

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1742525> since 2021-02-22T18:19:20Z

Published version:

DOI:10.1016/j.chroma.2019.460739

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(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
6 Marta Cialiè Rosso¹, Maria Mazzucotelli¹, Carlo Bicchi¹, Melanie Charron², Federica Manini², Roberto
7 Menta², Mauro Fontana², Stephen E. Reichenbach^{3,4} and Chiara Cordero^{1*}

8
9 ¹Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Turin, Italy

10 ²*Soremartec Italia Srl*, Ferrero Group, Alba (CN), Italy

11 ³Computer Science and Engineering Department, University of Nebraska – Lincoln, NE, USA

12 ⁴GC Image LCC, Lincoln, NE, USA

13

14 *Corresponding author:

15 Dr. Chiara Cordero - Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via Pietro
16 Giuria 9, I-10125 Torino, Italy – e-mail: chiara.cordero@unito.it; phone: +39 011 6707172

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% N₂ – 1% O₂) 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% N₂ – 21% O₂) 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^T) determination and α -tujone for volatiles internal standardization (IS) were from Merck (Milan,
111 Italy).

112 The mixture of *n*-alkanes for the I^T solution was prepared in cyclohexane at a concentration of 100
113 mg/L; internal standard (IS) α -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 H₂O/CH₃OH (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 μ 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 μ L of 4-chlorophenylalanine solution (4 mg/mL in CH₃OH) and dried under a gentle stream of
161 nitrogen into 1.5 mL glass vials. 45 μ 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 μ 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 μ L of 1,4-dibromobenzene (IS 1 g/L in CH₂Cl₂); an additional 75 μ L of CH₂Cl₂ were added up to a final
166 volume of 200 μ 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 (α -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 μ 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: ¹D DB-5 column (95% polydimethylsiloxane, 5% phenyl;
194 30 m × 0.25 mm d_c , 0.25 μ m d_f) coupled with a ²D OV1701 column (86% polydimethylsiloxane, 7% phenyl,
195 7% cyanopropyl; 2 m × 0.1 mm d_c , 0.10 μ m d_f), from J&W (Agilent, Little Falls, DE, USA). The first 0.80 m
196 of the ²D column, connected in series to the 1D column by a silTite μ -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 α -
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 α -dicarbonyls from Ile, Leu and Phe respectively [36].
259 Moreover, α -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 × 5 extraction/derivatization batches
302 × 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 ≥ 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 ≥ 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

493 **Funding**

494 The research was carried out thanks to the financial support of Soremartec Italia Srl, Alba (CN, Italy).

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

499 Italia Srl, Alba (CN, Italy).

500

501 **References**

- 502 [1] C. Cordero, J. Kiefl, S.E. Reichenbach, C. Bicchi, Characterization of odorant patterns by
503 comprehensive two-dimensional gas chromatography: A challenge in omic studies, *TrAC - Trends*
504 *Anal. Chem.* 113 (2019) 364–378. doi:10.1016/j.trac.2018.06.005.
- 505 [2] M. Adahchour, J. Beens, R.J.J. Vreuls, U.A.T. Brinkman, Recent developments in comprehensive
506 two-dimensional gas chromatography (GC x GC). IV. Further applications, conclusions and
507 perspectives, *TrAC - Trends Anal. Chem.* 25 (2006) 821–840. doi:10.1016/j.trac.2006.03.003.
- 508 [3] M. Adahchour, L.L.P. van Stee, J. Beens, R.J.J. Vreuls, M.A. Batenburg, U.A.T. Brinkman,
509 Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric
510 detection for the trace analysis of flavour compounds in food, *J. Chromatogr. A.* 1019 (2003) 157–
511 172. isi:000186503600014.
- 512 [4] C. Cordero, J. Kiefl, P. Schieberle, S.E. Reichenbach, C. Bicchi, Comprehensive two-dimensional gas
513 chromatography and food sensory properties: Potential and challenges, *Anal. Bioanal. Chem.* 407
514 (2015) 169–191. doi:10.1007/s00216-014-8248-z.
- 515 [5] P.Q. Tranchida, P. Donato, F. Cacciola, M. Beccaria, P. Dugo, L. Mondello, Potential of
516 comprehensive chromatography in food analysis, *TrAC - Trends Anal. Chem.* 52 (2013) 186–205.
517 doi:10.1016/j.trac.2013.07.008.
- 518 [6] P.Q. Tranchida, F.A. Franchina, P. Dugo, L. Mondello, Comprehensive two-dimensional gas
519 chromatography-mass spectrometry: Recent evolution and current trends, *Mass Spectrom. Rev.*
520 35 (2016) 524–534. doi:10.1002/mas.21443.
- 521 [7] F. Magagna, E. Liberto, S.E. Reichenbach, Q. Tao, A. Carretta, L. Cobelli, M. Giardina, C. Bicchi, C.
522 Cordero, Advanced fingerprinting of high-quality cocoa: Challenges in transferring methods from
523 thermal to differential-flow modulated comprehensive two dimensional gas chromatography, *J.*
524 *Chromatogr. A.* 1535 (2018) 122–136. doi:10.1016/j.chroma.2017.07.014.
- 525 [8] G. Purcaro, L. Barp, M. Beccaria, L.S. Conte, Fingerprinting of vegetable oil minor components by
526 multidimensional comprehensive gas chromatography with dual detection, *Anal. Bioanal. Chem.*
527 407 (2015) 309–319. doi:10.1007/s00216-014-8140-x.
- 528 [9] G. Purcaro, L. Barp, M. Beccaria, L.S. Conte, Characterisation of minor components in vegetable oil
529 by comprehensive gas chromatography with dual detection, *Food Chem.* 212 (2016) 730–738.
530 doi:10.1016/j.foodchem.2016.06.048.
- 531 [10] P.Q. Tranchida, A. Giannino, M. Mondello, D. Sciarrone, P. Dugo, G. Dugo, L. Mondello, Elucidation
532 of fatty acid profiles in vegetable oils exploiting group-type patterning and enhanced sensitivity of

533 comprehensive two-dimensional gas chromatography, *J. Sep. Sci.* 31 (2008) 1797–1802.
534 doi:10.1002/jssc.200800002.

535 [11] P.Q. Tranchida, S. Salivo, I. Bonaccorsi, A. Rotondo, P. Dugo, L. Mondello, Analysis of the
536 unsaponifiable fraction of lipids belonging to various milk-types by using comprehensive two-
537 dimensional gas chromatography with dual mass spectrometry/flame ionization detection and
538 with the support of high resolution time-of-flight mass , *J. Chromatogr. A.* 1313 (2013) 194–201.
539 doi:10.1016/j.chroma.2013.07.089.

540 [12] P.Q. Tranchida, S. Salivo, F.A. Franchina, I. Bonaccorsi, P. Dugo, L. Mondello, Qualitative and
541 quantitative analysis of the unsaponifiable fraction of vegetable oils by using comprehensive 2D
542 GC with dual MS/FID detection, *Anal. Bioanal. Chem.* 405 (2013) 4655–4663. doi:10.1007/s00216-
543 013-6704-9.

544 [13] C.H. Weinert, B. Egert, S.E. Kulling, On the applicability of comprehensive two-dimensional gas
545 chromatography combined with a fast-scanning quadrupole mass spectrometer for untargeted
546 large-scale metabolomics, *J. Chromatogr. A.* 1405 (2015) 156–167.
547 doi:10.1016/j.chroma.2015.04.011.

548 [14] C. Mack, D. Wefers, P. Schuster, C.H. Weinert, B. Egert, S. Bliedung, B. Trierweiler, C. Muhle-Goll,
549 M. Bunzel, B. Luy, S.E. Kulling, Untargeted multi-platform analysis of the metabolome and the non-
550 starch polysaccharides of kiwifruit during postharvest ripening, *Postharvest Biol. Technol.* 125
551 (2017) 65–76. doi:10.1016/j.postharvbio.2016.10.011.

552 [15] Markes International, Select-eV: The next generation of ion source technology, *Tech. Note.*
553 *Applicatio* (2016).

554 [16] C. Cordero, A. Guglielmetti, C. Bicchi, E. Liberto, L. Baroux, P. Merle, Q. Tao, S.E. Reichenbach,
555 Comprehensive two-dimensional gas chromatography coupled with time of flight mass
556 spectrometry featuring tandem ionization: challenges and opportunities for accurate
557 fingerprinting studies, *J. Chromatogr. A.* (2019). doi:10.1016/j.chroma.2019.03.025.

558 [17] M.S. Alam, C. Stark, R.M. Harrison, Using Variable Ionization Energy Time-of-Flight Mass
559 Spectrometry with Comprehensive GC??GC to Identify Isomeric Species, *Anal. Chem.* 88 (2016)
560 4211–4220. doi:10.1021/acs.analchem.5b03122.

561 [18] L.M. Dubois, K.A. Perrault, P.H. Stefanuto, S. Koschinski, M. Edwards, L. McGregor, J.F. Focant,
562 Thermal desorption comprehensive two-dimensional gas chromatography coupled to variable-
563 energy electron ionization time-of-flight mass spectrometry for monitoring subtle changes in
564 volatile organic compound profiles of human blood, *J. Chromatogr. A.* 1501 (2017) 117–127.

- 565 doi:10.1016/j.chroma.2017.04.026.
- 566 [19] C.E. Freye, N.R. Moore, R.E. Synovec, Enhancing the chemical selectivity in discovery-based analysis
567 with tandem ionization time-of-flight mass spectrometry detection for comprehensive two-
568 dimensional gas chromatography, *J. Chromatogr. A.* 1537 (2018) 99–108.
569 doi:10.1016/j.chroma.2018.01.008.
- 570 [20] F. Magagna, L. Valverde-Som, C. Ruíz-Samblás, L. Cuadros-Rodríguez, S.E. Reichenbach, C. Bicchi,
571 C. Cordero, Combined untargeted and targeted fingerprinting with comprehensive two-
572 dimensional chromatography for volatiles and ripening indicators in olive oil, *Anal. Chim. Acta.* 936
573 (2016) 245–258. doi:10.1016/j.aca.2016.07.005.
- 574 [21] D. Bressanello, E. Liberto, M. Collino, F. Chiazza, R. Mastrocola, S.E. Reichenbach, C. Bicchi, C.
575 Cordero, Combined untargeted and targeted fingerprinting by comprehensive two-dimensional
576 gas chromatography: revealing fructose-induced changes in mice urinary metabolic signatures,
577 *Anal. Bioanal. Chem.* 410 (2018) 2723–2737. doi:10.1007/s00216-018-0950-9.
- 578 [22] J.C. Giddings, Sample dimensionality: A predictor of order-disorder in component peak distribution
579 in multidimensional separation, *J. Chromatogr. A.* 703 (1995) 3–15. doi:10.1016/0021-
580 9673(95)00249-M.
- 581 [23] J. Kiefl, P. Schieberle, Evaluation of process parameters governing the aroma generation in three
582 hazelnut cultivars (*Corylus avellana* L.) by correlating quantitative key odorant profiling with
583 sensory evaluation, *J. Agric. Food Chem.* 61 (2013) 5236–5244. doi:10.1021/jf4008086.
- 584 [24] J. Kiefl, G. Pollner, P. Schieberle, Supporting Information Sensomics Analysis of Key Hazelnut
585 Odorants (*Corylus avellana* L., 'Tonda Gentile') Using Comprehensive Two-Dimensional Gas
586 Chromatography in Combination with Time-of-Flight-Mass Spectrometry (GC×GC/TOF-MS), *J.*
587 *Agric. Food Chem.* 4 (2013) 1–20.
- 588 [25] J. Kiefl, G. Pollner, P. Schieberle, Sensomics analysis of key hazelnut odorants (*Corylus avellana* L.
589 'Tonda Gentile') using comprehensive two-dimensional gas chromatography in combination with
590 time-of-flight mass spectrometry (GC×GC-TOF-MS), *J. Agric. Food Chem.* 61 (2013) 5226–5235.
591 doi:10.1021/jf400807w.
- 592 [26] J. Kiefl, Differentiation of Hazelnut Cultivars (*Corylus avellana* L.) by Metabolomics and Sensomics
593 Approaches Using Comprehensive Two-dimensional Gas Chromatography Time-Of-Flight Mass
594 Spectrometry (GC×GC/TOF-MS), 2013. doi:10.1017/CBO9781107415324.004.
- 595 [27] J. Kiefl, Differentiation of Hazelnut Cultivars (*Corylus avellana* L.) by Metabolomics and Sensomics
596 Approaches Using Comprehensive Two-dimensional Gas Chromatography Time-Of-Flight Mass

- 597 Spectrometry (GC×GC/TOF-MS), Verlag Deutsche Forschungsanstalt für Lebensmittelchemie
598 (DFA), Freising, 2013.
- 599 [28] M. Cialie Rosso, E. Liberto, N. Spigolon, M. Fontana, M. Somenzi, C. Bicchi, C. Cordero, Evolution of
600 potent odorants within the volatile metabolome of high-quality hazelnuts (*Corylus avellana* L.):
601 evaluation by comprehensive two-dimensional gas chromatography coupled with mass
602 spectrometry, *Anal. Bioanal. Chem.* 410 (2018) 3491–3506. doi:10.1007/s00216-017-0832-6.
- 603 [29] L. Nicolotti, C. Cordero, C. Bicchi, P. Rubiolo, B. Sgorbini, E. Liberto, Volatile profiling of high quality
604 hazelnuts (*Corylus avellana* L.): Chemical indices of roasting, *Food Chem.* 138 (2013) 1723–1733.
605 doi:10.1016/j.foodchem.2012.11.086.
- 606 [30] Eurachem, Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide
607 to Method Validation and Related Topics., 2014. doi:978-91-87461-59-0.
- 608 [31] C. Alasalvar, F. Shahidi, C.M. Liyanapathirana, T. Ohshima, Turkish Tombul Hazelnut (*Corylus*
609 *avellana* L.). 1. Compositional Characteristics, *J. Agric. Food Chem.* 51 (2007) 3790–3796.
610 doi:10.1021/jf0212385.
- 611 [32] F. Seyhan, G. Ozay, S. Saklar, E. Ertaş, G. Satir, C. Alasalvar, Chemical changes of three native Turkish
612 hazelnut varieties (*Corylus avellana* L.) during fruit development, *Food Chem.* 105 (2007) 590–596.
613 doi:10.1016/j.foodchem.2007.04.016.
- 614 [33] S.K. Chang, C. Alasalvar, B.W. Bolling, F. Shahidi, Nuts and their co-products: The impact of
615 processing (roasting) on phenolics, bioavailability, and health benefits - A comprehensive review,
616 *J. Funct. Foods.* 26 (2016) 88–122. doi:10.1016/j.jff.2016.06.029.
- 617 [34] C. Alasalvar, F. Shahidi, Tree Nuts: Composition, Phytochemicals, and Health Effects,
618 *Chromatographia.* 72 (2010) 589–589. doi:10.1365/s10337-010-1619-5.
- 619 [35] C. Alasalvar, B.W. Bolling, Review of nut phytochemicals, fat-soluble bioactives, antioxidant
620 components and health effects, *Br. J. Nutr.* 113 (2015) S68–S78. doi:10.1017/S0007114514003729.
- 621 [36] N. Göncüoğlu Taş, V. Gökmen, Maillard reaction and caramelization during hazelnut roasting: A
622 multiresponse kinetic study, *Food Chem.* 221 (2017) 1911–1922.
623 doi:10.1016/j.foodchem.2016.11.159.
- 624 [37] P.V. Guerra, V.A. Yaylayan, Dimerization of azomethine ylides: An alternate route to pyrazine
625 formation in the maillard reaction, *J. Agric. Food Chem.* 58 (2010) 12523–12529.
626 doi:10.1021/jf103194k.
- 627 [38] A. Burdack-Freitag, P. Schieberle, Changes in the key odorants of Italian hazelnuts (*Coryllus*
628 *avellana* L. Var. Tonda Romana) induced by roasting, *J. Agric. Food Chem.* 58 (2010) 6351–6359.

629 doi:10.1021/jf100692k.

630 [39] E.C.Y. Chan, K.K. Pasikanti, J.K. Nicholson, Global urinary metabolic profiling procedures using gas
631 chromatography-mass spectrometry, *Nat. Protoc.* 6 (2011) 1483–1499.
632 doi:10.1038/nprot.2011.375.

633 [40] M.M. Ulaszewska, C.H. Weinert, A. Trimigno, R. Portmann, C. Andres Lacueva, R. Badertscher, L.
634 Brennan, C. Brunius, A. Bub, F. Capozzi, M. Cialiè Rosso, C.E. Cordero, H. Daniel, S. Durand, B. Egert,
635 P.G. Ferrario, E.J.M. Feskens, P. Franceschi, M. Garcia-Aloy, F. Giacomoni, P. Giesbertz, R. González-
636 Domínguez, K. Hanhineva, L.Y. Hemeryck, J. Kopka, S.E. Kulling, R. Llorach, C. Manach, F. Mattivi,
637 C. Migné, L.H. Münger, B. Ott, G. Picone, G. Pimentel, E. Pujos-Guillot, S. Riccadonna, M.J. Rist, C.
638 Rombouts, J. Rubert, T. Skurk, P.S.C. Sri Harsha, L. Van Meulebroek, L. Vanhaecke, R. Vázquez-
639 Fresno, D. Wishart, G. Vergères, *Nutrimetabolomics: An Integrative Action for Metabolomic*
640 *Analyses in Human Nutritional Studies*, *Mol. Nutr. Food Res.* 63 (2019) 1–38.
641 doi:10.1002/mnfr.201800384.

642 [41] D. Chelius, K. Jing, A. Lueras, D.S. Rehder, T.M. Dillon, A. Vizel, R.S. Rajan, T. Li, M.J. Treuheit, P. V.
643 Bondarenko, Formation of pyroglutamic acid from N-terminal glutamic acid in immunoglobulin
644 gamma antibodies, *Anal. Chem.* 78 (2006) 2370–2376. doi:10.1021/ac051827k.

645 [42] S.E. Reichenbach, C.A. Zini, K.P. Nicolli, J.E. Welke, C. Cordero, Q. Tao, Benchmarking Machine
646 Learning Methods for Comprehensive Chemical Fingerprinting and Pattern Recognition, *J.*
647 *Chromatogr. A.* (2019). doi:10.1016/j.chroma.2019.02.027.

648 [43] NIST/EPA/NIH Mass Spectral Library with Search Program Data Version: NIST v17, (n.d.).

649 [44] S.E. Reichenbach, D.W. Rempe, Q. Tao, D. Bressanello, E. Liberto, C. Bicchi, S. Balducci, C. Cordero,
650 Alignment for Comprehensive Two-Dimensional Gas Chromatography with Dual Secondary
651 Columns and Detectors, *Anal. Chem.* 87 (2015) 10056–10063. doi:10.1021/acs.analchem.5b02718.

652 [45] R.D. W., R.S. E., T. Qingping, C. Chiara, R.W. E., Z.C. Alcaraz, Effectiveness of Global, Low-Degree
653 Polynomial Transformations for GCxGC Data Alignment, *Anal. Chem.* 88 (2016) 10028–10035.
654 doi:10.1021/acs.analchem.6b02254.

655 [46] S.E. Reichenbach, X. Tian, C. Cordero, Q. Tao, Features for non-targeted cross-sample analysis with
656 comprehensive two-dimensional chromatography, *J. Chromatogr. A.* 1226 (2012) 140–148.
657 doi:10.1016/j.chroma.2011.07.046.

658 [47] C. Cordero, E. Gabetti, C. Bicchi, P. Merle, E. Belhassen, GCxGC with parallel detection by FID and
659 TOF MS featuring tandem ionization: extra-dimensions for great flexibility in fragrance allergens
660 profiling, in: D.W. Armstrong, K.A. Schug (Eds.), 43rd Int. Symp. Capill. Chromatogr. 16th GCxGC

- 661 Symp., Fort Worth, Texas, USA, 2019: p. 1.
- 662 [48] C. Cordero, E. Liberto, C. Bicchi, P. Rubiolo, P. Schieberle, S.E. Reichenbach, Q. Tao, Profiling food
663 volatiles by comprehensive two-dimensional gas chromatography coupled with mass
664 spectrometry: Advanced fingerprinting approaches for comparative analysis of the volatile fraction
665 of roasted hazelnuts (*Corylus avellana* L.) from different origins, *J. Chromatogr. A.* 1217 (2010) 5848–
666 5858. doi:10.1016/j.chroma.2010.07.006.
- 667 [49] C. Cordero, C. Bicchi, P. Rubiolo, Group-type and fingerprint analysis of roasted food matrices
668 (coffee and hazelnut samples) by comprehensive two-dimensional gas chromatography, *J. Agric.*
669 *Food Chem.* 56 (2008) 7655–7666. doi:10.1021/jf801001z.
- 670 [50] A. Burdack-freitag, P. Schieberle, Characterization of the Key Odorants in Raw Italian Hazelnuts, *J.*
671 *Agric. Food Chem.* 60 (2012) 5057–5064.
- 672 [51] R.A. van den Berg, H.C.J. Hoefsloot, J.A. Westerhuis, A.K. Smilde, M.J. van der Werf, Centering,
673 scaling, and transformations: Improving the biological information content of metabolomics data,
674 *BMC Genomics.* 7 (2006) 1–15. doi:10.1186/1471-2164-7-142.
- 675

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 (¹*t_R* min, ²*t_R* sec), experimental and tabulated (NIST database [21]) *f_i*,
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).