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# **Untargeted and Targeted (*UT*) Fingerprinting of Extra Virgin Olive Oil Volatiles by Comprehensive Two-Dimensional Gas Chromatography with Mass Spectrometry: Challenges in Long-Term Studies**

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1 **Abstract**

2           Comprehensive two-dimensional gas chromatography coupled with mass spectrometric detection  
3 (GC×GC-MS) offers an information-rich basis for effective chemical fingerprinting of food. However, GC×GC-  
4 MS yields 2D-peak patterns (i.e., sample 2D fingerprints) whose consistency may be affected by variables  
5 related to either the analytical platform or to the experimental parameters adopted for the analysis.

6           This study focuses on the complex volatile fraction of extra-virgin olive oil and addresses 2D-peak  
7 patterns variations, including MS signal fluctuations, as they may occur in long-term studies where pedo-  
8 climatic, harvest year or shelf-life changes are studied.

9           2D-pattern misalignments are forced by changing chromatographic settings and MS acquisition. All  
10 procedural steps, preceding pattern recognition by template matching, are analyzed and a rational work-flow  
11 defined to accurately re-align patterns and analytes metadata.

12           Signal-to-noise ratio (SNR) detection threshold, reference spectra and similarity match factor threshold  
13 are critical to avoid false-negative matches. Distance thresholds and polynomial transform are key  
14 parameters for effective template transform. In targeted analysis (supervised work-flow) with optimized  
15 parameters method accuracy achieves 92.5% (i.e., % of true-positive matches) while for combined  
16 untargeted and targeted (*UT*) fingerprinting (unsupervised work-flow), accuracy reaches 97.9 %. Response  
17 normalization also is examined, evidencing good performance of multiple internal standard normalization  
18 that effectively compensates for discriminations occurring during injection of highly volatile compounds. The  
19 resulting work-flow is simple, effective, and time efficient.

20

21 **Key words**

22 Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry; extra-  
23 virgin olive oil volatiles; template matching; combined untargeted and targeted (UT) fingerprinting; data  
24 alignment in long-term studies

25

## 26 Introduction

27 Comprehensive two-dimensional gas chromatography (GC×GC) is one of the most informative  
28 separation techniques for chemical characterization of complex fractions of volatiles from food<sup>1-3</sup>. It enables  
29 highly effective fingerprinting<sup>4</sup> and, when combined with mass spectrometric detection (GC×GC-MS), it has  
30 the intrinsic potential to provide a detailed profiling, giving access to higher level information encrypted in  
31 complex patterns of volatiles, for example: sample origin, technological signature, and aroma<sup>2,5-8</sup>.

32 Each analytical run produces dense and multidimensional data, so that elaboration and  
33 interpretation of chemical information is challenging. Moreover, 2D-peak patterns representing the sample  
34 2D fingerprint, are defined by a series of variables also related to the analytical platform and to the  
35 experimental parameters adopted for the analysis. The choice of flow modulation instead of  
36 thermal/cryogenic modulation, MS detection by fast scanning quadrupoles vs. time-of-flight MS, low-  
37 resolution MS vs. high-resolution MS as well as GC×GC stationary phase combination, columns lengths and  
38 diameters, carrier gas linear-velocities, modulation period ( $P_M$ ) and oven temperature programming greatly  
39 impact on 2D-patterns signature and informational density.

40 Although most of these parameters, once fixed after method development and optimization, are  
41 kept constant (e.g., column set-up, carrier gas flows, and modulation parameters) or can be standardized as  
42 the MS tuning and optimization, some others represent a source of random variability that must be  
43 considered when fingerprinting and pattern recognition studies extend over time and/or across different  
44 platforms.

45 For mono-dimensional (1D) GC-MS applications, possible strategies for chromatographic alignment  
46 and data normalization are: (a) linear retention indexing (van Den Dool and Kratz or Kovats indices) or  
47 retention time locking methods based on pressure/flow adjustments (i.e., retention time locking<sup>9-11</sup>) to  
48 accurately locate target analytes along the analytical run; (b) chromatographic realignment<sup>12-14</sup>; (c) internal  
49 standardization for response normalization by single or multiple Internal Standards (IS) addition; and (d)  
50 external standard normalization by adopting single or multiple External Standards (ES). These strategies are  
51 effective and routinely adopted in peak-features based applications<sup>4</sup>. However, for GC×GC, these 1D-GC

52 strategies could be ineffective especially for retention inconsistencies that result from two, almost  
53 independent, separation steps. On the other hand, the peculiar nature of 2D-peak patterns offers the  
54 possibility of exploiting pattern recognition algorithms for prompt and effective fingerprinting. So, strategies  
55 for pattern alignment and normalization are needed.

56 Pattern recognition approaches based on peak-region features<sup>4</sup>, implemented with the smart  
57 template concept in commercial software<sup>15</sup>, use different transform functions capable of recognizing 2D peak  
58 patterns based on retention times coordinates, and establishing correspondences between 2D-peaks, or 2D-  
59 peak-regions, from a *reference* pattern to those in an *analyzed* pattern even in presence of retention times  
60 shifts<sup>15-17</sup> and/or when severe misalignments occur because of different modulation principles<sup>18,19</sup>.

61 Pattern correspondences are at the basis of the re-alignment of untargeted/targeted 2D-peaks or  
62 2D-peak-regions across a samples-set to enable fingerprinting investigations<sup>2</sup>. Furthermore, the specificity  
63 and reliability of pattern matching can be improved by including constraint functions, operating on the third  
64 dimension of the data, i.e., the MS signature. Typical functions are those that limit positive correspondences  
65 to 2D-peaks with spectral similarity match above a certain threshold or, more simply, for 2D-peaks that  
66 comply for specific m/z relative ratios between informative fragments of the spectrum.

67 In a scenario where food chemical fingerprinting has to be extended over long time-frames, as for  
68 example to cover different harvest years or shelf-life modifications, strategies and tools for data re-alignment  
69 and normalization are necessary<sup>20,21</sup> together with more rational strategies and intuitive operative  
70 protocols/work-flows to guide analysts over the actual limits posed by analytical data misalignment.

71 This study addresses 2D-peak patterns variations occurring in long-term studies that might affect the  
72 effectiveness of combined untargeted and targeted fingerprinting (*UT* fingerprinting). The procedural steps  
73 preceding template matching function are analyzed to define a rational work-flow enabling consistent  
74 pattern recognition. These steps, supervised by the analyst, aim at selecting the key-parameters to generate  
75 a targeted template for marker 2D-peaks or a *reliable* or *consensus* template covering the sample chemical  
76 dimensionality<sup>5</sup>, in particular: (a) 2D-peaks response thresholds (Signal-to-Noise ratio - SNR and Volume-to-  
77 Noise ratio – VNR); (b) MS reference signature to be used for spectral constraints; (c) MS similarity thresholds;

78 and (d) template matching transforms are considered, with their settings varied to compensate for 2D-peak  
79 patterns variability so as to achieve effective and comprehensive chemical fingerprinting.

80 The complex 2D patterns of volatiles from Extra Virgin Olive oil of different quality are studied. Volatiles  
81 are sampled by headspace solid-phase microextraction (HS-SPME) and analyzed by GC×GC-MS in a platform  
82 equipped with a loop-type thermal modulator adopting L<sub>2</sub> cryogenics. The 2D-patterns are obtained in a one-  
83 year study during which misalignments and inconsistencies were introduced by varying column lengths and  
84 restrictions, modulation period ( $P_M$ ), and operating with the time-of-flight (TOF) MS with different optimized  
85 parameters.

86

## 87 **Materials and methods**

### 88 ***Reference compounds and solvents***

89 Pure reference standards of  $\alpha$ - and  $\beta$ -thujone and methyl-2-octynoate used as Internal Standards  
90 (ISs), *n*-alkane standards (n-C7 to n-C25) used for linear retention index ( $I_s^T$ ) calibration and pure reference  
91 compounds for targeted analytes identity confirmation were supplied by Merk (Sigma-Aldrich srl Italy, Milan,  
92 Italy). Cyclohexane (HPLC grade) for *n*-alkane standards and pure dibutyl phthalate for ISTDs working  
93 solutions were from Merk.

### 94 ***Olive Oil samples***

95 Extra Virgin Olive oils (EVO oils), supplied by the University of Granada (Spain), Prof. Luis Cuadros-  
96 Rodríguez, were obtained from olives of the *Picual* cultivar, harvested in the regions of Granada *Altipiano*  
97 named *Baza* and *Benamaurel*, and grown under differing production and irrigation practices<sup>7</sup>. Samples were  
98 obtained by mixing olives from five different trees from the same plot in duplicate batches (A and B). Olives  
99 for oil production were collected at four different ripening stages: November 10-12, 2014; November 24-28,  
100 2014; December 16-17, 2014; and January 12-15, 2015, and classified by oil quality (Extra Virgin - EVOO,  
101 Virgin-VOO or *Lampante- LOO*). Samples acronyms and characteristics are summarized in Table 1. Oil  
102 qualifications were by a certified laboratory (ISO 17025:2018)<sup>7</sup> and according to Commission Regulation

103 (EEC) No 2568/91 of 11 July 1991. Some quality indices are reported in Table 1, including the sensory panel  
104 test results.

#### 105 ***Headspace solid-phase microextraction sampling devices and conditions***

106 Volatiles were extracted from samples by headspace (HS) solid-phase microextraction (SPME).  
107 DVB/CAR/PDMS  $d_f$  50/30  $\mu\text{m}$  1 cm length fiber (Supelco, Belle-fonte, PA, USA) was chosen based on previous  
108 studies<sup>8</sup> and conditioned before use as recommended by the manufacturer. The ISs were pre-loaded onto  
109 the SPME device<sup>22,23</sup>, before sampling, in a 20 mL headspace vial a 5.0  $\mu\text{L}$  of  $\alpha/\beta$ -thujone and methyl-2-  
110 octynoate at 100  $\text{mg L}^{-1}$  in dibutyl phthalate. ISs were equilibrated at 40°C and pre-loaded by exposing the  
111 SPME device for 5 min.

112 Sampling was carried out on  $0.100 \pm 0.005$  g of oil samples precisely weighed in 20 mL headspace  
113 vials. The very low amount of sample was chosen to comply with HS linearity conditions for most of the key-  
114 analytes responsible of samples discrimination<sup>7</sup>. Sampling was at 40°C for 60 min. After extraction, the SPME  
115 device was introduced into the S/SL injection port of the GC $\times$ GC system kept at 260°C for 5 min. Each sample  
116 was analyzed in triplicate.

#### 117 ***Instrument set-up and analysis conditions***

118 GC $\times$ GC analyses were performed on an Agilent 7890 GC unit (Agilent Technologies, Wilmington DE,  
119 USA) coupled to a Markes BenchTOF-Select™ featuring Tandem ionization™ (Markes International,  
120 Llantrisant, UK). The GC transfer line was at 270°C. TOF MS tuning parameters were set for single ionization  
121 at 70 eV and for tandem ionization at 70 and 12 eV; the scan range was set at 35-350  $m/z$  with a spectra  
122 acquisition frequency of 100 Hz for single eV and 50 Hz/channel for tandem ionization.

123 The system was equipped with a two-stage KT 2004 loop type thermal modulator (Zoex Corporation,  
124 Houston, TX) cooled with liquid nitrogen controlled by Optimode v2.0 (SRA Instruments, Cernusco sul Naviglio,  
125 Milan, Italy). Modulation periods ( $P_M$ ) and hot jet pulse times are detailed in Table 2, along with other  
126 parameters. A Mass Flow Controller (MFC) reduced the cold-jet stream from 45% to 8% of the total flow with  
127 a linear function along the run duration. A fused silica capillary loop (1.0 m  $\times$  0.1 mm id) was used in the  
128 modulator slit.

129 The column set was configured as follows: <sup>1</sup>D SolGel-Wax column (100% polyethylene glycol; 30 m ×  
130 0.25 mm  $d_c$  × 0.25 μm  $d_f$ ) from SGE Analytical Science (Ringwood, Australia) coupled with a <sup>2</sup>D OV1701 column  
131 (86% polydimethylsiloxane, 7% phenyl, 7% cyanopropyl; 1 m × 0.1 mm  $d_c$  × 0.10 μm  $d_f$ ) from Mega (Legnano,  
132 Milan, Italy). A capillary restriction towards MS was used to generate a differential pressure-drop influencing  
133 actual carrier gas linear velocities along the column in *Set-up 2* (1 m × 0.1 mm  $d_c$  deactivated silica). The GC  
134 Split/Splitless (S/SL) injector port was set at 260°C and operated in split mode with a split ratio 1:20.

135 The carrier gas was helium at a constant nominal flow of 1.3 mL/min. The oven temperature  
136 programming was set as follows: 40°C (2 min) to 240°C (10 min) at 3.5°C/min. Carrier gas average linear  
137 velocities (<sup>1</sup> $\bar{u}$  and <sup>2</sup> $\bar{u}$ ), pressure settings, and hold-up times are reported in **Table 2** and were obtained by basic  
138 calculations with a reference temperature of 60°C.

139 For  $I_T^S$  determination, 1 μL of the n-alkanes sample solution was injected with a split ratio 1:50.  
140 Data were acquired by TOF-DS™ (Markes International, Llantrisant, UK) and processed by GC Image ver 2.8  
141 (GC Image, LLC Lincoln, NE, USA).

#### 142 ***UT fingerprinting work-flow***

143 The distribution of detectable analytes over the 2D chromatographic space in a GC×GC separation is  
144 at the basis of pattern recognition based on the *smart template* concept<sup>24</sup>. The template is a pattern of 2D-  
145 peaks and/or graphic objects built over a *reference* image(s) (single or cumulative image<sup>25</sup>) and then used to  
146 recognize similar patterns of 2D-peaks in an *analyzed* image(s)<sup>26</sup>. Each template object (2D-peak and/or  
147 graphic) can carry various metadata including: compound chemical name, retention times,  $I_T^S$ , mass spectra,  
148 informative ions and their relative ratios, constraint functions to limit peak correspondences above certain  
149 thresholds, and qualifier functions.

150 In the presence of temporal inconsistencies and detector fluctuations *peak-region* features<sup>27</sup> are of  
151 great help. Peak-regions attempt to define one chromatographic region around each individual peak thereby  
152 achieving the one-feature-to-one-analyte selectivity but with greater robustness than can be achieved with  
153 single 2D-peak detection<sup>28</sup>. 2D-peaks and peak-regions are features adopted in the combined targeted and  
154 untargeted fingerprinting (*UT fingerprinting*) strategy<sup>7,18,25,29</sup>.

155 *UT* fingerprinting establishes a group of *reliable* peaks, positively matched across *all* or *most-of*  
156 chromatograms in set<sup>30</sup>, and then uses them to align chromatograms<sup>16</sup> for their combination into a single,  
157 composite chromatogram. From the composite chromatogram, 2D-peaks (i.e., the combination of the re-  
158 aligned responses in the 2D retention-times plane) are detected and their outlines are recorded to define  
159 peak-region objects. The set of reliable 2D-peaks and peak-regions objects are collected in the *feature*  
160 template, or *consensus* template, covering the whole sample-set variability and capable of cross-  
161 corresponding chemical feature patterns among samples.

162 To note, within all detected analytes, the sub-group of targeted compounds can be highlighted by  
163 completing their metadata fields (compound name, ion ratios,  $I^T_S$ ) and computed together with untargeted  
164 features during the data processing.

165 A schematic of the *UT* fingerprinting process is illustrated in the Supplementary Material –  
166 Supplementary Figure 1 together with some details on targeted and untargeted 2D peaks and peak-regions.

167

## 168 **Results and discussion**

169 In spite of the great potential of GC×GC in exploiting the chemical dimensionality of olive oil volatile  
170 fraction, just a few studies are available in this field and none of them address challenges posed by long-time  
171 frame studies. Vaz Freire *et al.*<sup>31</sup> adopted an image-features approach<sup>28</sup> to investigate characteristic  
172 distributions of volatiles. An open-source image analysis software (Image J, National Institutes of Health) was  
173 used to extract detector response information from 2D regions over the separation space. By Principal  
174 Component Analysis (PCA) image-features with a high discrimination potential were selected and targeted  
175 profiling was then combined to locate known analytes within most informative 2D regions.

176 Studies aimed at defining geographical origin indicators or cultivar markers are those by Cajka *et al.*<sup>32</sup>  
177 who adopted GC×GC-TOF-MS to identify 44 compounds able to discriminate extra-virgin olive oils based on  
178 their different geographical origin and production year and by Lukić *et al.*<sup>33</sup> who applied a peak-features  
179 approach to reveal compositional differences between oils obtained by different olive cultivars and  
180 geographical areas. They considered, as potentially informative, both untargeted and targeted analytes as

181 they were extracted from the raw data-set on the basis of relative retention and spectral features. Magagna  
182 et al.<sup>7</sup> were the first who developed an integrated strategy for *UT* fingerprinting based on template matching,  
183 to define olive ripening indicators while Purcaro et al.<sup>8</sup> combined targeted and untargeted analysis to  
184 delineate chemical blueprints of olive oil aroma defects.

185 In this scenario, intuitive and easily applicable strategies to extend the breath of comprehensive  
186 profiling and fingerprinting of olive oil volatiles over wider time frames, e.g. over harvest years or across the  
187 shelf-life of a product, are of great interest especially in the perspective of collecting data for authenticity  
188 and typicity databases as in the case of the Italian *Violin* project<sup>34</sup> aimed at the valorisation of the Italian  
189 extra-virgin olive oil.

190 The next sections will illustrate the strategy adopted to generate misaligned patterns and the  
191 subsequent work-flow designed to re-align templates for targeted and untargeted peak-features covering  
192 the entire volatile fraction of extra-virgin olive oil. Results are critically discussed in term of accuracy (i.e.,  
193 true positive matches) and data inter-batch transferability (i.e., response normalization).

194

### 195 ***Pattern misalignment challenges***

196 Chromatographic pattern distortions and misalignments were induced, generating the worst-case  
197 scenario, by changing the following parameters: (a) columns were from different commercial batches; (b) a  
198 post-column restriction was added generating a pressure drop between column inlet and outlet, influencing  
199 carrier gas linear velocities in both analytical dimensions; (c) the  $P_M$  was set at 4 or 3.5 s generating an  
200 absolute <sup>2</sup>D retention misalignment. All the other parameters, carrier gas nominal flow, oven temperature  
201 programming and injection conditions were kept constant. Analytical conditions for the two resulting set-ups  
202 (i.e., *Set-up 1* and *Set-up 2*) are summarized in Table 2 while Figure 1 shows the colorized plot of an oil sample  
203 obtained from olives at early ripening stage (*Baza-1-A*) analyzed by the two set-ups (Figure 1A for *Set-up 1*  
204 and Figure 1B for *Set-up 2*). Pattern differences are visible and are related to the different chromatographic  
205 efficiencies (peak-width – <sup>1</sup>*W* and <sup>2</sup>*W* – Table 2 data) that impact resolution and to the absolute retentions  
206 that affect system orthogonality<sup>35–37</sup>.

207 The global misalignment between peaks patterns is visualized in Figure 2 and evaluated by calculating  
208 analytes relative retention in both separation dimensions against reference peaks<sup>19</sup>. *(Z)*-3-hexen-1-ol acetate,  
209 which elutes in the middle area of the chromatographic plane, was arbitrarily chosen as reference/centroid,  
210 while *phenol*, the last-eluting marker analyte, was used to normalize analytes relative position *i*.

211 The <sup>1</sup>D relative retention (<sup>1</sup>D RR) is calculated by Equation 1:

$$212 \quad {}^1\text{D RR} = ({}^1t_{Ri} - {}^1t_{R(Z)\text{-Hexen-1-ol acetate}}) / {}^1t_{R\text{phenol}} \quad \text{Eq. 1}$$

213 where <sup>1</sup>t<sub>Ri</sub> corresponds to the first dimension retention time expressed in minutes for the targeted peak *i*, *(Z)*-  
214 *3-Hexen-1-ol acetate* is the reference peak, and *phenol* is the last eluting peak.

215 The <sup>2</sup>D relative retention (<sup>2</sup>D RR) is calculated through Equation 2:

$$216 \quad {}^2\text{D RR} = ({}^2t_{Ri} - {}^2t_{R(Z)\text{-Hexen-1-ol acetate}}) / P_M \quad \text{Eq. 2}$$

217 where <sup>2</sup>t<sub>Ri</sub> corresponds to the second dimension retention time expressed in minutes for the targeted peak *i*,  
218 *(Z)*-3-hexen-1-ol acetate is the reference peak, and *P<sub>M</sub>* the modulation time<sup>19</sup>.

219 As visualized in Figure 2, there is a dramatic impact on the <sup>2</sup>D absolute and relative retention. This  
220 effect is primarily due to the different *P<sub>M</sub>* applied (4 vs. 3.5 seconds) and to the actual carrier gas linear  
221 velocities (<sup>1</sup>ū and <sup>2</sup>ū) and operative pressures (initial head-pressure and mid-point pressure). Analytes falling  
222 in the third quadrant show an higher <sup>2</sup>D *k* in *Set-up 1* whereas this effect is minimized as a function of the  
223 increasing <sup>1</sup>D *k* (retention).

224 Interestingly, the two patterns, although misaligned on the normalized retention times space, keep  
225 coherent the group-type separation for homologous series. Normal alkanes (*n*-alkanes), shown with green  
226 indicators, mostly in the second quadrant; linear aldehydes, shown with orange indicators, spanning mostly  
227 across the first and third quadrants; and short chain fatty acids, shown with cyan indicators, appearing in the  
228 fourth quadrant, all are rationalized over the 2D space.

229 The next step of the study addresses detector response variations and examines threshold  
230 parameters for 2D-peaks descriptors to adopt for consistent template matching.

231

## 232 ***Supervised work-flow for reliable targeted template construction***

233 MS detector response fluctuations due to tuning, optimization, or by other factors directly impact on  
234 absolute response and background noise intensity. Such performance issues also may affect template  
235 matching effectiveness and analytes identity confirmation, as a consequence of the varying quality and  
236 reliability of 2D-peak spectra adopted as reference for matching. In this study, TOF MS was set differently: in  
237 *Set-up 1* it multiplexed between high and low ionization energies (Tandem Ionization™ - 70 and 12 eV) at 50  
238 Hz acquisition rate for each channel whereas in *Set-up 2* TOF MS operated in single electron energy  
239 acquisition mode (70 eV) at 100 Hz. Therefore, MS was tuned differently<sup>38</sup> and output signals exhibited  
240 different absolute response (total ion current) and background noise intensity.

241 The signals resulting from the same sample (*Baza-1-A*), whose patterns are illustrated in Figure 1A  
242 and 1B, have the following characteristics:

- 243 – background noise sampled in the middle of the chromatogram within a 50x50 acquisition-point window  
244 reported an average absolute intensity of 75,500 counts (RSD% <sup>1</sup>D 0.6 and <sup>2</sup>D 5.98) for *Set-up 1* and  
245 167,000 counts (RSD% <sup>1</sup>D 0.5 and <sup>2</sup>D 1.57) for *Set-up 2*. Supplementary data visually illustrates  
246 performance evaluation operations (Supplementary material – Supplementary Figure 2 – SF2);
- 247 – after background subtraction<sup>39</sup>, the average intensity was 38,000 counts (RSD% <sup>1</sup>D 1.19 and <sup>2</sup>D 8.9) for  
248 *Set-up 1* and 88,000 counts (RSD% <sup>1</sup>D 0.9 and <sup>2</sup>D 2.49) for *Set-up 2*;
- 249 – the number of detected 2D-peaks above a SNR value of 15 were 770 for *Set-up 1* and 500 for *Set-up 2*;
- 250 – within the detected 2D-peaks, SNR values ranged between 15-13,000 in *Set-up 1* and between 15-3,000  
251 in *Set-up 2*;
- 252 – volume-to-noise ratio (VNR) values ranged between 100-14,100 in *Set-up 1* and 100-6,700 in *Set-up 2*.

253 Experimental results indicate that MS, operating with a single ionization energy at 70 eV, results in  
254 higher absolute and relative background noise (e.g., 1.9 times) compared to the tandem ionization settings.  
255 Interestingly, the noise fluctuations are greater along the <sup>1</sup>D (RSD% values) where column bleeding increases  
256 as a function of temperature programming. Background noise subtraction has almost the same effect, in  
257 terms of noise suppression, and in both cases, signal intensity is halved compared to the initial values.

258 With respect to peak detection, *Set-up 1* exhibited better chromatographic efficiency (Table 3  $^1W_{0.1}$  and  $^2W_{0.1}$ )  
259 and resulted in a higher number of detected peaks over  $SNR \geq 15$ , with a wide range of variation, i.e., 15-  
260 13,000, whereas in *Set-up 2* maximum SNR achieved only a value of 3,000. On the other hand, VNR, which  
261 corresponds to the ratio of analyte 2D-peak volume to the standard error – SE ( $\sigma/\sqrt{n}$ ), is not so influenced by  
262 peak-width as SNR. The dynamic range of the MS response with *Set-up 1* is 10 times greater (up to 14,100 vs.  
263 up to 6,700). Although with *Set-up 1* the number of detected peaks over a SNR of 15 was higher, a greater  
264 volume standard deviation ( $\sigma$ ) was computed.

265 Based on the differences observed in the absolute response, spectral quality fluctuations were  
266 expected especially for low-intensity or threshold peaks. Therefore, the next step was to define threshold  
267 parameters for template construction with the objective of achieving 100% true-positive matches (accuracy)  
268 in presence of random variability over the application of the method in a short term and with the same  
269 method set-up. Therefore, these tests were performed between analytical replicates of the same oil sample  
270 to define benchmark values and then validated over a new sample acquired in the same conditions. Tests  
271 were done on the three analytical replicates of *Baza-4-A* acquired by *Set-up 1*; validation was on the three  
272 replicates of *Bena-4-A* acquired by the same set-up.

273 Threshold values for candidate peaks populating a template were set for SNR and NIST Similarity  
274 match factor (direct match factor - DMF); as reference, spectral signatures were tested for the average  
275 spectrum (named *blob spectrum*) and the highest modulation spectrum (named *peak spectrum*). SNR values  
276 were varied step-wise in a range between 10 and 100, covering 2D peaks with a percent response between  
277 0.01 and 0.04, while the DMF threshold was set at 800 or 700<sup>40</sup>. Templates were built with ten 2D-peaks with  
278 SNR within the fixed range and homogeneously distributed over the 2D space. Experimental results,  
279 expressed as % of true-positive matches, are reported in Table 4.

280 2D Peaks with SNR values below 50 are connoted by inconsistent MS spectral signatures resulting in  
281 false-negative matches even when DMF threshold is lowered from 800 to 700. For these peak, neither the  
282 *blob spectrum* nor the *peak spectrum* are sufficiently reliable to carry consistent information for identity  
283 confirmation. For 2D-peaks with a SNR within 50-100 in the reference chromatogram, the rate of positive

284 matches increases from 10% to 70% when MS constraints are kept at 800 DMF and *blob spectrum* considered.  
285 The rate of true-positive matches reaches 100% with the combination of lower DMF threshold at 700 and  
286 *peak spectrum* taken as reference MS. Note, in these cases no false positives were revealed, meaning that  
287 correspondences were just established between peaks generated by the same chemical entity.

288 Results suggest that a SNR cut-off should be defined, based on 2D data particulars, to limit  
289 inconsistencies at targeted identity confirmation level. The validation of these settings was by applying the  
290 templates resulting from the reference sample *Baza-4-A* to *Bena-4-A* replicates on the same column set-up.  
291 Results, reported in Table 4, confirmed the need of applying a SNR threshold of at least 50 with better  
292 performances at DMF threshold of 700 with a reference template spectrum collected from the highest  
293 modulation (*peak spectrum*.) In this case, some template peaks were unmatched (true-negative matches)  
294 because they were not detected in the analyzed sample (below method Limit of Detection LOD).

295 Rules for template peaks thresholds and reference spectra were then applied to build a reference  
296 *targeted template* of known analytes. This fully supervised approach aimed at characterizing the distribution  
297 of marker compounds known for their role in defining olive oil sensory quality or to refer about olives ripening  
298 status<sup>7,8</sup>. It was also the complement of the *UT* fingerprinting process and included targeted peaks listed in  
299 Table 3. Analytes identifications were by combining <sup>1</sup>D retention data ( $I^T_s$ ) with MS fragmentation pattern  
300 similarity above 900 DMF adopting commercial<sup>40,41</sup> and in-house databases or, when possible, by authentic  
301 standards.

302 The template of 126 2D-peaks was built by inspecting samples patterns obtained with *Set-up 1*;  
303 reference peaks inclusion was limited to those analytes showing a  $SNR \geq 100$ , the reference spectra was from  
304 the highest modulation (e.g., *peak spectrum*), and the MS constraint was set at 700 DMF and 700 Reverse  
305 Match Factor (RMF)<sup>40</sup>.

306 Results for targeted template matching are summarized in Table 5. The average matching within *Set-*  
307 *up 1* samples achieved 97% (122 over 126 peaks). Further comments will follow for template matching for  
308 *Set-up 2* patterns.

309

## 310 **Template transformation**

311           Once template construction was established with simple rules for confident identification and  
312 effective matching, the next step was the selection of matching algorithm (transform) and related  
313 parameters to effectively transform the template over the peak pattern showing severe misalignment. To  
314 approach this challenge, global polynomial transformation were tested, as it was successful in complex re-  
315 alignment problems such as those posed by method translation from thermal to differential flow modulated  
316 platforms<sup>16,19</sup>. Global, low-degree transformation functions (second-degree or third-degree polynomials) are  
317 successful when a sufficient number of alignment points, at least 10 for affine and 30 for second-degree  
318 polynomial, are available to guide the pattern re-alignment<sup>16</sup>.

319           The strategy here applied included the re-alignment of the targeted template built over *Set-up 1*  
320 analyses on those from *Set-up 2*. The first step included the adjustment of the *distance threshold* parameters  
321 for the <sup>1</sup>D and <sup>2</sup>D, which correspond to the horizontal and vertical distance threshold that limits the after-  
322 transformation distance between template and candidate 2D-peaks. These distances are expressed in inter-  
323 sample distances (i.e., pixel dimensions). To compensate for the greater <sup>2</sup>D misalignment the 2D distance  
324 threshold was step-wise incremented from a minimum value of 5 up to 25. On the <sup>1</sup>D, where the  
325 misalignment was minimal, a threshold of 10 was sufficient to avoid false-negative matches.

326           At the same time, affine and polynomial second-degree matching transforms were tested for  
327 performance. The *Set-up 1* targeted template was applied to the first (arbitrarily chosen) sample pattern (i.e.,  
328 *Baza-4-A*) obtained with *Set-up 2*. At first, the number of true-positive matches was higher for the second-  
329 degree transform, so the iterative process of *match-and-transform* was continued. Iterating the process of  
330 matching and template transform allows the template to be adapted to the actual pattern while increasing,  
331 step-by-step, the number of matched peaks up to the maximum number that corresponds to all targeted  
332 analytes actually detected/confirmed in the analyzed pattern. In practice, this operation increments the  
333 number of alignment points available step-by-step, thus improving the quality of the global template  
334 transformation at each step.

335 Experimental results for the application of the targeted template adapted to *Set-up 1* over the  
336 patterns of *Set-up 2* resulted in a 65 positive matches over 126 template peaks (51% - 5.4% RSD); after  
337 transformation by taking these 65 alignment points, the successive matching step achieved 95 positive  
338 matches with a 75% of matched peaks; then, the next step matched 110 peaks, 87%. The maximum number  
339 of matched peaks, shown in Table 5, was 121 (96%) and was achieved after one additional matching step. In  
340 practice, for a full and effective re-alignment of the targeted template, a variable number of iterations  
341 between 3 and 5 was applied.

342 All such results are listed in Table 5; benchmark values for maximum template matching performance  
343 are those corresponding to the application of the targeted template to *Set-up 1* samples (first column). On  
344 average matching performance was better for *Set-up 1* patterns (97% of true-positive matches), although the  
345 loss of accuracy on *Set-up 2* pattern was just of 5% (92.46% vs. 97.02%). These results present a solid  
346 foundation for the application of this experimented strategy to a fully-unsupervised approach as that for the  
347 reliable template construction.

348 The next section illustrates the process of *feature template* construction over samples patterns from  
349 *Set-up 1* and its successive alignment over *Set-up 2* patterns. Accuracy results are discussed as % of true-  
350 positive matches <sup>42</sup>.

351

### 352 ***UT fingerprinting: feature template construction***

353 The feature template was built over a subset of *Set-up 1* chromatograms with the first analytical  
354 replicate of all analyzed samples. The 2D chromatograms were pre-processed for rasterization, background  
355 subtraction, and 2D peaks detection above a SNR threshold of 10. Data processing was then conducted within  
356 a component of the GC Image software suite (Image Investigator™) using the previously validated settings:

- 357 – SNR $\geq$ 100 as threshold value for template peaks;
- 358 – *peak spectrum* as MS reference to upload in the template;
- 359 – DMF similarity threshold at 700;
- 360 – matching transform parameters with <sup>1</sup>D distance threshold of 10 and <sup>2</sup>D of 25.

361 Additional settings, specific for this process, included an option for reliable peak inclusion that was set as  
362 *most relaxed*: with this setting the algorithm considers as *reliable peaks* all 2D-peaks that match across at  
363 least half of the chromatograms. Reliable peaks are fundamental as they are used as alignment points for the  
364 transform function when the *feature template* is used to cross-align a large set of chromatograms including  
365 those obtained with a different set-up.

366 The final *feature template* accounted for 257 reliable peaks and 1500 peak-regions. The reliable  
367 template built over *Set-up 1* chromatograms is shown in Supplementary material – Supplementary Figure 3  
368 – SF3. Matching constraints for MS spectrum similarity were applied on 2D-peak features and results are  
369 summarized in Table 5. The average % of matching for *Set-up 1* chromatograms was 99.75 ( $\pm 0.45$  RSD%);  
370 when the feature template is transformed to match for *Set-up 2* patterns, the % of matching is slightly lower  
371 97.95 ( $\pm 0.99$  RSD%) but evidences the high accuracy of the process.

372 Once re-aligned almost all chemical features detected in all samples patterns, proceeding in a sort of  
373 data fusion, the final step aimed at defining the best 2D-peak response descriptor for cross-sample analysis.

374

### 375 ***Response normalization and samples clustering***

376 As evidenced by signal intensity evaluation and by 2D-peaks statistics (SNR and VNR distribution),  
377 pattern cross-alignment is not sufficient to compensate for random variations across measurement sessions  
378 and impacting detector response. Response normalization is mandatory to allow consistent cross-  
379 comparison of data set. The removal of unwanted intensity variation (i.e., normalization) is referred to as  
380 signal drift correction, removal of batch effect, scaling, and matrix effects removal, and can be approached  
381 differently as function of the study objectives<sup>21</sup>. Normalization is, in fact, a fundamental step because it may  
382 affect the outcomes of a study; the meaningfulness of differentially abundant analytes may vary depending  
383 on the normalization method<sup>21,39</sup>.

384 In this study, we tested three simple approaches within those generally adopted in volatiles profiling  
385 studies<sup>2,43</sup>. The first included multiple IS normalization with  $\alpha$  and  $\beta$  tujone and methyl octanoate that were  
386 pre-loaded into the SPME device before headspace sampling of olive oils<sup>23</sup>. As an alternative method, the

387 analyte % response (calculated on the 2D-Volume) was considered. It was obtained by: (a) normalizing  
388 analyte 2D-Volume over all detected 2D-peaks above fixed thresholds or (b) normalizing analyte 2D-Volume  
389 over all *UT* peaks included in the feature template. In this last approach 2D-peaks from column bleeding and  
390 from interferences were excluded.

391 Results are illustrated, for a selection of informative analytes covering different volatilities, polarities  
392 and amounts, in Figure 3. The bias is computed as Error %, according to Equation 3, and between 2D peaks  
393 response (Normalized 2D Volume over IS, % response over all 2D peaks detected or over *UT* peaks) taking  
394 *Set-up 2* as reference.

395

$$396 \text{ Error \%} = ((\text{Response}_i \text{ Set-up 2} - \text{Response}_i \text{ Set-up 1}) / \text{Response}_i \text{ Set-up 2}) * 100 \quad \text{Eq. 3}$$

397 Normalization by IS(s) performs, on average, more effectively than those on % response (Average  
398 Error % 11.2 vs. 16.2); it better compensates for response fluctuations derived by S/SL injection  
399 discriminations here impacting on highly volatiles (heptane and acetone) and due to the different operative  
400 head-pressures applied to the two set-up. For highly volatile analytes, the Error % computed for IS  
401 normalization drops below 16 while for % response it reaches 50-60%. On the other hand, % response  
402 indicators well compensate for detection variability on less abundant analytes as 1-octen-3 ol (0.01%), 2-  
403 butanone (0.01%), 3-penten-2-one (0.02%), 1-octanol (0.04%), and octanal (0.06%). Note, response  
404 variations also are influenced by chromatographic performance; 2D-peaks showing long tails (carbonyl  
405 derivatives or unsaturated alcohols) or distorted by overloading phenomena may be splitted in multiple 2D-  
406 peaks. In these cases, supervision is needed to merge all 2D-peaks belonging to the same analytes in a single  
407 one.

408 Although ISs normalization gave better results for analytes heavily discriminated, it requires a  
409 dedicated sample preparation with ISs pre-loading before sampling that may impact on the global analysis  
410 time. In addition, a careful selection of standards is necessary by focusing on compounds not present in the  
411 samples under study while covering the suitable range of volatility and polarity. In this scenario, % response  
412 normalization is attractive being simpler and less time consuming although it does not rule out the use of

413 quality control procedures as for example external standardization (ES) or multiple quality control samples  
414 analysis.

415 As a proof of concept, a PCA was run on normalized responses obtained from the two set-up. The  
416 data matrix consisted of 126 chemical variables corresponding to the targeted analytes listed in Table 2, and  
417 16 samples corresponding to 2 plots  $\times$  4 ripening stages  $\times$  2 set-up. Technical replicates were averaged;  
418 therefore, the final data matrix was  $126 \times 16$  dimensioned. Results are shown in Figure 4A; observations  
419 (samples) are rationally distributed over the Cartesian plane according to the ripening stage of olives (visible  
420 along the F1 from right to left) and in accordance to oil quality (i.e. extra-virgin, virgin or *lampante*). Results  
421 are in agreement with those from the original study by Magagna et al.<sup>7</sup> Measurements from the two set up  
422 are homogeneously distributed confirming the effectiveness of response normalization on the “batch effect”.  
423 The latter is clearly visible in Figure 4B where the PCA is conducted on analytes 2D volumes without  
424 normalization. Here the two independent clusters belonging to set up 1 and set up 2 are clearly visible and  
425 well discriminated along the F1.

426

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430

431 **Compliance with ethical standards Notes**

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

433

434

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- 552

553 **Caption to Figures**

554 **Figure 1:** colorized plot of the *Baza-1-A* sample analyzed with *Set-up 1 (1A)* and *Set-up 2 (1B)*. Red colored  
555 areas indicate the available separation space while green areas include all targeted peaks elution area. For  
556 details, see the text.

557

558 **Figure 2:** dispersion graph resulting from the relative position of targeted peak analytes from the two set-  
559 ups in the normalized retention times space: homologous series *n*-alkanes - green indicators, linear saturated  
560 aldehydes - orange indicators, an short chain fatty acids - cyan indicators.

561

562 **Figure 4:** Error %, according to Equation 3, calculated between 2D peaks response (Normalized 2D Volume  
563 over IS, % response over all 2D peaks detected or over UT peaks) taking *Set-up 2* as reference for a selection  
564 of informative analytes. Red dotted lines indicate boundaries for acceptance at  $\pm 20\%$ .

565

566 **Caption to Tables**

567 **Table 1:** List of samples together with acronym, harvest region, harvest stage, quality parameters according  
568 to COMMISSION REGULATION (EEC) No 2568/91 of 11 July 1991, sensory evaluation results (Md: median of  
569 defects – Mf: median of fruity notes) and commercial classification.

570

571 **Table 2:** *Set-up 1* and 2 columns characteristics, settings and operative pressures.

572

573 **Table 3:** List of all targeted analytes together with their elution order (#Rank) in the two set-ups, retention  
574 times ( $^1t_R$  and  $^2t_R$ ), relative standard deviation (RSD%) calculated over all analyzed samples, and peak-widths  
575 at 10% of peak height estimated on each dimension ( $^1W_{0.1}$  (min) and  $^2W_{0.1}$  (sec)).

576

577 **Table 4:** template matching results based on *Set-up 1* templates including 2D peaks with SNR ranging from  
578 10 to 100. Similarity DMF threshold applied is 800 or 700 and reference spectrum is *blob* (average 2D-peak  
579 spectrum) or *peak* (highest modulation spectrum). The upper part of the table refers to benchmark values  
580 obtained by applying *Set-up 1* templates over replicated analyses of the same sample (i.e., *Baza-4-A*); the  
581 lower part of the table refers about results of *Set-up 1* templates over replicated analyses of another sample  
582 (i.e., *Bena-4-A*).

583

584 **Table 5:** template matching results for targeted template (supervised work-flow) from *Set-up 1* applied to  
585 *Set-up 2* samples and for feature template (unsupervised and automatic work-flow) built over *Set-up 1*  
586 samples and applied on *Set-up 2* samples.

587

588 **Table 1:** List of samples together with acronym, harvest region, harvest stage, quality parameters according  
589 to COMMISSION REGULATION (EEC) No 2568/91 of 11 July 1991, sensory evaluation results (Md: median of  
590 defects – Mf: median of fruity notes) and commercial classification.  
591

Sample Acronym	Region	Harvest stage	Acidity (%)	Peroxide index (mEq O <sub>2</sub> /kg)	K <sub>232</sub>	K <sub>270</sub>	ΔK	Md	Mf	Classification
<i>Baza-1-A</i>	Baza	November 10-12	0.2	5	1.84	0.2	0	0	5	EVOO
<i>Baza-2-A</i>	Baza	November 24-28	0.2	3	1.6	0.2	0	0	4.1	EVOO
<i>Baza-3-A</i>	Baza	December 16-17	0.2	5	1.17	0.2	0	> 0.00	1.3	VOO
<i>Baza-4-A</i>	Baza	January 12-15	0.4	11	1.11	0.1	0	> 0.00	0	LOO
<i>Baza-1-B</i>	Baza	November 10-12	0.2	4	1.92	0.2	0	0	5.2	EVOO
<i>Baza-2-B</i>	Baza	November 24-28	0.1	3	1.65	0.2	0	0	3.8	EVOO
<i>Baza-3-B</i>	Baza	December 16-17	0.2	6	1.28	0.1	0	> 0.00	1.7	VOO
<i>Baza-4-B</i>	Baza	January 12-15	0.4	13	1.12	0.1	0	> 0.00	0	LOO
<i>Bena-1-A</i>	Benamaurel	November 10-12	0.2	5	1.61	0.2	0	0	4.4	EVOO
<i>Bena-2-A</i>	Benamaurel	November 24-28	0.2	4	1.53	0.2	0	0	4.3	EVOO
<i>Bena-3-A</i>	Benamaurel	December 16-17	0.2	8	1.19	0.1	0	0	3.1	EVOO
<i>Bena-4-A</i>	Benamaurel	January 12-15	0.4	19	1.05	0.1	0	> 0.00	0	LOO
<i>Bena-1-B</i>	Benamaurel	November 10-12	0.1	4	1.64	0.4	0	0	4.2	EVOO
<i>Bena-2-B</i>	Benamaurel	November 24-28	0.2	3	1.48	0.2	0	0	4.3	EVOO
<i>Bena-3-B</i>	Benamaurel	December 16-17	0.2	6	1.51	0.1	0	0	2.9	EVOO
<i>Bena-4-B</i>	Benamaurel	January 12-15	0.2	14	1.05	0.1	0	> 0.00	0	LOO

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593 **Table 2:** Set-up 1 and 2 columns characteristics, settings and operative pressures.

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	<b>Set-up 1</b>	<b>Set-up 2</b>
<b><sup>1</sup>D Columns</b>	<sup>1</sup> D: SolGelWax™ (30 m, 0.25mm d <sub>c</sub> , 0.25 μm d <sub>f</sub> ) Batch N° 1238274C06	<sup>1</sup> D: SolGelWax™ (30 m, 0.25mm d <sub>c</sub> , 0.25 μm d <sub>f</sub> ) Batch N° 1315621E03
<b><sup>1</sup>D Carrier settings</b>	gas He carrier @ 1.3 mL/min - constant flow conditions Average velocity ( <sup>1</sup> ū): 15.3 cm/s Initial head-pressure (relative) 234 kPa Outlet pressure (absolute) 285 kPa Hold-up 3.27 min	gas He carrier @ 1.3 mL/min - constant flow conditions Average velocity ( <sup>1</sup> ū): 12.8 cm/s Initial head-pressure (relative) 290 kPa Outlet pressure (absolute) 349 kPa Hold-up 3.89 min
<b><sup>2</sup>D Columns</b>	<sup>2</sup> D: OV1701 Mega (1.0 m, 0.10 mm d <sub>c</sub> , 0.10 μm d <sub>f</sub> ) Loop-capillary: deactivated fused silica (1.0 m, 0.10 mm d <sub>c</sub> ) Restriction toward MS: none	<sup>2</sup> D: OV1701 Mega (1.0 m, 0.10 mm d <sub>c</sub> , 0.10 μm d <sub>f</sub> ) Loop-capillary: deactivated fused silica (1.0 m, 0.10 mm d <sub>c</sub> ) Restriction toward MS: deactivated fused silica (1 m, 0.10 mm d <sub>c</sub> )
<b><sup>2</sup>D Carrier settings</b>	gas He carrier @ 1.3 mL/min - constant flow conditions Average velocity ( <sup>2</sup> ū): 157 cm/s Mid-point pressure (relative) 184 kPa Hold-up 1.28 s	gas He carrier @ 1.3 mL/min - constant flow conditions Average velocity ( <sup>2</sup> ū): 128 cm/s Mid-point pressure (relative) 248 kPa Hold-up 2.35 s
<b>Modulation</b>	P <sub>M</sub> : 3.5 s - Hot-Jet pulse time: 250 ms	P <sub>M</sub> : 4s - Hot-Jet pulse time: 250 ms

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597 **Table 3:** List of all targeted analytes together with their experimental  $I^T_s$ , identification criterion (a) authentic reference compound or (b)  $I^T_s \pm 20$  and spectral  
598 similarity direct match  $\geq 850$ , elution order (#Rank) in the two Set-up; retention times ( $^1t_R$  and  $^2t_R$ ) and relative standard deviation (RSD%) calculated over all  
599 analyzed samples, peak-width at 10% of peak height estimated on each dimension ( $^1W_{0.1}$  (min) and  $^2W_{0.1}$  (sec)).

Compounds	Exp $I^T_s$	Identification	Set-up 1						Set-up 2							
			# Rank	$^1t_R$ (min)	RSD%	$^2t_R$ (sec)	RSD%	$^1W_{0.1}$ (min)	$^2W_{0.1}$ (sec)	# Rank	$^1t_R$ (min)	RSD%	$^2t_R$ (sec)	RSD%	$^1W_{0.1}$ (min)	$^2W_{0.1}$ (sec)
Hexane	778	a	1	4.36	0.77	0.33	3.53	0.12	0.05	1	4.80	8.45	1.06	5.80	0.40	0.15
2,2,4-Trimethylpentane	789	b	2	4.63	0.73	0.56	0.00	0.10	0.06	2	5.44	3.53	0.83	3.07	0.36	0.13
Heptane	795	a	3	4.78	0.00	0.58	0.00	0.10	0.05	3	5.53	3.61	0.60	5.38	0.38	0.16
Cyclohexane	802	b	4	4.96	0.00	0.49	2.34	0.08	0.04	6	5.88	3.49	0.96	7.99	0.29	0.11
1,3-Pentadiene	813	b	5	5.25	0.00	0.42	0.00	0.37	0.17	4	5.60	0.00	0.54	4.26	0.53	0.17
Propanal	819	a	6	5.44	0.62	0.28	0.00	0.25	0.12	5	5.82	0.00	0.52	7.62	0.40	0.15
Octane	830	a	7	5.70	0.59	1.06	0.00	0.10	0.09	8	6.28	1.18	1.68	1.04	0.33	0.15
Acetone	831	a	8	5.74	0.59	0.29	4.03	0.25	0.11	10	6.67	0.00	0.74	8.48	0.53	0.37
1-Octene	850	b	9	6.26	0.54	1.01	1.15	0.12	0.08	7	6.26	0.62	1.01	3.78	0.40	0.23
Tetrahydrofuran	858	b	10	6.46	0.52	0.48	0.00	0.23	0.16	9	6.33	1.82	0.48	13.50	0.36	0.18
Butanal	867	a	11	6.67	0.51	0.42	0.00	0.23	0.13	11	7.47	2.36	0.91	8.41	0.42	0.23
Ethyl acetate	873	a	12	6.86	0.49	0.43	2.71	0.18	0.05	13	7.74	0.01	1.08	0.05	0.33	0.16
2,3-Dimethylheptane	893	b	13	7.43	0.45	1.76	1.14	0.23	0.17	12	7.73	0.86	1.99	11.30	0.38	0.13
2-Methylbutanal	895	a	14	7.44	0.01	0.60	0.21	0.22	0.07	15	8.27	8.41	1.16	8.24	0.43	0.24
Ethanol	913	a	15	7.78	0.43	0.26	0.00	0.19	0.09	14	7.78	2.47	0.38	3.53	0.42	0.29
1-Methoxyhexane	932	b	16	8.42	0.40	1.15	1.01	0.14	0.09	17	9.09	0.43	2.21	2.66	0.29	0.31
2-Ethylfuran	932	b	17	8.42	0.40	0.54	0.00	0.29	0.07	18	9.11	0.74	1.10	3.64	0.38	0.17
2-Methylnonane	947	b	18	8.85	0.38	2.29	2.02	0.31	0.24	16	9.04	0.43	2.85	2.84	0.49	0.23
2,3-Butanedione	955	a	19	8.94	0.38	0.37	3.09	0.37	0.05	19	9.18	3.71	0.45	11.80	0.42	0.13
Pentanal	956	a	20	9.03	0.00	0.65	0.02	0.30	0.23	21	10.00	0.04	1.26	0.05	0.37	0.16
3-Methylnonane	960	b	21	9.20	0.37	2.33	0.50	0.31	0.27	20	9.65	0.69	2.48	12.00	0.51	0.19
Acetonitrile	980	b	22	9.69	0.00	0.27	0.04	0.15	0.10	22	10.27	0.03	0.86	0.29	0.30	0.32
(Z)-1-Methoxy-3-hexene	991	b	23	10.01	0.34	1.03	1.12	0.14	0.09	24	10.66	0.36	1.99	4.07	0.24	0.24
Decane	997	a	24	10.18	0.01	2.51	0.03	0.18	0.08	23	10.48	0.06	3.31	0.09	0.32	0.15
1-Penten-3-one	1000	a	25	10.25	0.33	0.56	0.00	0.21	0.06	25	10.96	0.70	1.11	3.74	0.42	0.23
Propan-1-ol	1011	a	26	10.65	0.00	0.29	0.04	0.19	0.08	28	11.43	0.02	0.65	0.11	0.34	0.18
$\alpha$ -Pinene	1011	a	27	10.66	0.00	1.62	0.01	0.20	0.06	27	11.18	0.01	2.77	0.17	0.28	0.16
(E)-2-Butenal	1017	a	28	10.95	1.11	0.50	4.00	0.21	0.15	26	11.05	1.51	0.59	6.55	0.40	0.15
Toluene	1020	a	29	10.99	0.31	0.68	2.94	0.18	0.07	29	11.61	0.67	1.37	4.68	0.33	0.17
1-Decene	1032	b	30	11.41	0.00	2.06	0.01	0.17	0.05	30	11.98	0.38	2.98	0.53	0.43	0.20
2,3-Pentanedione	1033	a	31	11.50	0.00	0.52	0.00	0.21	0.06	32	12.19	0.32	1.16	0.27	0.36	0.23
4-Methyldecane	1051	b	32	12.04	0.56	2.83	0.41	0.29	0.31	31	12.02	0.56	2.93	2.44	0.53	0.38

Hexanal	1059	a	33	12.39	0.27	0.86	0.00	0.21	0.07	33	13.09	1.28	1.72	6.23	0.42	0.19
Isobutyl alcohol	1061	a	34	12.46	0.27	0.34	0.00	0.16	0.04	34	13.10	0.88	0.78	6.51	0.29	0.17
2,4,6-Trimethyldecane	1090	b	35	13.26	0.25	3.11	0.37	0.29	0.33	38	13.99	1.93	3.26	8.52	0.44	0.35
β-Pinene	1093	a	36	13.59	0.00	1.58	0.00	0.17	0.05	36	13.77	0.01	1.70	0.22	0.25	0.12
Undecane	1097	a	37	13.75	0.00	2.85	0.01	0.19	0.09	42	14.30	0.01	3.61	0.09	0.32	0.16
3-Penten-2-one	1100	a	38	13.88	0.00	0.60	0.00	0.27	0.14	35	13.73	1.70	0.69	6.19	0.42	0.22
1-Methoxy-2-propanol	1101	a	39	13.90	0.01	0.39	0.03	0.38	0.09	39	14.10	0.02	0.48	0.74	0.41	0.22
3-Methylbutyl acetate	1101	a	40	13.90	0.24	1.02	0.00	0.14	0.10	37	13.95	3.01	1.34	9.21	0.69	0.18
1-Methoxy-1-propanol	1103	b	41	13.98	0.24	0.39	2.94	0.39	0.16	40	14.12	1.84	0.66	10.90	0.36	0.32
Ethylbenzene	1106	b	42	14.12	0.00	0.86	0.00	0.16	0.07	41	14.24	1.51	1.31	7.54	0.42	0.30
1,4-Dimethylbenzene	1115	b	43	14.41	0.00	0.87	1.32	0.16	0.17	43	14.59	0.53	0.95	10.60	0.47	0.25
1-Butanol	1115	a	44	14.41	0.00	0.36	0.00	0.21	0.06	44	14.98	1.81	1.22	8.66	0.36	0.23
1,3-Dimethylbenzene	1121	b	45	14.64	0.00	0.84	0.00	0.14	0.06	45	15.09	0.44	1.75	6.31	0.64	0.35
Butyl 2-methylpropanoate	1128	b	46	14.94	0.00	1.36	0.02	0.12	0.08	47	15.62	0.01	2.12	0.05	0.33	0.15
1-Penten-3-ol	1130	a	47	15.01	0.22	0.36	0.00	0.16	0.05	46	15.53	0.86	0.99	12.50	0.36	0.24
2-Methylpropyl butyrate	1141	a	48	15.40	0.00	1.33	0.87	0.31	0.21	48	15.74	1.30	1.55	8.44	0.38	0.15
β-Myrcene	1147	a	49	15.65	0.22	1.33	0.87	0.14	0.09	49	15.74	1.07	1.55	6.74	0.34	0.32
Heptanal	1162	a	50	16.28	0.00	1.02	0.00	0.19	0.08	52	17.07	2.64	2.00	6.10	0.44	0.18
1,3-Xylene	1163	b	51	16.33	0.00	0.83	1.39	0.18	0.11	50	16.61	0.97	1.34	4.40	0.44	0.29
2-Ethylhexanal	1166	a	52	16.45	0.00	1.33	0.87	0.14	0.09	53	17.17	3.19	2.30	13.60	0.51	0.29
3-Methyl-2-butenal	1172	a	53	16.68	0.00	0.61	1.88	0.16	0.07	51	16.98	2.24	0.97	14.10	0.47	0.32
3-Methyl-1-butanol	1177	b	54	16.86	0.00	0.79	1.47	0.16	0.07	54	17.50	3.19	1.45	13.60	0.51	0.29
2-Methyl-1-butanol	1177	b	55	16.86	0.00	0.41	0.03	0.16	0.08	56	17.69	0.01	1.10	0.25	0.38	0.25
Limonene	1182	a	56	17.09	0.00	1.41	0.82	0.16	0.09	55	17.57	0.44	2.87	19.40	0.36	0.26
Eucalyptol	1190	a	57	17.37	0.00	1.54	0.01	0.32	0.15	58	17.94	0.01	2.43	0.12	0.37	0.24
(E)-2-Hexenal	1193	a	58	17.50	0.00	0.81	1.43	0.31	0.12	57	17.80	2.43	1.50	3.66	0.31	0.19
Dodecane	1197	a	59	17.68	0.00	3.02	0.01	0.19	0.09	59	18.18	0.01	3.68	0.08	0.33	0.16
Terpinene	1229	a	60	18.92	0.18	1.35	0.85	0.12	0.09	60	19.20	0.34	1.71	3.07	0.20	0.30
(E)-β-Ocimene	1234	a	61	19.08	0.00	1.27	1.82	0.16	0.11	62	19.49	0.35	2.64	5.06	0.27	0.21
1-Pentanol	1236	a	62	19.15	0.18	0.11	6.74	0.33	0.17	61	19.33	2.60	0.92	7.57	0.69	0.31
Styrene	1244	b	63	19.57	0.01	0.62	0.01	0.31	0.22	63	19.98	0.01	1.39	0.10	0.58	0.43
1-Dodecene	1249	b	64	19.74	0.17	2.67	0.87	0.27	0.32	64	19.98	1.75	2.74	12.80	0.33	0.30
Hexyl acetate	1250	a	65	19.78	0.00	1.13	1.02	0.18	0.09	67	20.59	0.97	2.35	15.40	0.42	0.22
3-Hydroxy-2-butanone	1256	a	66	19.93	0.17	0.63	1.84	0.37	0.21	66	20.58	1.16	1.02	4.55	0.36	0.21
1,2,3-Trimethylbenzene	1261	b	67	20.18	0.00	0.96	0.00	0.27	0.09	65	20.35	2.01	1.06	3.73	0.38	0.27
2-Ethyl-2-hexenal	1278	a	68	20.77	0.00	1.14	0.02	0.17	0.09	68	21.34	0.02	2.13	0.08	0.48	0.32
(Z)-3-Hexenyl acetate	1300	a	69	21.43	0.16	0.94	0.00	0.14	0.09	69	21.95	0.47	1.98	1.01	0.27	0.33
N,N-Dimethylformamide	1302	b	70	21.53	0.00	0.50	0.00	0.21	0.15	71	22.28	1.95	1.47	13.30	0.36	0.37
(Z)-2-Heptenal	1306	a	71	21.66	0.16	0.90	0.00	0.25	0.08	70	22.24	1.55	1.38	5.59	0.29	0.29
6-Methyl-5-hepten-2-one	1319	a	72	22.17	0.00	0.91	1.26	0.18	0.08	72	22.86	0.45	1.87	1.22	0.20	0.29
(Z)-3-Hexen-1-ol	1346	a	73	23.74	0.00	0.42	0.00	0.21	0.11	73	23.73	0.85	1.03	6.28	0.22	0.24
(E,E)-2,4-Hexadienal	1369	a	74	24.38	0.00	0.58	0.00	0.18	0.05	76	25.39	0.26	1.22	2.84	0.33	0.49
Nonanal	1370	a	75	24.44	0.00	1.24	1.61	0.29	0.10	75	24.77	1.48	1.77	14.10	0.49	0.31

(E)-2-Hexen-1-ol	1373	a	76	24.56	0.00	0.40	0.00	0.21	0.09	74	24.71	2.20	0.91	3.37	0.31	0.31
(E)-3-Octen-2-one	1382	a	77	24.85	0.00	0.96	0.01	0.28	0.10	77	25.41	0.01	1.82	0.03	0.50	0.33
$\alpha$ -Thujone (ISTD)	1402	a	78	25.55	0.00	1.24	0.02	0.25	0.16	79	26.47	0.02	2.20	0.13	0.31	0.31
(E)-2-Octenal	1415	a	79	25.73	0.00	0.98	2.04	0.27	0.14	78	25.83	1.13	1.01	11.90	0.80	0.24
1-Octen-3-ol	1417	a	80	25.78	0.00	0.34	0.00	0.18	0.08	81	26.83	0.01	1.15	0.09	0.20	0.29
$\beta$ -Thujone (ISTD)	1424	a	81	26.26	0.00	1.21	0.01	0.15	0.09	80	26.59	0.01	2.21	0.10	0.21	0.20
1-Heptanol	1429	a	82	26.43	0.00	0.53	2.19	0.18	0.05	84	27.19	0.28	1.17	2.65	0.27	0.30
1-Ethenyl-4-ethyl-benzene	1430	b	83	26.48	0.00	0.86	0.00	0.37	0.13	82	26.87	0.72	1.81	1.68	0.51	0.25
Furfural	1434	a	84	26.77	0.00	0.34	0.00	0.17	0.06	86	27.54	0.01	1.17	0.22	0.32	0.30
(E,E)-2,4-Heptadienal	1437	a	85	26.89	0.00	0.69	1.67	0.14	0.06	87	27.80	1.06	1.44	0.00	0.62	0.25
5-Methyl-2-(1-methylethyl)-cyclohexanone	1446	b	86	27.13	0.00	1.31	0.88	0.21	0.13	83	27.05	0.28	1.37	2.41	0.33	0.25
Butyl-2-ethylhexanoate	1453	a	87	27.42	0.00	1.89	1.22	0.39	0.29	85	27.33	0.88	1.97	7.99	0.40	0.19
2-Ethyl-1-hexanol	1460	a	88	27.72	0.00	0.60	0.00	0.21	0.11	88	28.06	0.02	1.46	0.23	0.34	0.20
(Z)-Hepten-4-ol	1469	a	89	28.18	0.00	0.46	0.02	0.19	0.08	90	28.93	0.01	1.17	0.23	0.39	0.15
Decanal	1474	a	90	28.35	0.00	1.30	1.54	0.25	0.09	89	28.60	1.50	1.89	14.30	0.33	0.25
(E)-2-Hepten-1-ol	1476	a	91	28.41	0.00	0.44	0.02	0.41	0.16	91	29.20	0.00	0.97	0.03	0.55	0.33
3,5-Octadien-2-one	1485	a	92	28.88	0.00	0.77	0.01	0.21	0.12	92	29.52	0.02	1.56	0.10	0.60	0.31
Benzaldehyde	1494	a	93	29.03	0.12	0.49	2.34	0.18	0.04	95	29.95	0.47	1.06	15.40	0.44	0.22
6-Undecanone	1505	a	94	29.38	0.00	1.53	0.01	0.38	0.14	96	29.96	0.01	2.30	0.05	0.44	0.25
Propanoic acid	1506	a	95	29.40	0.01	0.14	0.06	0.35	0.09	97	30.29	0.02	0.96	0.12	0.47	0.23
(E)-2-Nonenal	1509	a	96	29.58	0.00	1.05	1.10	0.41	0.17	93	29.66	1.42	1.16	16.50	0.36	0.15
Linalool	1514	a	97	29.75	0.00	0.67	1.73	0.18	0.06	94	29.90	0.26	0.72	2.90	0.38	0.12
1-Octanol	1525	a	98	30.16	0.00	0.57	2.01	0.14	0.05	98	30.69	0.25	1.57	2.40	0.16	0.22
Nonyl acetate	1567	b	99	31.15	0.00	1.33	0.01	0.25	0.16	99	30.96	0.00	1.29	0.06	0.40	0.28
5-Methyl-2(5H)-furanone	1589	b	100	31.46	0.11	0.46	0.00	0.54	0.07	100	32.49	0.24	1.02	3.08	0.62	0.25
Undecanal	1598	a	101	32.08	0.00	1.37	1.69	0.53	0.28	102	32.76	0.12	2.72	0.71	0.78	0.50
Butanoic acid	1610	a	102	32.57	0.10	0.16	0.00	0.39	0.09	101	32.64	0.42	1.45	7.74	0.47	0.23
Butyrolactone	1611	a	103	32.61	0.00	0.44	0.00	0.35	0.09	103	32.78	0.12	0.94	3.34	0.24	0.27
1-Nonanol	1628	a	104	33.66	0.00	0.63	1.82	0.16	0.05	104	34.22	0.11	1.43	2.20	0.20	0.23
Ethyl benzoate	1637	a	105	34.03	0.10	0.67	1.71	0.12	0.06	105	34.53	0.84	1.35	10.90	0.40	0.28
5-Ethyl-2(5H)-furanone	1643	b	106	34.28	0.20	0.41	10.20	0.25	0.16	106	35.04	0.61	1.24	8.38	0.36	0.33
(Z)-3-Nonen-1-ol	1647	a	107	34.44	0.00	0.57	0.02	0.21	0.07	107	35.04	0.01	1.11	0.26	0.44	0.30
Dodecanal	1684	a	108	35.64	0.00	1.42	1.41	0.51	0.21	108	36.25	0.38	2.49	8.17	0.47	0.36
$\alpha$ -Muurolene	1701	a	109	35.99	0.00	1.61	1.89	0.16	0.13	110	36.86	0.28	2.52	12.80	0.40	0.30
3,4-Dimethyl-2,5-furandione	1701	b	110	35.99	0.00	0.54	0.02	0.25	0.11	111	36.96	0.01	1.41	0.12	0.55	0.28
Pentadecanoic acid	1710	a	111	36.16	0.00	0.17	0.21	0.32	0.20	109	36.80	0.01	0.94	0.14	0.60	0.34
1,4-Cyclohex-2-enedione	1710	b	112	36.16	0.00	0.43	0.03	0.33	0.19	112	37.13	0.00	1.28	0.21	0.49	0.22
$\alpha$ -Farnesene	1725	a	113	36.98	0.00	1.43	0.02	0.15	0.06	113	37.13	0.01	2.16	0.11	0.28	0.15
(E,E)-2,4-Decadienal	1732	a	114	37.20	0.09	0.91	2.55	0.37	0.20	114	37.64	0.10	0.95	3.20	0.64	0.35
Methyl salicylate	1743	b	115	37.57	0.00	0.57	2.04	0.25	0.11	115	38.11	1.57	1.16	9.73	0.56	0.41
2-(2-Butoxyethoxy)-ethanol	1754	a	116	37.92	0.00	0.52	0.00	0.25	0.16	116	38.39	0.70	1.31	5.33	0.31	0.35
$\delta$ -Pentalactone	1767	b	117	38.33	0.00	0.52	0.00	0.39	0.16	117	39.36	0.00	1.18	2.74	0.60	0.32

3-Phenyl-2-propenal	1794	b	118	39.20	0.00	0.52	0.00	0.21	0.07	118	40.26	0.10	1.14	3.51	0.40	0.29
Hexadecanoic acid	1805	a	119	39.51	0.09	0.20	0.00	0.31	0.22	119	40.47	0.10	0.83	7.78	0.58	0.43
(Z)-6,10-Dimethyl-5,9-undecadien-2-one	1821	b	120	40.02	0.00	1.13	2.05	0.35	0.20	120	40.70	0.09	2.12	0.99	0.31	0.29
Butyl benzoate	1830	b	121	40.31	0.00	0.82	0.02	0.41	0.27	121	41.27	0.00	1.69	0.14	0.59	0.30
Benzyl alcohol	1832	a	122	40.37	0.00	0.28	0.00	0.28	0.14	122	41.29	0.00	0.99	0.26	0.46	0.27
Phenylethyl alcohol	1869	a	123	41.44	0.16	0.34	0.00	0.29	0.11	123	41.42	0.16	0.83	12.00	0.56	0.34
4-Phenyl-3-buten-2-one	1914	b	124	42.82	0.00	0.56	0.00	0.40	0.14	124	43.77	0.09	1.12	1.61	0.64	0.25
1-Dodecanol	1928	a	125	43.23	0.00	0.81	1.42	0.23	0.13	125	43.89	0.32	1.68	8.43	0.38	0.31
Phenol	1956	a	126	44.04	0.00	1.27	3.97	0.43	0.34	126	44.84	1.51	1.66	18.30	0.53	0.41
<i>Average</i>			-	-	0.12	-	0.86	0.24	0.12	-	-	0.90	-	5.03	0.42	0.25
<i>Min</i>			-	-	0.00	-	0.00	0.08	0.04	-	-	0.00	-	0.00	0.16	0.11
<i>Max</i>			-	-	1.11	-	10.20	0.54	0.34	-	-	8.45	-	19.40	1.04	0.50

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602 **Table 4:** template matching results based on *Set-up 1* templates including 2D peaks with SNR ranging from 10 to 100. Similarity DMF threshold applied are 800  
603 or 700 while reference spectrum is *blob* (average 2D-peak spectrum) or *peak* (highest modulation spectrum). The upper part of the table refers to benchmark  
604 values obtained by applying *Set-up 1* templates over replicated analyses of the same sample (i.e., *Baza-4-A*); lower part of the table refers about results of *Set-*  
605 *up 1* templates over replicated analyses of another sample (i.e., *Bena-4-A*).  
606

**Baza-4-A Set-up 1 – three replicates**

607

SNR	% Response	Peaks n°	Similarity threshold 800 – <i>Blob</i> MS		Similarity threshold 700 – <i>Peak</i> MS	
			% Matching	Matched peaks n°	% Matching	Matched peaks n°
10 ± 2	0.01	Column bleeding or interferences				
30 ± 2	0.02	10	10.00 %	1	40.00 %	4
50 ± 2	0.02	10	10.00 %	1	90.00 %	9
70 ± 2	0.03	10	40.00 %	4	100.00 %	10
90 ± 2	0.04	10	40.00 %	4	100.00 %	10
100 ± 2	0.04	10	70.00 %	7	100.00 %	10

**Baza 4-A Set-up 1 over Bena-4-A Set-up 1**

SNR	% Response	Peaks n°	Similarity threshold 800 – <i>Blob</i> MS		Similarity threshold 700 – <i>Peak</i> MS	
			% Matching	Matched peaks n°	% Matching	Matched peaks n°
10 ± 2	0.01	Column bleeding or interferences				
30 ± 2	0.02	10	10.00 %	1	10.00 %	1
50 ± 2	0.02	10	10.00 %	1	20.00 %	1
70 ± 2	0.03	8	25.00 %	2	87.50 %	7
90 ± 2	0.04	5	40.00 %	2	100.00 %	5
100 ± 2	0.04	7	42.86 %	3	100.00 %	7

609

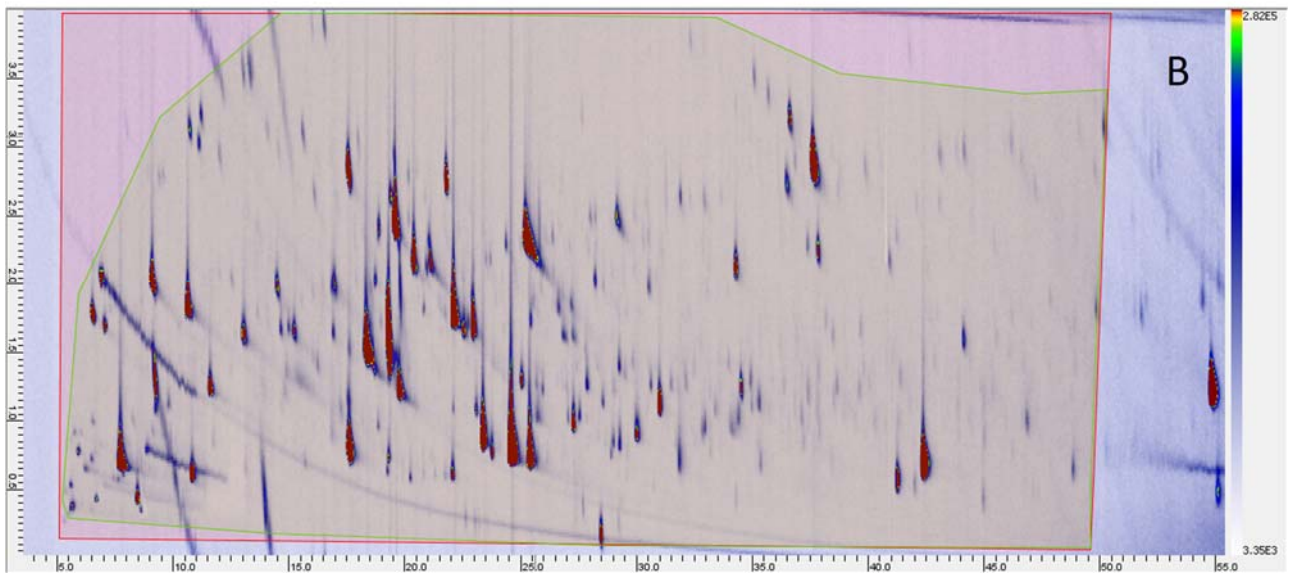
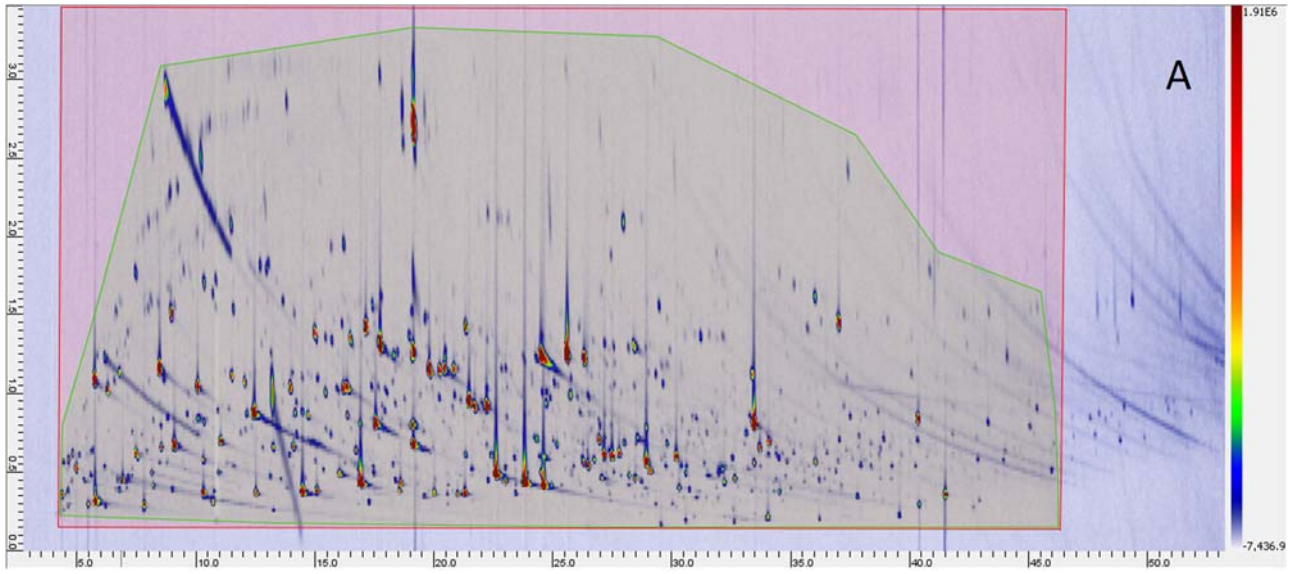
610 **Table 5:** template matching results for targeted template (supervised work-flow) from *Set-up 1* applied to *Set-up 2* samples and for feature template  
 611 (unsupervised and automatic work-flow) built over *Set-up 1* samples and applied on *Set-up 2* samples.  
 612

Samples	Targeted template (126 peaks)				Feature template (257 reliable peaks)			
	Set-up 1		Set-up 2		Set-up 1		Set-up 2	
	%	Peaks n°	%	Peaks n°	%	Peaks n°	%	Peaks n°
Baza-1	94.44	119	91.27	115	100.00	257	97.28	250
Baza-2	97.62	123	92.06	116	100.00	257	98.44	253
Baza-3	98.41	124	95.24	120	100.00	257	98.83	254
Baza-4	100.00	126	96.03	121	99.22	255	96.86	247
Bena-1	93.65	118	88.89	112	100.00	257	98.44	253
Bena-2	96.03	121	91.27	115	100.00	257	96.50	248
Bena-3	97.62	123	92.06	116	98.83	254	99.22	256
Bena-4	98.41	124	92.86	117	100.00	257	98.05	252
<i>Average</i>	<i>97.02</i>	<i>122</i>	<i>92.46</i>	<i>116</i>	<i>99.75</i>	<i>256</i>	<i>97.95</i>	<i>252</i>
<i>RSD%</i>	<i>2.22</i>	<i>2.22</i>	<i>2.47</i>	<i>2.47</i>	<i>0.45</i>	<i>0.46</i>	<i>0.99</i>	<i>1.22</i>

626

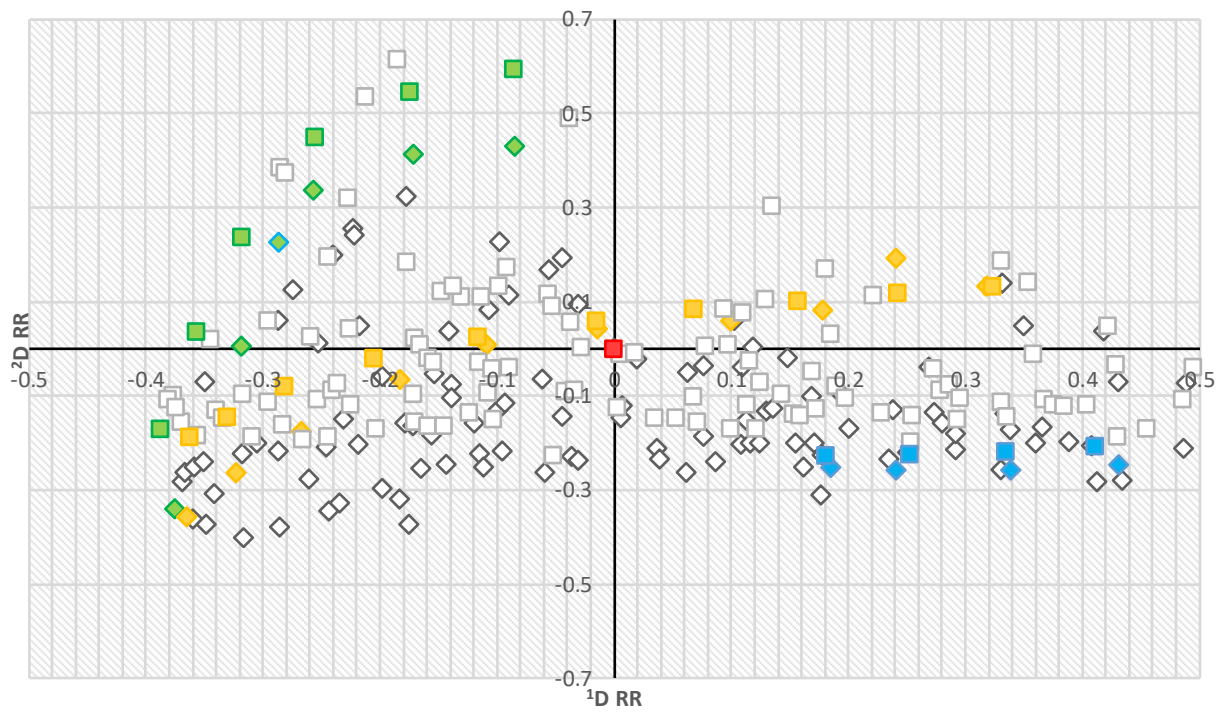
627

628 **Figure 1**



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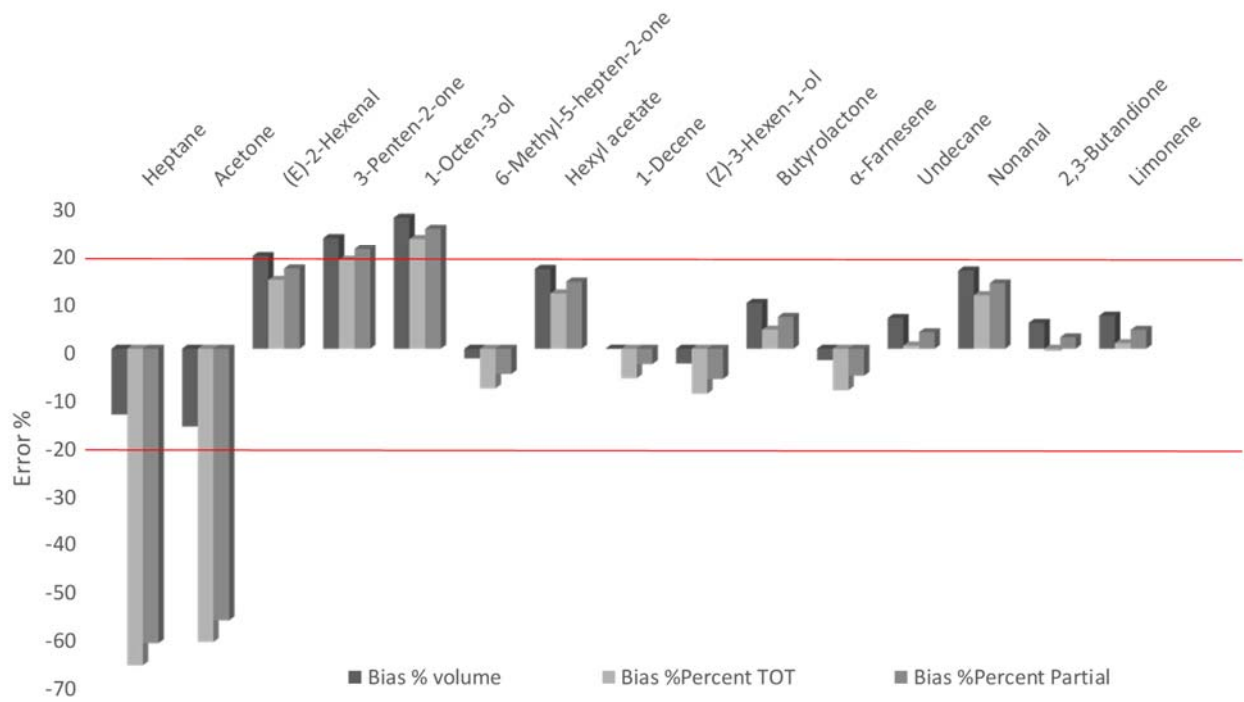


- *Set-up 1*    ◇ *Set-up 2*    ■ (Z)-3-Hexen-1-ol acetate
- Hydrocarbons *Set-up 1*    ◆ Hydrocarbons *Set-up 2*
- Linear aldehydes *Set-up 1*    ◆ Linear aldehydes *Set-up 2*
- Fatty acids *Set-up 1*    ◆ Fatty acids *Set-up 2*

632

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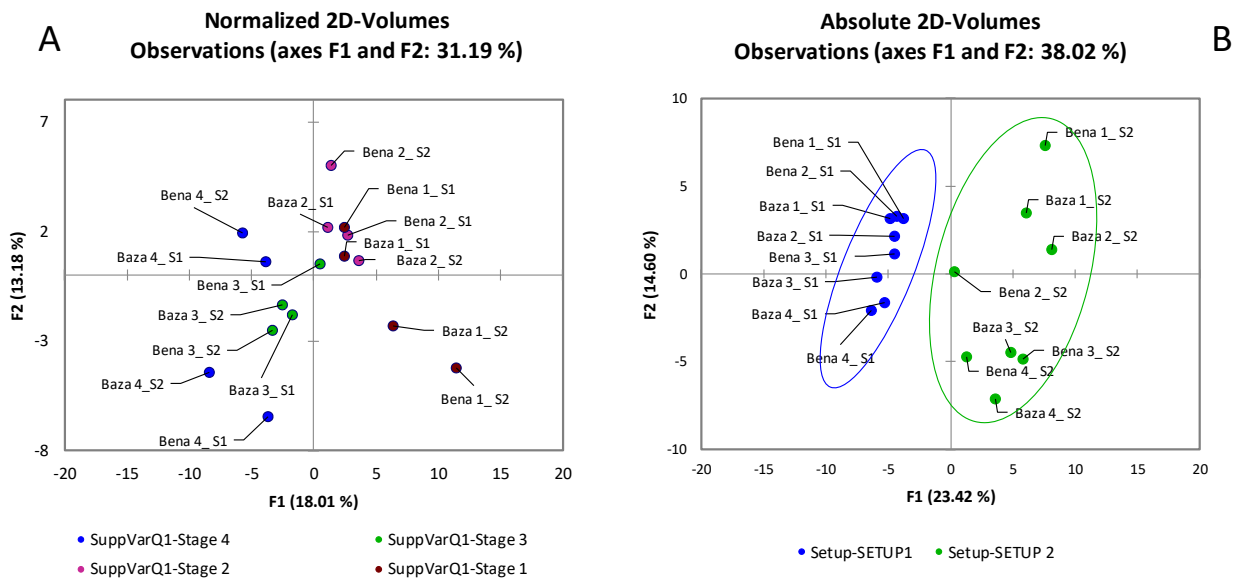
634 Figure 3



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