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Comprehensive two-dimensional gas chromatography coupled with time of flight mass spectrometry featuring tandem ionization: Challenges and opportunities for accurate fingerprinting studies

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1 **Comprehensive two-dimensional gas chromatography coupled with time of**
2 **flight mass spectrometry featuring tandem ionization: challenges and**
3 **opportunities for accurate fingerprinting studies**

4
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19

20 **Abstract**

21 The capture of volatiles patterns from food gives access to a high level of information related to
22 the role of several functional variables (origin, processing, shelf-life etc.) on sample composition and
23 quality. This analytical process is a type of *fingerprinting* that captures signals revealing a sample's
24 unique traits in order to make effective comparisons. When the focus is on food volatiles,
25 comprehensive two-dimensional gas chromatography combined with time-of-flight mass spectrometry
26 (GC×GC-TOF MS) is undoubtedly the most effective technique for comprehensive fingerprinting studies.
27 TOF MS combined with Electron Ionization (EI) gives access to characteristic fragmentation patterns that
28 enables high confident analyte identification.

29 A recently patented ion source, featuring variable-energy EI, when operated at low energies (10
30 eV, 12 eV, 14 eV), claims enhanced intensity of structure-indicating ions while minimizing the inherent
31 loss of sensitivity traditionally experienced at low EI energies. The acquisition, done by multiplexing
32 between two ionization energies in a single analytical run, generates tandem data streams with
33 complementary natures in terms of both MS pattern signature and relative response.

34 This study explores the potentials of combined untargeted/targeted (*UT*) fingerprinting based on
35 template matching (i.e., *UT fingerprinting* work-flow) with tandem signals. As a challenging bench-test,
36 the complex volatile fractions of high quality cocoas are analyzed and exploited for discrimination.

37 The quality of the spectra at 70 eV is confirmed by similarity match factors above the
38 acceptability threshold, fixed at 950, while spectral differences between hard (70 eV) and soft (12 eV, 14
39 eV) ionization are evaluated in terms of spectral profiles (similarity match factor) and signal-to-noise
40 ratio (SNR). Tandem signals are processed independently and after their fusion in a single stream
41 (summed signal) by the *UT fingerprinting* work-flow. Signal characteristics and 2D-peak indicators (SNR,
42 detectable 2D peaks, spectral peak intensities) are computed and evaluated to define the best strategy.

43 Classification performance, directed to discriminate raw from roasted cocoa from four different
44 origins, is validated by cross-comparing supervised pattern recognition results (Linear Discriminant
45 Analysis and Partial Least Squares Discriminant Analysis) on the most discriminant 2D-peak features as
46 they are revealed by single ionization channels or from fused data streams. Cross-matching untargeted
47 and targeted data provides additional validation. Classification results indicate the potential for superior
48 performances of *UT fingerprinting* with fused data streams (summed signals), while signal characteristics
49 at low ionization energies not only offer additional elements to better discriminate isomeric analytes but
50 also the chance to achieve wider dynamic range of exploration.

51 **Key-words**

52 UT fingerprinting, template matching, tandem ionization, comprehensive two-dimensional gas
53 chromatography, fused data streams
54

55 1. Introduction

56 The capture of volatiles patterns from food is a process that, although challenging, gives access
57 to a high level of information related to important variables such as sample composition, origin,
58 processing, shelf-life, and product quality. This process is a type of *fingerprinting* in that it records
59 analytical signals as a sample's distinctive traits, e.g., to make comparisons [1–3]. Therefore, analytical
60 fingerprinting should utilize technologies or platforms that are capable of informing about analytes
61 identities and relative abundances (or quantities) while providing their complete resolution to
62 effectively exploit sample chemical dimensionality [4].

63 Analytical platforms that combine multidimensional chromatography (MDC) with mass
64 spectrometric detection deliver on these requirements and, if the focus of the research is food
65 volatiles, comprehensive two-dimensional gas chromatography combined with time-of-flight mass
66 spectrometry (GC×GC-TOF MS) is undoubtedly the most effective technique [2,3,5,6]. GC×GC-TOFMS
67 yields highly resolved 2D patterns of volatiles that are distinctive signatures encoding fundamental
68 information about individual samples, particularly analytes identities and relative amounts.

69 MS with Electron Ionization (EI) produces characteristic fragmentation patterns that, thanks to
70 the availability of general or dedicated commercial databases [7,8], when combined with relative
71 retention data (i.e., linear retention indexes I'_S), enable reliable identifications of targeted compounds.
72 TOF MS with low mass resolution is the most common detector adopted in combination with GC×GC [5],
73 although qMS with advanced high efficiency sources has gained popularity for its compatibility with
74 routine applications [9,10]. Within the available GC×GC-MS solutions, recent studies, dealing with food
75 sensometabolome or characteristic odorants in natural extracts [11–13], discussed the advantages
76 provided by high-resolution MS (HRMS) that produces exact masses, specific fragments, or mass defects
77 data even when co-elution issues affect chromatographic resolution and therefore quality of the
78 spectral data.

79 In such systems, soft ionization techniques have the potential to help solving identification
80 ambiguities in those cases where EI produces very similar fragmentation patterns, as for structural
81 isomers. In general, soft ionization preserves information about molecular ions while minimizing
82 associated structural fragmentation [14]. Most of the available soft-ionization techniques, i.e., chemical
83 ionization (CI), field ionization (FI), and photoionization (PI), require dedicated instrumentation and/or
84 ion sources, e.g., to switch from standard EI to CI acquisition. However, recent instrumental solutions
85 perform variable-energy EI feature tandem ionization across single analytical runs.

86 A recently patented ion source, featuring variable-energy EI, also referred to as Tandem
87 Ionization (TI) [Select eV™ - US patent number 9,786,480], claims enhanced intensity of structure-
88 indicating ions and minimize the inherent loss of sensitivity traditionally experienced at low-energy EI.
89 The ion source applies a high potential difference to accelerate the electrons away from the filament,
90 but then reduces their energy before they arrive in the ion chamber [15]. The acquisition is done by
91 time-switching between two ionization energies in a single analytical run so that two data streams are
92 generated and acquired simultaneously.

93 Experimental data demonstrated that variable-energy EI was successful to distinguish and
94 identify large isomeric species in unresolved complex mixture (UCM) of motor oil samples [14]. The
95 authors combined data from tandem signals, acquired at 70 eV and 14 eV, together with rationalized 2D
96 retention patterns of aliphatic and aromatic hydrocarbons in the range between C₁₂-C₃₆, to achieve an
97 almost complete chemical characterization of samples.

98 Dubois et al. [16] explored the composition of light volatile organic compounds (VOCs) mixtures
99 from human blood and tested the beneficial effect of low ionization energies (12, 14 and 16 eV) on
100 analyte fragmentations and on the presence of structurally meaningful ions including molecular ions.
101 The authors confirmed previous evidence of the additional confidence in peak identification, especially
102 for closely eluting isomers, often observed in the profiling of the headspace of blood.

103 A recent paper by Freye et al. [17] moved a step ahead and provided a proof of concept on tandem
104 ionization at 14 eV and 70 eV, discussing the complementary nature of tandem data streams with
105 respect to data processing opportunities. The authors applied a tile-based Fisher ratio analysis and
106 designed a discovery-based investigation by spiking diesel fuel samples with a mixture of twelve
107 analytes at a nominal concentration of 50 ppm. They were successful in detecting eleven of twelve
108 exogenous analytes by processing the data after fusion of tandem signals.

109 In this study, we explore, for the first time, the potentials and limitations of pattern recognition
110 approaches based on template matching (i.e., *UT* fingerprinting work-flow [18,19]) applied to tandem
111 signals provided by hard and soft ionization. In particular, this work begins to develop a work-flow that
112 exploits information from hard and soft ionization data streams while keeping the advantages of
113 comprehensively mapping the distributions of known and unknown compounds across samples with
114 great confidence. As a challenging test case, high quality cocoa from different origins and in two stages
115 of processing are considered. The cocoa volatile metabolome, with its high chemical dimensionality [4],
116 poses several challenges for both detailed profiling and comprehensive fingerprinting.

117

118 2. Experimental

119 2.1 Chemicals and cocoa samples

120 The internal standard (IS) α -thujone for chromatographic areas/volumes normalization was from
121 Sigma Aldrich (Milan, Italy) and dissolved in diethyl phthalate (Sigma Aldrich 99% of purity) at a
122 concentration of 100 mg/L.

123 The mixture of *n*-alkanes (*n*-C9 to *n*-C25) for calibrating linear retention indices (I_s^T) in the first
124 dimension was from Sigma-Aldrich. The I_s^T solution was prepared in cyclohexane at a concentration of
125 100 mg/L.

126 Cocoa samples were provided by Gobino srl (Turin, Italy). Samples were selected on the basis of
127 their specific sensory profile from high-quality productions of different geographic origins. Roasting
128 conditions (time and temperature) were set to achieve optimal flavor. The list of samples, together with
129 their origin, supplier, and harvest year are reported in **Table 1**.

130

131 2.2 Headspace Solid Phase Microextraction devices and sampling conditions

132 The divinylbenzene/carboxen/polydimethylsiloxane 1 cm SPME fiber was from Supelco
133 (Bellefonte, PA, USA) and used for HS-SPME sampling. The standard in-fiber procedure [20] was adopted
134 to preload the IS (α -thujone) onto the fiber before sampling. A 5.0 μ L solution of IS (α -thujone at 100 mg
135 L⁻¹ in diethyl phthalate) was placed into a 20 mL glass vial and subjected to HS-SPME at 50°C for 5 min.
136 After the IS loading step, the SPME device was exposed to 500 mg of cocoa in a headspace glass vials (20
137 mL) for 30 min at 50°C. Extracted analytes were recovered by thermal desorption of the fiber into the
138 S/SL injection port of the GC system at 250°C for 5 min.

139

140 2.3 GC×GC-TOF MS featuring Tandem Ionization: instrument set-up and conditions

141 GC×GC analyses were performed on an Agilent 7890B GC unit coupled with a Bench TOF-Select™
142 system (Markes International, Llantrisant, UK) featuring Tandem EI. For the purposes of this study, hard
143 ionization at 70 eV was set for identity confirmation while lower electron ionization energies were
144 explored in the range 12-16 eV to find optimal conditions for tandem acquisitions. The ion source and
145 transfer line were set at 270°C. The MS optimization option was set to operate in Tandem Ionization
146 with a mass range between 40 and 300 m/z; data acquisition frequency was 50 Hz per channel; filament
147 voltage was set at 1.60 V.

148 The system was equipped with a two-stage KT 2004 loop thermal modulator (Zoex Corporation,
149 Houston, TX) cooled with liquid nitrogen controlled by Optimode™ V.2 (SRA Instruments, Cernusco sul

150 Naviglio, MI, Italy). The hot jet pulse time was set at 250 ms, modulation period was 4 s, and cold-jet
151 total flow was progressively reduced with a linear function from 40% of Mass Flow Controller (MFC) at
152 initial conditions to 8% at the end of the run.

153

154 **2.4 GC×GC columns and settings**

155 The column set was configured as follows: ¹D SolGel-Wax column (100% polyethylene glycol; 30
156 m × 0.25 mm d_c, 0.25 μm d_f) from SGE Analytical Science (Ringwood, Australia) coupled with a ²D
157 OV1701 column (86% polydimethylsiloxane, 7% phenyl, 7% cyanopropyl; 2 m × 0.1 mm d_c, 0.10 μm d_f),
158 from J&W (Agilent, Little Falls, DE, USA). SPME thermal desorption into the GC injector port was under
159 the following conditions: split/splitless injector in split mode at 250°C, split ratio 1:20. The carrier gas
160 was helium at a constant flow of 1.3 mL/min. The oven temperature program was from 40°C (2 min) to
161 240°C at 3.5°C/min (10 min).

162 The *n*-alkanes liquid sample solution for I^T_S determination was analyzed under the following
163 conditions: split/splitless injector in split mode, split ratio 1:50, injector temperature 250°C, and
164 injection volume 1 μL.

165

166 **2.5 Data acquisition and 2D data processing**

167 Data were acquired by TOF-DS software (Markes International, Llantrisant, UK) and processed
168 using GC Image GC×GC Software, ver 2.8 (GC Image, LLC, Lincoln NE, USA).

169

170 **3. Results and Discussion**

171 In this study, the cocoa volatile metabolome was used as challenging bench test to evaluate
172 potentials and opportunities provided by tandem hard and soft electron ionization in terms of detailed
173 and informative profiling and accurate fingerprinting with template matching algorithms. Based on the
174 outcomes of previous studies aimed at capturing diagnostic fingerprints from high-quality cocoa of
175 different geographical and botanical origin [21–24], it was clear that GC×GC-MS can be employed to
176 deeply explore the multiple chemical dimensions encrypted on the volatile metabolome. Within this
177 fraction are several chemical classes, including informative homologues that are formed through known
178 and unknown chemical and enzymatic pathways during post-harvest and industrial processing.
179 Therefore, the possibility to map and collect informative 2D patterns, i.e., characteristic quali-
180 quantitative distributions of analytes in the multidimensional analytical space, is fundamental. The
181 hyphenation of GC×GC with TOF MS featuring tandem ionization adds a further dimension at the

182 detection level, providing additional information while opening new opportunities at the data
183 elaboration level [17].

184 The next subsection describes some preliminary steps designed to evaluate tandem ionization
185 detection reliability and to better understand the complementary nature of tandem signals at 70 eV and
186 lower energies for cocoa application. Spectral quality at 70 eV and spectral similarity/dissimilarity are
187 computed to set optimal parameters for tandem acquisition.

188 Once tandem ionization acquisition parameters are defined, samples are run in a single
189 analytical batch and 2D data processed by UT fingerprinting [18,19]. A schematic diagram of the UT
190 fingerprinting workflow is illustrated in **Supplementary Figure 1**. Informational features (untargeted and
191 targeted peaks) covering the entire cocoa volatile metabolome, are then computed and some statistical
192 descriptors (2D peaks detection thresholds, signal levels, and Signal-to-Noise Ratio (SNR)) are evaluated.
193 Finally, classification is run on untargeted 2D-peak features from single ionization channels and on fused
194 data streams; then, results are discussed and cross-validated with targeted peak features.

195

196 **3.1 Tandem ionization: spectral quality at 70 eV and cocoa volatiles information dimensions**

197 As a preliminary step, the reliability of 70 eV spectra acquired featuring tandem ionization at 50
198 Hz per channel was evaluated. Quality matches were calculated by matching candidate spectra at 70 eV
199 with those collected in commercial databases (NIST 2014 and Wiley 7n) and in in-house databases
200 established from regular single quadrupole measurement, with the positive identification threshold set
201 at 950 of Direct Match Factor (DMF). Linear retention indices I_s^T (± 20 units tolerance) also were
202 considered for identification. In case of co-elutions, spectral deconvolution by the AMDIS algorithm [8]
203 and/or manual inspection with spectral subtraction were performed before identification.

204 **Supplementary Table 1** lists all 193 targeted analytes plus the IS together with their retention
205 times (1t_R min, 2t_R sec), experimentally determined I_s^T values, and NIST Identity Search algorithm Match
206 Factors: DMF and Reverse Match Factor (RMF), obtained by considering the Peak Apex average
207 spectrum.

208 **Figure 1** illustrates, as a bar chart of the summed DMF and RMF values (ordered by DMF) for the
209 192 targeted analytes. On average, DMF achieved 930 similarity and RMF achieved 960. The latter
210 excludes from the computation those m/z fragments that are not present in the reference spectra.
211 Peaks with Peak Apex DMF below 950 were affected by co-elution issues. In those cases, by
212 deconvolution and/or manual spectral subtraction, matches above 950 were obtained (data not shown)
213 confirming that tandem ionization acquisition does not preclude confident identification while adding

214 further information in the soft ionization data stream. Further comments on this aspect are reported in
215 *Section 3.2.*

216

217 **Insert Figure 1 here**

218

219 Within the 193 targeted analytes are several informative chemicals known for their role in the
220 description of cocoa aroma (key-aroma compounds and potent odorants), post-harvest practices, and
221 technological impacts. Within the list of sensory active compounds [25,26] concurring in the definition
222 of cocoa aroma blueprint, twenty were successfully identified: 2-methyl-butanal, ethyl 2-
223 methylbutanoate, 2-heptanol, dimethyl trisulfide, 2,3,5-trimethylpyrazine, acetic acid, 2-ethyl-3,6-
224 dimethyl-pyrazine, 2-ethyl-3,5-dimethyl-pyrazine, 2,3-diethyl-5-methylpyrazine, linalool, 2-methyl
225 propanoic acid, ethyl 2-methylpropanoate, butanoic acid, phenyl acetaldehyde, 3-methyl butanoic acid,
226 1-phenyl ethanol, 2-phenyl ethyl acetate, phenylethyl alcohol, δ -2-decenolactone, and 4-hydroxy-2,5-
227 dimethyl-3(2h)-furanone. Their signature (quali-quantitative distribution) informs about cocoa flavor,
228 imparting characteristic notes as: *earthy, roasted, rancid, sour, sweaty, malty, cocoa, buttery, flowery,*
229 *honey-like, fruity, green, fatty, sulfury, and phenolic.*

230 Furthermore, important technological markers [21] and origin tracers [18,26] also were
231 identified: 2,3-butanedione, 2,3-pentanedione, dimethyl disulfide, methyl pyrazine, 3-hydroxy-2-
232 butanone, 1-hydroxy-2-propanone, 2,5-dimethylpyrazine, 2,3-octanedione, 2,6-dimethylpyrazine,
233 ethylpyrazine, 2,3-dimethyl pyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, 1-acetyloxy-2-
234 propanone, furfural, tetramethylpyrazine, 2-furan methanol, and (e)-2-phenyl-2-butenal. The residual
235 150 targeted analytes complete the characteristic volatiles signatures, carrying information about
236 additional variables (covering most of the key steps impacting on cocoa chemical composition along the
237 production chain) although their specific roles are not yet validated.

238 Such a comprehensive chemical characterization of the sample volatilome is greatly attractive,
239 especially for those studies of interactions of multiple variables. Therefore, some key-performance
240 parameters have to be evaluated for their impact on fingerprinting effectiveness: specificity, sensitivity,
241 and dynamic range of the response are fundamental since tandem ionization could provide additional
242 advantages if properly set.

243 Since spectral quality at 70 eV was confirmed as satisfactory, providing proofs on adequate
244 method reliability at the detection level, the successive step was the selection of tandem acquisition
245 ionization energies capable of providing complementary information at spectral level [14,17,27]In this

246 perspective, spectral similarity/dissimilarity between different ionization energies was considered to
247 define the best conditions. The next section focuses, for a selection of informative chemicals, on spectral
248 differences at 12 and 14 eV and discusses dissimilarity between hard and soft ionization spectra,
249 including some considerations about SNR values and consequent method sensitivity and dynamic range.

250

251 **3.2 Tandem ionization: spectral dissimilarity and complementary nature of tandem signals**

252 The effect of different ionization voltages on spectral profiles was evaluated by recording
253 spectra at 12 and 14 eV. Lower energies (10 eV) were excluded because of a dramatic drop in signal
254 intensities. **Table 2** lists DMF and RMF values for a series of targeted analytes representative of different
255 chemical classes or series of homologues, for spectral comparisons between: a) 70 eV vs. database
256 (Wiley 7n or NIST 2014); b) 12 eV vs. 14 eV; c) 12 eV vs. 70 eV; d) 14 eV vs. 70 eV.

257 Results indicate, as expected, that on average, the spectral dissimilarity between 12 eV and 70
258 eV is higher compared to that between 14 and 70 eV. The average DMF was 779 at 12 eV and 830 at 14
259 eV. Interestingly, several analytes spectra at 12 and 14 eV are characterized by the same fragments
260 although with different relative abundance; this is true for those analytes that reported identical values
261 for DMF and RMF: 2,3-pentanedione, 3-penten-2-one, limonene, benzaldehyde, 2-furan methanol, and
262 benzyl alcohol. The same situation is evident also between spectra at 12 and 14 eV vs. 70 eV for 2,3-
263 pentanedione, 3-penten-2-one, benzaldehyde, 2-furan methanol, benzyl alcohol, γ -octalactone, 1h-
264 pyrrole-2-carboxaldehyde, and γ -nonalactone.

265 Within the analytes that showed the most dissimilar patterns (lower DMF values), nonanal and
266 limonene are illustrated in **Figure 2**. For nonanal (**Fig. 2A**), lower ionization energies revealed the
267 molecular ion (i.e., 142 m/z) that was not present at 70 eV. Additionally, on the spectrum at 12 eV, the
268 base peak was 98 m/z while at 14 eV and 70 eV, the most abundant fragment was 57 m/z. For limonene
269 (**Fig. 2B**), a terpenoid derivative, lower ionization energies produced higher relative abundances for
270 fragments with higher m/z ratios (i.e., 93, 107 and 121 m/z) and the molecular ion (i.e., 136 m/z) is
271 enhanced.

272

273 **Insert Figure 2 here**

274

275 Lower ionization energies produce fewer fragments, therefore resulting in lower spectral/signal
276 intensities. However, for those analytes that showed reduced fragmentation at lower eV, the resulting
277 signals are enhanced and consequently SNR may be improved compared to higher energies.

278 **Table 2** reports the SNR values for a selection of targets registered from tandem signals at 70
279 and 12 eV from a roasted Ecuador cocoa. Strecker aldehydes (2-, and 3- methylbutanal), furan derivatives
280 (furfural and 2-furan methanol), and benzaldehyde have higher relative intensities at 12 eV. This
281 interesting pattern, also seen for other analytes (data not shown), evidences the complementary nature
282 of tandem ionization signals and, in this case as quantitative indicator, suggests that lower ionization
283 energies may be beneficial for fingerprinting sensitivity extending the dynamic range of detection. For
284 analytes where 70 eV produces higher SNRs, detector saturation may therefore be a limiting factor and,
285 in these cases, the tandem signal at lower eV may compensate for this.

286 In this perspective, where the complementary nature of tandem signals has been established by
287 comparing several analytes features, it is of interest to test the effectiveness of comprehensive
288 chromatographic fingerprinting conducted on tandem signals independently or after their fusion in
289 single data streams. The next section evaluates informational features over the entire volatile
290 metabolome (untargeted and targeted peaks) through some statistical descriptors: 2D peaks detection
291 thresholds, signal levels, and SNR.

292

293 **3.3 Tandem signals informational features**

294 Cocoa samples from four different origins and two processing stages (raw and roasted) analyzed in
295 duplicate are considered here for the processing. Each of the 16 runs (4×2×2) produced a chromatogram
296 for each ionization level (12eV and 70eV), resulting in 32 directly acquired chromatograms. To assess the
297 possibility of increasing performance by combining the data for the two ionization levels (i.e., data
298 fusion), an additional 16 chromatograms were created by adding the two directly acquired
299 chromatograms for each run. So, in total, 48 chromatograms from 16 runs on eight samples were
300 analyzed.

301

302 **3.3.1 2D-peak detection**

303 An important step in the *UT fingerprinting* workflow [18,19] is to establish a set of reliable peaks that
304 can be used for alignment, in order to obtain consistent features across a set chromatograms, even in
305 the presence of retention-times variations. In this step, a relatively high SNR peak-detection threshold of
306 100 was applied as the acceptable limit to the ratio of the total intensity count (TIC) of the apex
307 spectrum to the standard deviation of background noise TIC. Then, composite chromatograms were
308 computed as the sums of the sets of aligned chromatograms for 12 eV, 70 eV, and summed data. These
309 three composite chromatograms are shown in **Figure 3**. Note that adding the chromatograms to create

310 a composite not only yields a single chromatogram to which all compounds in all samples contribute,
311 but also attenuates random-noise variations, thereby increasing SNR.

312

313 **Insert Figure 3 here**

314

315 With the threshold $\text{SNR} \geq 100$, 335 2D-peaks (blobs) were detected in the composite of 12 eV
316 chromatograms, 491 blobs were detected in the composite of 70 eV chromatograms, and 498 blobs
317 were detected in the composite of summed chromatograms. These results, shown in the top row of
318 **Table 3**, indicate that more high-SNR 2D-peaks are produced by 70 eV ionization than by 12 eV
319 ionization. The number of detected high-SNR 2D-peaks was largest in the composite of summed
320 chromatograms.

321 When analyzing individual chromatograms (e.g., to be matched with a UT template), a relatively low
322 SNR peak-detection threshold may be appropriate so as not to miss compound peaks even at the cost of
323 false detections. The lower part of **Table 3** shows the number of 2D-peaks (or blobs) detected in each of
324 the individual chromatograms with the $\text{SNR} \geq 20$, as well as the averages by ionization energy and
325 sample source region. With the low SNR threshold, about 70% more 2D-peaks were detected, on
326 average, in the 12 eV chromatograms than in the 70 eV chromatograms, with an average of 777 2D-
327 peaks detected with 12 eV and 451 2D-peaks detected with 70 eV. However, as shown by the example
328 chromatograms in **Figure 4**, many of the additional 2D-peaks are in noisy regions and appear to be false
329 detections (**Fig. 4A**). In the summed chromatograms (**Fig. 4C**), more 2D-peaks are detected than with 70
330 eV, but fewer than with 12 eV. As detailed below, the average signal intensities in the 12 eV
331 chromatograms are lower than in the 70 eV chromatograms.

332

333 **Insert Figure 4 here**

334

335 With respect to the cocoa volatiles patterns, on average, a few more 2D-peaks were detected from
336 the roasted samples than the raw samples. However, although more 2D-peaks were detected in the
337 roasted samples from Colombia and Ecuador, more 2D-peaks were detected in the raw samples from
338 Mexico and Sao Tome. The Sao Tome samples yielded the most 2D-peaks, indicating a higher chemical
339 complexity, followed by samples from Mexico, Colombia, and Ecuador, with about 16% more 2D-peaks
340 in the Sao Tome samples than those from Ecuador. Differences of volatiles signatures are in line with
341 previous studies [21] where it was confirmed the pre-eminent role of botanical/geographical origin over

342 processing in the chemical dimensionality of samples. Roasting on cocoa triggers several chemical
343 reactions that result in more “quantitative” changes on volatiles signatures rather than “qualitative”
344 differences.

345

346 3.3.2 *Signal intensity*

347 Signal levels were analyzed in each of the peak-regions derived from the composite of summed
348 chromatograms (shown in **Figure 3C**). Signal levels were substantially greater in the 70 eV
349 chromatograms than in the 12 eV chromatograms. In the individual chromatograms, on average, the
350 peak-region TIC apexes in the 12 eV chromatograms were only about 30% of the same peak-region TIC
351 apexes in the 70 eV chromatograms. The peak-region TIC apex in the summed chromatograms averaged
352 126% of the peak-region apex TIC with 70 eV. On average, the peak-region TIC volumes in the 12 eV
353 chromatograms were only 40% of the same peak-region volumes in the 70 eV chromatograms. The
354 peak-region TIC volumes in the summed chromatograms averaged 124% of the peak-region volume with
355 70 eV. It appears that the ratios of the 12 eV to 70 eV peak-region volumes (40%) are larger than the
356 ratios of the 12 eV to 70 eV apexes (30%) because lower intensity spectra are relatively less different
357 between the two ionization energies (so, the off-apex spectra are less different than are the apex
358 spectra). Spectral differences are discussed below.

359

360 3.3.3 *Spectral peak intensities*

361 From each peak-region in the composite of summed chromatograms, two spectral channels were
362 selected to evaluate spectral intensities: (1) the channel of the base peak (i.e., the m/z of the largest
363 intensity component in apex spectrum), and (2) a large-mass candidate for the molecular ion (selected
364 as the largest m/z with relative intensity of at least 10% of the base peak intensity, with the additional
365 constraint to filter isotopes that the unit mass interval just below did not have a larger intensity peak).

366 Just as for the TIC intensities, the base peak intensities were substantially greater in the 70 eV
367 chromatograms than in the 12 eV chromatograms. On average, peak-region apex base-peak intensities
368 in the 12 eV chromatograms were only 29% of the peak-region apex base-peak intensities in the 70 eV
369 chromatograms. The peak-region apex base-peak intensities in summed chromatograms averaged 128%
370 of the peak-region apex base-peak intensities with 70 eV. On average, peak-region base-peak volumes in
371 the 12 eV chromatograms were only 17% of the peak-region base-peak volumes in the 70 eV
372 chromatograms. The peak-region base-peak volume in the summed chromatograms averaged 116% of
373 the peak-region base-peak volume with 70 eV.

374 Similarly, the large-mass-peak intensities were substantially greater in the 70 eV chromatograms
375 than in the 12eV chromatograms, but the difference was smaller than for the base-peak intensities. On
376 average, peak-region apex large-mass-peak intensities in the 12 eV chromatograms were 60% of the
377 peak-region apex large-mass-peak intensity in the 70 eV chromatograms. The peak-region apex large-
378 mass-peak intensities in summed chromatograms averaged 158% of the peak-region apex large-mass-
379 peak intensities with 70eV. On average, peak-region large-mass-peak volumes in the 12 eV
380 chromatograms were only 43% of the peak-region large-mass-peak volumes in the 70 eV
381 chromatograms. The peak-region large-mass-peak volume in the summed chromatograms averaged
382 142% of the peak-region large-mass-peak volume with 70 eV.

383 This is an interesting result if we consider the higher informative power of large-mass-peaks in a
384 spectrum. This characteristic relates to the specificity of lower ionization energies and so provides
385 foundation for the adoption of low eV data stream for effective fingerprinting as well as for the added
386 value it brings when summed to the 70 eV channel.

387

388 *3.3.4 Signal-to-noise: SNR and VNR*

389 On average, the TIC background noise for 12 eV was approximately 33% of the noise for 70 eV and
390 the noise in the summed chromatograms was about 105% of the noise for 70eV. With the lower signal
391 and noise levels for 12 eV, the average peak SNR (TIC apex intensity to noise standard deviation) for 12
392 eV was about 86% of the SNR with 70 eV. However, the average volume-to-noise ratio (VNR) with 12 eV
393 was about 134% of the VNR with 70 eV. The SNR of the summed chromatograms was about 115% of
394 the SNR with 70eV and the VNR of the summed chromatograms about 117% of the VNR with 70 eV.

395 The spectral background noise was fairly consistent at most m/z levels, but, at some m/z levels,
396 especially smaller m/z levels, the raw spectral background noise was substantially larger with 70 eV than
397 with 12 eV, as illustrated in **Supplementary Figure 2**. However, baseline correction can detect and
398 attenuate large baseline spectral values, as illustrated in **Supplementary Figure 3**.

399

400 **3.4 Classification performance**

401 *3.4.1 Fisher Discriminant Ratios of Individual Features in Individual Peak-Regions*

402 The potential of individual features for classification is indicated by the Fisher Discriminant Ratio
403 (FDR), the ratio of the scatter between classes to the scatter within classes. Classification was part of the
404 automated work-flow applied on single and summed data streams within the Image Investigator™ (GC
405 Image). For the 498 peak-regions extracted from untargeted analysis of the composite of summed

406 chromatograms, various features could be used for discrimination. Seven computed peak-region
407 features were analyzed: volume (summed response for all modulated peaks included in a defined peak-
408 region), percent response (volume to total chromatogram response), apex response (highest modulation
409 response), base-peak apex response (response related to most intense m/z fragment from highest
410 modulation), base-peak volume (peak-region volume related to most intense m/z fragment), large-mass
411 apex response (response related to largest m/z fragment from highest modulation), and large-mass
412 volume (peak-region volume related to most intense m/z fragment).

413 For all of these features, most peak-regions have relatively small FDR, i.e., are weak indicators of
414 class differences. As shown in **Table 4**, the average FDR for different features ranged from 0.31 for apex
415 response with 12 eV to 0.54 for percent response with 12 eV. The median FDR was far below the mean,
416 ranging from 0.02 for volume with 12 eV to 0.32 for percent response with 12 eV. For five of the seven
417 computed features, the average FDR with 70 eV was larger than with 12 eV, whereas only one feature
418 had a larger average FDR with 12 eV (and the average FDRs were nearly identical for each feature). The
419 average FDR for the summed chromatograms was about the same as with 70 eV.

420 Although there can be useful information in many weakly indicative features, most of the potential
421 for classification exists within a relatively small number of features. The maximum FDR ranged from 2.92
422 for the large-mass peak intensity with 12 eV to 14.01 for the percent response with 70 eV. For six of the
423 seven features, the maximum FDR with 70 eV was larger than with 12 eV and about the same as with
424 the summed chromatograms. The average of the top-ten FDRs for each feature ranged from 3.01 for the
425 large-mass peak intensity with 12 eV to 5.51 for the percent response with 70 eV. For six of the seven
426 features, the top-ten FDR average was greater with 70 eV than with 12 eV. The top-ten FDR average for
427 the summed chromatograms was larger than with 70 eV for four of the seven features.

428 As the basis for classification, percent response is clearly the most promising of the seven features,
429 producing the largest FDR (14.01 with 70 eV), the largest top-ten FDR average (5.51 with 70 eV), and
430 largest mean FDR (0.54 with 12 eV). However, base-peak volume was the best performing feature with
431 12 eV for maximum FDR and top-ten FDR average (7.80 and 4.96, respectively).

432 Percent response also has some intrinsic advantages being a peak feature that refers to normalized
433 data therefore enabling consistent comparative analysis even without external standard/internal
434 standard normalization of analytes responses. On the other side, base-peak volume is more sensitive to
435 “true” quantitative response variations across chromatograms and has the advantage of informing
436 about single analyte fluctuations in more detail.

437

438 3.4.2 Linear Discriminant Analysis on untargeted features from tandem signals

439 As anticipated by the FDR analysis, percent response provided the best basis for classification by
440 linear discriminant analysis (LDA), and was the only feature to support 100% classification accuracy in
441 leave-one-out trials with the chromatograms from raw and roasted samples. The next best classification
442 accuracy was 93.75% for both base-peak and large-mass volume with 12eV. The LDA scores with
443 inferential Gaussian distributions for the leave-one-out trials are shown in **Supplementary Figure 4**. In
444 the cross-validation experiments, replicates were left out together to prevent bias. The Fisher ratio for
445 the LDA scores were 2.99 for the summed chromatograms, 2.75 with 70 eV, and 2.71 with 12 eV, which
446 is not a very large range, but does indicate that better discrimination can be achieved with the
447 fused/summed data.

448 Concerning cocoa analysis, the roasted samples had much more variable LDA scores than did the raw
449 samples (as seen in **Supplementary Figure 4**). Within the roasted samples, the LDA scores of samples
450 from Mexico were the most different from the raw samples scores (leftmost in **Supplementary Figure 4**)
451 and the LDA scores of the samples from Ecuador were the closest to the raw samples scores. Many of
452 the same peak-regions were significant (large score-weighted standard deviations) with 12 eV, 70 eV,
453 and the summed chromatograms, with six features in the top-10 for all three classification schemes and
454 four other features in the top-10 for two of the three classification schemes. The top-ten peak-regions
455 for LDA classification with summed chromatograms are listed in **Table 5**. Of the other four peak-regions
456 that appeared in the top-ten of only one scheme, all were in the top-40 for the other two schemes.
457 However, two of the top-10 features with both 70 eV and summed chromatograms were not highly
458 significant with the 12 eV data: peak-regions #68 and #102 ranked 138 and 177 among 12 eV features.

459

460 3.5 Untargeted-Targeted UT fingerprinting: results validation

461 Results obtained by untargeted fingerprinting on tandem signals elaborated separately or after their
462 summation in a derived data stream were validated against the targeted approach. This step enables
463 objective evidence on discrimination performances of tandem data after fusion taking as benchmark
464 results obtained through the well-established work-flow based on template matching of targeted
465 analytes guided by analyst supervision [28,29]. Supervision was here necessary for those analytes that
466 were affected by co-elution issues (section 3.1); therein, deconvolution and/or manual spectral
467 subtraction were performed to achieve confident identification while informative fragments were
468 selected to isolate analytes response from low-resolved peak-regions. The data matrix for the targeted
469 analytes was obtained by collecting percent responses (the 2D-peak/2D peak-region feature connoted

470 by a higher information potential – section 3.3.1) for the 193 reliable analytes at 70 and 12 eV ionization
471 energies.

472 Partial Least Squares – Discriminant Analysis (PLS-DA) was adopted at this stage and, to define
473 informative variables, Variable Importance on the Projections (VIPs) was used to rank targeted analytes
474 on the basis of their power to discriminate between raw and roasted cocoa samples. **Figure 5** shows the
475 first 20 target analytes ranked for their relevance in the discrimination and deriving from the elaboration
476 of 70 and 12 eV data streams independently. In parentheses are the LDA ranking obtained from the
477 untargeted processing of the summed signals (from **Table 5**).

478

479 **Insert Figure 5 here**

480

481 Notably, the targeted approach validates the information power of those analytes that were
482 selected by LDA on untargeted features distribution on summed signals (**Table 5**): the top-10 most
483 relevant variables were included in the list of the first top-20 targets with the highest informative power.
484 In addition, within the top-20 analytes highly ranked after PLS-DA on targetetes, 3-hydroxy-2-butanone,
485 2,3-pentanedione, 1-hydroxy-2-propanone, 2,3-dimethyl pyrazine, tetramethylpyrazine, and 2-furan
486 methanol have their informative power cross-validated between tandem data streams. At 12 eV, within
487 the top-20 additional analytes, additional targets are evidenced: 5-methyl-2(5H)-furanone, dodecanal,
488 ethyl 2-methylbutanoate, and 2,6-dimethylpyrazine while at 70 eV those with high relevance are 2-
489 hydroxy-3-pentanone, methyl 2-hydroxypropanoate, dimethyl disulfide, and 2-methyl pyrazine.

490 The cross-validation of fingerprinting results confirms, once again, the complementary nature of
491 tandem signals: whichever is the data stream (12 or 70 eV) treated as such or as sum of signals, or the
492 approach (untargeted/unsupervised or targeted/supervised) there is univocal identification of
493 discriminant features (untargeted peaks or known analytes) even in such a complex context where
494 confounding variables play a great role (origin and post-harvest practices above all). The advantages of
495 supervised elaborations, as in the case of targeted analysis, are evident for those analytes where co-
496 elution occur; in these cases, single analyte response has to be isolated from co-eluent to achieve
497 adequate specificity. The differential response between tandem signals extends dynamic range of the
498 detector resulting in a larger group of candidates, when tandem signals are combined, to be screened
499 for their information power.

500

501 **4. Conclusions**

502 The present study gives foundations for a full exploitation of the complementary nature of tandem
503 signals obtained by adopting variable EI energies for UT fingerprinting. The cross-comparison of several
504 2D peak features and signal characteristics demonstrates that signal fusion (i.e., summed signals)
505 enables effective untargeted fingerprinting leading also to a good discrimination potential of the
506 methodology even in very complex samples. The targeted fingerprinting, driven by analyte supervision
507 during data pre-processing, better exploits the complementary nature of tandem signals due to their
508 differential informative content. In addition, multiplexing tandem ionization during a single analytical
509 run, does not impact on confident analytes identification while offering additional elements to better
510 discriminate isomeric analytes. The improved SNR registered for some analytes at lower ionization
511 energies, is an interesting performance characteristic of the method that can achieve a wider dynamic
512 range of exploration.

513

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516

517 **Compliance with ethical standards Notes**

518 Prof. Stephen E. Reichenbach and Dr. Qingping Tao have a financial interest in GC Image, LLC.

519 Lucie Baroux and Philippe Merle are employees of Firmenich S.A. Geneva, Switzerland.

520

521

522 **References**

- 523 [1] S.E. Reichenbach, X. Tian, C. Cordero, Q. Tao, Features for non-targeted cross-sample analysis
524 with comprehensive two-dimensional chromatography, *J. Chromatogr. A.* 1226 (2012) 140–148.
525 doi:10.1016/j.chroma.2011.07.046.
- 526 [2] C. Cordero, J. Kiefl, P. Schieberle, S.E. Reichenbach, C. Bicchi, Comprehensive two-dimensional
527 gas chromatography and food sensory properties: Potential and challenges, *Anal. Bioanal. Chem.*
528 407 (2015) 169–191. doi:10.1007/s00216-014-8248-z.
- 529 [3] C. Cordero, J. Kiefl, S.E. Reichenbach, C. Bicchi, Characterization of odorant patterns by
530 comprehensive two-dimensional gas chromatography: a challenge in omic studies, *Trends Anal.*
531 *Chem.* (2018).
- 532 [4] J.C. Giddings, Sample dimensionality: A predictor of order-disorder in component peak
533 distribution in multidimensional separation, *J. Chromatogr. A.* 703 (1995) 3–15.
534 doi:10.1016/0021-9673(95)00249-M.
- 535 [5] P.Q. Tranchida, I. Aloisi, B. Giocastro, L. Mondello, Current state of comprehensive two-
536 dimensional gas chromatography-mass spectrometry with focus on processes of ionization, *TrAC*
537 *- Trends Anal. Chem.* 105 (2018) 360–366. doi:10.1016/j.trac.2018.05.016.
- 538 [6] C. Cordero, H.G. Schmarr, S.E. Reichenbach, C. Bicchi, Current Developments in Analyzing Food
539 Volatiles by Multidimensional Gas Chromatographic Techniques, *J. Agric. Food Chem.* 66 (2018)
540 2226–2236. doi:10.1021/acs.jafc.6b04997.
- 541 [7] R.P. Adams, *Identification of Essential Oil Components by Gas Chromatography—Mass*
542 *Spectroscopy*, Allured Publishing, New York, 1995.
- 543 [8] NIST/EPA/NIH Mass Spectral Library with Search Program Data Version: NIST v17, (n.d.).
- 544 [9] E. Belhassen, D. Bressanello, P. Merle, E. Raynaud, C. Bicchi, A. Chaintreau, C. Cordero, Routine
545 quantification of 54 allergens in fragrances using comprehensive two-dimensional gas
546 chromatography-quadrupole mass spectrometry with dual parallel secondary columns. Part I:
547 Method development, *Flavour Fragr. J.* 33 (2018) 63–74. doi:10.1002/ffj.3416.
- 548 [10] C. Cordero, P. Rubiolo, S.E. Reichenbach, A. Carretta, L. Cobelli, M. Giardina, C. Bicchi, Method
549 translation and full metadata transfer from thermal to differential flow modulated
550 comprehensive two dimensional gas chromatography: Profiling of suspected fragrance allergens,
551 *J. Chromatogr. A.* 1480 (2017) 70–82. doi:10.1016/j.chroma.2016.12.011.
- 552 [11] Y. Duan, F. Zheng, H. Chen, M. Huang, J. Xie, F. Chen, B. Sun, Analysis of volatiles in Dezhou
553 Braised Chicken by comprehensive two-dimensional gas chromatography/high resolution-time of

554 flight mass spectrometry, *LWT - Food Sci. Technol.* 60 (2015) 1235–1242.
555 doi:10.1016/j.lwt.2014.09.006.

556 [12] Y.F. Wong, P. Perlmutter, P.J. Marriott, Untargeted metabolic profiling of *Eucalyptus* spp. leaf oils
557 using comprehensive two-dimensional gas chromatography with high resolution mass
558 spectrometry: Expanding the metabolic coverage, *Metabolomics*. 13 (2017) 1–17.
559 doi:10.1007/s11306-017-1173-3.

560 [13] D.D. Yan, Y.F. Wong, L. Tedone, R.A. Shellie, P.J. Marriott, S.P. Whittock, A. Koutoulis,
561 Chemotyping of new hop (*Humulus lupulus* L.) genotypes using comprehensive two-dimensional
562 gas chromatography with quadrupole accurate mass time-of-flight mass spectrometry, *J.*
563 *Chromatogr. A.* (2017). doi:10.1016/j.chroma.2017.08.020.

564 [14] M.S. Alam, C. Stark, R.M. Harrison, Using Variable Ionization Energy Time-of-Flight Mass
565 Spectrometry with Comprehensive GC-MS to Identify Isomeric Species, *Anal. Chem.* 88 (2016)
566 4211–4220. doi:10.1021/acs.analchem.5b03122.

567 [15] Markes International, Select-eV: The next generation of ion source technology, *Tech. Note.*
568 *Applicatio* (2016).

569 [16] L.M. Dubois, K.A. Perrault, P.H. Stefanuto, S. Koschinski, M. Edwards, L. McGregor, J.F. Focant,
570 Thermal desorption comprehensive two-dimensional gas chromatography coupled to variable-
571 energy electron ionization time-of-flight mass spectrometry for monitoring subtle changes in
572 volatile organic compound profiles of human blood, *J. Chromatogr. A.* 1501 (2017) 117–127.
573 doi:10.1016/j.chroma.2017.04.026.

574 [17] C.E. Freye, N.R. Moore, R.E. Synovec, Enhancing the chemical selectivity in discovery-based
575 analysis with tandem ionization time-of-flight mass spectrometry detection for comprehensive
576 two-dimensional gas chromatography, *J. Chromatogr. A.* 1537 (2018) 99–108.
577 doi:10.1016/j.chroma.2018.01.008.

578 [18] F. Magagna, L. Valverde-Som, C. Ruíz-Samblás, L. Cuadros-Rodríguez, S.E. Reichenbach, C. Bicchi,
579 C. Cordero, Combined untargeted and targeted fingerprinting with comprehensive two-
580 dimensional chromatography for volatiles and ripening indicators in olive oil, *Anal. Chim. Acta.*
581 936 (2016) 245–258. doi:10.1016/j.aca.2016.07.005.

582 [19] D. Bressanello, E. Liberto, M. Collino, F. Chiazza, R. Mastrocola, S.E. Reichenbach, C. Bicchi, C.
583 Cordero, Combined untargeted and targeted fingerprinting by comprehensive two-dimensional
584 gas chromatography: revealing fructose-induced changes in mice urinary metabolic signatures,
585 *Anal. Bioanal. Chem.* (2018). doi:10.1007/s00216-018-0950-9.

- 586 [20] Y. Wang, J. O'Reilly, Y. Chen, J. Pawliszyn, Equilibrium in-fibre standardisation technique for solid-
587 phase microextraction, *J. Chromatogr. A.* 1072 (2005) 13–17. doi:10.1016/j.chroma.2004.12.084.
- 588 [21] F. Magagna, A. Guglielmetti, E. Liberto, S.E. Reichenbach, E. Allegrucci, G. Gobino, C. Bicchi, C.
589 Cordero, Comprehensive Chemical Fingerprinting of High-Quality Cocoa at Early Stages of
590 Processing: Effectiveness of Combined Untargeted and Targeted Approaches for Classification
591 and Discrimination, *J. Agric. Food Chem.* 65 (2017) 6329–6341. doi:10.1021/acs.jafc.7b02167.
- 592 [22] F. Magagna, E. Liberto, S.E. Reichenbach, Q. Tao, A. Carretta, L. Cobelli, M. Giardina, C. Bicchi, C.
593 Cordero, Advanced fingerprinting of high-quality cocoa: Challenges in transferring methods from
594 thermal to differential-flow modulated comprehensive two dimensional gas chromatography, *J.*
595 *Chromatogr. A.* (2018). doi:10.1016/j.chroma.2017.07.014.
- 596 [23] E.M. Humston, Y. Zhang, G.F. Brabeck, A. McShea, R.E. Synovec, Development of a GCxGC-TOFMS
597 method using SPME to determine volatile compounds in cacao beans, *J. Sep. Sci.* 32 (2009) 2289–
598 2295. doi:10.1002/jssc.200900143.
- 599 [24] L.F. Oliveira, S.C.G.N. Braga, F. Augusto, J.C. Hashimoto, P. Efraim, R.J. Poppi, Differentiation of
600 cocoa nibs from distinct origins using comprehensive two-dimensional gas chromatography and
601 multivariate analysis, *Food Res. Int.* 90 (2016) 133–138. doi:10.1016/j.foodres.2016.10.047.
- 602 [25] F. Frauendorfer, P. Schieberle, P.E.S. Chieberle, Changes in Key Aroma Compounds of Criollo
603 Cocoa Beans During Roasting Changes in Key Aroma Compounds of Criollo Cocoa Beans During
604 Roasting, *J. Agric. Food Chem.* 56 (2008) 10244–10251. doi:10.1021/jf802098f.
- 605 [26] E.O. Afoakwa, A. Paterson, M. Fowler, A. Ryan, Flavor formation and character in cocoa and
606 chocolate: A critical review, *Crit. Rev. Food Sci. Nutr.* 48 (2008) 840–857.
607 doi:10.1080/10408390701719272.
- 608 [27] E. Commission, Commission Decision 2002/657/EC implementing Council Directive 96/23/EC
609 concerning the performance of analytical methods and the interpretation of results, *Off. J. Eur.*
610 *Union.* L221 (2002) 8–36. [http://www.ecolex.org/ecolex/ledge/view/RecordDetails?id=LEX-
611 FAOC049615&index=documents%5Cnfile://g/R&D/PAPE/articles/Mass Spec/European directive
612 MS IP.pdf](http://www.ecolex.org/ecolex/ledge/view/RecordDetails?id=LEX-FAOC049615&index=documents%5Cnfile://g/R&D/PAPE/articles/Mass Spec/European directive MS IP.pdf).
- 613 [28] J. Kiefl, C. Cordero, L. Nicolotti, P. Schieberle, S.E. Reichenbach, C. Bicchi, Performance evaluation
614 of non-targeted peak-based cross-sample analysis for comprehensive two-dimensional gas
615 chromatography-mass spectrometry data and application to processed hazelnut profiling, *J.*
616 *Chromatogr. A.* 1243 (2012) 81–90. doi:10.1016/j.chroma.2012.04.048.
- 617 [29] C. Cordero, C. Bicchi, P. Rubiolo, Group-type and fingerprint analysis of roasted food matrices

618 (coffee and hazelnut samples) by comprehensive two-dimensional gas chromatography, *J. Agric.*
619 *Food Chem.* 56 (2008) 7655–7666. doi:10.1021/jf801001z.
620

621 **Figure Captions**

622 **Figure 1:** bar chart showing the sum of NIST Identity Search algorithm Match Factors: Direct Match
623 Factor (DMF – blue bars) and Reverse Match Factor (RMF – orange bars) for the 192 targeted analytes.
624 Ordering follows descending order of DMF values.

625
626 **Figure 2:** Spectral profiles for nonanal (**2A**) and limonene (**2B**) at 70 eV, 12 eV and 14 eV. Spectral
627 comparisons are between 12 eV and 14 eV (**2A-I** and **2B-I**); between 12 eV and 70 eV (**2A-II** and **2B-II**) and
628 between 14 eV and 70 eV (**2A-III** and **2B-III**). Green text below spectra refers DMF and RMF values.

629
630 **Figure 3:** composite 2D chromatograms obtained by summing single ionization energies channels (12 eV
631 and 70 eV) runs (**3A** and **3B**) or both channels (12 eV + 70 eV) after alignment. 2D-peaks connoted by a
632 cyan rounded shapes are those positively matched in all-but-one chromatogram of the set, e.g., reliable
633 peaks while red rounded shapes indicate 2D-peaks detected in just few chromatograms.

634
635 **Figure 4:** single channel chromatograms from Ecuador samples acquired at 12 eV (**4A**) at 70 eV (**4B**) or
636 after single data stream fusion (**4C**). Effect of SNR variable threshold on 2D-peaks detection. See text for
637 details.

638
639 **Figure 5:** histograms reporting the first twenty most informative analytes (Variable Importance for
640 Projections VIPs) revealed by PLS-DA on targeted peaks (roasted vs. raw cocoa). Analytes are reported
641 together with their ranking (in parentheses Sum#n) as resulted by classification analysis of untargeted
642 features on fused data streams. Blue bars refer to 70 eV data while red bars are from 12 eV data,

643
644

645 **Table Captions:**

646 **Table 1:** Cocoa samples under study, together with their origin, supplier, and harvest year.

647

648 **Table 2:** Direct and Reverse Match Factor (DMF and RMF) values for a series of targeted analytes
649 representing different functionalities. Data refers of spectral similarity between 70 eV vs. database
650 (Wiley 7n or NIST 2014); b) 12 eV vs. 14 eV; c) 12 eV vs. 70 eV; d) 14 eV vs. 70 eV. Signal-to-noise ratio
651 (SNR) values are those corresponding to peak-apex and recorded at 12 and 70 eV. Their ratio (12 eV / 70
652 eV) is also reported to facilitate comparisons.

653

654 **Table 3:** number of detected 2D-peaks (blobs) above a certain SNR threshold from composite
655 chromatograms ($\text{SNR} \geq 100$ for 70 eV, 12 eV, and summed signals) and from single analytical runs ($\text{SNR} \geq$
656 20 for 70 eV, 12 eV, and summed signals).

657

658 **Table 4:** average Fisher Discriminant Ratio (FDR) values for different peak-region features at 12 and 70
659 eV and on summed signals. Q1 is the spectral base-peak quantifier ion and Q2 is the large-mass-peak
660 quantifier ion.

661

662 **Table 5:** list of the ten most discriminant 2D-peak regions as indicated by LDA analysis of the summed
663 chromatograms. Data is reported together with unique 2D-peak regions identification numbering (#n),
664 compound name, retention times, and ordinal significance rank within the classification scenarios
665 (Summed, 70 eV, and 12 eV).

666

Figure 1

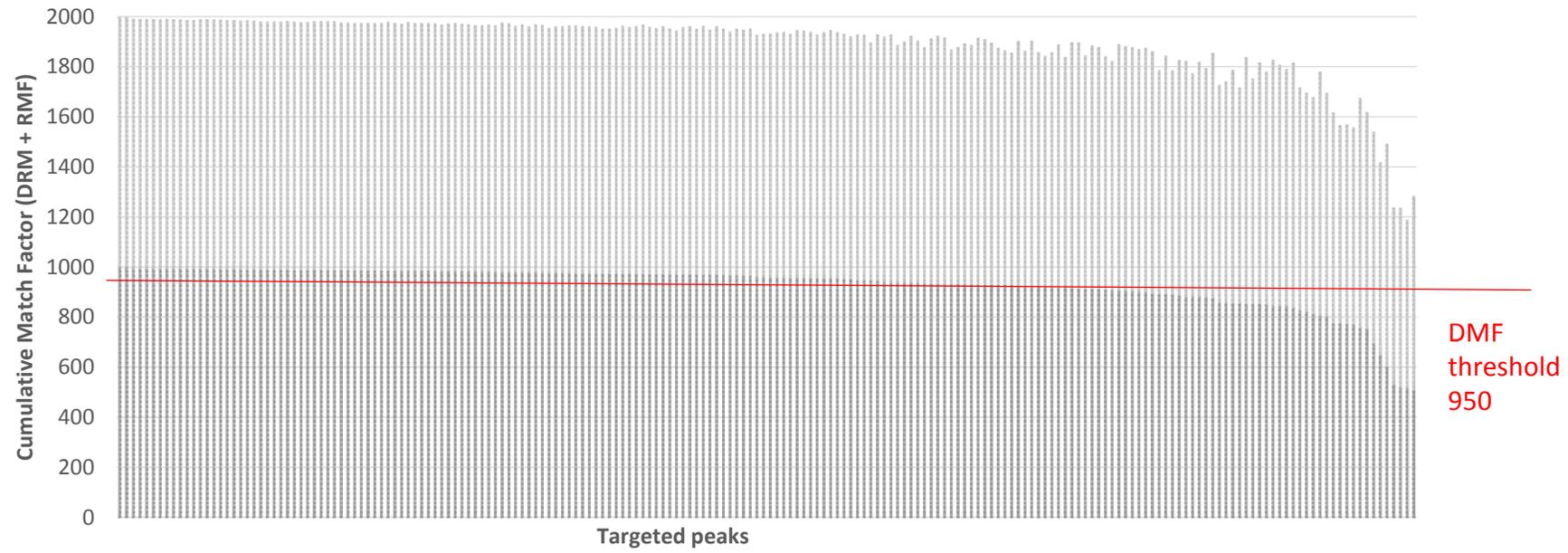


Figure 2

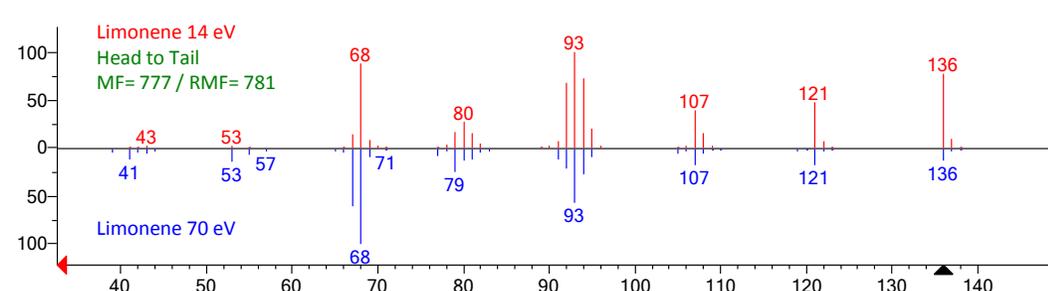
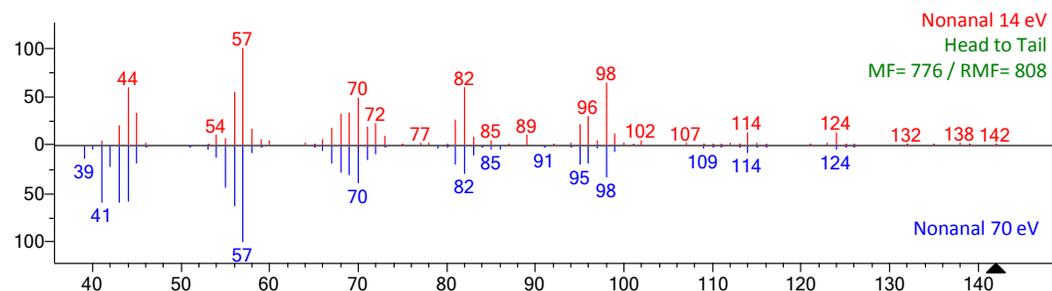
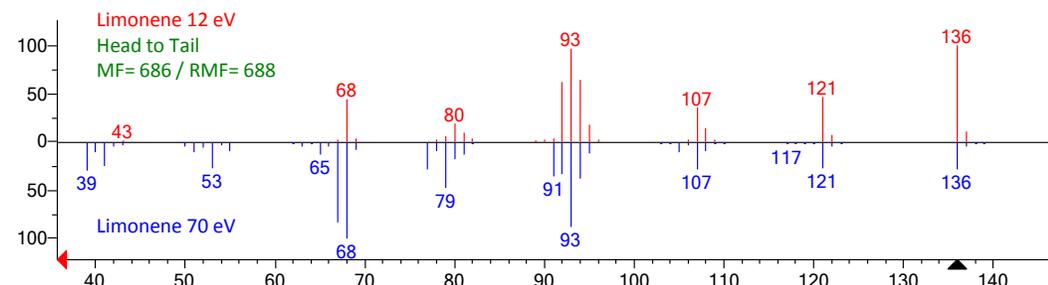
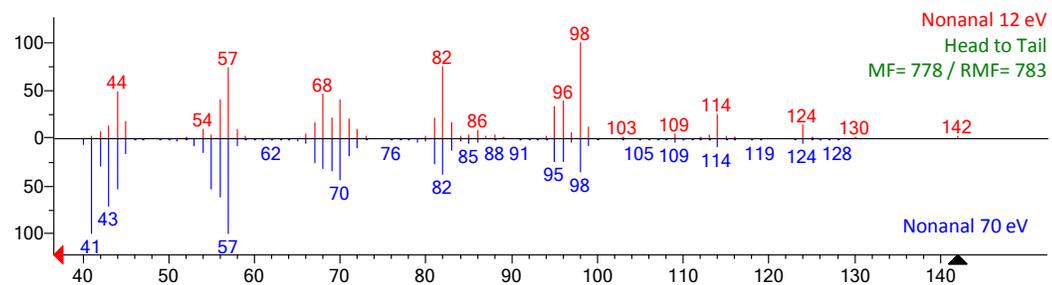
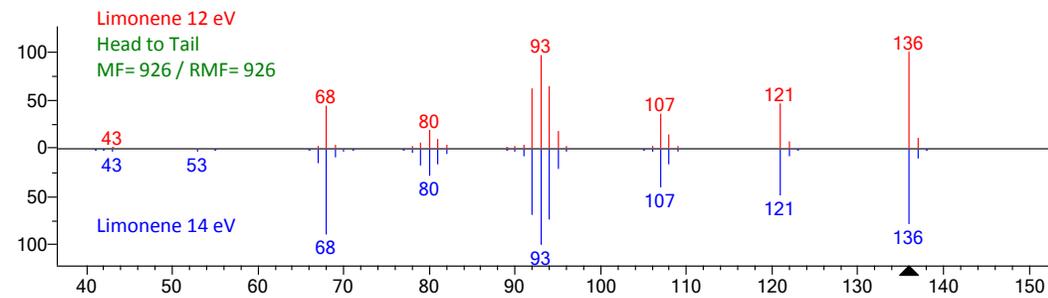
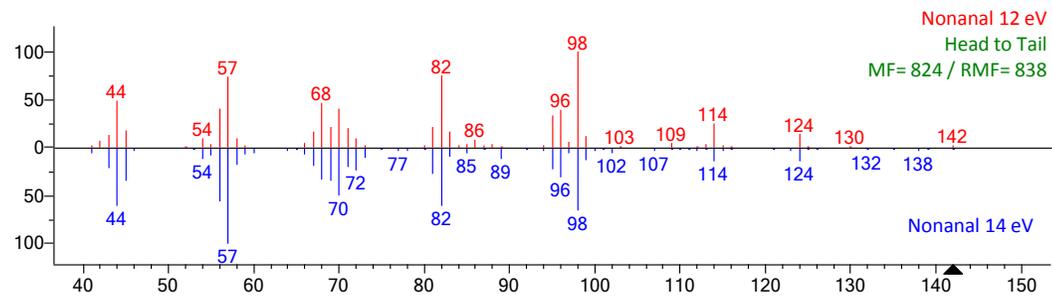


Figure 2

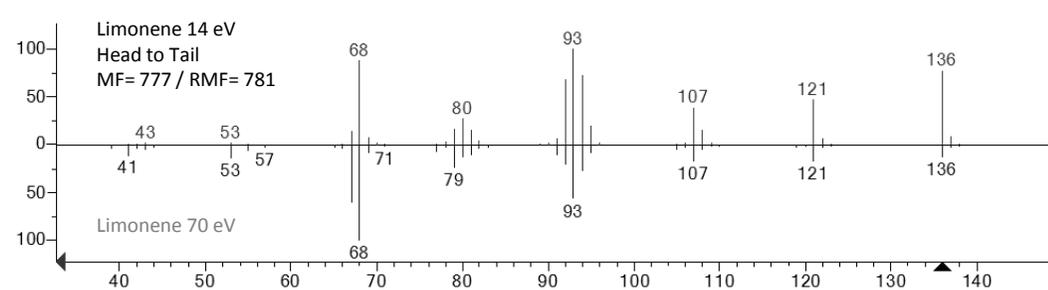
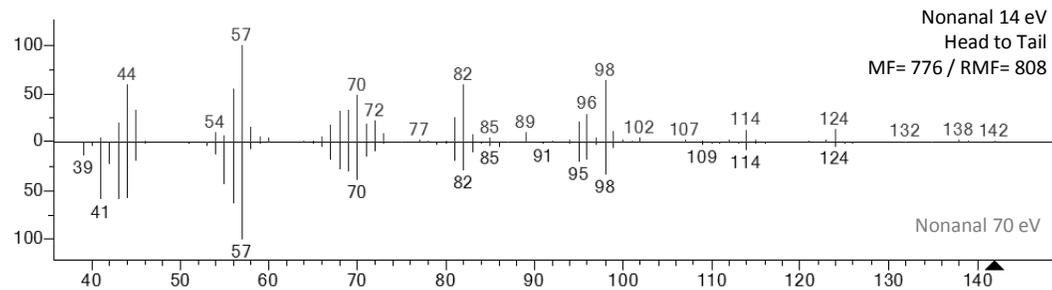
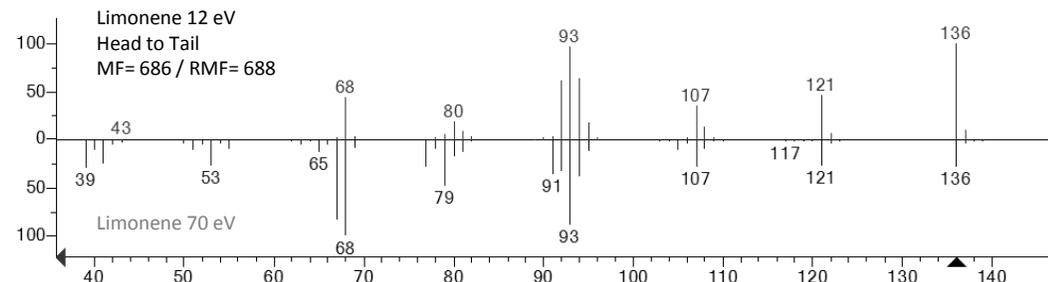
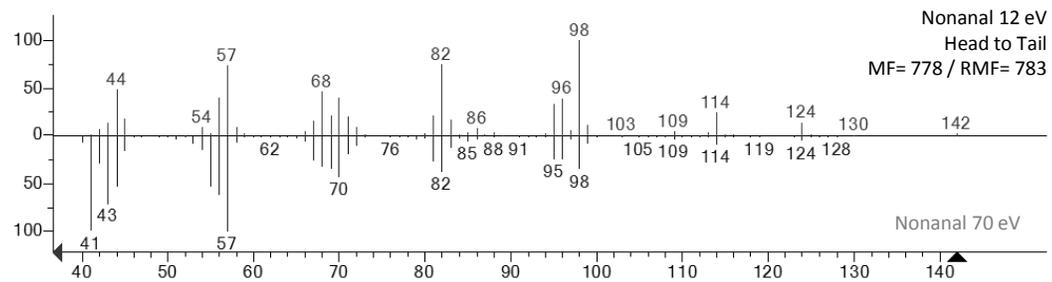
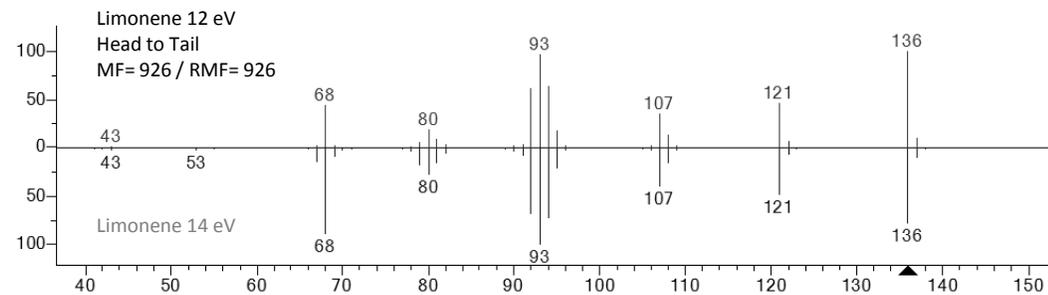
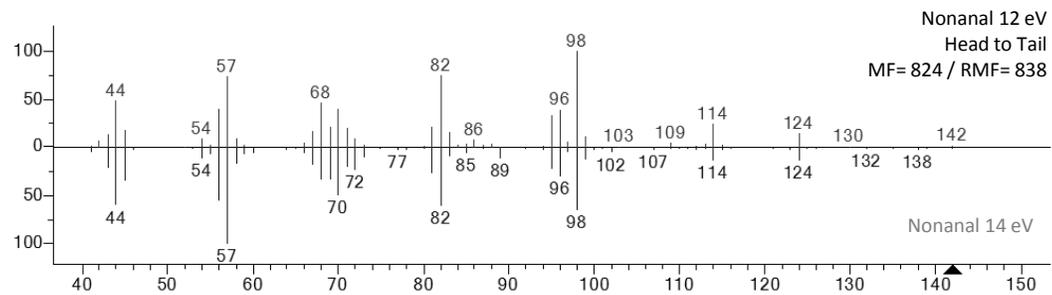
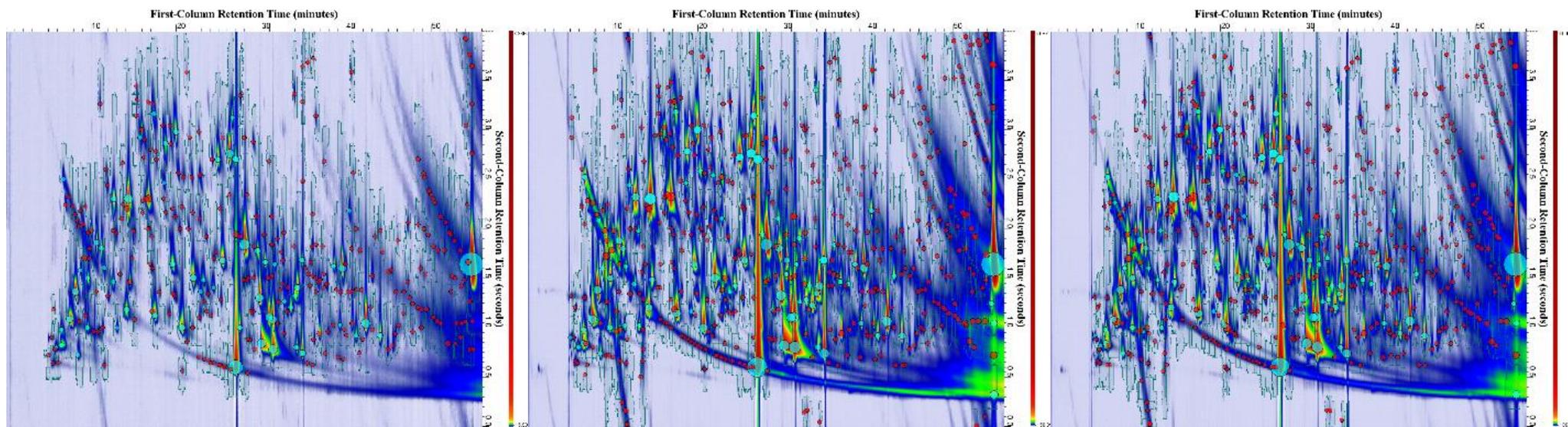


Figure 3

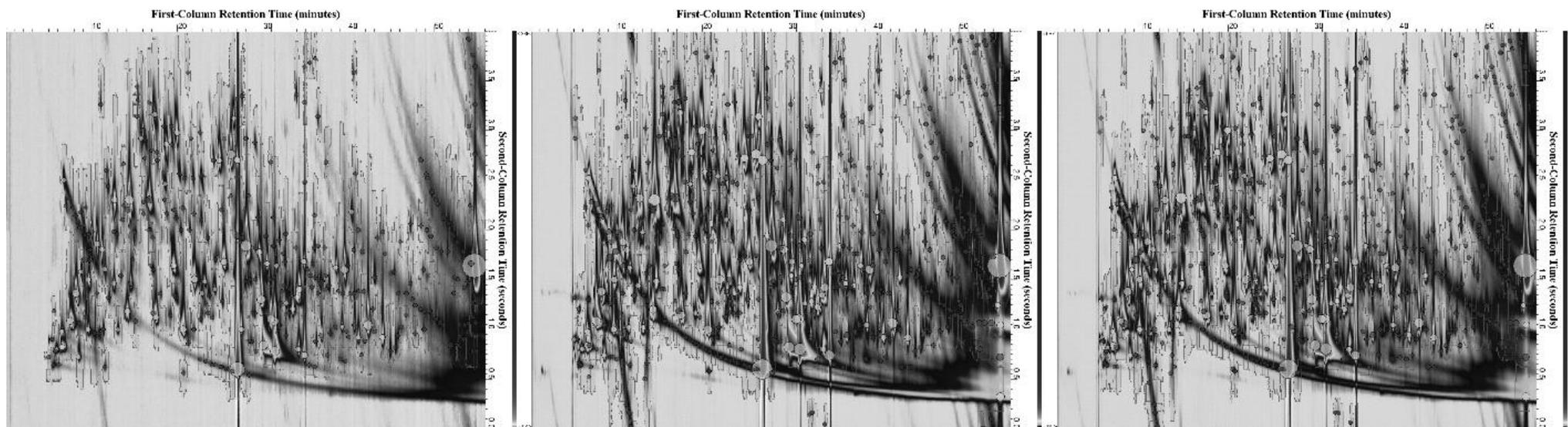


A. 12eV Composite

B. 70eV Composite

C. Summed Composite

Figure 3

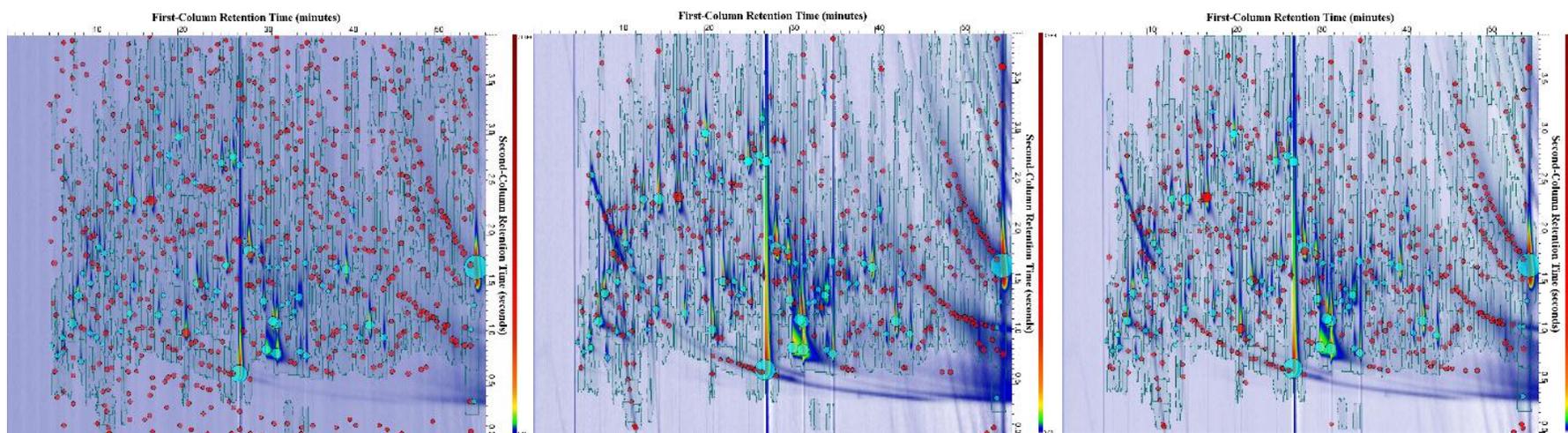


A. 12eV Composite

B. 70eV Composite

C. Summed Composite

Figure 4

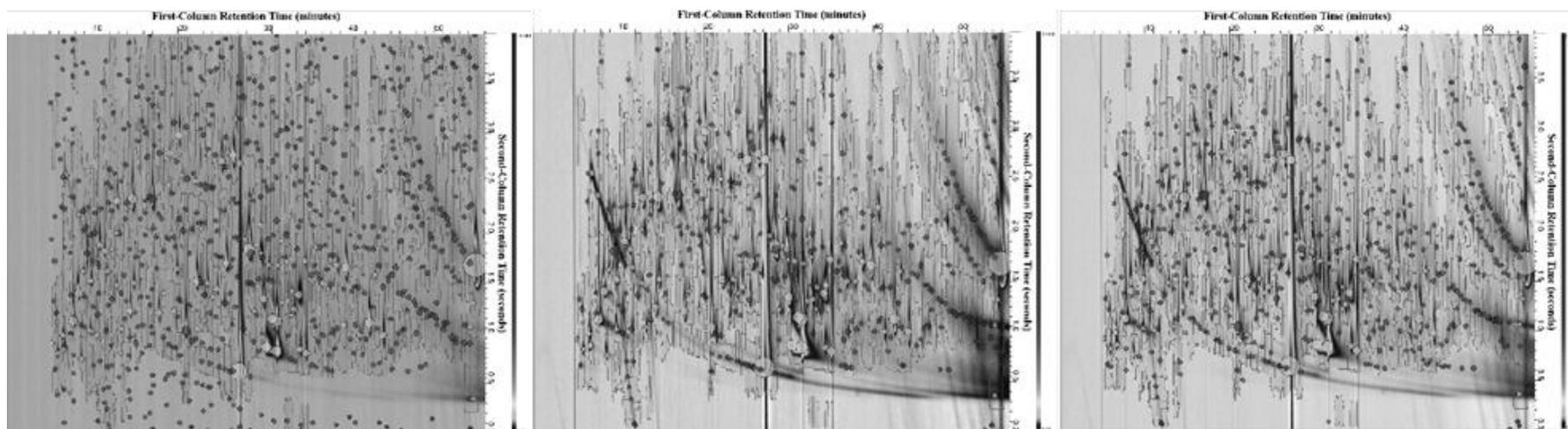


A. 12eV Chromatogram

B. 70eV Chromatogram

C. Summed Chromatogram

Figure 4



A. 12eV Chromatogram

B. 70eV Chromatogram

C. Summed Chromatogram

Figure 5

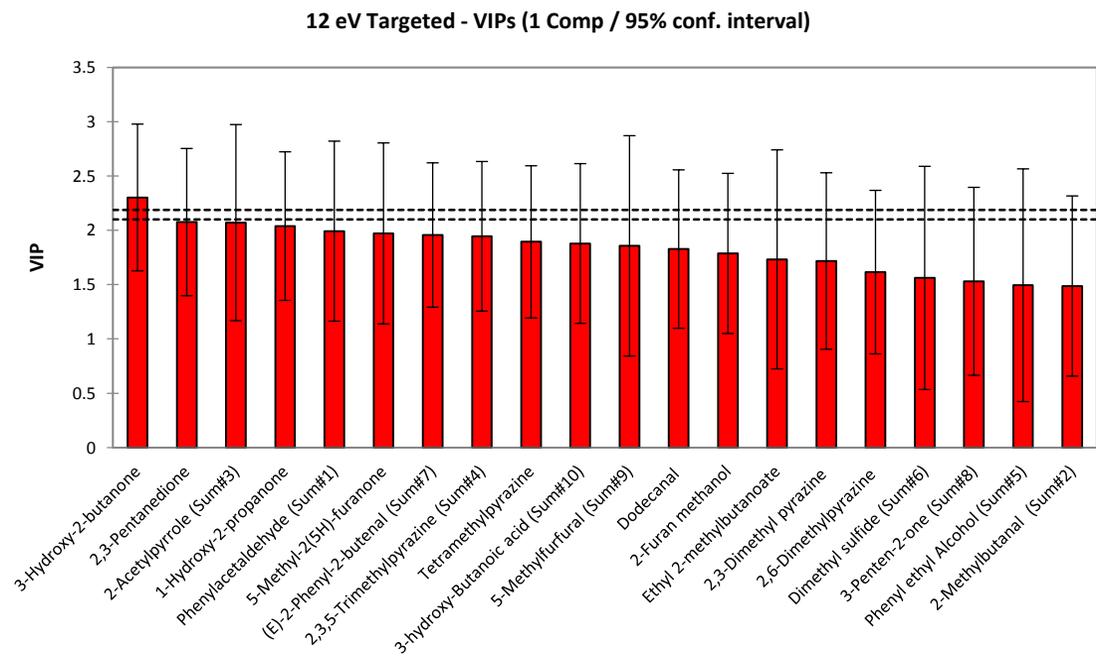
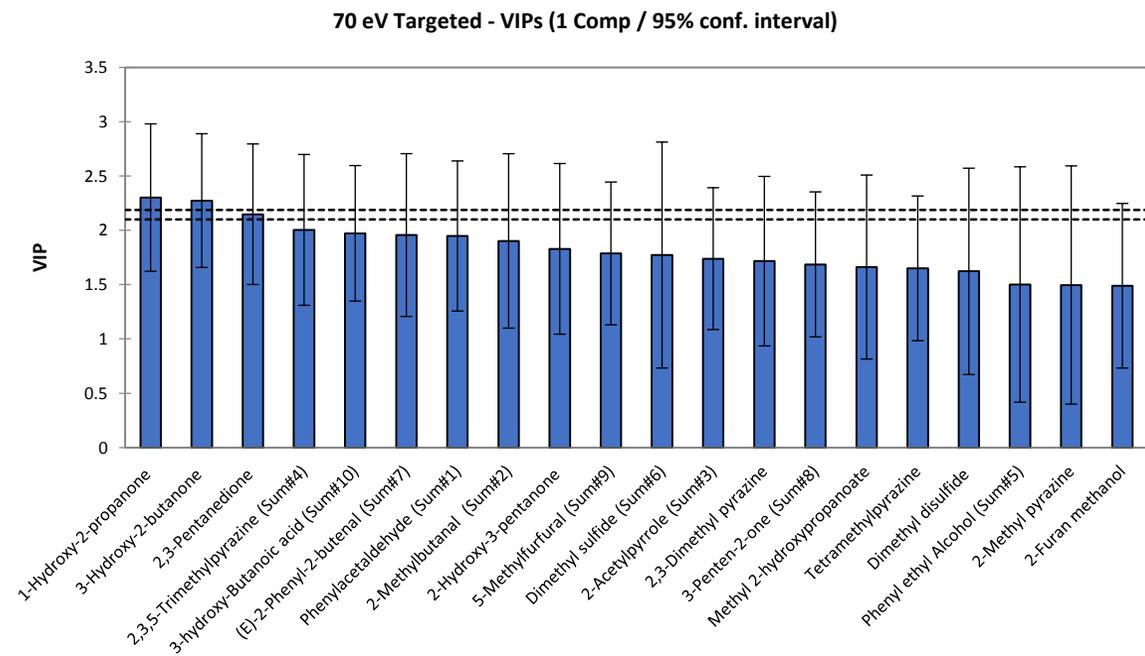


Figure 5

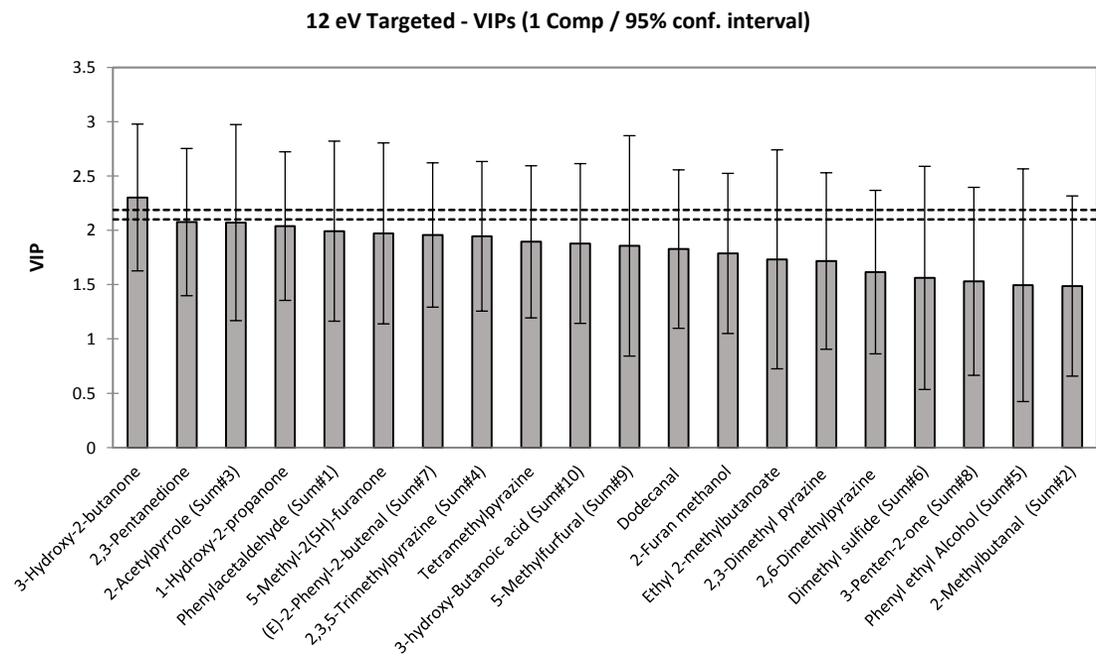
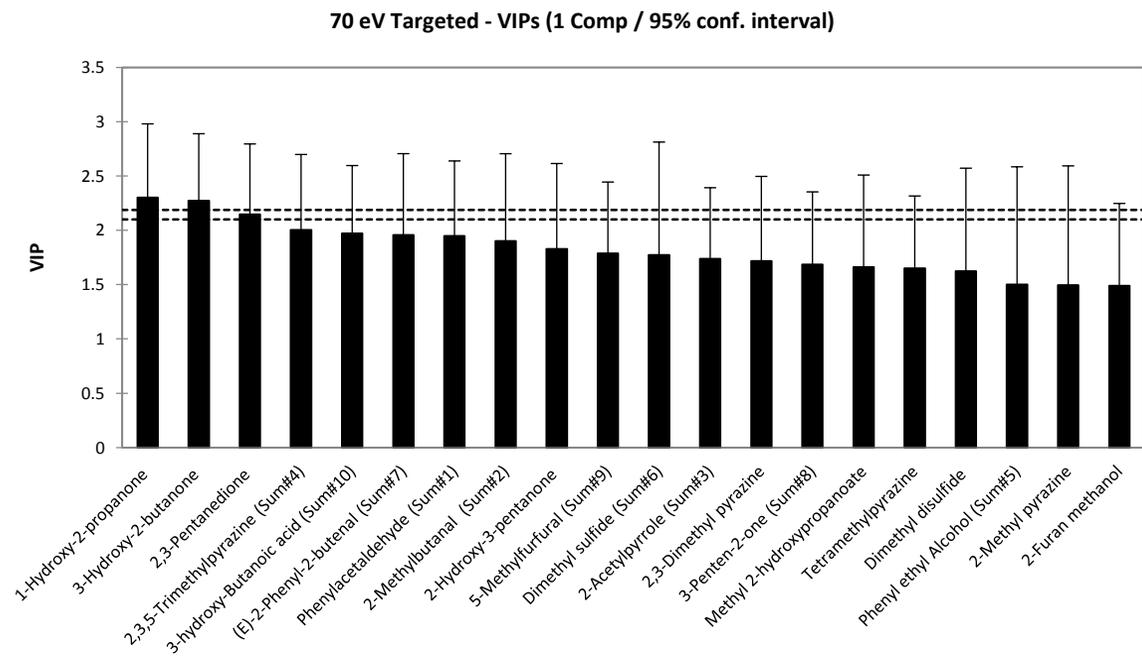


Table 1

Origin	Commercial description	Supplier - Trader	Harvest year
Mexico	<i>Chontalpa Cacao fermentado seco calidad Baluarte</i>	"Mercados alternativos y solidarios para productos del campo S. de RL. de CV" Calle Exterior Manzana 17 Lote 18 Colonia Fracc. Lomas de Ocuilzapotlan localidad Villa de Ocuilzapotlan referencia Tabasco Mexico http://www.lacoperacha.org.mx	2016
Colombia	Fino de Aroma Colombia Premium 1	Newchem Srl, Via M.F. Quintiliano 30 20138 Milan, Italy http://www.newchem.it	2016
Sao Tomè	Superior Cacau Fino, good fermented	Satocao LDA -Morro Peixe, Distrito de Lobata São Tomé e Príncipe - CP 762 http://www.satocao.com	2016
Ecuador	Ecuador ASS (Arriba Superior Selecto)	Domori S.r.l. - Via Pinerolo 72-74 10060 None (Torino), Italy	2016

Table 2

Compound name	¹ t _R min	² t _R sec	I _S ^T	70 eV vs. lib		12 vs.14 eV		12 vs. 70 eV		14 vs.70 eV		SNR		Ratio
				DMF	RMF	DMF	RMF	DMF	RMF	DMF	RMF	70 eV	12 eV	(12/70)
3-Methyl-butanal	8.40	1.32	911	963	963	827	833	696	694	798	805	2309	4300	1.86
2-Methyl-butanal	8.40	1.34	911	970	981	883	902	878	909	839	868	3791	5920	1.56
2,3-Pentanedione	12.40	1.24	1050	973	995	914	914	810	810	817	822	512	280	0.55
Hexanal	13.33	1.82	1077	992	993	881	880	786	789	868	872	237	69	0.29
β-Pinene	14.27	3.20	1104	974	979	918	924	693	694	796	796	43	31	0.72
3-Penten-2-one	15.20	1.52	1128	983	989	850	850	682	684	835	836	765	384	0.50
Limonene	17.80	1.04	1197	985	986	926	926	717	724	773	786	277	150	0.54
Hexyl acetate	21.07	0.92	1280	982	985	922	928	737	745	911	912	529	142	0.27
Octanal	21.60	0.80	1293	990	990	848	853	805	840	793	820	50	18	0.36
Nonanal	25.60	1.16	1397	974	989	824	838	778	783	776	808	658	455	0.69
Furfural	27.93	1.78	1459	985	990	876	877	803	805	863	883	529	590	1.12
Benzaldehyde	30.60	0.58	1533	974	984	964	964	837	837	717	723	2296	2640	1.15
2(E)-Nonenal	30.80	1.74	1538	975	976	922	931	805	840	832	837	419	197	0.47
Linalool	31.20	1.36	1550	978	988	919	923	771	776	851	853	605	394	0.65
1-Octanol	31.67	0.62	1563	996	996	887	889	815	829	864	889	294	105	0.36
2-Furan methanol	34.60	1.30	1648	984	989	897	897	735	735	813	813	1350	2100	1.56
Benzyl alcohol	41.53	1.26	1862	994	995	901	901	783	787	789	789	713	450	0.63
γ-octalactone	43.33	3.04	1920	990	992	941	945	857	857	890	890	29	16	0.55
1H-Pyrrole-2-carboxaldehyde	46.13	0.84	2017	860	869	924	930	780	780	924	927	120	113	0.94
γ-Nonalactone	46.40	0.90	2026	877	978	939	949	817	817	857	862	204	95	0.47

Table 3

	70eV Composite			12eV Composite			Summed Composite		
<i>Detected 2D-peaks in composite chromatograms with SNR ≥ 100</i>	491			335			498		
<i>Detected 2D-peaks in individual chromatograms with SNR ≥ 20</i>	70eV			12eV			Summed		
	Run 1	Run 2	Avg.	Run 1	Run 2	Avg.	Run 1	Run 2	Avg.
Colombia Raw	456	357	407	747	811	779	497	445	471
Ecuador Raw	364	321	343	706	761	734	471	383	427
Mexico Raw	516	505	511	793	780	787	614	622	618
Sao Tome Raw	497	527	512	796	813	805	584	633	609
Average Raw	443			776			531		
Colombia Roasted	394	495	445	860	765	813	453	557	505
Ecuador Roasted	405	417	411	714	786	750	521	492	507
Mexico Roasted	470	479	475	761	761	761	510	571	541
Sao Tome Roasted	527	484	506	801	784	793	624	545	585
Average Roasted	459			779			534		

Table 4

Feature	Mean	Median	Max	Mean 10
<i>Apex Response 12eV</i>	0.31	0.09	6.08	3.34
<i>Apex Response 70eV</i>	0.40	0.17	8.12	3.74
<i>Apex Response Sum</i>	0.41	0.18	7.29	3.86
<i>Volume 12eV</i>	0.19	0.02	6.02	3.59
<i>Volume 70eV</i>	0.53	0.20	13.08	4.83
<i>Volume Sum</i>	0.53	0.18	12.16	5.30
<i>% Resp. 12eV</i>	0.54	0.32	6.31	3.90
<i>% Resp. 70 eV</i>	0.42	0.14	14.01	5.51
<i>% Resp. Sum</i>	0.40	0.15	12.75	5.23
<i>Base Peak Apex Response 12eV</i>	0.32	0.09	5.63	3.43
<i>Base Peak Apex Response 70eV</i>	0.38	0.16	8.83	3.96
<i>Base Peak Apex Response Sum</i>	0.37	0.14	8.73	3.81
<i>Base Peak Volume 12eV</i>	0.37	0.09	7.80	4.96
<i>Base Peak Volume 70eV</i>	0.42	0.14	7.52	4.37
<i>Base Peak Volume Sum</i>	0.41	0.13	7.77	4.41
<i>Large-mass Apex Response 12eV</i>	0.29	0.08	2.92	3.01
<i>Large-mass Apex Response 70eV</i>	0.30	0.09	5.58	3.27
<i>Large-mass Apex Response Sum</i>	0.30	0.08	4.95	3.07
<i>Large-mass Volume 12eV</i>	0.34	0.08	6.31	3.87
<i>Large-mass Volume 70eV</i>	0.34	0.08	6.42	4.00
<i>Large-mass Volume Sum</i>	0.35	0.08	6.31	4.42

Table 5

Area ID	Compound name	¹ t _R min - ² t _R sec	Significance Rank		
			Sum	70eV	12eV
37	Phenylacetaldehyde	(33.98, 1.33)	1	1	2
14	2-Methylbutanal	(8.18, 1.36)	2	2	1
38	2-Acetylpyrrole	(43.99, 0.91)	3	3	4
26	2,3,5-Trimethylpyrazine	(25.43, 1.68)	4	4	6
4	Phenyl ethyl alcohol	(42.28, 1.05)	5	5	3
134	Dimethyl sulfide	(6.08, 2.80)	6	7	5
68	(E)-2-Phenyl-2-butenal	(43.03, 1.53)	7	6	138
52	3-Penten-2-one	(14.60, 1.52)	8	11	10
86	5-Methylfurfural	(31.58, 1.64)	9	9	29
102	3-hydroxy-Butanoic acid	(32.03, 1.25)	10	8	177