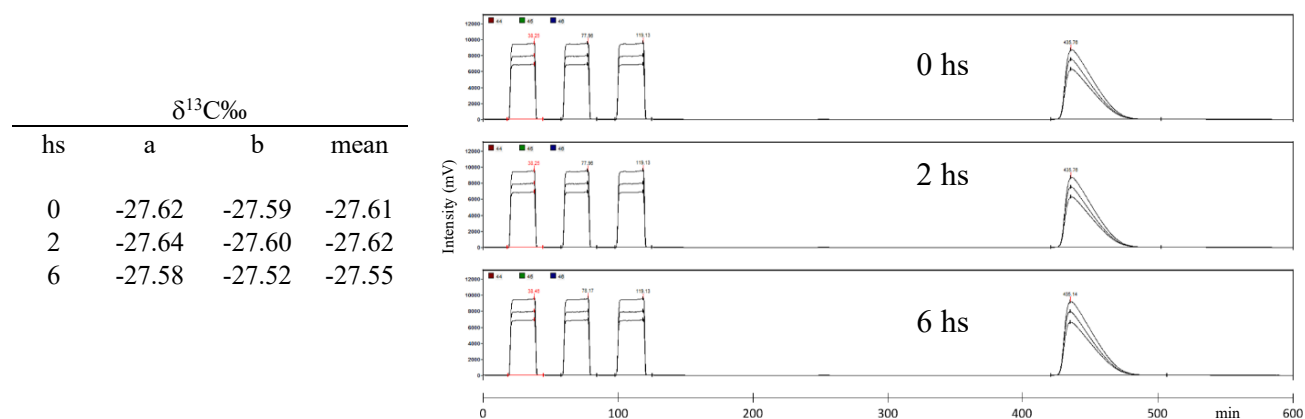
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Table 1.  $\delta^{13}\text{C}$  values measured for EA-IRMS analysis on natural (N) and synthetic (S) molecules, with different volatilities and boiling points between 99 °C and 285 °C, with the absorption method on zeolite. The standard deviation values demonstrate the high repeatability of the results.

compound	CAS	b.p. (°C)	$\delta^{13}\text{C}$ (‰)	s.d.
ethyl propionate N	105-37-3	99.1	-21.75	0.02
ethyl propionate S	105-37-3	99.1	-24.98	0.14
ethyl butyrate N	105-54-4	121.5	-27.61	0.03
ethyl butyrate S	105-54-4	121.5	-28.01	0.07
isoamyl acetate N	123-92-2	142.5	-22.34	0.11
isoamyl acetate S	123-92-2	142.5	-17.32	0.02
ethyl heptanoate N	106-30-9	188.5	-30.38	0.09
ethyl octanoate N	106-32-1	208.5	-28.76	0.04
ethyl octanoate S	106-32-1	208.5	-32.87	0.19
vanillin ex fer ac	121-33-5	285.0	-37.01	0.04



Figure 1. EA-IRMS traces for ethyl butyrate N analyzed at 0, 2, and 6 hours (the corresponding values are reported on the left). The description of the sample preparation is reported in section 2.2.



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