Performance evaluation of non-targeted peak-based cross-sample analysis for comprehensive two-dimensional gas chromatography–mass spectrometry data and application to processed hazelnut profiling

Performance evaluation of non-targeted peak-based cross-sample analysis for Comprehensive Two-Dimensional Gas Chromatography-Mass Spectrometry data and application to processed hazelnut profiling.

Johannes Kiefl[1], Chiara Cordero[2*], Luca Nicolotti[2], Peter Schieberle[1], Stephen E. Reichenbach[3] and Carlo Bicchi[2]

[1]Deutsche Forschungsanstalt für Lebensmittelchemie, Lise-Meitner-Straße 34, 85354 Freising, Germany

[2]Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria 9, I-10125 Torino, Italy

[3]Computer Science and Engineering Department, University of Nebraska , 1400 R Street,Lincoln, NE 68588-0115, USA

* Address for correspondence:

Dr. Chiara Cordero - Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria 9, I-10125 Torino, Italy – e-mail: chiara.cordero@unito.it ; phone: +39 011 670 7662; fax: +39 011 2367662

Abstract

The continuous interest in non-targeted profiling induced the development of tools for automated cross-sample analysis. Such tools were found to be selective or not comprehensive thus delivering a biased view on the qualitative/quantitative peak distribution across 2D sample chromatograms. Therefore, the performance of non-targeted approaches needs to be critically evaluated. This study focused on the development of a validation procedure for non-targeted, peak-based, GC×GC-MS data profiling. The procedure introduced performance parameters such as specificity, precision, accuracy, and uncertainty for a profiling method known as *Comprehensive Template Matching*. The performance was assessed by applying a three-week validation protocol based on CITAC/EURACHEM guidelines. Optimized ^{1}D and ^{2}D retention times search windows, MS match factor threshold, detection threshold, and template threshold were evolved from two training sets by a semi-automated learning process. The effectiveness of proposed settings to consistently match 2D peak patterns was established by evaluating the rate of mismatched peaks and was expressed in terms of results accuracy. The study utilized 23 different 2D peak patterns providing the chemical fingerprints of raw and roasted hazelnuts (*Corylus avellana* L.) from different geographical origins, of diverse varieties and different roasting degrees. The validation results show that non-targeted peak-based profiling can be reliable with error rates lower than 10 % independent of the degree of analytical variance.

The optimized *Comprehensive Template Matching* procedure was employed to study hazelnut roasting profiles and in particular to find marker compounds strongly dependent on the thermal treatment, and to establish the correlation of potential marker compounds to geographical origin and variety/cultivar and finally to reveal the characteristic release of aroma active compounds.

Keywords: GC×GC-MS; validation; comprehensive template matching fingerprinting; roasted hazelnut; *Corylus avellana* L. volatile fraction; key aroma markers.

1. Introduction

Comprehensive two-dimensional gas chromatography coupled with mass spectrometry (GC×GC-MS) is a powerful tool for targeted and non-targeted analysis of complex mixtures of volatile compounds due to the enhanced peak capacity compared to one-dimensional GC [1-3]. Non-targeted fingerprint analysis can reveal qualitative/quantitative differences in chemical compositions facilitating the identification of potential marker compounds [4,5] and grouping or classification of samples [6,7]. Recent publications have described the development of non-targeted, peak-based fingerprinting tools to exploit the informative content of three dimensional GC×GC-MS data sets characterized by first (^{1}D) and second (^{2}D) dimension retention times and mass fragmentation patterns [5,6,8-12]. Such tools (name of the tool is set in brackets) were developed by Oh et al. (MSort [8]), Wang et al. (DISCO [9]), Kim et al. (mSPA [12]), Castillo et al. (Guineu [11]), Almstetter et al. (INCA [5]), and Leco Corporation (Statistical Compare, St. Joseph, USA). The latter provides commercially available software that was tested by Almstetter et al. [10]. In a recent review, Reichenbach et al. [13] characterized such approaches as *peak feature analysis*. *Peak features* collate the response data of individual analytes across chromatograms; for comparative analysis, analyte peaks must therefore be consistently matched across 2D chromatograms. However, consistent *peak feature analysis* remains challenging because retention times and mass spectra are subject to run-to-run variations due to random or systematic errors depending on sample preparation, injection, chromatographic and mass spectrometric conditions. This process of peak matching is a critical step of data elaboration, because matching errors produce false qualitative and quantitative differences [13], thus complicating the ongoing data interpretation.

The extent of variability, generated during analysis, can be minimized by adopting automated sample preparation techniques, validated protocols for sample processing, and robust instruments. On the other side, software for data processing should reliably match corresponding analyte peaks, within a set of sample chromatograms, by accounting for the remaining variation on, for example, ^{1}D and ^{2}D retention times and fragmentation pattern intensities. This variability can be propagated during data processing, if the *feature* content (i.e., response values of one analyte matched across many chromatograms) is erroneously computed and/or elaborated by the software. Data processing errors can occur at different stages and have partially been addressed: (a) removal of background [14], (b) peak detection

84 [15], (*c*) recognition of retention time shifts [16], and (*d*) peak alignment [5,8-12,16-18]. For
85 example, peak detection thresholds are used to detect and integrate peaks above a certain
86 signal-to-noise ratio (S/N). Therefore, trace peaks may be detected in some samples, but not
87 in others [13]. This inconsistent peak detection may be propagated by the peak alignment
88 routine by matching just detected peaks while ignoring corresponding analyte peaks with
89 lower S/N. Approaches to eliminate such propagation of errors have scarcely been
90 evaluated, but this issue is critical for the challenging task of a consistent and unbiased *peak*
91 *feature analysis*. A further example is given by Castillo et al. [11]; the authors compared sixty
92 2D chromatograms of serum samples getting an aligned data file with 14756 compounds.
93 However, only 1540 compounds were aligned across minimum six 2D chromatograms [11].
94 This indicates that 90 % of the detected peaks were lost during data processing and these
95 might have included diagnostic analyte peaks.
96 Non-targeted, peak-based fingerprint analysis should produce reliable data on the
97 qualitative/quantitative distribution of compounds within a sample set. Then, results can be
98 interpreted and research can provide compound identifications, structure elucidation of
99 unknowns, or quantitative information. It is proposed that the quality of such investigations
100 can be assured by applying an appropriate validation procedure. The analytical protocol
101 adopted in the present study was designed and validated in accordance with general
102 protocols (Eurachem/Citac guidelines [19,20]) to systematically investigate method
103 performance parameters (i.e., specificity, repeatability and intermediate precision, accuracy
104 and uncertainty) as a function of the most critical variables (data acquisition and data
105 elaboration levels). An existing alignment tool was adopted using *Comprehensive Template*
106 *Matching* as introduced by Reichenbach et al. [21]. The validated *Comprehensive Template*
107 *Matching* procedure was then applied to a food-type intervention study designed to identify
108 marker peaks highly informative for the hazelnuts roasting process. Various raw hazelnuts
109 were subjected to a roasting protocol to induce chemical changes which were recorded with
110 the help of the alignment routine. Hazelnuts are an ideal model system, because different
111 known and unknown formation pathways are generating odorless volatiles and odorants
112 with different kinetics [22,23]. Therefore, non-targeted analysis is useful for studying the
113 biological and/or chemical response after perturbation or technological intervention and
114 provides significant and valid information about interaction-relationships in a food system.
115

116 **2. Materials and Methods**

117 **2.1 Reference compounds and solvents**

118 Pure reference compounds for identity confirmation and *n*-alkanes (*n*-C9 to *n*-C25)
119 were supplied by Sigma-Aldrich (Taufkirchen, Germany) except α/β-thujone (α/β-1-
120 isopropyl-4-methylbicyclo[3.1.0]hexan-3-one, 95/5 weight ratio) supplied by Fluka (Milan,
121 Italy). A standard stock solution of α/β-thujone diluted to 45 ng/mL was prepared in
122 ultrapure water and the solution was stored in a sealed vial at 5 °C. Both, α- and β-thujone,
123 were used as internal standards for peak response normalization (ISTD) adopting the ISTD
124 loading procedure [24,25]. Solvents (cyclohexane, n-hexane, dichloromethane) were all
125 HPLC-grade from Riedel-de Haen (Seelze, Germany). 3-Methyl-4-heptanone was synthesized
126 according to [22].

127

128 **2.2 Hazelnut samples and roasting**

129 Raw and shelled hazelnuts of *Corylus avellana* L. (harvest 2009, supplied by
130 Marchisio, Cortemilia- CN, Italy) from different geographical origins and varieties/cultivars
131 were roasted at 160°C in a ventilated oven for 7, 12, 17 and 23 minutes. Tonda Gentile
132 Romana (*Romana*, Lazio, Italy), Tonda Gentile delle Langhe (*Gentile*, Piedmont, Italy), Tonda
133 di Giffoni (*Giffoni*, Campania, Italy) were monovarieties and Azerbajian hazelnuts were a
134 blend of different locally grown cultivars. Roasting was conducted every day and the
135 hazelnuts of a uniform dimension (caliber within 12-13 mm) were left at room temperature
136 to cool down. No storage of manually roasted hazelnuts was necessary, thereby avoiding an
137 alteration of the volatile fraction. The hazelnut samples were manually ground prior to vial
138 filling, and the particle size was compared to a ground reference sample.

139 Standard roasted hazelnuts (harvest 2009, supplied by Soremartec Italia SpA, Alba-
140 CN, Italy) of Romana, Gentile and Giffoni were submitted to roasting in an industrial plant at
141 different time/temperature ratios consistent with their desirable final sensory
142 characteristics. These samples were hermetically sealed under vacuum in non-permeable
143 polypropylene/aluminium/polyethylene packages and stored at -20°C prior to analysis.

144

145

146

147

148 **2.3 Headspace Solid Phase Microextraction (HS-SPME) devices and sampling conditions**

149 The SPME device and fibers were from Supelco (Bellefonte, PA, USA). A
150 Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) df 50/30 µm, 2 cm length
151 fiber was chosen and conditioned before use as recommended by the manufacturer.
152 Roasted hazelnuts (1.5 g) were ground, sealed in a 20 mL vial, and equilibrated for 20 min at
153 50°C before sampling. The internal standard (ISTD) loading procedure onto the SPME fiber
154 [24,25] was as follows: the SPME device was manually inserted into a 20 mL sealed vial
155 containing 1 mL of ISTD (α/β-thujone) standard solution at 45 ng/mL, then the fiber was
156 exposed to the headspace at 50°C for 20 min. After ISTD loading, the fiber was exposed to
157 the matrix headspace at 50°C for another 20 min. Just the lower part of the vial, filled with
158 the solid sample, was heated to improve the vapor phase/fiber coating distribution
159 coefficient. The SPME device was directly introduced into the GC injector for thermal
160 desorption for 10 min at 250 °C. Samples for validation purposes were analyzed in triplicate
161 each week. Relative standard deviations (RSD %) for first and second retention times and 2D-
162 peak normalized volume (i.e., cumulative 2D peak area) for 24 identified target analytes are
163 given (**Table 1 and S1).**

164

165 **2.4 GC×GC-qMS instrument set-up**

166 GC×GC analyses were performed on an Agilent 6890 GC unit coupled with an Agilent
167 5975 MS detector operating in the EI mode at 70 eV (Agilent, Little Falls, DE, USA). The
168 transfer line was set at 280°C. A *Standard Tune* was used and the scan range was set at *m/z*
169 35-250 with a scan rate of 10,000 amu/s to obtain a suitable number of data points for each
170 chromatographic peak for reliable identification and quantitation. The system was equipped
171 with a two-stage KT 2004 loop thermal modulator (Zoex Corporation, Houston, TX) cooled
172 with liquid nitrogen and with the hot jet pulse time set at 250 ms with a modulation time of
173 4 s adopted for all experiments. Fused silica capillary loop dimensions were 1.0 m length and
174 100 µm inner diameter. The column set was configured as follows: ^{1}D Carbowax CW20M
175 column (100% polyethylene glycol)(30 m × 0.25 mm i.d., 0.25 µm df) coupled with a ^{2}D
176 OV1701 column (86% polydimethylsiloxane, 7% phenyl, 7% cyanopropyl) (1 m × 0.1 mmi.d.,
177 0.10 µm df). Columns were from Mega (Legnano, Milan, Italy).

178 One microliter of the *n*-alkane sample solution was automatically injected with an
179 Agilent ALS 7683B injection system under the following conditions: split/splitless injector,

split mode, split ratio 1:50, injector temperature 280°C. The HS-SPME sampled analytes were thermally desorbed from the fiber for 10 min into the GC injector using the following conditions: split/splitless in split mode, split ratio 1:20, injector temperature 250°C. The carrier gas was helium at a constant flow of 0.7 mL/min (initial head pressure 260 KPa). The temperature program was 40°C (2 min) to 180°C at 2.5°C/min and to 250°C at 20°C/min (5 min). Data were acquired by Agilent MSD ChemStation ver D.02.00.275 and processed using GC Image GC×GC Software version 2.1b1 (GC Image, LLC Lincoln NE, USA). Statistical analysis was performed with SPSS 14.0 (SPSS Inc. Chicago, Illinois, USA).

2.5 Validation protocol and data elaboration

The validation protocol was designed and applied to characterize the following performance parameters: specificity, repeatability and intermediate precision of the analytical method (sample preparation and separation), and accuracy of results related to the *Comprehensive Template Matching* methodology. The five-days/three-weeks validation scheme is summarized in **Table S2** .

Raw data files were loaded into the software and were background corrected according to [14]. The 2D peaks were automatically detected by fixing a *peak signal-to-noise ratio* (S/N) threshold of 10 and a footprint-area threshold of 10. The peak detection uses the watershed algorithm [15]. A set of 24 target peaks (**Table 1**), selected over the GC×GC patterns to cover homogeneously the chromatographic run, were matched over nine replicates of standard roasted Romana samples and over nine replicates of self-roasted Romana samples to evaluate the inter- and intra-week variability of retention times, 2D peak normalized volumes (normalization was done over the ISTDs 2D peak volume), and mass spectra match factors. Target analysis was performed as described in [3]. Normality of the distribution of normalized 2D peak volumes and normalized S/N values was tested with Kolmogorov-Smirnov and Shapiro-Wilk test with $p = 0.05$ (Origin 6.1, OriginLab Corporation MA, USA).

To determine optimized peak matching parameters, ^{1}D and ^{2}D retention times search windows were fixed on the basis of the average standard deviations of retention-times data (**Table 1**). The thresholds were set to three times the standard deviation for each dimension giving a retention time window of 5 modulations for the ^{1}D and 0.17 seconds for the ^{2}D dimension. Therefore, the peaks could be expected to elute within this time window with a

212 high probability (here 99.7 %) considering run-to-run retention time shifts. Based on the
213 reported MS match factors (**Table 1**), a match factor threshold of 600 was used (see below)
214 to confirm target identity, accepting again a run-to-run match factor variability of three
215 times the standard deviation.

216 Validation was performed *(a)* to assess the fingerprinting method specificity by
217 determining false-positive and false-negative error rates of the peak matching process; *(b)* to
218 assess precision (repeatability and intermediate precision of standard roasted Romana
219 samples) and to estimate the contribution of manual roasting to data dispersion (self-
220 roasted Romana samples); *(c)* to evaluate fingerprinting method accuracy; and finally *(d)* to
221 assess the fingerprinting method uncertainty.

222 After the validation step, *Comprehensive Template Matching* fingerprinting was
223 adopted to investigate chemical changes on extended data set samples of different
224 geographical origins and roasting conditions. A schematic work-flow of the procedure is
225 reported in **Figure 1** and details are discussed in the next paragraphs. The 23 sample
226 patterns were processed to build a *consensus template* by adding all the detected peaks,
227 exceeding fixed S/N and area thresholds from one randomly selected chromatogram within
228 the set to an empty template. Then, this template was matched with a second
229 chromatogram to add unmatched peaks, and the resulting "updated template" was matched
230 with a third chromatogram of the set and this was repeated with all patterns [6]. An
231 automatic retention time alignment was here used to compensate for pattern shifts due to
232 the intrinsic variation of chromatographic performance [16].

233 Each non-target peak included in the *consensus template* was saved with its
234 chromatographic descriptors (see above) and every entry was labeled with a unique number
235 to unequivocally identify each non-target peak.

236

237 **3. Results and Discussion**

238 This section is divided into two parts. The first part describes the routine adopted to
239 assess performance parameters of the fingerprinting method. The second part is dedicated
240 to a practical example where advanced fingerprinting is applied to profile the roasting
241 process of hazelnuts of different origin and variety/cultivar altered by increasing roasting
242 time at constant temperature.

243

244 **3.1 Assessment of method performance parameters**

245 **3.1.1 Specificity**

246 Specificity is strictly related to the chromatographic separation and requires that a
247 peak is correctly assigned to an analyte [19]. From a chromatographic point of view, the
248 separation of hazelnut volatiles presented in this study is the result of an optimization
249 procedure based on systematic columns selection aimed at finding the best orthogonal
250 column combination that minimizes the number of coelutions and maximizes the 2D peak
251 spreading over the chromatographic space [7]. With a view on data elaboration,, peak-based
252 *features* evaluate the analyte responses across many chromatograms, where one feature
253 can contain information of spuriously matched peaks. Specificity was evaluated in three
254 steps: *(a)* optimization of peak matching parameters (i.e., ^{1}D and ^{2}D retention times search
255 windows and MS match factors threshold); *(b)* matching of selected targets reported in
256 **Table 1** across nine replicate samples to establish peak correspondences and to verify peak
257 identities; and (*c*) analysis of blank samples to locate and remove interfering peaks.

258 The optimum peak matching involves low levels of false-positive and false-negative
259 hits, so that an analyte is consistently matched across several chromatograms and not
260 mismatched with impurity/blank peaks or other interfering analytes. **Figure 2** illustrates the
261 effect of different matching criteria on the peak matching specificity. When a template with
262 8 peaks (thin circles) is matched by defining just ^{1}D and ^{2}D retention times constraints
263 (**Figure 2a**), analyte peaks are positively matched if the Euclidean Distance between sample
264 and template peaks does not exceed the user-defined retention time window threshold
265 (here of 5 modulations for the ^{1}D and 0.17 seconds for the ^{2}D dimension). In this case, false-
266 positive matches are denoted for peaks 1, 2 and 5-8. When a MS constraint with 600 match
267 factor is added, peak matching gives consistent assignment (**Figure 2b**). However, the
268 average MS match factors and the corresponding relative standard deviations (**Table 1**)

269 indicate that an increasing MS threshold causes an increasing number of analyte peaks to fail
270 the matching criterion, thus producing false-negative hits. **Figure 2c** illustrates the effect of a
271 too restrictive peak matching obtained by increasing the MS threshold to 774 (**Table 1**): only
272 3 peaks of 8 template peaks are now correctly matched.

273 Results reported in **Table 1** confirm that the method is specific for matching an
274 extended set of target analytes containing resolved – unresolved peaks as well as high and
275 low abundant peaks. Peak 15 (nonanal), for example, showed a higher variation of
276 normalized volume, because of its long-tail and consequent difficult peak integration. False-
277 positive and false-negative error rates were zero percent and the true-positive rate was
278 100 % for 24 target analytes matched across nine replicate chromatograms analyzed over
279 three weeks, supporting the effectiveness of the settings for the selected retention time
280 search window and minimum match factor.

281 On average, 179 2D peaks were detected above S/N 10 in blank sample runs (**Table**
282 **S2**) and were associated to fiber bleeding, column bleeding or impurities derived from
283 solvents and ISTDs reference material. Templates were matched against the blank runs and
284 matched peaks were removed to obtain pruned templates which were then matched against
285 sample chromatograms.

286 Literature dealing with peak-based comparative analysis also aimed at improving the
287 specificity of the data elaboration. For example, Oh et al. [8] determined true-positive and
288 false-positive error rates by pair-wise matching the mass spectra of 46 derivatized standards
289 in 16 samples. A standard was positively matched when the Pearson correlation coefficient
290 was near one; a true-positive rate of 92 % and a false-positive error incidence of 11 % was
291 obtained. Retention times, however, were not used as constraints. Wang et al. [9] spiked 6
292 analytes and correctly matched 5 analytes across 5 replicate chromatograms. Kim et al. [12]
293 used mass spectra and retention times to match corresponding peaks across metabolite
294 samples from rat plasma; a true-positive rate of 70 % was determined. Almstetter et al. [5]
295 spiked 20 standard compounds to *E. coli* extracts and optimized the peak matching
296 procedure until true-positive rate was 100 %. This is in accordance with results reported
297 here, except that our peak matching optimization calculates threshold values using simple
298 and intuitive peak descriptors, i.e., retention time standard deviations and mass spectrum
299 match factor standard deviations, while other approaches adopted iteratively measured
300 threshold values requiring extensive computational work [5,8,9,12]. Thus, optimization of

301 matching parameters can be done routinely using a training set of sample chromatograms
302 and relative standard deviations of matching parameters calculated from peak features.
303
304 **3.1.2 Precision**
305 Two levels of precision [26], *repeatability* and *intermediate precision*, were evaluated.
306 The *repeatability* of the separation method was assessed by analyzing standard roasted
307 Romana and self-roasted Romana samples in single weeks, with the same instrument,
308 laboratory, and operator, and the *intermediate precision* was calculated over a period of
309 three weeks. *Repeatability* and *Intermediate precision* were calculated as relative standard
310 deviations (RSD %) for chromatographic peak volumes (normalized volume *vs.* ISTD) and
311 retention times (^{1}D and ^{2}D t_R) for each analyte in each validation week and over all weeks.
312 Results showed a good intermediate precision for retention times, with maximum averaged
313 RSD values of 1.0 % and 3.2 % for ^{1}D and ^{2}D respectively (**Table 1)**.
314 A higher dispersion is evident for quantitative data (normalized volumes). The
315 normalized volumes for standard roasted Romana samples were submitted to the Analysis
316 of Variance (ANOVA) to evaluate the comparability of data between weeks. The One-Way
317 ANOVA on the nine sample replicates collected over the three-weeks revealed that the null
318 hypothesis, "there is no difference between normalized volume values measured in different
319 weeks" could be accepted for each of the 22 target compounds (excluding the ISTD) with
320 $p <= 0.05$. The RSD % on normalized volumes showed an average value of 12.4 %, which is an
321 acceptable intermediate precision (**Table 1)**. The highest RSD % value was 47.6 % for peak 15
322 (nonanal) probably related to its long tailing, as already mentioned above.
323 The average RSD % of normalized volumes of the self-roasted Romana samples is,
324 however, larger (i.e. 46 %). This increase of dispersion is remarkable and originates from
325 sample processing, and not from the analytical procedure. With the designed validation
326 protocol, it has been possible: *(a)* to estimate the magnitude of this external source of
327 variation and *(b)* to evaluate better fingerprinting accuracy in the cross-comparison of real-
328 world samples. The dispersion registered for the self-roasted Romana samples was
329 considered by assigning a suitable uncertainty interval to fingerprinting results.
330
331 **3.1.3 Accuracy**

332 The accuracy of the *Comprehensive Template Matching* fingerprinting data was
333 verified [20] on nine standard roasted Romana samples and nine self-roasted Romana
334 samples acquired over the entire validation period and considering all separated and
335 detected 2D peaks. The extension of the validation procedure to the entire chemical pattern
336 (i.e., all 2D peaks above the fixed threshold) is required to evaluate fingerprinting accuracy
337 as a function of the chromatographic performance (specificity and precision) and data
338 elaboration parameters (specificity). Therefore, chromatograms were processed with an
339 optimized procedure to match consistently all detected analyte peaks across GC×GC
340 patterns collected on different days, within three weeks.

341 First, a *Sample Template* for each chromatogram of the set was created by including
342 all detected peaks exceeding the fixed S/N and area threshold (see section *Validation*
343 *protocol and data elaboration*). Each non-targeted peak included in each *Sample Template*
344 was saved with its ^{1}D and ^{2}D retention times, detector response, mass fragmentation
345 pattern, match factor threshold and with a unique number. This number was assigned to a
346 non-targeted analyte peak just after successful matching (**Figure 1**).

347 However, small variations of overall peak intensities were observed within the set.
348 **Figure 3** shows the distribution of S/N values of detected peaks across replicate
349 chromatograms. The histogram evidences that: (*a*) the S/N-threshold for peak detection
350 should be sufficiently low, otherwise most of the peak information is discarded, and (*b*) the
351 S/N values, like 2D peak volumes, are normally dispersed. This means that S/N values of low
352 abundant peaks can scatter around the peak detection limit and hence can sometimes be
353 detected/matched and sometimes not (**Figure 4a**). Thus, only analytes with S/N values
354 always above the peak-detection limit were uploaded into the *Sample Template* to keep a
355 consistent peak-matching. This was automatically done using internal standardization and
356 template thresholds that are higher than the peak detection limit (**Figure 4b**). This procedure
357 enables reduction of the rate of mismatches resulting from template peaks that cannot be
358 matched with the corresponding analyte peaks, because they were not detected. The
359 template threshold was calculated for every chromatogram adopting the S/N of α- and β-
360 thujone (ISTDs). The value was fixed as three times the standard deviation of S/N values
361 from the least intense chromatogram peaks (i.e., those peaks just above the peak detection
362 limit) multiplied by the ISTD S/N abundance. The resulting thresholds are reported in **Table**
363 **2**.

364 The closeness of agreement (i.e., accuracy) for the fingerprinting was verified through
365 a *pair-wise comparison* showing that the method achieves a high similarity rate, which is
366 always within a fixed interval and always above a certain value (**Table 2**) for samples of the
367 same origin and roasting conditions acquired over the entire validation period. The similarity
368 rate is here expressed as percentage of matched peaks between sample pairs: a maximum of
369 100% is expected for patterns obtained by analyzing the same sample even over an
370 extended time period.

371 The results show that arbitrarily selected *Sample Templates* reliably match the
372 chromatograms of all replicates (**Table 2**). Accuracy results, expressed as percentage of
373 matched peaks, ranged from a minimum of 91 % (for the sample acquired in week-one, day-
374 two) to a maximum of 95 % with 2 % RSD (standard roasted hazelnut samples). The
375 percentages of matched peaks of self-roasted hazelnut samples ranged from a minimum of
376 85 % (for the sample acquired in week-three, day-three) to a maximum of 95 % with 3 %
377 RSD. As a consequence, the peak matching performance proved to be robust for samples
378 with high dispersive quantitative values (standard roasted samples 12.4 RSD % and self-
379 roasted samples 46.2 RSD % on normalized peak volumes).

380 The qualitative differences in peak patterns are important and should consistently be
381 "extractable" by compensating retention time shifts (cf. 3.1.1) and dispersive response
382 values through appropriate template threshold values. On the contrary, problems with
383 consistent peak matching are often reported in literature. Oh et al. [8] compared eight
384 replicate chromatograms of a derivatized fatty acid/organic acid mixture with eight replicate
385 chromatograms of a derivatized fatty acid/amino acid mixture and resulted in a list with
386 8683 features, while only 46 were expected to be generated from standard compounds.
387 Performing a metabolite profiling on wild-type *vs.* double mutant *E. coli* strains, Almstetter
388 et al. [5] obtained a list of 2259 features from nine replicates per sample group (a peak
389 detection threshold of 500 was fixed). To limit the number of entries, the authors excluded
390 those features that could not be matched in at least 9 of 18 samples resulting in 398 peaks.
391 Castillo et al. [11] studied the metabolic profile of 60 human serum samples, from which
392 14756 features were extracted, but only 1013 features were found to be useful. Kim et al.
393 [12] aligned 5 replicate samples of rat plasma with an average of 446 analyte peaks per 2D
394 chromatogram, but just 146 peaks could be matched throughout all replicates. Although
395 these works might have handled differently complex peak patterns (depending on sample

396 composition, sample preparation and injection technique), the evaluation and optimization
397 of fingerprinting accuracy could help to reliably extract their true qualitative differences.
398 In our study, analyte peaks (on average 166 for each standard roasted sample
399 chromatogram) could successfully be extracted from the larger number of detected peaks (in
400 average 387 for standard roasted samples) after a systematic optimization of data
401 elaboration parameters. As a consequence, analyte peaks could reliably be matched across
402 replicate chromatograms acquired over three weeks with an average of 94 % of true-positive
403 peak matches. Manual investigation of the missing peak matches revealed that especially
404 coeluting compounds were not reliably detected by the software (**Figure 2**). This limit might
405 be overcome by adopting a suitable deconvolution/unmixing algorithm for unresolved
406 peaks.
407
408 **3.1.4 Uncertainty**
409 The goal of the fingerprinting is to reveal qualitative and quantitative differences
410 within a set of samples. As a consequence, the uncertainty [20,21] should account for the
411 dispersion of the quantitative data, mainly influenced by sample preparation and
412 chromatographic separation, and the consistency of the qualitative data (% of matched
413 peaks) mostly influenced by method specificity and accuracy.
414 The combined standard uncertainty can be calculated through the classical equation [20]:
415
416 $[u(y)/y]^2 = [u(y)_A/y]^2 + [u(c)_B/c]^2 + [u(d)_B/d]^2 + [u(f)_B/f]^2 + [u(p)_B/p]^2$
417 Equation 1
418 where $u(y)/y$ is the combined standard uncertainty for the measurand y; $u(y)_A/y$ is the
419 uncertainty referred to as repeatability and intermediate precision data; $u(c)_B/c$ is the
420 uncertainty derived from calibration data; $u(d)_B/d$ is the uncertainty derived from dilution;
421 $u(f)_B/f$ is the uncertainty derived from the efficiency of the method; and $u(p)_B/p$ is the
422 uncertainty derived from errors on weight.
423 The contribution to the combined uncertainty of the method of sample weighing and
424 dilution were negligible being respectively 1.0E-6 and 1.0E-4. Calibration was not included in
425 this analytical procedure; thus, the uncertainty range, to be associated to normalized
426 volumes after successful peak matching, was expressed through $u(y)_A/y$ (i.e., *Repeatability* of

427 the method). The relative uncertainty associated with the quantitative results was calculated
428 as +/-12.4 %.
429 On the other hand, the standard uncertainty for the *Comprehensive Template*
430 *Matching* fingerprinting to be associated with the result of the cross-comparison of samples
431 and expressed as percent of matched peaks, was referred to as the accuracy of the data (i.e.,
432 % error) of the peak-based fingerprinting. The relative uncertainty associated with the
433 fingerprinting results was +/-6 %.
434
435 **3.2 Application of the validated pair-wise Comprehensive Template Matching**
436 **fingerprinting in profiling the roasting process of hazelnuts.**
437 *Comprehensive Template Matching* fingerprinting has been demonstrated to be a
438 general tool to compare sample fingerprints without any knowledge of sample composition.
439 To be truly comprehensive, the fingerprinting procedure should evaluate the complete peak
440 information and, as a consequence, all 2D analyte peaks of all sample chromatograms should
441 accurately be aligned.
442 This is demonstrated by studying the effect of roasting at different time-temperature
443 profiles on hazelnut volatile development. In view of this, the validated *pair-wise*
444 comparison method was applied to extract temperature-sensitive *features* from a
445 representative sample subset (here Tonda Gentile). The resulting *consensus template* then
446 was used to extract qualitative/quantitative data on analytes in all 23 chromatograms. This
447 was done to verify the independence of the potential marker compounds from the
448 geographical origin and variety/cultivar of the hazelnuts. The *consensus template* was
449 created according to the procedure described in *validation protocol and data elaboration*
450 section (**Figure 1**).
451 Marker compounds indicating roasting should be detectable at an early stage of heat
452 treatment. The potential marker compounds are detectable within 7 minutes of roasting,
453 making it reasonable to create a *consensus template* for the Tonda Gentile with samples of 0
454 and 7 minutes roasting time. The c*onsensus template* of the sample pair was next matched
455 with all chromatograms of the Tonda Gentile subset. The average matching rate was 88 %
456 (*n*=5, i.e., 0, 7, 12, 17 and 23 minutes roasting) across all chromatograms. Peak data were
457 automatically aligned. The resulting list, containing the respective normalized peak volume
458 values, was sieved to define significant *features* (**Table 3**). The fold changes of increasing

459 response values were calculated relative to the peak responses of the 7 min roasted
460 hazelnut sample. A two-fold increase (corresponds to minimum +200%; **Table 1**) was
461 required to define a significant *feature* for self-roasted hazelnut samples. Finally, 24 of the
462 83 features were regarded as "significant indicators" for the roasting process of Tonda
463 Gentile samples. Ongoing data reduction addressed potential roasting markers that were
464 independent of geographical origin or variety. Thus, normalized peak volume values of the
465 selected 24 supposed markers were extracted from all chromatograms of the entire sample
466 set, using specific quantifier ions (**Table S3**), and submitted to a Principal Component
467 Analysis (PCA)

468 PCA was used for an unsupervised analysis and was performed initially on each
469 variety/origin independently (**Figure S1**) to show the degree of correlation between
470 potential marker compounds and roasting time. The first principal component (roasting
471 degree) explained on average 71 % of the total variance. Several marker *features* had
472 loadings >+/-0.6 on component 2, indicating dependence on an unknown factor. *Features*
473 with loadings of more than 0.6 for component 1 and loadings of <+/-0.6 for component 2
474 were deemed strong markers for "roasting degree" thus enabling the removal of visually
475 recognizable "outliers". The number of potential markers was thereby reduced from 24 to
476 11 (**Table 3**). Linear regression analysis on normalized volume values of the proposed 11
477 marker compounds for all hazelnut samples (**Figure 5**) revealed a nearly proportional
478 relationship between increased roasting time and increased normalized peak volume values,
479 with R^2 of 0.8147. A look backward into raw data (**Figure 3**) revealed that normalized
480 volumes of markers, e.g., 2,3-pentandione and unknown compound **81**, steadily increased
481 with roasting time, whereas 5-methyl-(E)-2-hepten-4-one and unknown compound **79**,
482 excluded on the basis of the PCA screening, reached their maximum after 12 minutes (**Figure**
483 **S1, S3**).

484 This *Comprehensive Template Matching* fingerprinting method combined with a
485 food-type intervention study has proven to be reliable and straightforward to select
486 significant peak data from a set of 23 unique sample files. The simple linear model derived
487 from marker compounds, for example, allows an interpolation and prediction of roasting
488 time, thereby facilitating automated food processing. With manual roasting, the marker
489 compound responses considerably vary (Figure 5) limiting the possibility to distinguish
490 between smaller time intervals. These results also provide deep insights into the formation

491 of volatile compounds during roasting and differences emerging from different varieties of
492 different origin. Alasalvar et al. reported on the formation of 71 volatile compounds from
493 hazelnuts from Tombul (Turkey) [27]. Burdack-Freitag and Schieberle [22] recently
494 investigated the formation of key aroma compounds during roasting of Tonda Romana (Italy)
495 hazelnuts, showing that 2-methyl-butanal (**8**, malty), 2,3-pentandione (**19**, buttery),
496 phenylacetaldehyde (**77**, flowery), and 5-methyl-(E)-2-hepten-4-one (**52**, filbertone, nutty)
497 have an important impact on the aroma of roasted hazelnuts. However, GC×GC-qMS has
498 been used in this study to explore systematically the effect of roasting at different time
499 intervals on the formation of odor-active as well as odorless volatile compounds.
500 Concentrations of identified odorants **8**, **19**, **77** (2-methylbutanal, 2,3-pentandione,
501 phenylacetaldehyde) increase almost linearly with roasting time independently on variety
502 suggesting them as suitable marker compounds to predict the degree of roasting. Future
503 studies could investigate whether these odorants can evoke different distinct aromas
504 despite their similar release profiles across different hazelnuts. 1-Methyl-pyrrole (**29**),
505 pyridine (**35**), 3-hydroxy-2-butanone (**50**), 2-ethyl-pyrazine (**54**), furfural (**66**) and the three
506 unknowns **23**, **73**, **81** belong to the group of proposed roasting markers (**Table 3**). Some of
507 them, e.g., furfural and pyrazines are known Mailliard reaction products [28,29]. It is
508 assumed that the reaction of amino compounds and reducing sugars is mediated by heat
509 and fits well with our observation of almost linear increase with increasing roasting time. In
510 contrast, a second group of temperature-sensitive compounds has been identified (**13**, **22**,
511 **36**, **41**, **45**, **52**, **58**, **63**, **71**, **74**, **78**, **79**, **80**). They are not roasting marker compounds, because
512 they lack in the typical release profile at least in one variety (**Table 3**). For example, 5-
513 methyl-(E)-2-hepten-4-one is similarly released in the Gentile and Giffoni samples, whereas
514 its level in Azerbaijan sample remains low (**Figure S3**). According to this, compound *79* tracks
515 approximately the same profile for the Gentile, Giffoni and Romana samples, but not for the
516 Azerbaijan hazelnuts (**Figure S3**). The release profiles of these components show that their
517 normalized volume values rapidly increase and remain constant or decrease with longer
518 roasting times (17-23 min). The formation of these ketones is still unclear, although **52** was
519 suggested to originate from a yet unknown precursor [23].
520

4.Conclusions

This study presents a systematic approach to evaluate the *fitness for purpose* of a peak-based fingerprinting method, Comprehensive Template Matching fingerprinting. The reliability of the proposed method was confirmed by employing performance parameters such as specificity, precision, accuracy, and uncertainty [30], and following a general validation protocol based on Eurachem/Citac guidelines [19,20].

The results of the comparative 2D data analysis were improved by properly compensating the dispersion of detector response values through an appropriate tuning of the main data elaboration parameters (i.e., ^{1}D and ^{2}D retention times search windows, MS match factor threshold, detection threshold, and template threshold) with two sets of training samples showing different degrees of analytical variance (i.e., industrially and manually prepared sample material). Optimization was done by non-iterative standardized procedures that could be fully automated by the GC-Image software.

The validated non-targeted, peak-based fingerprinting method has successfully been applied to elucidate the generation of volatile compounds during roasting in a set of 23 hazelnut samples, where 11 roasting markers were identified, and to study the release of key aroma compounds showing specific profiles as a function of variety/origin of hazelnut samples.

Further investigations are under way: a) to study the effect of data elaboration settings on the result quality when, for example, high density data from high frequency MS detectors or high resolution MS are studied and b) to investigate a possible correlation between concentration changes of character-impact compounds with overall odor impressions, to identify further unknown significant *features* and to clarify formation pathways explaining observed trends.

Acknowledgments

The results reported in this chapter regarding work in the authors' laboratory were obtained within the "ITACA" and "ECOFOOD" projects of the POR-FESR "Competitività regionale e occupazione" 2007/2013, Asse 1, Misura I.1.1, "Piattaforme innovative" of the Piedmont Region (Italy).

References

[1] M. Adahchour, J. Beens, U.A.T. Brinkman, J. Chromatogr., A 1186 (2008) 67.

[2] K.M. Pierce, J.C. Hoggard, R.E. Mohler, R.E. Synovec, J. Chromatogr., A 1184 (2008) 341.

[3] C. Cordero, E. Liberto, C. Bicchi, P. Rubiolo, S.E. Reichenbach, X. Tian, Q. Tao, J. Chromatogr. Sci. 48 (2010) 251.

[4] X. Li, Z. Xu, X. Lu, X. Yang, P. Yin, H. Kong, Y. Yu, G. Xu, Anal. Chim. Acta 633 (2009) 257.

[5] M.F. Almstetter, I.J. Appel, M.A. Gruber, C. Lottaz, B. Timischl, R. Spang, K. Dettmer, P.J. Oefner, Anal. Chem. (Washington, DC, U. S.) 81 (2009) 5731.

[6] C. Cordero, E. Liberto, C. Bicchi, P. Rubiolo, P. Schieberle, S.E. Reichenbach, Q. Tao, J. Chromatogr., A 1217 (2010) 5848.

[7] C. Cordero, C. Bicchi, P. Rubiolo, J. Agric. Food Chem. 56 (2008) 7655.

[8] C. Oh, X. Huang, F.E. Regnier, C. Buck, X. Zhang, J. Chromatogr., A 1179 (2008) 205.

[9] B. Wang, A. Fang, J. Heim, B. Bogdanov, S. Pugh, M. Libardoni, X. Zhang, Anal. Chem. (Washington, DC, U. S.) 82 (2010) 5069.

[10] M.F. Almstetter, I.J. Appel, K. Dettmer, M.A. Gruber, P.J. Oefner, J. Chromatogr., A 1218 (2011) 7031.

[11] S. Castillo, I. Mattila, J. Miettinen, M. Oresic, T. Hyotylainen, Anal. Chem. (Washington, DC, U. S.) 83 (2011) 3058.

[12] S. Kim, A. Fang, B. Wang, J. Jeong, X. Zhang, Bioinformatics 27 (2011) 1660.

[13] S.E. Reichenbach, X. Tian, C. Cordero, Q. Tao, Journal of Chromatography A, In press:DOI: 10.1016/j.chroma.2011.07.046 (2011).

[14] S.E. Reichenbach, M. Ni, D. Zhang, E.B. Ledford, J. Chromatogr., A 985 (2003) 47.

[15] I. Latha, S.E. Reichenbach, Q. Tao, J. Chromatogr., A 1218 (2011) 6792.

[16] M. Ni, S.E. Reichenbach, A. Visvanathan, J. TerMaat, E.B. Ledford, J. Chromatogr., A 1086 (2005) 165.

[17] C.G. Fraga, B.J. Prazen, R.E. Synovec, Anal. Chem. 73 (2001) 5833.

[18] D. Zhang, X. Huang, F.E. Regnier, M. Zhang, Anal. Chem. (Washington, DC, U. S.) 80 (2008) 2664.

[19] The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics., Eurachem Guide, 1998, p. http://www.eurachem.org/guides/pdf/valid.pdf.

[20] Guide to Quality in Analytical Chemistry, CITAC/Eurachem Guide, 2002, p. http://www.eurachem.org/guides/pdf/CITAC%20EURACHEM%20GUIDE.pdf.

[21] S.E. Reichenbach, P.W. Carr, D.R. Stoll, Q. Tao, J. Chromatogr., A 1216 (2009) 3458.

[22] A. Burdack-Freitag, P. Schieberle, J. Agric. Food Chem. 58 (2010) 6351.

[23] W. Silberzahn, R. Tressl, Prog. Flavour Precursor Stud. Proc. Int. Conf. (1993) 147.

[24] Y. Wang, J. O'Reilly, Y. Chen, J. Pawliszyn, J. Chromatogr., A 1072 (2005) 13.

[25] L. Setkova, S. Risticevic, C.M. Linton, G. Ouyang, L.M. Bragg, J. Pawliszyn, Anal. Chim. Acta 581 (2007) 221.

[26] ISO 3534-1, Statistics - Vocabulary and Symbols, International Organization for Standardization, 1998.

[27] C. Alasalvar, F. Shahidi, K.R. Cadwallader, J. Agric. Food Chem. 51 (2003) 5067.

[28] C. Cerny, W. Grosch, Z. Lebensm.-Unters. Forsch. 198 (1994) 210.

[29] H.-D. Belitz, W. Grosch, P. Schieberle, Food Chemistry, Springer, Berlin Heidelberg, 2004.

[30] C. Cordero, C. Bicchi, D. Joulain, P. Rubiolo, J. Chromatogr., A 1150 (2007) 37.

Figure legends

Figure 1: Work flow adopted for the *Comprehensive Template Matching* fingerprinting of hazelnut samples under study.

Figure 2: Effect of user-defined retention time window and MS match factor thresholds on consistent peak matching. 2a) Only a retention time window constraint was defined: 2 of 8 overlaid template peaks (thin circles) were correctly matched and 6 were erroneously matched; 2b) The retention time window and a MS match factor threshold (600) were defined: all analyte peaks were correctly matched; 2c) Restrictive rules have been applied (retention time window and MS match factor threshold of 774): only 3 of 8 peaks were correctly matched.

Figure 3: Histogram reporting the dispersion of signal-to-noise ratios (S/N) of all detected peaks of six replicate sample chromatograms of Standard Roasted Romana hazelnuts acquired over the three weeks of validation.

Figure 4: a) Signal-to-noise-ratio (S/N) of a low abundant peak measured on nine days in three weeks. A peak can be consistently matched across the nine 2D chromatograms, if the peak is always detected, which means that the peak's S/N is always above the user-defined peak detection limit (here S/N 10 indicated by the lower line). Such peaks are recognized using template thresholds for Sample Template development (indicated by upper line at S/N 30) . Asterisks label peaks that would be detected and matched, if the peak detection limit would be set to S/N 30 (inconsistent peak matching indicating false marker compounds); b) S/N of the internal standard α-thujone was used for calculating the template threshold for each 2D chromatogram.

Figure 5: Linear correlation of 11 potential marker compounds with roasting time (0-7-12-17-23 min, 11x4 data points) from four different hazelnut varieties/origins.

Captions to Tables

Table 1: Target analytes adopted for method performance evaluation on Standard Roasted and Self-roasted Romana samples over a period of three weeks*.

Table 2: Results of the pair-wise comparison of replicate analyses by *Comprehensive Template Matching*. Replicates were acquired during the three-week long validation period at different days in the week (1-3).

Table 3: Primary output of the non-targeted analysis of differently roasted hazelnuts (7-12-17-23 minutes) using *Comprehensive Template Matching* on Gentile sample subset.

Supplementary files

Table S1: detailed RSD % data (week 1, week 2, week 3) according to table 1.

Table S2: Experimental design giving the number of replicates per sample and total number of analyses runs. Standard and Self-roasted Romana samples as well as blank and alkane runs were acquired for validation purposes.

Table S3: secondary data output from non-targeted analysis. Peak features were identified and markers were confirmed using selective quantifier ions.

Figure S1: PCA-plots of four different hazelnut samples a) Azerbaijan b) Tonda Gentile from Piedmont c) Tonda Romana from Lazio and d) Tonda Giffoni from Campania. The 2D Normalized Volume values of compounds (numbered rectangular) showing increasing concentrations during hazelnut roasting were analyzed. Some compounds show consistently high correlation with the factor “roasting degree” (component 1) despite the variety/origin.

Figure S2: Ten most temperature-sensitive roasting markers (cf. table 3 for peak numbering) of Gentile hazelnuts. Fold changes were calculated relative to 7 min normalized volume value (7 min = 1).

Figure S3: Release profiles of potential roasting markers 2,3-pentanedione and unknown 81. 5-Methyl-(E)-2-hepten-4-one and unknown compound 79 were excluded from marker screening, because releases were inconsistent across all hazelnut samples (cf. 3.2).

Table 1: Target analytes[#] adopted for method performance evaluation on Standard Roasted and Self Roasted Romana samples over a period of three weeks*.

			absolute retention times				normalized response		match factor	
ID	**compound**	I^T_S	**^{1}D t_r (min)**	**week 1-3 (RSD %)**	**^{2}D t_r (s)**	**week 1-3 (RSD %)**	**peak volume**	**week 1-3 (RSD %)**	**MS spectra**	**week 1-3 (RSD %)**
1	2,3-pentanedione[#]	1041	8.6	1.2	1.1	2	0.4	9.1	767	5
2	2,2-dimethyl-3-hexanone	1107	10.5	1	1.8	1.9	0.09	17.2	771	14
3	(E)-3-penten-2-one	1115	10.8	0.9	1.3	2.4	1.29	7.6	765	17
4	pyridine	1169	13	0.9	1.2	3.2	0.17	15.6	781	3
5	5-methyl-(Z)-2-hepten-4-one[#]	1182	13.5	0.8	2.2	1.4	0.13	11.7	848	6
6	3-methyl-3-penten-2-one	1185	13.7	0.8	1.6	1.6	0.14	13.9	771	12
7	1-pentanol	1238	16	0.8	1.2	3.7	0.11	9	839	2
8	2-methylpyrazine	1252	16.6	0.7	1.3	2.9	0.96	7.6	871	2
9	5-methyl-(E)- 2-hepten-4-one[#]	1280	17.9	0.6	2.3	2.1	1.44	12	867	6
10	2,5-dimethylpyrazine[#]	1311	19.3	0.7	1.6	2.8	0.55	8.1	816	13
11	2-ethylpyrazine	1321	19.8	0.6	1.6	3	0.37	10.2	829	2
12	2,3-dimethylpyrazine	1334	20.4	0.6	1.6	3.7	0.08	7.5	813	3
13	1-hydroxy-2-butanone	1362	21.8	0.6	1.2	4	0.09	8.2	861	5
14	2-ethyl-5-methyl-pyrazine	1373	22.3	0.6	1.9	2.9	0.15	11.9	838	2
15	nonanal[#]	1383	22.8	0.5	2.9	1.9	0.28	47.6	779	11
16	2,3,5-trimethylpyrazine[#]	1393	23.3	0.5	1.8	3.2	0.15	7.6	680	3
17	3-ethyl-2,5-dimethyl-pyrazine[#]	1434	25.3	0.5	2.1	2.9	0.11	11.1	804	3
18	furan-2-carbaldehyde	1451	26.1	0.5	1.2	5	1.6	12.5	901	6
19	benzaldehyde[#]	1509	28.9	0.4	1.5	3.9	0.18	17.7	680	10
20	2-phenylacetaldehyde[#]	1628	34.4	0.5	1.6	4.1	0.14	17.4	730	3
21	2-furanmethanol	1649	35.4	0.4	1.1	5.4	0.78	13.6	766	10
22	phenylmethanol	1859	44.5	0.4	1.4	7.4	0.03	12.3	722	5
23	α-thujone[#]	1409	24	0.5	3	2.7	1	0	880	2
24	β-thujone[#]	1428	24.9	0.5	2.9	2.2	0.07	7.5	848	8
	α-thujone raw peak volume							*23.5*		
	β-thujone raw peak volume							*29.7*		
	average of standard roasted Romana			0.6		3.2		12.4	801	6
	average of self roasted Romana			1		2.8		46.2	774	9

[#]: Analytes were identified with authentic standards (linear retention index (I^T_S), ^{2}D absolute retention time, EI mass spectrum); all other analytes were tentatively identified (linear retention indices and mass spectra identical to data reported in literature).

* Detailed RSD % data on each single week (w1, w2 and w3) are shown in Supplementary Information Table 1.

Table 2: Results of the pair-wise comparison of replicate analyses by *Comprehensive Template Matching*. Replicates were acquired during the three-week long validation period at different days in the week (1-3).

Validation Week	**1**	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	
Validation Day	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**RSD %**
Replicate analysis of standard roasted Romana samples										
Number of peaks above peak detection limit[a]	487	395	470	431	321	325	357	342	354	
Template threshold [S/N][b]	54	30	51	31	33	25	30	33	30	29
Number of template peaks[c]	172	168	162	205	150	159	156	155	165	10
Percentage of matched peaks [%][d]	95	91	95	94	92	93	95	94	93	2
Error [%][e]	5	9	5	6	8	7	5	6	7	
Replicate analysis of self roasted Romana samples										
Number of peaks above peak detection limit[a]	478	640	436	579	552	624	605	618	501	
Template threshold [S/N][b]	47	144	30	157	85	93	132	90	98	43
Number of template peaks[c]	68	50	67	64	72	72	55	66	48	15
Percentage of matched peaks [%][d]	92	92	86	95	89	89	90	91	85	3
Error [%][e]	8	8	14	5	11	11	11	9	15	

a: Peak detection limit was fixed at S/N > 10.

b: Template threshold values were calculated relative to S/N of the ISTDs.

c: Number of template peaks, i.e. peaks exceeding the template threshold were loaded into the template after blank sample removal.

d: Percentage of matched peaks is the arithmetic mean of the pair-wise comparison of arbitrarily chosen templates with the other eight 2D chromatograms of the set (n=3).

e: Error % represents fingerprinting accuracy.

Table 3: Primary output of the non-targeted analysis of differently roasted hazelnuts (7-12-17-23 minutes) using *Comprehensive Template Matching* on Gentile sample subset.

no.[a]	1D t_r (min)	2D t_r (s)	fold change[b]				no.[a]	1D t_r (min)	2D t_r (s)	fold change[b]				no.[a]	1D t_r (min)	2D t_r (s)	fold change[b]			
			7	**12**	**17**	**23**				**7**	**12**	**17**	**23**				**7**	**12**	**17**	**23**
1	4.2	0.97	1.0	1.2	0.5	0.4	30	11.4	1.47	1.0	1.2	1.4	1.7	59	22.2	1.47	1.0	0.7	0.8	1.2
2	4.7	1.47	1.0	1.3	0.6	0.6	31	11.5	1.94	1.0	1.0	0.6	1.2	60	22.6	1.73	1.0	0.7	0.6	0.8
3	4.8	0.67	1.0	0.4	0.9	1.1	32	11.8	2.44	1.0	1.2	1.1	1.2	61	23.7	1.73	1.0	0.8	0.6	0.7
4	5.1	1.81	1.0	1.4	0.6	0.6	33	12.4	1.56	1.0	1.8	1.1	1.4	62	25.3	1.81	1.0	2.6	2.0	2.0
5	5.2	0.76	1.0	1.2	2.6	2.7	34	12.5	1.81	1.0	0.9	1.4	1.2	63	25.6	1.81	1.0	2.4	2.0	3.5
6	5.2	1.05	1.0	0.7	1.4	2.6	*35	13.1	1.18	1.0	2.3	3.2	5.5	64	25.7	0.88	1.0	2.9	2.3	2.8
7	5.4	1.64	1.0	1.4	0.7	0.6	36	13.6	2.15	1.0	11.4	4.5	8.9	65	25.8	1.60	1.0	1.0	2.3	1.7
*8	5.8	0.97	1.0	5.2	4.3	6.5	37	13.8	1.60	1.0	2.3	1.4	2.3	*66	26.0	1.22	1.0	6.5	8.4	11.6
9	6.3	1.01	1.0	2.1	2.8	2.0	38	13.8	2.23	1.0	1.9	2.7	2.6	67	26.8	1.81	1.0	1.5	1.3	2.6
10	6.6	1.56	1.0	1.7	1.2	1.6	39	14.0	1.85	1.0	1.5	2.0	2.2	68	27.1	1.98	1.0	1.3	1.4	1.2
11	6.8	1.14	1.0	0.5	0.4	0.5	40	14.1	1.14	1.0	1.2	0.5	1.2	69	27.4	1.94	1.0	0.3	0.7	0.9
12	7.4	1.47	1.0	1.3	1.6	2.4	41	14.4	2.15	1.0	6.8	2.6	4.3	70	27.6	1.73	1.0	1.4	0.9	1.1
13	7.6	1.05	1.0	1.5	3.7	3.8	42	14.5	1.64	1.0	0.6	0.2	0.2	71	27.8	1.98	1.0	2.8	3.4	2.6
14	7.6	1.39	1.0	1.7	1.5	2.6	43	15.8	1.94	1.0	1.8	1.7	2.3	72	29.2	1.64	1.0	1.0	1.0	0.6
15	7.7	0.93	1.0	0.6	0.5	0.3	44	15.9	1.09	1.0	1.8	1.4	1.4	*73	29.5	1.89	1.0	5.6	3.9	3.9
16	7.9	1.39	1.0	1.3	1.8	1.1	45	16.0	1.22	1.0	1.7	1.8	3.0	74	32.1	1.98	1.0	1.4	1.4	3.2
17	8.2	0.63	1.0	1.8	0.8	0.8	46	16.2	2.44	1.0	1.4	0.7	1.8	75	32.8	1.68	1.0	1.8	2.7	2.9
18	8.6	1.43	1.0	0.3	0.2	0.3	47	16.3	1.85	1.0	2.0	1.7	2.9	76	33.4	2.06	1.0	1.8	0.8	2.1
*19	8.7	1.05	1.0	2.3	3.3	3.5	48	17.0	1.98	1.0	1.1	1.5	1.4	*77	34.2	1.64	1.0	22.8	27.1	38.5
20	9.0	1.85	1.0	0.7	0.7	0.9	49	17.4	1.52	1.0	0.9	0.5	2.0	78	34.9	1.39	1.0	3.5	2.9	2.5
21	9.0	1.35	1.0	0.9	0.9	0.9	*50	17.6	1.14	1.0	2.7	2.9	3.9	79	37.1	1.52	1.0	4.9	1.4	4.0
22	9.8	1.26	1.0	2.0	2.0	3.8	51	17.6	1.64	1.0	0.8	0.8	1.1	80	37.2	1.22	1.0	1.4	2.1	3.9
*23	10.4	1.64	1.0	2.1	3.2	3.9	52	17.9	2.31	1.0	4.0	2.9	3.9	*81	38.0	1.26	1.0	2.0	0.9	6.2
24	10.6	1.73	1.0	1.2	1.7	1.6	53	18.4	2.02	1.0	2.2	2.4	2.2	82	44.6	1.09	1.0	0.6	0.6	0.8
25	10.7	1.05	1.0	0.3	0.3	0.5	*54	19.8	1.60	1.0	6.8	16.2	26.7	83	45.8	1.56	1.0	2.3	1.7	2.5
26	10.9	2.02	1.0	0.8	0.5	0.8	55	20.2	1.26	1.0	0.7	1.0	1.3							
27	11.0	1.26	1.0	1.5	1.5	1.6	56	20.9	1.43	1.0	1.4	2.1	1.5							
28	11.0	1.64	1.0	1.9	1.7	1.7	57	21.0	1.26	1.0	0.8	0.9	1.3							
*29	11.3	1.18	1.0	1.5	3.5	10.7	58	21.2	1.77	1.0	2.2	1.7	3.5							

Table 3 continued:

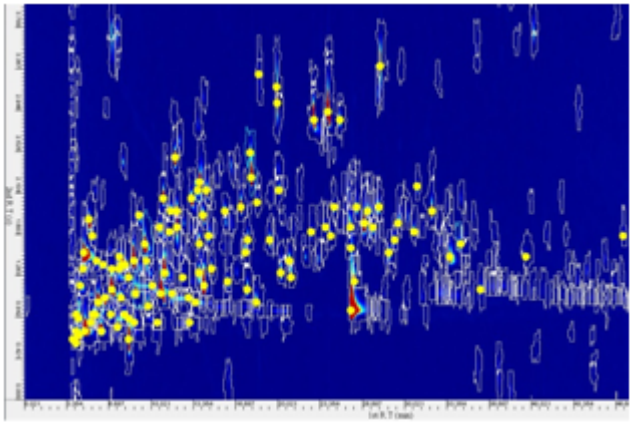
a: Features are reported together with the template peak numbering. A feature characterizes an analyte peak with ^{1}D and ^{2}D retention times and EI mass spectra.
b: Relative increase of Normalized Peak Volumes of 7 minutes roasted sample versus 12, 17 and 23 minutes roasted samples.
* An asterisk indicates analytes which were identified as roasting markers.

Figure 1

GC Image™
Background substraction
Blob detection above certain S/N ratio

Consensus template development [3,6],
every template peak gets an unique number

-> remove template peaks of blank samples
-> remove multiple entries



GC Project™
Consensus template is consecutivley matched with all chromatograms,
template peak number is assigned to positive matched analyte

-> apply correct 1st, 2nd rentention time window for peak pattern matching
-> apply correct MS match factor threshold
-> apply correct template threshold

Peak tables are automatically aligned according to the unique numbers,
response data of same analytes are sorted in one row

Figure 2

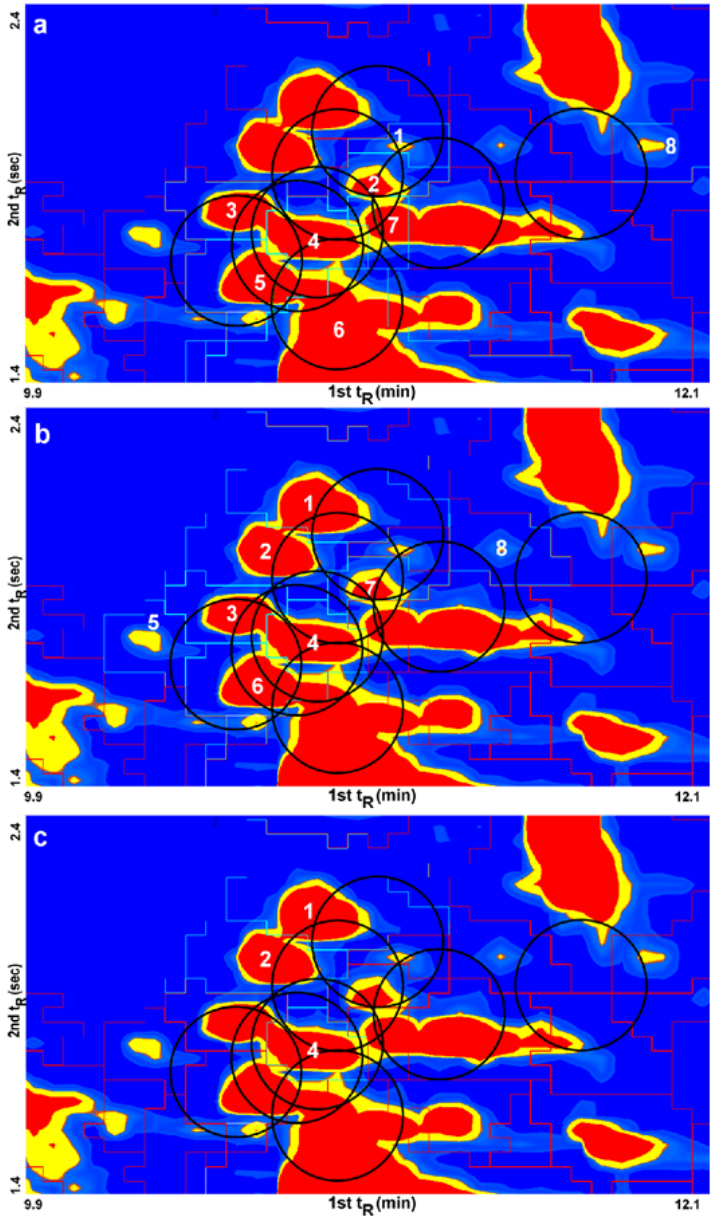


Figure 3

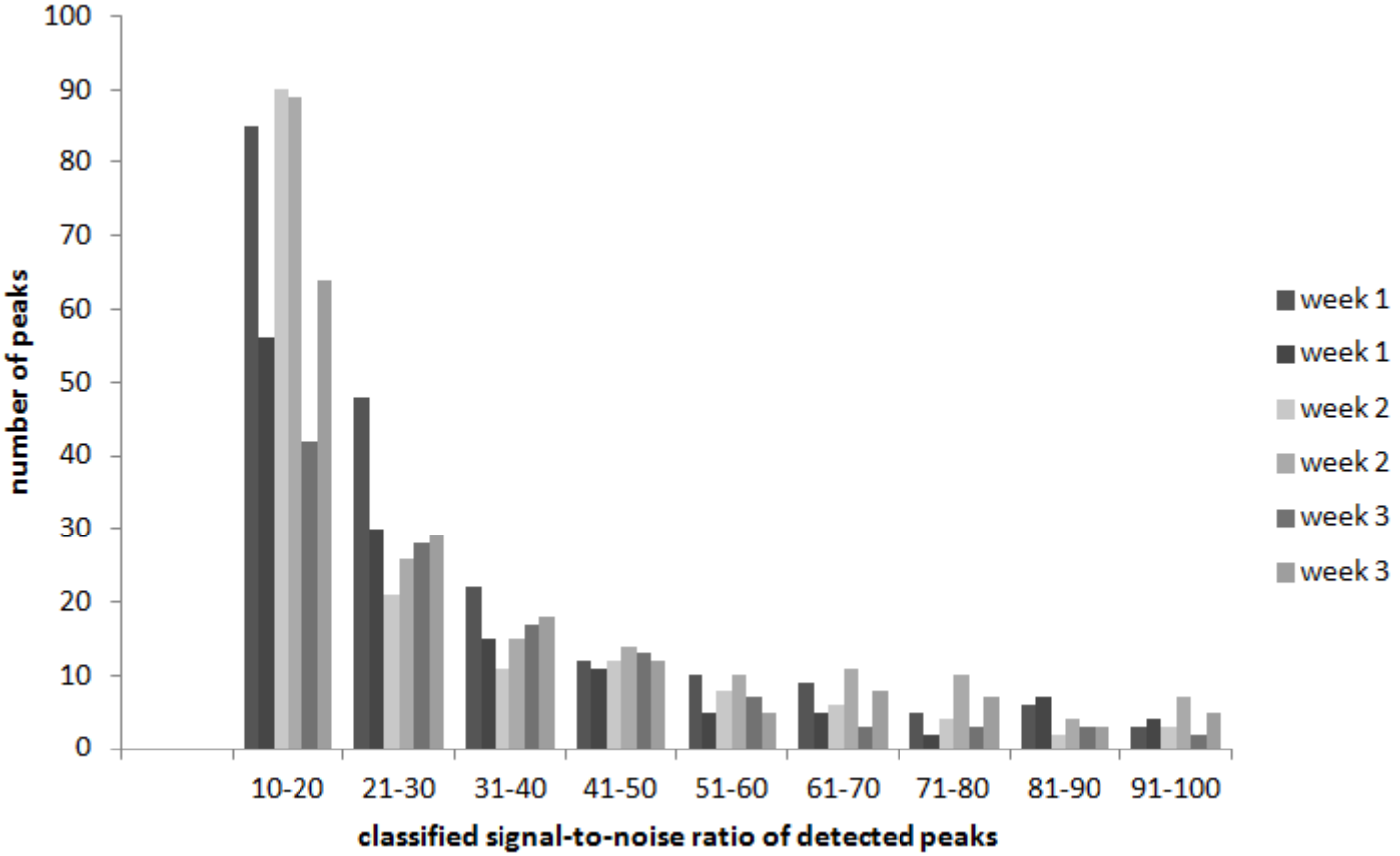
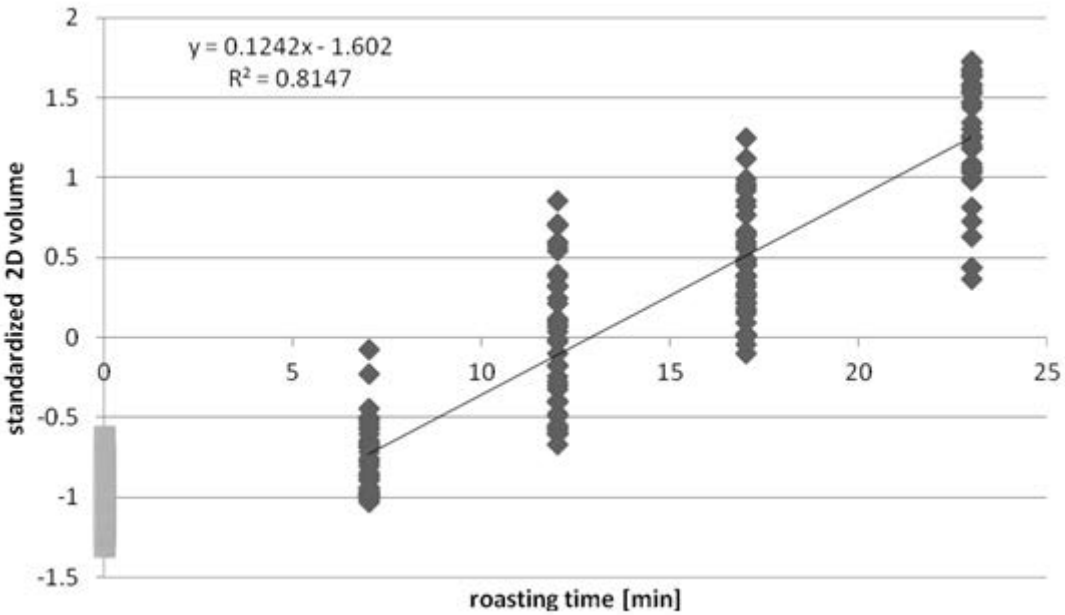
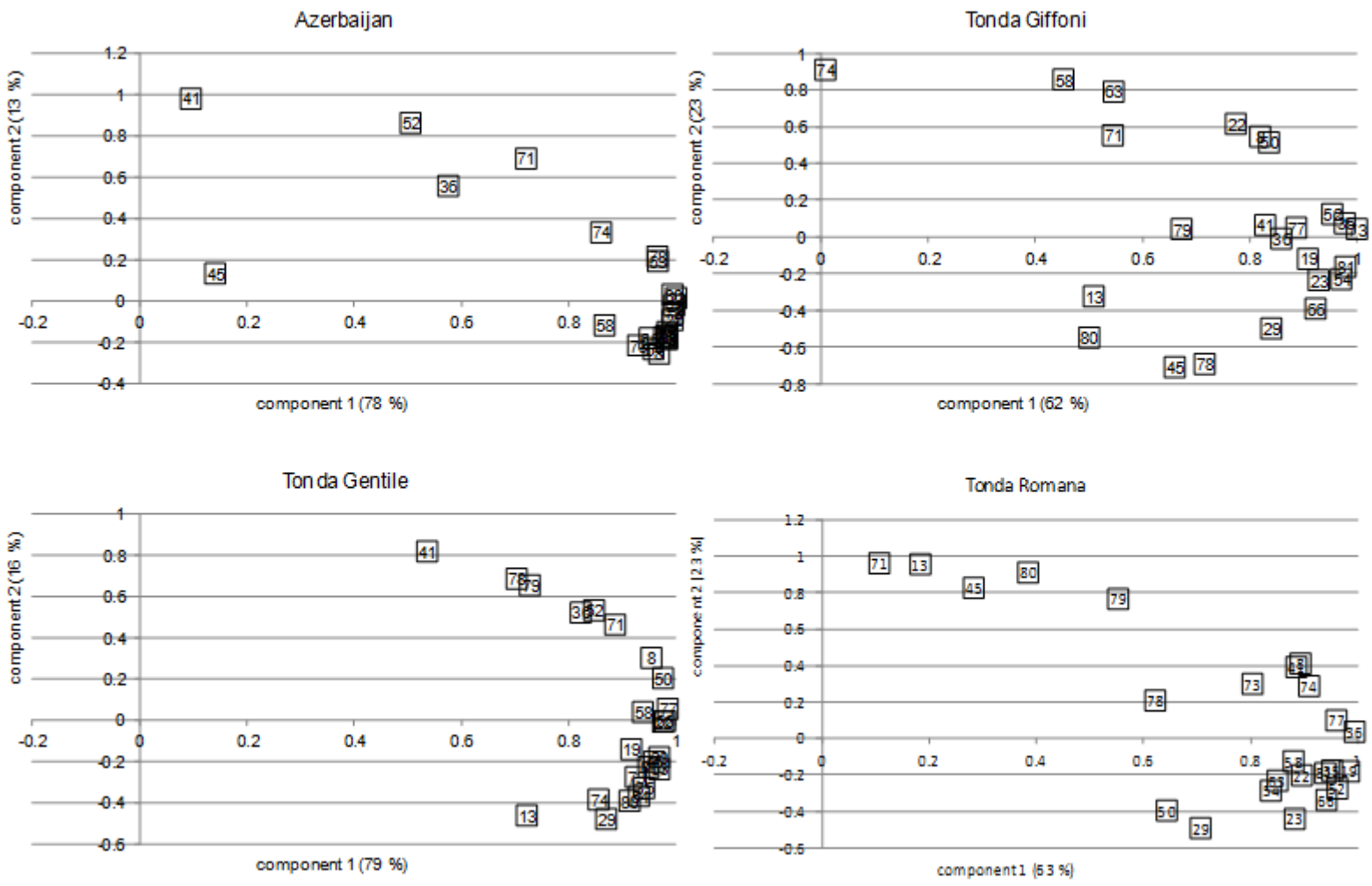

100
90
80
70
60
50
40
30
20
10
0
number of peaks
10-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
classified signal-to-noise ratio of detected peaks
week 1
week 1
week 2
week 2
week 3
week 3

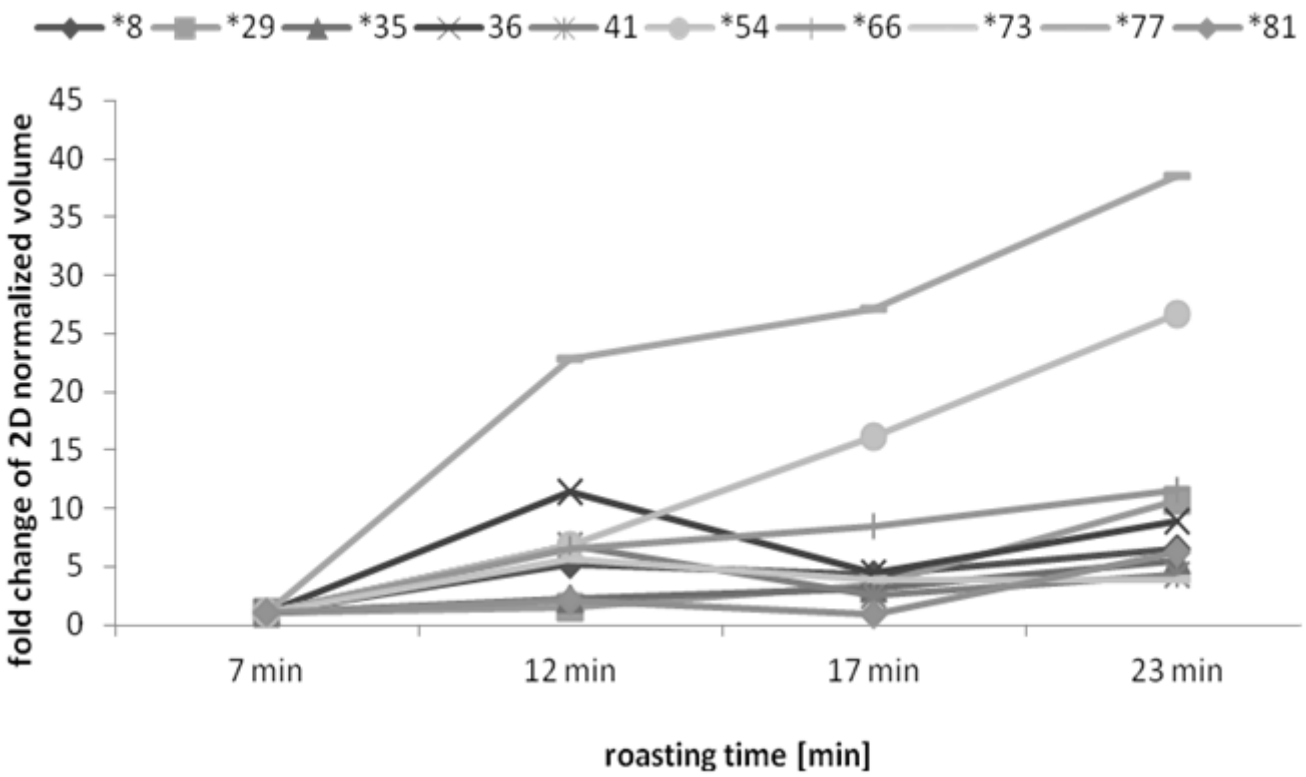
Figure 4


y = 0.1242x - 1.602
R² = 0.8147
standardized 2D volume
roasting time [min]
2
1.5
1
0.5
0
-0.5
-1
-1.5
0
5
10
15
20
25

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3