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**Adding extra-dimensions to hazelnuts primary metabolome fingerprinting by comprehensive two-dimensional gas chromatography combined with time-of-flight mass spectrometry featuring tandem ionization: Insights on the aroma potential**

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(Article begins on next page)

1 **Adding extra-dimensions to hazelnuts primary metabolome fingerprinting by**  
2 **comprehensive two-dimensional gas chromatography combined with time-of-**  
3 **flight mass spectrometry featuring tandem ionization: insights on the aroma**  
4 **potential**

5  
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17

18

19 **Abstract**

20 The information potential of comprehensive two-dimensional gas chromatography combined  
21 with time of flight mass spectrometry (GC×GC-TOFMS) featuring tandem hard (70 eV) and soft (12 eV)  
22 electron ionization is here applied to accurately delineate high-quality hazelnuts (*Corylus avellana* L.)  
23 primary metabolome fingerprints. The information provided by tandem signals for untargeted and  
24 targeted 2D-peaks is examined and exploited with pattern recognition based on template matching  
25 algorithms. EI-MS fragmentation pattern similarity, base-peak  $m/z$  values at the two examined energies  
26 (i.e., 12 and 70 eV) and response relative sensitivity are adopted to evaluate the complementary nature  
27 of signals.

28 As challenging bench test, the hazelnut primary metabolome has a large chemical dimensionality  
29 that includes various chemical classes such as mono- and disaccharides, amino acids, low-molecular  
30 weight acids, and amines, further complicated by oximation/silylation to obtain volatile derivatives.

31 Tandem ionization provides notable benefits including larger relative ratio of structural informing  
32 ions due to limited fragmentation at low energies (12 eV), meaningful spectral dissimilarity between 12  
33 and 70 eV (direct match factor values range 222-783) and, for several analytes, enhanced relative  
34 sensitivity at lower energies. The complementary information provided by tandem ionization is exploited  
35 by untargeted/targeted (*UT*) fingerprinting on samples from different cultivars and geographical origins.  
36 The responses of 138 *UT*-peak-regions are explored to delineate informative patterns by univariate and  
37 multivariate statistics, providing insights on correlations between known precursors and (key)-aroma  
38 compounds and potent odorants. Strong positive correlations between non-volatile precursors and  
39 odorants are highlighted with some interesting linear trends for: 3-methylbutanal with isoleucine ( $R^2$   
40 0.9284); 2,3-butanedione/2,3-pentanedione with monosaccharides (fructose/glucose derivatives) ( $R^2$   
41 0.8543 and 0.8860); 2,5-dimethylpyrazine with alanine ( $R^2$  0.8822); and pyrroles (1H-pyrrole, 3-methyl-  
42 1H-pyrrole, and 1H-pyrrole-2-carboxaldehyde) with ornithine and alanine derivatives ( $R^2$  0.8604). The  
43 analytical work-flow provides a solid foundation for a new strategy for hazelnuts quality assessment  
44 because aroma potential could be derived from precursors' chemical fingerprints.

45

46 **Key-words**

47 comprehensive two-dimensional gas chromatography, *UT* fingerprinting, template matching, time-of-  
48 flight mass spectrometry featuring tandem ionization, high quality hazelnuts, aroma potential

49

50 **1. Introduction**

51 Since its introduction, comprehensive two-dimensional gas chromatography (GC×GC) coupled  
52 with mass spectrometry (MS) has shown great potential in profiling and fingerprinting investigations [1]  
53 in food [2–6]. 2D patterns of separated compounds well describe sample chemical dimensionality [7].

54 Although GC×GC is the technique of election to investigate food volatiles, it also enables effective  
55 studies on semi-volatiles and on non-volatiles suitably, after derivatization to improve thermal stability  
56 and volatility. Examples of research in this direction are the characterization of lipid fractions by GC×GC-  
57 MS/FID [8,9], fatty acids methyl esters (FAMES) profiling [10–12], and primary metabolites in function of  
58 harvesting and storage practices [13,14].

59 The current study aims at developing a methodology based on GC×GC-TOF MS that combines  
60 profiling and fingerprinting strategies [1] based on 2D-data patterns, to comprehensively map hazelnuts  
61 key-aroma compounds precursors and relevant primary metabolites (amino acids, reducing sugars and  
62 polyalcohols, organic acids etc..) from raw fruits. To deal with the great complexity of primary  
63 metabolome, variable electron ionization (EI) MS is examined. The patented technology, termed tandem  
64 ionization™ [Select eV™ - US patent number 9,786,480], operates with variable-energy EI across single  
65 analytical runs. The acquisition is therefore done by time-switching between two ionization energies so  
66 that tandem data streams are generated and acquired iteratively. In the ion source, a high potential  
67 difference accelerates electrons away from the filament while reducing their energy before their entrance  
68 in the ion chamber [15]. This results in a more efficient ionization and a reduced loss of sensitivity at low-  
69 energy EI with enhanced intensity for structure-diagnostic ions [16].

70 Recent studies, have demonstrated that tandem ionization combining 14 and 70 eV provided  
71 successful discrimination of isomeric species in complex mixtures of motor oil samples [17] and in blood  
72 volatiles [18]. Additional benefits are derived from dedicated tandem data processing procedures. Freye  
73 *et al.* [19] applied tile-based Fisher ratio analysis on fused tandem signals to detect analytes spiked at 50  
74 mg/kg level in diesel samples. Cordero *et al.* [16] extended the combined untargeted/targeted  
75 fingerprinting (*UT* fingerprinting) approach [20,21] to cocoa volatilome analysis. They confirmed that  
76 tandem signals, when summed together, gave better performances, in terms of samples discrimination  
77 and classification. In addition, analytes with a reduced fragmentation at 12 and 14 eV gave higher signal-  
78 to-noise ratio at lower ionization voltages also extending the dynamic range of measurements.

79  
80 Challenges posed by hazelnuts primary metabolome fingerprinting are related to the large  
81 chemical dimensionality [22], that includes different chemical classes as mono- and disaccharides, amino

82 acids, low-molecular weight acids, and amines, and are furtherly complicated by oximation/silylation to  
83 obtain volatile derivatives. However, the information embedded in primary metabolites chemical  
84 signatures could be of high interest if correlated with hazelnuts hedonic qualities. Several aroma active  
85 compounds [23–26] have known mechanisms of formation from non-volatile precursors [25–27].

86 Intriguing empirical evidence on the instability, and/or evolution, of the volatile metabolome  
87 during shelf-life and storage of hazelnuts was discussed by Cialiè Rosso *et al.* [28]. In that study, GC×GC-  
88 MS, combined with advanced fingerprinting based on template matching, highlighted informative  
89 patterns of odorants strongly correlated, in raw hazelnuts, to post-harvest drying practices and storage  
90 conditions within a 12-month shelf-life [28]. At the same time, the aroma potential of nuts was studied  
91 by mapping volatiles fingerprints after lab-scale roasting across the shelf-life (e.g., 12 months after  
92 harvest). Results obtained with two origins (Turkish *Ordu* and Italian Nocciola Romana or Tonda Gentile  
93 Romana PDO - IT/PDO/0005/0573), showed that volatile patterns were greatly impacted by storage  
94 conditions. Samples stored under modified atmosphere (99% N<sub>2</sub> – 1% O<sub>2</sub>) at 5°C were characterized by  
95 higher abundance of alkyl pyrazines, Strecker aldehydes, and ketones compared to samples kept in a  
96 storage atmosphere richer in oxygen (78% N<sub>2</sub> – 21% O<sub>2</sub>) and/or at ambient temperature (18°C). Most  
97 components are Maillard reaction products and/or formed by thermal degradation of reducing sugars, so  
98 it was hypothesized that storage would have impacted the distribution/availability of some non-volatile  
99 precursors in the fruits.

100 In this study, for the first time, GC×GC-TOF MS featuring tandem ionization is tested for its  
101 effectiveness on hazelnuts primary metabolome fingerprinting. In particular, spectral  
102 similarity/dissimilarity between high (70 eV) and low (12 eV) ionization energy is examined, including  
103 complementary information potential, sensitivity, and dynamic range of response. The analytical strategy  
104 is then validated for its informative potential and reliability in providing robust data for correlation studies  
105 with potent odorants distribution.

106

## 107 2. Experimental

### 108 2.1 Chemicals and reference solutions

109 Pure standards of *n*-alkanes (from n-C9 to n-C25) for system evaluation and linear retention  
110 indexes (*I*<sup>T</sup>) determination and  $\alpha$ -tujone for volatiles internal standardization (IS) were from Merck (Milan,  
111 Italy).

112 The mixture of *n*-alkanes for the *I*<sup>T</sup> solution was prepared in cyclohexane at a concentration of 100  
113 mg/L; internal standard (IS)  $\alpha$ -tujone solution was prepared in diethyl phthalate (Sigma Aldrich 99% of  
114 purity) at a concentration of 100 mg/L.

115 Pure standards for identity confirmation of pyruvic acid, lactic acid, malonic acid, acetoacetic acid,  
116 phosphoric acid, succinic acid, glyceric acid, fumaric acid, malic acid, citric acid, Alanine-Ala, Asparagine-  
117 Asn, Aspartic acid-Asp, Cysteine-Cys, Glutamic acid-Glu, Glycine-Gln, Isoleucine-Ile, Leucine-Leu, Lysine-  
118 Lys, Methionine-Met, Ornithine-Orn, Phenylalanine-Phe, Proline-Pro, Serine-Ser, Threonine-Thr,  
119 Tryptophan-Trp, Tyrosine-Tyr, Valine-Val, glycerol, xylitol, mannitol, myo-inositol, fructose, glucose,  
120 saccharose, and the internal standards (IS), 4- chlorophenylalanine (quality control - QC for derivatization)  
121 and 1,4-dibromobenzene (QC for GC normalization), were from Merck (Milan, Italy).

122 Derivatization reagents and HPLC grade solvents: O-methylhydroxylamine hydro- chloride (MOX),  
123 (N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) methanol, pyridine, *n*-hexane, di- chloromethane, and  
124 toluene were from Merck (Milan, Italy).

### 125 2.2 Hazelnut samples

126 Commercial grade samples of raw hazelnuts (*Corylus avellana* L.) with uniform caliber of 13 mm  
127 and harvested in 2016 were supplied by Soremartec Italia Srl (Alba-CN, Italy). They were from different  
128 geographical areas: (a) mono-cultivar Nocciola Romana (TR), also known as Tonda Gentile Romana, a  
129 Protected Denomination Origin (PDO) product (EU Quality registration code IT/PDO/0005/0573) here  
130 defined as *Roman*; (b) mono-cultivar Tonda Gentile delle Langhe (TGL) or Nocciola del Piemonte, a  
131 Protected Geographical Indication (IGP) product here defined as *Piedmont* and, (c) Turkish blend  
132 harvested in the Ordu (OR) region with *Tombul*, *Palaz* and *Çakıldak* cultivars here defined as *Ordu*.

133 Raw hazelnuts were grinded after freezing with liquid nitrogen and stored at -18 °C before  
134 analysis. Lab-scale roasting was by a ventilated oven for 15 minutes at 160 °C on 100 g aliquots; these  
135 conditions were set in a previous study and optimized to develop major key-odorants [26] and roasting  
136 marker compounds [29].

## 137 **2.3 Primary metabolites extraction and derivatization**

### 138 *2.3.1 Defatting*

139 Micro-scale defatting was conducted in sealed glass vials (20 mL volume) on 1.00 g of ground  
140 hazelnuts aliquots with 5.0 mL of *n*-hexane as extraction solvent at ambient temperature and with the aid  
141 of ultrasound (US - 40 KHz  $\pm$  5%). Steps of 15' were set and exhaustiveness was verified after 6-7 successive  
142 extractions with fresh *n*-hexane aliquots. To avoid any contamination of the hydrophilic phase and to  
143 ensure efficient derivatization in the next sample preparation step, 10 extractions were set in the final  
144 protocol for samples defatting. A schematic diagram of the extraction/derivatization procedure is  
145 provided as **Supplementary Figure 1 – SF1** together with experimental results on fat fraction extraction  
146 yields on a representative sample (**Supplementary Figure 2 – SF2**). The defatted hazelnut powder was  
147 then kept under a gentle stream of nitrogen to remove residual solvent and stored at -18°C before  
148 extraction and derivatization.

### 149 *2.3.2 Extraction of primary metabolites*

150 An aliquot of 0.100 g of defatted hazelnuts was placed in a centrifuge glass tube with 5.0 mL of  
151 H<sub>2</sub>O/CH<sub>3</sub>OH (98:2 v/v) mixture. Extraction was conducted at ambient temperature and with the aid of  
152 ultrasound (US - 40 KHz  $\pm$  5%) for 15'. Exhaustiveness was verified after 3-10 successive extractions,  
153 depending on the targeted metabolite, with fresh solvent aliquots. A schematic diagram of the  
154 extraction/derivatization procedure is provided as **Supplementary Figure 1 – SF1** together with  
155 experimental results on targeted primary metabolites extraction yields **Supplementary Figure 2- SF2**.  
156 After extraction, centrifugation was carried out at 5,500 rpm for 10 min, the supernatant was then  
157 carefully collected and filtered with Nylon HPLC filters with 20  $\mu$ m pores.

### 158 *2.3.3 Derivatization*

159 1 mL of the water-methanol extract from the first four extractions were collected together, spiked  
160 with 20  $\mu$ L of 4-chlorophenylalanine solution (4 mg/mL in CH<sub>3</sub>OH) and dried under a gentle stream of  
161 nitrogen into 1.5 mL glass vials. 45  $\mu$ L of MOX solution (20 mg/mL in pyridine) were added and  
162 methoximation reaction was carried out at 60 °C for 2 h. Lastly, 60  $\mu$ L of (N,O-  
163 bis(trimethylsilyl)trifluoroacetamide - BSTFA were added to the reaction mixture. The silylation reaction  
164 was carried out at 60 °C for 1 h. At the end of the derivatization step, the reaction mixture was spiked with  
165 20  $\mu$ L of 1,4-dibromobenzene (IS 1 g/L in CH<sub>2</sub>Cl<sub>2</sub>); an additional 75  $\mu$ L of CH<sub>2</sub>Cl<sub>2</sub> were added up to a final  
166 volume of 200  $\mu$ L.

167 For primary metabolites identity confirmation, 1.00 mL of primary metabolites standards mixture  
168 (listed in *section 2.1*) was submitted to the derivatization procedure and analyzed under conditions  
169 described in *section 2.5*.

#### 170 **2.4 Headspace Solid Phase Microextraction devices and sampling conditions**

171 Automated HS-SPME sampling was performed using a MPS-2 multipurpose sampler (Gerstel,  
172 Mülheim a/d Ruhr, Germany) installed on the GC×GC–TOF-MS system. SPME fibers,  
173 divinylbenzene/carboxen/polydimethyl siloxane (DVB/CAR/PDMS) df50/30m – 2 cm, were from Supelco  
174 (Bellefonte, PA, USA). Fibers were conditioned before their use as recommended by the manufacturer.  
175 The IS ( $\alpha$ -thujone) used for peak response normalization was pre-loaded into SPME fiber before sampling  
176 by exposing the extraction device (e.g., the SPME fiber) to 5  $\mu$ L of ISTD standard stock solution for 20 min  
177 at 50°C. Hazelnut samples were frozen before milling, using liquid nitrogen, to ensure homogeneous  
178 particle size distribution thus stored at –80°C until analyzed. Samples were exactly weighed (1.50 g) in  
179 headspace glass vials (20 mL) and submitted to headspace sampling for 40 min at 50°C.

#### 180 **2.5 GC×GC-TOF MS featuring Tandem Ionization: instrument set-up and experimental conditions**

181 GC×GC analyses were performed on an Agilent 7890B GC unit coupled with a Bench TOF-Select™  
182 system (Markes International, Llantrisant, UK) featuring Tandem EI. Hard ionization at 70 eV was set for  
183 identity confirmation while 12 eV was applied to explore spectral complementarity. The ion source and  
184 transfer line were set at 290°C. The MS optimization option was set to operate in Tandem Ionization with  
185 a mass range between 35 and 550  $m/z$ ; data acquisition frequency was 50 Hz per channel; filament voltage  
186 was set at 1.60 V.

187 The system was equipped with a two-stage KT 2004 loop thermal modulator (Zoex Corporation, Houston,  
188 TX) cooled with liquid nitrogen controlled by Optimode™ V.2 (SRA Instruments, Cernusco sul Naviglio, MI,  
189 Italy). The hot jet pulse time was set at 280 ms, modulation period was 5 s, and cold-jet total flow was  
190 progressively reduced with a linear function from 30% of Mass Flow Controller (MFC) at initial conditions  
191 to 5% at the end of the run.

#### 192 **2.6 GC×GC columns and settings for primary metabolites profiling**

193 The column set was configured as follows: <sup>1</sup>D DB-5 column (95% polydimethylsiloxane, 5% phenyl;  
194 30 m × 0.25 mm  $d_c$ , 0.25  $\mu$ m  $d_f$ ) coupled with a <sup>2</sup>D OV1701 column (86% polydimethylsiloxane, 7% phenyl,  
195 7% cyanopropyl; 2 m × 0.1 mm  $d_c$ , 0.10  $\mu$ m  $d_f$ ), from J&W (Agilent, Little Falls, DE, USA). The first 0.80 m  
196 of the <sup>2</sup>D column, connected in series to the 1D column by a silTite  $\mu$ -union (Trajan Scientific and Medical,  
197 Ringwood, Victoria, Australia), were wrapped in the modulator slit and used as loop-capillary for cryogenic



198 modulation. The carrier gas was helium at a constant flow of 1.6 mL/min. The oven temperature program  
199 was from 75°C (1 min) to 290°C (15 min) at 4°C/min.

200 For primary metabolites profiling, 2.0 µL of the derivatized solution (section 2.3.3) was analyzed under  
201 the following conditions: split/splitless injector in split mode, split ratio 1:20, injector temperature 290°C.  
202 The *n*-alkanes liquid sample solution for  $I^T$  determination was analyzed under the following conditions:  
203 split/splitless injector in split mode, split ratio 1:50, injector temperature 290°C, and injection volume 1  
204 µL.

## 205 **2.7 GC-MS configuration and settings for volatiles profiling**

206 GC-MS profiling of volatiles from lab-scale roasted samples was by a system configured as follows:  
207 Agilent 7890 GC unit coupled to an Agilent 5977B MS detector (Agilent, Little Falls, DE, USA) with High  
208 Efficiency Source (HES) operating in EI mode at 70 eV. The transfer line was set to 270°C, HES Tune was  
209 applied and the scan range was set to *m/z* 35-350 with a scanning rate of 2,500 *amu/s*.

210 A Solgel-Wax™ capillary column was used (100% polyethylene glycol, 30 m × 0.25 mm dc, 0.25 µm  
211 df) Trajan Scientific and Medical (Ringwood, Victoria, Australia).

212 The analytes sampled by HS-SPME were thermally desorbed from the fiber for 5 min, directly into  
213 the GC injector, under the following conditions: split mode, split ratio 1/20, injector temperature 250°C.  
214 The carrier gas was helium, at a constant flow rate of 1.0 mL/min. The oven temperature program was  
215 40°C (1 min) to 180°C at 3°C/min and to 240°C at 15°C/min (5 min). MS source was set at 250°C while MS  
216 quadrupole was set at 180°C.

## 217 **2.8 Method performance parameters: retention times and response repeatability**

218 Method validation was run on a three-weeks basis and aimed at the evaluation of repeatability of  
219 retention times and *UT* peaks response precision [30]. Retention times in both chromatographic  
220 dimensions ( $^1t_R$  and  $^2t_R$ ) were collected from the *UT* peak-regions at 70 eV of analyzed samples (3  
221 cultivar/origins × 5 extraction/derivatization batches × 2 analytical replicates) for a total of 30 analytical  
222 runs processed. Results are reported as relative standard deviation (RSD) in **Supplementary Table 1 – ST1**  
223 with quite good retention-times stability with an average RSD of 4.78E-03 for  $^1t_R$  and 1.80E-02 for  $^2t_R$ .  
224 Response repeatability was calculated on all *UT* peak-regions at 70 eV and was based on normalized  
225 responses from Piedmont samples (5 extraction/derivatization batches × 2 analytical replicates). As  
226 reported in in **Supplementary Table 1 – ST1**, repeatability RSD averaged of 0.10, with a minimal of 0.03  
227 for feature (35) and a maximum of 0.20 for (238), both near the end of the run.

## 228 **2.9 Data acquisition and 2D data processing**

229 GC×GC data were acquired by TOF-DS software (Markes International, Llantrisant, UK), 1D-GC-MS data  
230 were acquired and processed by Enhanced MassHunter (Agilent, Little Falls, DE, USA). GC×GC-TOF MS  
231 data were processed using GC Image GC×GC Edition, ver 2.8 (GC Image, LLC, Lincoln NE, USA). Data  
232 elaboration and results visualization were by XL-Stat (Addinsoft Inc, New York, USA) and by open source  
233 Gene-E (Broadinstitute.org).

### 234 **3. Results and Discussion**

235 In this study, hazelnut primary metabolome fingerprints are, for the first time, explored by  
236 GC×GC-TOF MS featuring tandem ionization and the information potential embedded in 2D-patterns  
237 evaluated in terms of both discrimination power between cultivars and origins and aroma precursors  
238 distribution. In the panorama of existing studies aimed at delineating hazelnuts nutritional quality, there  
239 is a lack of systematic investigations on non-volatile aroma precursors distribution studied for their  
240 correlation to potent odorants formed after technological roasting. Alasalvar *et al.* [31] studied the  
241 proximate composition, minerals, vitamins, dietary fiber, amino acids, and taste active components (free  
242 amino acids, sugars, and organic acids) distribution in *Tombul* hazelnuts. They applied a multi-platform  
243 analytical approach to profile major and essential nutrients, writing that the *Tombul* hazelnut would  
244 “serve as a good source of vital nutrients and taste active components” [31]. The study then was extended  
245 to other cultivars/origins [32,33] and also to other tree nuts [34] by also integrating the analytical profiling  
246 with bio-active phytochemicals [35].

247 The first attempt to correlate hazelnut metabolome and its volatile composition was by Kiefl [26],  
248 who, based on available literature, reviewed the most relevant non-volatile precursors responsible of the  
249 formation of key-aroma compounds and the reaction pathways in hazelnuts after dry-roasting. However,  
250 the study mostly focused on the volatile metabolome, lacking experimental evidence of the expected  
251 strong correlations between precursors and reaction products.

252 In this study, we designed an analytical approach based on a single analytical platform, to map  
253 the characteristic distribution (quali-quantitative) of informative analytes selected from those playing a  
254 major role in defining hazelnuts aroma potential and sensory quality. Reactions, promoted by dry-  
255 roasting, include carbohydrates caramelization, consisting of dehydration and isomerization to form  $\alpha$ -  
256 dicarbonyl compounds (2,3-butanedione and 2,3-pentanedione), and furanones (5-hydroxymethylfurfural  
257 and 4-hydroxy-2,5-dimethyl-3(2H)-furanone). 2-/3-Methylbutanal and phenylacetaldehyde are formed by  
258 Strecker degradation of amino acids in presence of  $\alpha$ -dicarbonyls from Ile, Leu and Phe respectively [36].  
259 Moreover,  $\alpha$ -amino carbonyl compounds, resulting from Strecker reaction, are pyrazine precursors; their  
260 dimerization leads to the formation of dihydropyrazines that can either oxidize into pyrazines or react

261 with aldehydes to generate substituted pyrazines [37]. Additionally, reactions between Ala, Arg, Lys, Pro  
262 and Orn, and sugar degradation products (i.e., deoxyosones) forms 2-acetyl and 2-propionylpyrroline that  
263 can be further oxidized to pyrrole derivatives [38]. Finally, thermal degradation of primary metabolites  
264 also can affect the lipid fraction, promoting autoxidation and formation of secondary products such as  
265 saturated and unsaturated aldehydes (hexanal, octanal, (Z)-2-octenal, (E)-2-octenal, (Z)-2-nonenal, (Z)-2-  
266 decenal and (E,E)-2,4-nonadienal, etc.).

267 The analytical strategy includes multi-analyte profiling and fingerprinting by GC×GC-TOF MS after  
268 oximation/silylation of non-volatile precursors. Tandem ionization is evaluated by combining 70 and 12  
269 eV ionization energies to improve the confidence on analytes identification and to validate the  
270 comparative capacity of 2D-fingerprints. The high energy (70eV) enables confident identification based  
271 on the similarity of characteristic fragmentation patterns; the lower energy (12eV) provides  
272 complementary information and maximizes the relative intensity of structural informative fragments [16–  
273 18].

274 Experimental data is based on a selection of three relevant hazelnuts cultivar/origins (*Roman*,  
275 *Piedmont*, and *Ordu*) from harvest year 2015 analyzed at time zero (i.e. after in-field post-harvest drying).  
276 Five different subsets of samples which were independently defatted/extracted/derivatized and two  
277 analytical replicates were run for each.

278 The next sections include: (a) a discussion of the informative power of the approach and on the  
279 coverage of the non-volatile precursors, e.g., primary metabolites, of interest; (b) an evaluation of the  
280 complementary nature of tandem signals and their synergy in revealing compositional differences  
281 between samples; (c) an evaluation of existing correlations between primary metabolites and volatiles  
282 generated by lab-scale roasting with some insights on specific compounds generating key-aromas.

### 283 **3.1 Fingerprinting information power**

284 In the development of the analytical protocol, reference studies based on GC-MS and GC×GC-MS  
285 [13,14,39–41] were considered for their potential to cover the chemical dimensions [22] of interest.  
286 Extraction and derivatization were therefore validated for their exhaustiveness and efficacy toward  
287 aminoacids, sugars and polyalcohols and some low-molecular weight acids. Exhaustiveness was evaluated  
288 by analysing each aliquot of the ten successive extractions until none of the targeted primary metabolites  
289 (including all silylated forms) were detected. Results are illustrated visually in **Supplementary Figure 2 -**  
290 **SF2** as normalized responses (Normalized 2D-Peak Volume) while some considerations about  
291 derivatization issues are commented and supported by dedicated references.

292

293 B

294 Experimental results indicate that AA are almost 100% recovered after four extractions while for  
295 most of the acids, the exhaustive recovery is after 5 extractions. Exceptions are phosphoric acid, malic  
296 acid, lactic acid, and citric acid. According to literature, sugars and polyalcohols are major metabolites  
297 (excluding the fat components) and, for most of them, a dedicated procedure would be necessary to  
298 achieve their complete extraction. However, the first four aliquots were collected and unified before  
299 oximation/silylation to obtain more representative extracts.

300 The application of the combined untargeted/targeted (*UT*) fingerprinting procedure [20,42] on  
301 the set of the 30 2D-patterns acquired at 70 eV (3 cultivars/origins  $\times$  5 extraction/derivatization batches  
302  $\times$  2 analytical replicates) revealed 138 *UT* peak-regions included in a *UT feature* template. They contained  
303 108 targeted peaks putatively identified through their 70 eV EI-MS fragmentation pattern, by comparing  
304 them to those collected in commercial and in-house databases and for which a Direct Match Factor (DMF)  
305  $\geq 900$  was obtained by applying the NIST Similarity match algorithm [43] on 2D-peakspectrum. The latter  
306 is the average spectrum obtained from the highest modulation within the 2D-peak region. Positive  
307 identifications were accepted for candidates with an experimental  $^1D I^T \pm 15$  units tolerance. Within this  
308 list of 110 known analytes, 25 were confirmed by reference standards (see *section 2.1*). The identities of  
309 30 analytes could not be confirmed and are tagged with unique numerical identifiers [(#)] Their re-  
310 alignment across the 30 2D-patterns was by template matching and by applying retention times and mass  
311 spectral similarity constraints ( $^1t_R$  and  $^2t_R \pm 15\%$  and  $DMF \geq 800$ ) with affine transformation [44,45]. The  
312 complete list of *UT* peak-regions together with their average retention times ( $^1t_R$  min,  $^2t_R$  sec),  
313 experimentally determined  $^1D I^T$  values and tabulated ones (NIST database [43]), is reported in the  
314 **Supplementary Table 1 – ST1.**

315 The next section discusses the complementary nature of tandem signals acquired at 70 and 12 eV  
316 and their information power in the chemical fingerprinting of hazelnut primary metabolome.

### 317 **3.2 Tandem ionization: the complementary nature of tandem signals**

318 The impact of different ionization voltages on spectral profiles and, thereby, on total ion response  
319 can be highlighted by applying datapoint features fingerprinting [46]. The approach compares the  
320 response, or the relative response, between image pairs based on a pixel-basis and promptly evidences  
321 those pattern regions in which detector responses greatly varies between the two data streams. **Figure 1**  
322 reports the comparative visualization, rendered as the *colorized fuzzy ratio*, between the 12 eV data  
323 stream (*analyzed image*) and the 70 eV signal (*reference image*) from a *Piedmont* hazelnut extract. Analyte  
324 relative response in the two detector signals is highlighted by color coding (green, red and light-grey).

325 Regions colored in green indicate analytes for which the relative intensity (Signal-to-Noise) was higher in  
326 the *analyzed* image (12 eV) and those colored in red indicate analytes with a higher relative intensity in  
327 the *reference* image (70 eV).

328 **Insert Figure 1 here**

329 *UT* peak-regions corresponding to some monosaccharides (glucose and fructose derivatives),  
330 sucrose and polyalcohols (xylitol and myo-inositol), and AA, showed a higher absolute and relative  
331 response on the 12 eV signal. This suggests that the lower ionization energy would have a great impact  
332 on analyte fragmentations. This evidence has been confirmed by calculating DMF and Reverse Match  
333 Factors (RMF) values on a selection of analytes at 12 eV and 70 eV. They are listed in **Table 1** together  
334 with base peak (BP) information at the two ionization energies and SNR values.

335 The DMF values averaged 608 with minimal values for Valine 2TMS derivative – 222, Serine 3TMS  
336 – 253, Valine TMS derivative – 254, and Alanine 2TMS – 317. The RMF averaged 657, slightly higher  
337 because the similarity computation does not include the analyzed spectra (12 eV) fragments not present  
338 in the reference at 70 eV. Smaller RMF values were for Valine 2TMS derivative – 228, Serine 3TMS – 374,  
339 Alanine 2TMS – 376, and Glutaric acid 2TMS – 389. Compared to average values registered in a previous  
340 study focused on the cocoa volatilome [16], for which DMF averaged 779 and RMF 787, spectral  
341 dissimilarity is higher emphasizing the complementary nature of high and low ionization energies. This  
342 evidence suggests that a differential impact on sensitivity has to be expected (see below).

343 An additional parameter considered in this comparative process was the base peak (BP)  $m/z$  value.  
344 *UT*-peaks spectra were manually inspected, background subtraction and deconvolution were applied,  
345 when necessary, to isolate a clean reference spectrum from which BP could be defined. Results are  
346 reported in **Table 1**. For 53 of 72 *2D*-peaks (74%), the BP at 70 eV had lower  $m/z$  value when compared to  
347 that recorded at 12 eV (shown as negative values in the *BP Diff* column). This result is quite interesting; it  
348 indicates that structure-informing fragments are more prevalent at lower ionization, thus providing an  
349 additional basis to identify analytes and, furthermore, additional specificity could be explored when the  
350 two data streams are combined. To note, just 3 analytes (Glucopyranose 5TMS, Glycine 2TMS, and Serine  
351 3TMS) presented a larger intensity BP at 70 eV (shown as a difference positive).

352 To further evaluate the complementary nature of tandem signals, relative sensitivity was  
353 considered through the ratio of the experimental SNR values registered at 12 and 70 eV data streams  
354 (data is the average value between data streams and replicates). Results indicate that for 38 of 138 *UT*-  
355 peaks (27%) listed in **Supplementary Table 1 – ST1**, the lower ionization energy signal has a larger relative  
356 intensity including several targeted analytes of interest for their role as aroma precursors. They include

357 sucrose 8TMS (SNR ratio 13), fructose 5TMS *syn* (SNR ratio 5), glucose 5TMS (SNR ratio 4), xylitol, 5TMS  
358 derivative (SNR ratio 4), malic acid 3TMS (SNR ratio 4), myo-inositol 6TMS derivative (SNR ratio 3), glycerol  
359 3TMS (SNR ratio 3), glycine 3TMS (SNR ratio 2), ornithine 4TMS derivative (SNR ratio 2), and citric acid  
360 4TMS derivative (SNR ratio 2).

361 **Figure 2** reports the spectra of four analytes showing dissimilar fragmentation patterns: glucose  
362 in its cyclic form (i.e., glucopyranose 5TMS), Gly 3TMS, citric acid 4TMS, and succinic acid 2TMS. In all  
363 cases, the molecular ion was detected at both 70 and 12 eV. In the glucopyranose spectrum at lower  
364 ionization energy, the molecular ion (i.e, 540  $m/z$ ) was slightly larger (1.0E-02 vs. 9.8E-04 percent  
365 intensity). In general, spectra at 12 eV are dominated by structurally informing fragments whereas those  
366 at 70 eV are dominated by the fragments of silylating agent (i.e., TMS–73  $m/z$ ) with low informing power.  
367 In the case of glucose and citric acid derivatives, the base peak (BP) between 12 (red) and 70 (blue) eV  
368 spectra is different. These results confirm the complementary nature of tandem ionization signals, a  
369 characteristic that is very useful for the accurate fingerprinting of complex samples. For analytes showing  
370 larger SNR values at 12 eV, the relative sensitivity of the method increases at lower energies and additional  
371 benefits could be also expected on the linearity range. At lower energies the amount of ionized molecules  
372 is reduced, compared to 70 eV, and the risk of detector saturation is minimized. Experimental evidence  
373 on the extended linearity range of responses for volatile compounds is discussed in an application of  
374 interest for the flavor and fragrance field [47].

375 **Insert Figure 2 here**

376 The *UT feature template* was applied to the 30 2D-patterns obtained at 70 eV and re-aligned to  
377 the 12 eV data streams, then response information from all *UT* peak-regions was extracted to exploit the  
378 complementary information from tandem signals in terms of fingerprinting information potential. The  
379 final data matrix was 60×144 (60 2D-patterns [3 cultivar/origins × 5 extraction/derivatization batches × 2  
380 ionization energies × 2 analytical replicates] × 144 *UT* peak-regions). Results are visualized as heat-map in  
381 **Figure 3. Figure 3A** shows the 70 eV data stream and represents, in a color scale from blue to red, the  
382 normalized *UT* peak-regions volume distribution; **Figure 3B** shows, in a color scale from green to red, the  
383 12 eV *UT* peak-regions. Hierarchical clustering (HC) is based on Euclidean distances and was applied after  
384 Z-score normalization of the data (i.e., subtract mean and divide by standard deviation).

385 **Insert Figure 3 here**

386 Primary metabolites fingerprints can independently cluster samples based on their cultivar/origin.  
387 The two data streams show a high consistency in their discrimination potential: both data streams closely  
388 cluster *Piedmont* and *Roman* samples although informative *UT* peaks contributing to this classification are

389 different (see rows/UT peaks HC clusters). In particular, *Ordu* samples are connoted by a lower relative  
390 concentration of primary metabolites (confirmed by both data streams). Only a few analytes show a  
391 higher relative abundance: Gly and tartaric acid within the known compounds set and (1199) and (1153)  
392 within the unknown compounds set. On the other hand, the discrimination between *Piedmont* and *Roman*  
393 samples is mainly driven by: (a) sugars that are more abundant in the *Piedmont* cultivar (e.g. glucose,  
394 galactose, maltose, and fructose (considering all derivatives); (b) AA like Trp, Orn, and Tyr more abundant  
395 in *Piedmont* and Leu, Ile, Met, Val, Phe, Pro, and pyroglutamic acid with a more intense response in the  
396 *Roman* samples; (c) acids such as lactic acid (monomer 2TMS and dimer 2TMS), glutaric acid, galacturonic  
397 acid, fumaric acid, tartaric acid, and oxalic acid which are more abundant in *Roman* samples. Univariate  
398 statistics, in box-plot diagrams, for a selection of *UT* peaks are reported in the **Supplementary Figure 3 –**  
399 **SF3**.

400 Tandem signals, when independently processed, enable cross validation of fingerprinting results.  
401 For some analytes, linear regression analysis between absolute responses recorded at 12 eV (dependent  
402 variable) and at 70 eV (independent variable) give good results ( $R^2 \geq 0.90$ ) as shown in **Figure 4** for some  
403 aroma precursors: glucose, Orn, Leu, and Ile. The coherent trend of analytes response validate the  
404 differential distribution of markers within sample set, while the different response factors between  
405 ionization channels, described by regression functions with slopes  $\neq 1$ , confirms their complementary  
406 nature.

407 The next section deals with the correlation between primary metabolites signatures and hazelnuts  
408 volatile metabolome, in particular with key-aroma compounds and marker analytes distribution after lab-  
409 scale roasting.

410 **Insert Figure 4 here**

411

### 412 **3.3 Hazelnuts aroma potential and correlation with primary metabolites signatures**

413 Samples were submitted to lab-scale roasting, designed in a previous study, to evaluate the aroma  
414 quality of roasted hazelnuts. The protocol includes a dry-roasting in a ventilated oven at 160°C for 15  
415 minutes [28]. Roasted samples then were submitted to HS-SPME sampling to extract volatiles and GC-MS  
416 profiling to monitor quali-quantitative changes in volatile markers including potent odorants [26,29,48–  
417 50].

418 The list of targeted volatiles is provided in the **Supplementary Table 2 – ST2**, together with the  
419 experimental  $I^T$ , odor quality and odor threshold for markers. They include key-aroma compounds  
420 validated by sensomics [23]: 2- and 3-methylbutanal, with *malty* notes; 2,3-butanedione and 2,3-

421 pentanedione, with *buttery* odor quality; 3-methyl-4-heptanone, 5-methyl-(Z)-2-hepten-4-one and 5-  
422 methyl-(E)-2-hepten-4-one (i.e., filbertone), with characteristic *nutty* and *fruity* odors; the *earthy*  
423 pyrazines 2,3,5-trimethylpyrazine and 3,5-dimethyl-2-ethylpyrazine; acetic acid, responsible of the *sour*  
424 note; phenylacetaldehyde, with *honey* and *flowery* odors; 2-methylbutyric acid, with a *sweaty* note; and  
425 4-hydroxy-2,5-dimethyl-3(2H)-furanone (i.e., furaneol), with the characteristic *sweet* and *caramel-like*  
426 note. Within the volatile fraction of roasted samples, several other compounds were targeted. They  
427 belong to the group of roasting indicators, i.e., compounds formed during thermal processing and for  
428 which a meaningful increase was registered during lab-scale roasting [29]. They include: carbonyl  
429 derivatives (2-methylpropanal, 2-butenal and 3-hydroxy-2-butanone), alkyl pyrazines (pyrazine, 2-  
430 methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, ethylpyrazine, 2,3-dimethylpyrazine, 2-  
431 ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, 2,3,5-trimethylpyrazine and 3-ethyl-2,5-  
432 dimethylpyrazine), furanones (furfural, 5-methylfurfural, dihydro-2(3H)-furanone and furfuryl alcohol),  
433 and pyrroles (1(H)-pyrrole, 1-methyl-1(H)pyrrole and 1(H)-pyrrole-2-carboxaldehyde). The present  
434 discussion does not include secondary products of lipid oxidation that contribute to some odor qualities  
435 and inform about shelf-life stability, although a dedicated procedure to study the fat fraction has been  
436 developed.

437 The squared Pearson correlation coefficient ( $r$ ) was calculated to evaluate the existence of  
438 positive correlations between primary metabolites and volatiles. The  $p$ -values computed for each  
439 coefficient afford testing the null hypothesis that  $r$  values are not significantly different from 0 and informs  
440 about the relevance of the correlation.

441 The data matrix included the normalized responses (i.e. normalized 2D peak-regions from GC×GC  
442 data and normalized chromatographic areas for 1D-GC data) for targeted primary metabolites and all  
443 volatiles respectively (see **ST1** and **ST2**). The resulting matrix was 30×162 features (30 2D-patterns [3  
444 cultivar/origins × 5 extractions/derivatization batches × 1 ionization energy 70 eV × 2 analytical replicates]  
445 × 108 targeted peak-regions + 54 volatiles). Before processing, data were scaled by Pareto scaling [51] in  
446 order to reduce the relevance of large values while keeping data structure almost intact. The resulting  
447 Pearson correlation matrix, showing  $r$  values, is illustrated as heat-map in **Figure 5**. The color scale, from  
448 blue to red, has been adapted to emphasize (red color)  $r$  values  $\geq 0.8$ , arbitrarily considered meaningful  
449 in this specific data-set for notable correlations. HC is based on “one minus Pearson correlation” value as  
450 metrics.

451 **Insert Figure 5 here**



452 Results show: (a) high  $r$  values (accompanied by meaningful  $p$ -values –  $\alpha=0.05$ ) within primary  
453 metabolites (**Figure 5 – box 1**) confirming fingerprinting results where *Piedmont* and *Roman* samples  
454 showed a higher relative abundance for most of the non-volatile precursors and metabolites compared  
455 to *Ordu* origin (see **Fig. 3A** and **SF3**); (b) high correlation values ( $r \geq 0.8$  - **Figure 5 - box 2**) within volatiles  
456 including 3-methyl-4-heptanone, 2,3-pentanedione, (*E*)-3-penten-2-one, 3-hydroxy-2-butanone,  
457 benzenemethanol, furaneol, furfuryl alcohol, 3-methylbutanal, 1H-pyrrole, 2,3-dimethylpyrazine,  
458 ethylpyrazine, methylpyrazine, and trimethyl pyrazine; and (c) between primary metabolites (and/or non-  
459 volatile precursors) and volatiles with a high informing power in terms of aroma quality. Further details  
460 on these last correlations are discussed below.

461 These correlations were tested for their significance ( $p$ -value) and linearity; the coefficients of  
462 determination ( $R^2$ ) of the regression were calculated, considering the precursor(s) as independent variable  
463 ( $x$ ) and key-volatiles as dependent variable ( $y$ ). **Figure 6A** reports the regression function between 3-  
464 methylbutanal and Leu ( $R^2$  0.9577), **Fig. 6B** between 3-methylbutanal and Ile ( $R^2$  0.9284), **Fig. 6C-D**  
465 between 2,3-butanedione and 2,3-pentanedione and the sum of fructose (Fructose 5TMS *syn*- and *anti*-  
466 forms) and glucose (Glucopyranose 5TMS and Glucose 5TMS) derivatives ( $R^2$  0.8543 and 0.8860), **Fig. 6E**  
467 between 2,5-dimethylpyrazine and Ala ( $R^2$  0.8822) and pyrroles (1H-pyrrole, 3-methyl-1H-pyrrole and 1H-  
468 pyrrole-2-carboxaldehyde) and the sum of Orn and Ala derivatives ( $R^2$  0.8604).

#### 469 **Insert Figure 6 here**

470 These results are interesting, and evidence a solid foundation for adoption of the proposed approach for  
471 a comprehensive primary metabolome fingerprinting as an informative tool to characterize aroma  
472 potential of hazelnuts. However, the approach needs validation through a more extensive sampling that  
473 includes further variables influencing primary metabolites distribution in hazelnuts: cultivar and origins  
474 should be accompanied by a selection of multiple harvest years, shelf-life, and storage conditions that  
475 also are relevant because of their known impact on the aroma potential [28].

476

#### 477 **4. Conclusions**

478 The present study is focused on variable EI energy TOF MS adopted to extend the analytical  
479 dimensions of GC×GC for hazelnut primary metabolites profiling. By combining standard 70 eV and 12 eV,  
480 tandem data streams are generated and spectra show a complementary nature. This characteristic can  
481 be exploited in identification and structural elucidation studies. At low energy, structure informing  
482 fragments prevail while overall fragmentation is reduced, achieving, for some analytes, a higher relative  
483 sensitivity that results in a wider dynamic range of the method. Tandem data responses from *UT*-peaks in

484 complex chemical signatures are strongly correlated but exhibit different response factors that open  
485 interesting perspectives for quantitative studies.

486 Response data recorded from primary metabolites fingerprints in different cultivars/origins show  
487 good correlation to volatiles formed during dry-roasting and validate empirical observations from  
488 previous experiments [28]. Linear regressions with meaningful  $R^2$  give solid foundations to the causal  
489 relationship between metabolome and sensobolome signatures. The proposed approach, once validated  
490 over a wider sampling design, would introduce a new concept in hazelnuts quality assessment while  
491 opening new perspectives for breeding studies and shelf-life quality evaluation.

492

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495

#### 496 **Compliance with ethical standards Notes**

497 Prof. Stephen E. Reichenbach has a financial interest in GC Image, LLC.

498 Melanie Charron, Federica Manini, Roberto Menta and Mauro Fontana are employees of Soremartec

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500

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676 **Figure Captions**

677 **Figure 1:** comparative visualization, rendered as the *colorized fuzzy ratio*, between the 12 eV data stream  
678 (*analyzed image*) and the 70 eV signal (*reference image*) from a *Piedmont* hazelnut extract. Green  
679 highlighted areas indicate peak-regions where the differential response between channels was higher on  
680 12 eV ionization energy.

681  
682 **Figure 2:** fragmentation patterns recorded for a selection of analytes at 12 eV (red trace) and 70 eV (blue  
683 trace) ionization energy.

684  
685 **Figure 3:** heat-map visualization of the *UT* peak-regions normalized response from 70 eV (Fig. 3A) and 12  
686 eV (Fig. 3B) data streams. Analytical replicates were averaged resulting in a (15+15) × 138 data matrix.  
687 Hierarchical clustering (HC) is based Euclidean distances - Z-score normalization of the data (i.e., subtract  
688 median and divide by standard deviation).

689  
690 **Figure 4:** regression analysis between absolute responses recorded at 12 eV (dependent variable) and at  
691 70 eV (independent variable) for aroma precursors: glucose, Orn, Leu and Ile.

692  
693 **Figure 5:** heat-map illustrating Pearson correlation matrix results referred as *r* values. Color scale, from  
694 blue-to-red, is adapted to emphasize (red color) *r* values ≥ 0.8; HC is based on “one minus Pearson  
695 correlation and green squares indicates analytes pairs commented in the text.

696  
697 **Figure 6:** regression functions between 3-methylbutanal and Leu ( $R^2$  0.9577), 3-methylbutanal and Ile ( $R^2$   
698 0.9284), 2,3-butanedione and 2,3-pentanedione and the sum of fructose (Fructose 5TMS *syn*- and *anti*-  
699 forms) and glucose (Glucopyranose 5TMS and Glucose 5TMS) derivatives ( $R^2$  0.8543 and 0.8860), 2,5-  
700 dimethylpyrazine and Ala ( $R^2$  0.8822) and pyrroles (1H-pyrrole, 3-methyl-1H-pyrrole and 1H-pyrrole-2-  
701 carboxaldehyde) and the sum of Orn and Ala derivatives ( $R^2$  0.8604). Graph legend (—) linear regression  
702 function, (---) confidence interval mean 95%, (—) confidence interval observ. 95%

703

704

705 **Table Captions:**

706 **Table 1:** selected targeted *UT* peak-regions corresponding to informative primary metabolites listed  
707 together with their average retention times (<sup>1</sup>*t<sub>R</sub>* min, <sup>2</sup>*t<sub>R</sub>* sec), experimental and tabulated (NIST database [21]) *f<sub>i</sub>*,  
708 base peak (BP) *m/z* values recorded from the 2D-apex spectrum and their difference (BP diff), direct match factor  
709 (DMF) and reverse match factors (RMF) values calculated between 12 eV vs. 70 eV and SNR value between  
710 12 and 70 eV channels (values in bold highlight analytes where SNR at 12 eV was higher compared to 70  
711 eV).