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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1753103> since 2021-02-23T10:23:57Z

Published version:

DOI:10.1016/j.foodchem.2020.127691

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(Article begins on next page)

1 **Cocoa smoky off-flavour: a MS-based analytical decision maker for routine**
2 **controls**

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8

10 Abstract

11 Cocoa smoky off-flavour is generated from an inappropriate artificial drying applied on beans to
12 speeding up the post-harvest process and it can affect the quality of the chocolate. The sensory
13 tests are time-consuming, and at present, a fast analytical method to detect this defect in raw
14 materials is not yet available. This study applies a HS-SPME-MS-enose in combination with
15 chemometrics to obtain diagnostic mass-spectral patterns to detect smoked samples and/or as
16 analytical decision maker. SIMCA models provide the best classification results, compared to PLS-
17 DA, with sensitivities exceeding 90% and a high class specificity range of 89-100% depending on
18 the matrix investigated (beans or liquors). Resulting diagnostic ions were related to phenolic
19 derivatives. The discrimination ability of the method has been confirmed by a quantitative
20 analysis through HS-SPME-GC-MS. HS-SPME-MS-enose turned out to be a fast, cost-effective and
21 objective approach for high throughput analytical screening to discard defective cocoa samples.

22

23

24 **Keywords:** cocoa volatiles; smoky off-flavour; phenolic derivatives; HS-SPME-MS-enose;
25 chemometrics; HS-SPME-GC-MS

26 1. INTRODUCTION

27 The quality of food is consumer-oriented and characterised by four closely interrelated
28 dimensions: hedonic profile, healthy characteristics, economic convenience and the production
29 process. Differences in quality assessment may have unfavourable consequences and lead to a
30 product not fulfilling the expectations of consumers. Flavour is a key food quality attribute (e.g.
31 safety and wholesomeness, physical characteristics, colour etc.) in cocoa-derived products. The
32 flavour of chocolate heavily influences the pleasure derived from its consumption and evokes
33 emotions, while also positively influencing the consumer's mood (Macht & Mueller, 2007;
34 Spence, 2017; Wagner, Ahlstrom, Redden, Vickers, & Mann, 2014). The evaluation of flavour and
35 its quality includes an appraisal of cocoa/chocolate intensity, particular flavour notes and the
36 absence of flavour defects. Quality is dependent on how food products are handled at every
37 touch point throughout the cocoa chain. Climate change, together with the global market
38 pressure in response to growing demand (Eghbal, 2018) influence post-harvest processing and
39 therefore affect the final flavour quality of the beans. The smoky off-flavour, in particular, can be
40 generated from the inappropriate or poorly controlled artificial drying of the beans, which is
41 performed to speed up moisture reduction (CABISCO/ECA/FCC, 2015; Perotti *et al.*, 2020; Serra
42 Bonvehí & Ventura Coll, 1998). The smoky off-flavour also strongly affects the quality of finished
43 chocolate (chocolate or confectionary) and cannot be eliminated during processing. This is
44 predominantly a problem for cocoa beans from Cameroon, in West-Africa, where cocoa
45 production originates from several small family farms, which must increasingly combat
46 unfavourable climate change to make a profit (Eghbal, 2018; Statista, 2020; Wessel & Quist-
47 Wessel, 2015). Some phenolic compounds, predominantly derived from lignin degradation by
48 pyrolysis, have been related to the smoky note (Janairo & Amalin, 2018; Serra Bonvehí, 1998;
49 Wang, Chambers, & Kan, 2018; Perotti *et al.*, 2020). Of the volatiles that make up the smoky note,

50 some components, such as guaiacols and methylphenols, have also been found to be cocoa key-
51 aroma compounds, but their presence at high concentrations can negatively affect cocoa's
52 sensory properties (Frauendorfer & Schieberle, 2006). In a previous article Perotti *et al.* (2020)
53 chemically characterised the smoky off-flavour of cocoa beans and liquors using a metabolomic
54 approach and a top-down strategy; HS-SPME was coupled with comprehensive GC equipped with
55 a time of flight mass spectrometer (HS-SPME-GC×GC-TOF-MS) for use as a screening platform to
56 identify informative odorants within a set of samples characterized as smoky and non-smoky by
57 an industrial sensory panel (Perotti *et al.*, 2020). 1D-HS-SPME-GC-MS, which is used in a fully
58 automated set-up coupled to both supervised and unsupervised chemometrics, was then used
59 to monitor targeted discriminating compounds. Targeted smoky volatiles were then quantified
60 using multiple headspace extraction (MHE) in cocoa beans, and a quantitative range of
61 acceptability/rejection for the incoming cocoa beans was defined.

62 Despite the good overall performance of HS-SPME-GC-MS, the main drawback for quality control
63 is still the long analysis time, which is unfavourable for high throughput data collection. Fast
64 analytical methods that are based on direct injection and mass spectrometry (MS-based
65 electronic nose or MS-enose) may be a solution for the rapid discrimination of smoky from non-
66 smoky samples (Biasioli, 2016; Biasioli, Yeretziyan, Märk, Dewulf, & Van Langenhove, 2011;
67 Deucher *et al.*, 2019; Liberto *et al.*, 2013; Liberto *et al.*, 2019). Unlike traditional electronic noses
68 that are based on solid-state sensors, an MS-enose uses m/z ratios as chemical sensors and is
69 more robust and reproducible, while also being unaffected by sample moisture (Loutfi,
70 Coradeschi, Mani, Shankar, & Rayappan, 2015). The lack of specificity of an MS-enose, when
71 compared to systems that include chromatographic separation, is compensated by its
72 effectiveness, fast response, non-invasive operations and adequate sensitivity even without
73 sample pre-treatment. On the other hand, the mass spectral fingerprints that are generated have

74 to be diagnostic of the sampled volatile fractions, and must provide characterising fragments
75 with abundances suitable to discriminate and modelling the categories of the investigated
76 samples (e.g. smoky vs non-smoky).

77 This study is therefore a step ahead of the top-down approach previously mentioned (Perotti *et*
78 *al.*, 2020) to meet industry needs for speeding up analytical controls for primary materials
79 acceptance, for industrial chocolate manufacturing, while supporting rejections with objective
80 measurements and reliable data.

81 With this contribution, we would verify the following two hypotheses:

- 82 1. whether the contribute of spectral masses of the smoky volatiles, within a rich complex mass
83 spectral fingerprint, is sufficiently diagnostic to be able to discriminate samples
- 84 2. whether this analytical approach, in combination with a discriminant mathematical model,
85 can be exploited as an analytical decision maker (ADM) in a first screening control.

86 This study evaluates whether a HS-SPME-MS-enose is able to discriminate smoky from non-
87 smoky cocoa in a significant set of samples. Our driving hypothesis is that, if diagnostic ions from
88 smoky volatiles are produced at a suitable intensity, the proposed analytical approach may be a
89 suitable ADM for cocoa batches received at the factory.

90

91

92 2. MATERIALS AND METHODS

93 2.1 Samples:

94 The sample set included 48 bean samples (n=23 smoky/rejected and n=25 non-smoky) and 176
95 cocoa liquors (n= 42 smoky/rejected and n= 134 non-smoky) (*Theobroma cacao* L. main crop).
96 Rejected and accepted samples here analysed have been selected based on the sensory tests. All
97 samples were of commercial grade and compliant with the industrial quality control of
98 Soremartec Italia srl (Alba, Italy). Cocoa-bean quality that was in agreement with FCC rules
99 (Federation of Cocoa Commerce) and ISO 2451 was classified “standard beans”(FCC; ISO). Beans
100 and liquors were directly sampled at the processing plants.

101

102 2.2 Head Space Solid Phase Micro Extraction sampling:

103 Volatiles were sampled using an automatic HS-SPME system installed on an MPS-2 multipurpose
104 sampler controlled by Gerstel Maestro software (Gerstel, Mülheim a/d Ruhr, Germany), which
105 was combined on-line with an Agilent 7890A GC coupled to a 5975B MS detector (Agilent, Little
106 Falls, DE, USA).

107 Cocoa samples were ground in liquid nitrogen to give a homogeneous powder and then stored
108 at -80°C until analysis. Cocoa powder (1.00 g) was weighed in headspace glass vials (20 mL),
109 equilibrated for 5 min at 80°C and then sampled using HS-SPME for 10 min at 80 °C at a stirring
110 speed of 350 rpm. **Sampling conditions and their optimization for smoky volatiles extraction were**
111 **in agreement with the experimental results reported in a previous article (Perotti *et al.*, 2020).**

112 SPME fiber: Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) df 50/30 µm - 2
113 cm length from Merck (Bellefonte, PA, USA). Fibers were conditioned before use as
114 recommended by the manufacturer. After sampling, the recovered analytes were thermally

115 desorbed, by heating the fibre for 3 min at 250 °C, into the GC injector body from where they
116 were transferred on-line to the transfer capillary (Cordero *et al.*, 2019; Magnagna *et al.*, 2017,
117 Magagna *et al.*,2018). All samples were analysed in duplicate.

118

119 2.3 MS-enose instrument set-up:

120 The GC oven and injector were maintained at 250 °C; injection mode, split; split ratio, 1/10;
121 carrier gas, helium; flow rate, 0.4 mL/min; fibre desorption time and reconditioning, 3 min. The
122 transfer column was uncoated deactivated fused silica tubing (dc = 0.10 mm, length = 6.70 m)
123 from MEGA (Legnano, Italy).

124 MSD Conditions: ionisation, EI mode at 70 eV; temperatures: ion source: 230 °C, transfer line:
125 280°C. Standard tuning was used and the scan range was set at m/z 35–350 with a scanning rate
126 of 1,000 amu/s.

127

128 2.4 Data acquisition and elaboration

129 Data were acquired and processed using an Agilent MSD Chem Station ver. E.02.01.1177 (Agilent,
130 Little Falls, DE, USA). Raw data were transformed using RapidDataInterpretation software by
131 Gerstel (Gerstel, Mülheim a/d Ruhr, Germany). This is a post-run macro derived from the Gerstel
132 Chemsensor add-on tool, which expands the scope of function of the Agilent ChemStation
133 software. This step allows the 3-dimensional raw data supplied by mass spectrometry (retention
134 time, m/z fragmentation and intensities) to be reduced to 2-dimensional data that can then be
135 properly used by statistical software for further elaboration. Data obtained can be in the form of
136 a *.TIC file and/or a *.DAT file (Mass Spectral Fingerprint). In both cases, sample intensities are
137 summed; with the TIC method, as a function of the scans, and with the DAT method, as a function
138 of the masses. The influence of retention time is removed from the data by summing the
139 intensities of m/z value over the complete time period of the run. The intensities of a sample are

140 added as a function of the masses. The mass axis is very stable when compared to the retention
141 time. The result is a diagram in which an intensity is assigned exactly to each mass (**Figure 1 SM**
142 in supplementary material). When several samples are analysed, *.DAT-file processing collects
143 data of the whole sample set and provides a data matrix in which the rows report the samples
144 and the columns report the intensity assigned to each mass fragment (Heiden *et al.*, 2002). Data
145 matrices of 15,120 data points for beans (48 objects x 315 variables) and 110,880 data points for
146 liquors (352 objects x 315 variables) were collected and elaborated further.

147 The *.DAT file was used for data interpretation in this work. Raw data underwent pre-treatment
148 that consisted of noise subtraction and internal normalisation of the signal from each sample
149 versus the most intense ions (m/z 43, basic peak taken as 1); they were subsequently pre-
150 processed.

151 Chemometric analyses, Principal component analysis (PCA), SIMCA (Soft-Independent modelling
152 of Class Analogy) and Partial Least Square Discriminant Analysis (PLS-DA) were carried out using
153 Pirouette® (Comprehensive Chemometrics Modelling Software, version 4.5-2014) (Infometrix,
154 Inc. Bothell, WA).

155

156

157 **3. RESULTS AND DISCUSSION**

158 The challenge in metabolomic studies is to simultaneously analyse a high number of metabolites
159 that are of very different size, molecular weight, polarity and stability. Cocoa-bean volatiles are
160 the final stage of the expression of changes in the metabolome that is the result of the production
161 chain (Biasoli, 2016; Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009;
162 Ellis, 2019). They are influenced by external factors, e.g. climate and environmental conditions,

163 ripening, time of harvest, fermentation and post-harvest processing (and roasting for liquors).
164 The physical and chemical interactions of all the compounds present in the volatile fraction
165 therefore result in the final attributes of the product, which include sensory acceptability, quality,
166 safety and shelf life (Acierno, Yener, Alewijn, Biasioli, & Van Ruth, 2016; Charles *et al.*, 2015;
167 Liberto *et al.*, 2019). The identification of the volatiles and the interactions that are responsible
168 for the characteristics of a food therefore facilitates the improved control and understanding of
169 the food processes and systems that influence final-product characteristics (Gloess *et al.*, 2014;
170 Liberto *et al.*, 2013; Lindinger *et al.*, 2008). However, chromatographic profiling and
171 fingerprinting cannot be performed quickly enough to meet the requirements of a routine
172 control system for the acceptability of incoming raw material at the production plant. This issue
173 can be addressed by the direct injection of the sampled headspace into a mass spectrometer,
174 which is here based on a quadrupole mass analyser (MS-enose).

175 As previously reported by Perotti *et al.* (Perotti *et al.*, 2020), in cocoa, smoky volatiles are, in
176 general, present at trace levels, and diagnostic fragments are mandatory if their presence is to
177 be confirmed; possible interference from isobaric ions that derive from components present in
178 higher amounts may hamper the correct discrimination of smoky from non-smoky samples
179 (hypothesis-driven approach). If their differentiation is still possible, other volatiles that are
180 correlated to the smoky components must then be considered (hypothesis-generating
181 approach). This last hypothesis is challenging and would require an in-depth investigation into
182 the relationships between these components/ions, the smoky volatiles and the chemical
183 fragmentation involved.

184 An explorative investigation by PCA was first carried out on bean and liquor samples to better
185 understand the real potential, and possible limits, of the informative power of MS-enose
186 fingerprinting in the description of samples, with or without the smoky defect.

187 *3.1 Explorative unsupervised data analysis on beans and liquors*

188 PCA results show fairly good separation between smoky (in red) and non-smoky (green) bean
189 samples with an explained variance of 63.9% (**Figure 1a**). This is acceptable discrimination if we
190 consider that the sample set was representative of different harvesting years (2017 and 2018),
191 (evidenced along the first PC1) and of different harvest regions (Cameroon and Ecuador). In this
192 case, the functional variables (harvest year and region) for the samples were kept to verify MS-
193 enose fingerprinting's ability to discriminate the smoky defect even in presence of confounding
194 external factors. The loadings plot (**Figure 1b**) shows several ions that are linked to the smoky
195 characteristic, in particular on PC2, together with the harvest time on the first PC. On the other
196 hand, the PCA on the whole data set of liquors (n=176) shows lower informative potential, in
197 grouping, on the first two PCs, but demonstrates fairly good discrimination between smoky and
198 non-smoky samples when further PCs are considered (**Figure 1c**). A possible explanation is that
199 the roasting and the grinding processes heavily influence the presence of these components,
200 either because of the effect of temperature and pressure or because of the physical changes in
201 the matrix that are caused by the melting of the cocoa butter to give the cocoa pastes. However,
202 the ions that describe the smoky clusters are the same regardless of whether beans or liquors
203 are considered (**Figure 1b and 1d**).

204 The clusters shown in PCA suggest that supervised approaches should be adopted to define
205 models for sample classification. In the next sections, two supervised approaches for the
206 classification of bean and liquor samples, PLS-DA and SIMCA, will be considered.

207 *3.2 Supervised data analysis on beans and liquors*

208 PLS-DA is a discriminant classification that uses regression that is constructed between X, used
209 as the predictor matrix the (m/z) variables, and the response Y, which indicates the category (ki);
210 in this case, $K=1$ for smoky and $K=2$ for non-smoky beans. The discrimination rule is based on the

211 comparison of each row of the predicted matrix \hat{Y} with each pattern response vector. A sample,
212 i , is categorised into the class, k , whose pattern is the closest match. The matrix \hat{Y} is treated as
213 the input data set for classification to evaluate the distance between a sample and a class pattern.
214 However, this cannot be done directly because this matrix has a rank of $K-1$, and the
215 corresponding covariance matrix is singular. Matrix \hat{Y} is decomposed using PCA, which reduces
216 its dimension to $K-1$, to resolve this singularity. The scores matrix T represents a new data set to
217 which a classification method can be applied.

218 SIMCA is considered soft class modelling because there are no hypotheses for the distribution of
219 variables and their independence because each category model is developed independently and
220 no information from the other categories is used. The mathematical model of each class is based
221 on the principal components of the category, generally obtained as eigenvectors of the
222 correlation coefficient matrix of the category. For a given class, the model dimensions are
223 described either by a line (for one PC), by a plane (for two PCs) or by a hyper-plane (for more
224 than two PCs). The range of scores onto such significant PCs defines the class space. New samples
225 are projected in each PC space, which describes a specific class, and the F-test is used to evaluate
226 the Euclidean distances of the objects from the model.

227 Class modelling differs from discriminant classification mainly because the focus is on a single
228 category; the modelling approach characterises the class of interest against all the others. Class
229 modelling may give a composite answer: 1) compliance with the model for one category only; 2)
230 multiple compliance, with models of several categories; 3) non-classification: a new sample may
231 be rejected by the models of all the categories under study.

232

233 3.2.1 Bean classification

234 To develop the classification model, the sample set was divided into a training set (n=41) and an
235 external test set (n=24). PLS-DA results are reported in **Figure 2a and b**, which displays the scores
236 and loadings plots of the training-set sample classification. The ion fragments with higher
237 discriminant power for smoky beans are highlighted in yellow. These (m/z)s were found to be
238 characteristic for several phenolic compounds and, in the current mode, they have a role in the
239 description of the smoky-bean flavour. The classification ability for the calibration of the PLS-DA
240 cross-validated model (CV=5) is 95.1%, as can be extrapolated from the confusion matrix in **Figure**
241 **3**. Despite the good results on the training set, the model shows a correct prediction classification
242 rate of only 83.3% when applied to the external test set, which was not used to train the model.
243 However, the prediction displays good specificity for both classes and a sensitivity of 80% for the
244 classification of the smoky flavour. This means that the model has quite a good ability to predict
245 class for unknown samples with high specificity for the smoky flavour, but a lower specificity for
246 non-smoky flavours, which can generate false negatives, i.e. indicating good samples when they
247 are not. This must be taken into consideration as a risk for subsequent processing.

248 SIMCA modelling results are displayed in **Figure 4a**, which shows that the two groups are well
249 recognizable. **Figure 4b** shows the class distance between smoky (CS1) and non-smoky (CS2)
250 samples; Cooman's plot displays good separation between classes without overlapping, with the
251 exception of sample "NSBCm2_2" (i.e., non-smoky beans from Cameroon). The variables (i.e.,
252 m/z ions) that have little or high importance for any class in the training set are shown in the
253 modelling power in **Figure 4c**. Variables close to 1 have a high impact on the description of the
254 training set. At the same time, it may be useful to know the best variables to classify the samples
255 categories through the discriminant power. In particular, for each variable, it compares the

256 average residual variance of each class fit to all other classes, and the residual variance of all
257 classes fit to themselves, thus providing an indication of how a variable discriminates between a
258 “correct” and “incorrect” classification. variable value close to 0 indicates low discrimination
259 ability, while a value that is much larger than 1 implies high discrimination power. **Figure 4d**
260 shows the ion fragments with the highest discriminant power in the two sample categories. Of
261 the high modelling and discriminant variables, some fragments ((m/z)) that are characteristic of
262 several phenolic compounds have a role in the description of the smoky beans flavour. These
263 compounds have also been identified as being responsible for smoky-hammy notes in other food
264 matrices (Aprotosoaie, Vlad Luca, & Miron, 2016; CABISCO/ECA/FCC, 2015; Petričević, Marušić
265 Radovčić, Lukić, Listeš, & Medić, 2018; Ridgway, Lalljie, & Smith, 2010; Serra Bonvehí & Ventura
266 Coll, 1998). In particular, m/z 107-108-109, 124 and 138 are diagnostic for isomers of methyl
267 phenols (*cresol isomers*) and *guaiacol*, while m/z 125-152-154 are diagnostic for *p-ethyl guaiacol*,
268 *4-methyl-2,6-dimethoxyphenol* and *2,6-dimethoxyphenol*, and m/z 128 for *naphthalene* (Perotti
269 *et al.*, 2020). The smoky-compound-related ions account for 40%, on average, of the whole gas
270 chromatographic profile acquired using 1D-GC-MS.

271 Classification ability in calibration, for the SIMCA model, was 100%, as shown in **Figure 3a**. The
272 developed model was applied to the external test set (not used to train the model) and showed
273 global prediction classification with a sensitivity of 91.6% and a specificity of 100%, as shown in
274 **Figure 3b** by the confusion matrix. In addition, the class specificity is excellent and the sensitivity
275 of the model for both classes is above 90%. This means that class modelling is robust, without
276 giving false-positive or false-negative sample classifications, and that only 8.40% of the samples
277 should be investigated further by a sensory panel or by a confirmatory method to verify their
278 acceptability (Perotti *et al.*, 2020).

279

280 *3.2.2 Liquor classification*

281 Liquor classification also need to be investigated because primary materials may either be beans
282 or liquors at the cocoa production plant, depending on the country of origin and supplier.

283 The liquor sample set was unbalanced as it contained a higher number of the non-smoky
284 category. Both classification approaches, however, showed similar prediction abilities, 92% and
285 97% for PLS-DA and SIMCA respectively (**Figure 3b** and **Figure 5b** and **5e**). SIMCA training and
286 test-set prediction is depicted in the hyper-plane of the first 3 PCs in **Figure 5a-b**, while PLS-DA
287 prediction on the test set is displayed by the prediction plots of **Figure 5d** and **5e**. In the prediction
288 plot of **Figure 5d**, reference lines (in light green) indicate the decision criterion space for class
289 membership; only samples with Y values greater than 0.5 are called category members.
290 Therefore, the red samples in the plot are all above the membership criterion for CS2, that is,
291 class 2 (i.e., non-smoky liquors), which is on the X axis, while the green samples fall above the
292 decision criterion for class 1 (e.g., smoky liquors), on the Y axis. **Figure 5e** clearly shows the
293 predicted y values and the samples that are classified outside their group in the blue circles.

294 Discriminant variables for SIMCA, as shown in **Figure 5c**, were found to be m/z 152 and 154,
295 which represent *phenethyl alcohol* and *p-ethyl guaiacol* (m/z=152) at 96% and *2,6-*
296 *dimethoxyphenol* (m/z=154) at 81% on the 1D-GC-MS chromatographic profile. The same
297 variables and m/z 107, 123, 137, 138 are diagnostic for isomers of *phenol*, *methyl phenols* (*cresol*
298 *isomers*) and *p-ethylguaiacol* **Figure 5f**, whose abundance in the 1D-GC-MS chromatographic
299 pattern of liquors for the above-cited volatiles is 62% (**Figure 2 SM**) (Perotti *et al.*, 2020). The
300 mass spectra of several smoky components present in commercial mass spectral libraries (i.e.
301 Wiley7N and NIST2014) are displayed in **Figure 3 SM** in the supplementary file.

302 Despite the unbalanced number of samples in the classes, the two supervised approaches show
303 similar classification/modelling performance. However, SIMCA modelling has higher specificity
304 for both classes and the highest sensitivity for CS1 (i.e. smoky samples). This means that a model
305 for discrimination between smoky and non-smoky liquors is feasible and that it may be exploited
306 as a second filter through the chocolate processing chain, for instance. Discrimination between
307 good and bad beans can be considered the first step for conformity to the qualitative standard
308 in cocoa-bean acceptance, also for economic reasons, while the second filter can be used on
309 liquors to reinforce the reduction of the impact of the off-flavour on the final product, while
310 maintaining its standard quality.

311 Samples that are excluded from the model or unclearly classified can be submitted to a
312 confirmatory validated method (Cordero *et al.*, 2019; Perotti *et al.*, 2020).

313

314 *3.3 Qualitative and quantitative confirmation analytical method*

315 A confirmatory method that is based on a reference standard material is not feasible due to the
316 lack of a cocoa smoky reference standard. Furthermore, several smoky volatiles are also
317 endogenous components in beans and even more so in liquors. Spiking methods, performed via
318 the addition of standard reference compounds, would falsify the quantitation due to their non-
319 homogeneous distribution in the cocoa (beans and liquors), because of the heterogeneous
320 nature of the solid matrix. Therefore, the sensory-driven screening method applied above has
321 been cross-validated using a confirmatory qualitative-quantitative analysis; multiple
322 headspace extraction (MHS-SPME) combined with 1D-GC-MS (Perotti *et al.*, 2020; Sgorbini *et al.*,
323 2019). MHS-SPME-1D-GC-MS was validated in terms of its repeatability (intra-day repeatability)
324 and intermediate precision (inter-day repeatability) for beans and liquors. The ISTD-normalised

325 analyte area responses of a set of 15 compounds were then processed using one-way ANOVA
326 and Tukeys' comparative analysis on the data acquired from a quality-control smoky sample of
327 both beans and liquors (QCs), on four different days in different weeks, analysed in triplicate.
328 **Table 1** displays the intra-day (repeatability) and inter-day precision for the quantified smoky
329 markers. Precision is expressed as RSD% on analyte normalized area responses. Results indicate
330 good intra/inter-day precision for both beans and liquors. The Limit of Detection (LOD) was
331 determined from the standard calibration curve, as $LOD=3*S_a/b$, where S_a is the standard
332 deviation of the response and b is the slope of the calibration curve, while the Limit of
333 Quantification LOQ was calculated as $3.3*LOD$. The LOD and LOQ of the smoky markers are also
334 reported in **Table 1** together with the odour qualities, odour thresholds, target and qualifier ions
335 that were used for their quantitation and qualification. The accuracy of the screening method
336 was evaluated by cross-comparison with the quantitative results obtained using MHE-SPME-1D-
337 GC-MS. **Figures 4 and 5 SM** display box plots of the quantitative results on non-matched or false
338 positive samples (doubtful) that were obtained by class modelling. The quantitation of the
339 selected markers allows an operative limit, of below 10 ng/g for beans and 100 ng/g for liquors,
340 to be adopted for the acceptance of incoming cocoa samples, **Figure 6 SM** and **Table 1**.

341

342 **4. CONCLUSION**

343 The HS-SPME-MS-enose, in combination with chemometric tools, delineate a successful work-
344 flow for the detection of defective "smoky" samples (beans or liquors) and their discrimination
345 from "non-smoky" ones, thus confirming our driving hypothesis. The validation of the models
346 was performed at two levels: via internal validation (cross-validation), using a training set, and
347 via an external validation test set. Diagnostic fragments of phenolic derivatives correlated to
348 these models enable to assess the classification ability of the MS-enose method through a cross-

349 verified evaluation of the actual concentration of smoky compounds using MHE-SPME-1D-GC-
350 MS. Despite 1) the compositional complexity of the bean and liquor volatilomes, 2) the low
351 concentration of the smoky targeted markers compared to the major volatiles, and 3) the
352 possible co-contribution of several different analytes to the fragment intensities, the sensitivity
353 of the technique's multi-channel nature is sufficiently diagnostic, making it possible to
354 mathematically model the variation in mass spectral fingerprints using multivariate regression
355 procedures. In particular, the high specificity of the SIMCA models indicates that there is a low
356 probability of false positive/negative classifications, although this occurred to a lesser extent for
357 the smoky class in liquors. These features mean that the MS-nose can be exploited as an
358 analytical decision maker for screening controls of both beans and liquors. Possibly unclear or
359 non-classified samples can be reasonably verified using a conventional analytical confirmation
360 method via the quantitation of the smoky components. Moreover, the analytical system is
361 versatile since it can be used for both a conventional setting for GC-MS and in MS-nose mode
362 and is therefore suitable for a high-throughput, objective and cost-effective quality control.

363 **Acknowledgments**

364 The research was financially supported by Soremartec Italia s.r.l Alba (CN), Italy

365

366 **Table caption**

367 **Table 1.** Average concentrations of targeted smoky markers, respectively for smoky and non-
368 smoky samples, together with their odour quality (The Good Scents Company Information
369 System, 2018), odour threshold (ng/g orthonasal from oily matrix) as reported in the literature
370 (Vichi, Romero, Tous, Tamames, & Buxaderas, 2008); *OT in water (Biotechnology National
371 Center, 2020; Buckpitt, Alan, Kephelopoulos, Stylianos, Koistinen, Kimmo, Kotzias, Dimitrios,
372 Morawska, Lidia and Sagunski, 2010); Target ion (Ti) and qualifiers (Q1-Q2), adopted for their
373 quantitation and identification, intra- inter-day precision, LOD and LOQ.

374 **Figure Captions**

375 **Figure 1.** PCA score plots of a) beans and b) liquors and the corresponding loading plots c) and
376 d). Data were logarithmically (Log10) transformed and pre-processed by autoscaling. Red spots
377 indicate smoky samples, green spots are non-smoky.

378 **Figure 2.** a) and b) PLS-DA score plot and loading plot of beans using an internal cross-validated
379 method (CV=5), c) classification prediction of the external test set of samples. Data were
380 transformed by autoscaling. Red spots indicate smoky samples, green spots are non-smoky.

381 **Figure 3.** Confusion matrices of bean and liquor classification in calibration (a) and in prediction
382 on the test set (b), respectively for SIMCA and PLS-DA, together with sensitivity, specificity and
383 correct classification rate values. CS1: Class 1, smoky beans, CS2: Class 2, non-smoky beans.

384 **Figure 4.** a) SIMCA beans classification scores plot. Data were transformed using a logarithmic
385 scale and autoscaled. Explained variance on the first 3 PCs: 66.9%. b) Interclass distances
386 between smoky and non-smoky samples, c) variables that impact on the modelling and d)
387 discriminant variables in class classification. Red spots indicate smoky samples, green spots are
388 non-smoky.

389 **Figure 5.** a-c) Liquor modelling using the SIMCA training set, test set and the discriminant
390 variables of the model. d-f) PLS-DA prediction plot, predicted members in the two classes and
391 discriminant variables for the classification. CS1: Class 1 smoky beans, CS2: Class 2 non-smoky
392 beans.

393

394 **Supplementary material**

395 **Figure 1 SM.** Flow-chart from raw-data acquisition to the raw-data matrix for suitable
396 chemometrics. Total ion chromatogram (TIC) of bean-volatile fingerprints represents the 3-D
397 data that is transformed, by the rapid data interpretation software, into 2-D data the Mass
398 spectral fingerprint (*.DAT) and the subsequent data matrix obtained from several mass spectral
399 fingerprints.

400 **Figure 2 SM.** a) Liquor HS-SPME-GC-MS patterns of the TIC (Total ion current), and b) MIC (Mixed
401 Ion Chromatogram: m/z 107, 123, 137, 138, 152, 154) with the recognised volatiles that
402 contained diagnostic ions, set in the MIC.

403 **Figure 3 SM.** Mass-spectra fragments of smoky components from commercial mass spectral
404 libraries (Wiley7N and NIST14)

405 **Figure 4.SM.** Box plots of the quantified smoky markers in false positive smoky beans

406 **Figure 5 SM.** Box plots of the quantified smoky markers in false positive smoky liquors

407 **Figure 6 SM.** Quantitative rejection ranges of the smoky markers (brown colour) for a) beans and
408 b) liquors

409

410

411

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534

Compounds	Odor quality	OT (ng/g)	Ti and Q1- Q2 (m/z)s	Beans				Liquors				LOQ (ng/g)	LOD (ng/g)
				Average conc (ng/g)		Precision		Average conc (ng/g)		Precision			
				smoky	non-smoky	Intraday RSD%	Interday RSD%	smoky	non-smoky	Intraday RSD%	Interday RSD%		
Naphthalene	Mothball-like	80*	128 ;64-102	32.5 ± 10.7	4.8 ± 3.3	3.0	6.7	41.8 ± 18.8	6.2 ± 1.3	0.3	3.6	3.0	0.9
Guaiacol	medicinal, smoky, woody	10	109 ;81-124	68.6 ± 25.0	8.2 ± 3.8	4.6	8.0	364.8 ± 117.9	73.6 ± 15.0	1.7	3.4	3.1	0.9
2-Methoxy-4-methylphenol	phenolic, smoke-like	90*	138 ;95-123	63.8 ± 20.1	-	1.8	6.0	370.7 ± 100.7	22.9 ± 12.8	1.7	3.4	5.8	1.7
Phenol	phenolic, plastic rubber	100	94 ;66-95	721.7 ± 482.2	5.7 ± 6.2	1.1	5.4	669.6 ± 149.3	59.2 ± 10.0	1.1	3.0	1.0	0.3
<i>p</i> -Ethylguaiacol	smoky bacon	50	137 ;122-152	82.9 ± 8.0	-	3.5	7.6	346.6 ± 111.1	38.7 ± 20.2	1.8	7.0	32.5	9.7
<i>p</i> -Cresol	phenolic	25	107 ;77-108	143 ± 47.9	-	4.4	7.7	675.4 ± 200.4	12.9 ± 6.9	1.4	2.7	24.1	7.2

Figure 1

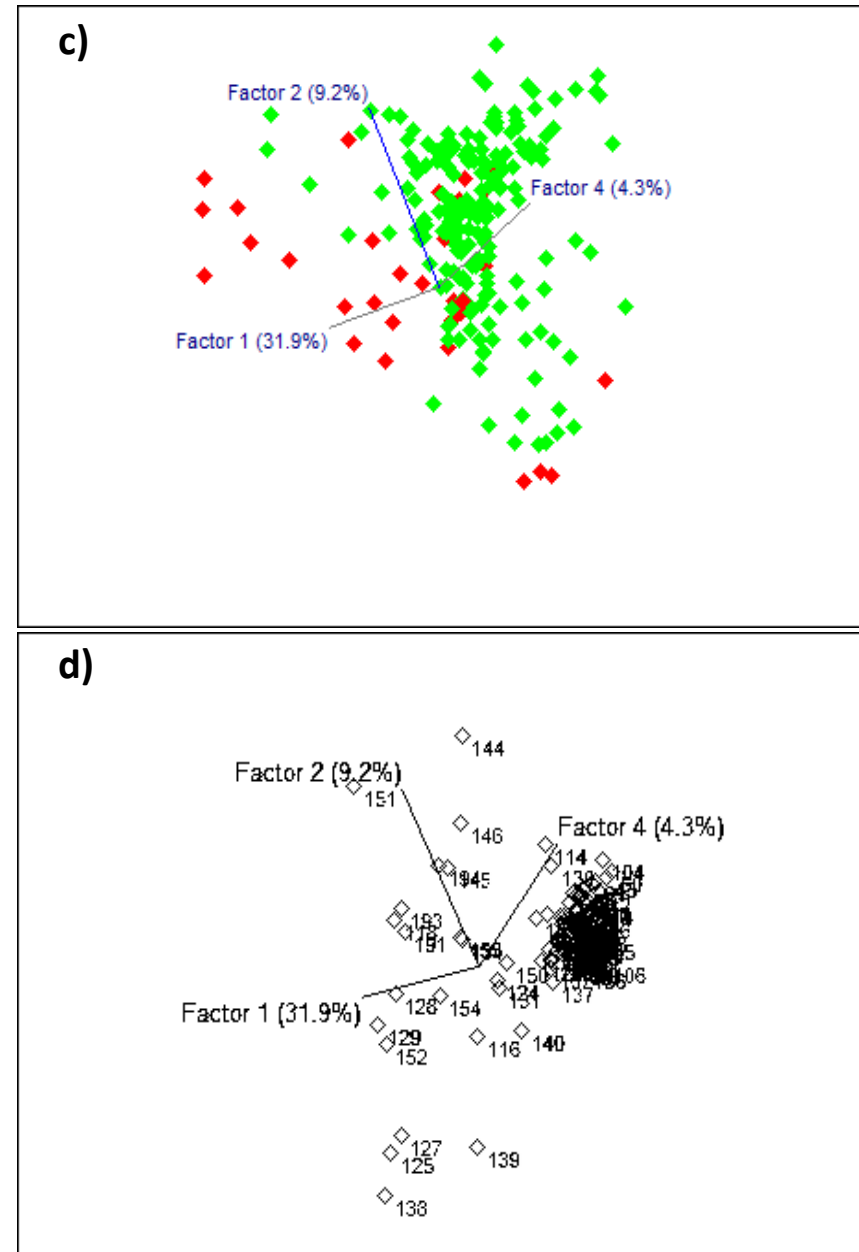
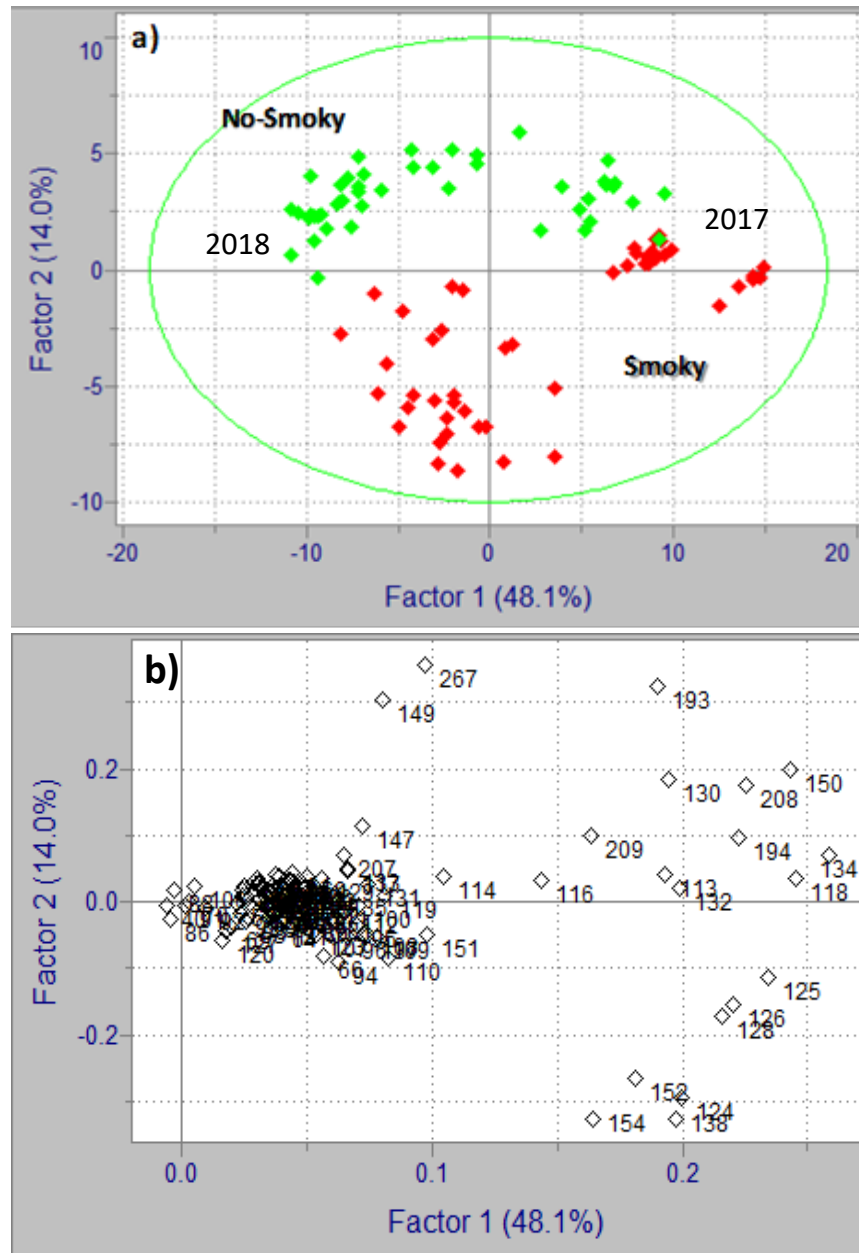


Figure 2

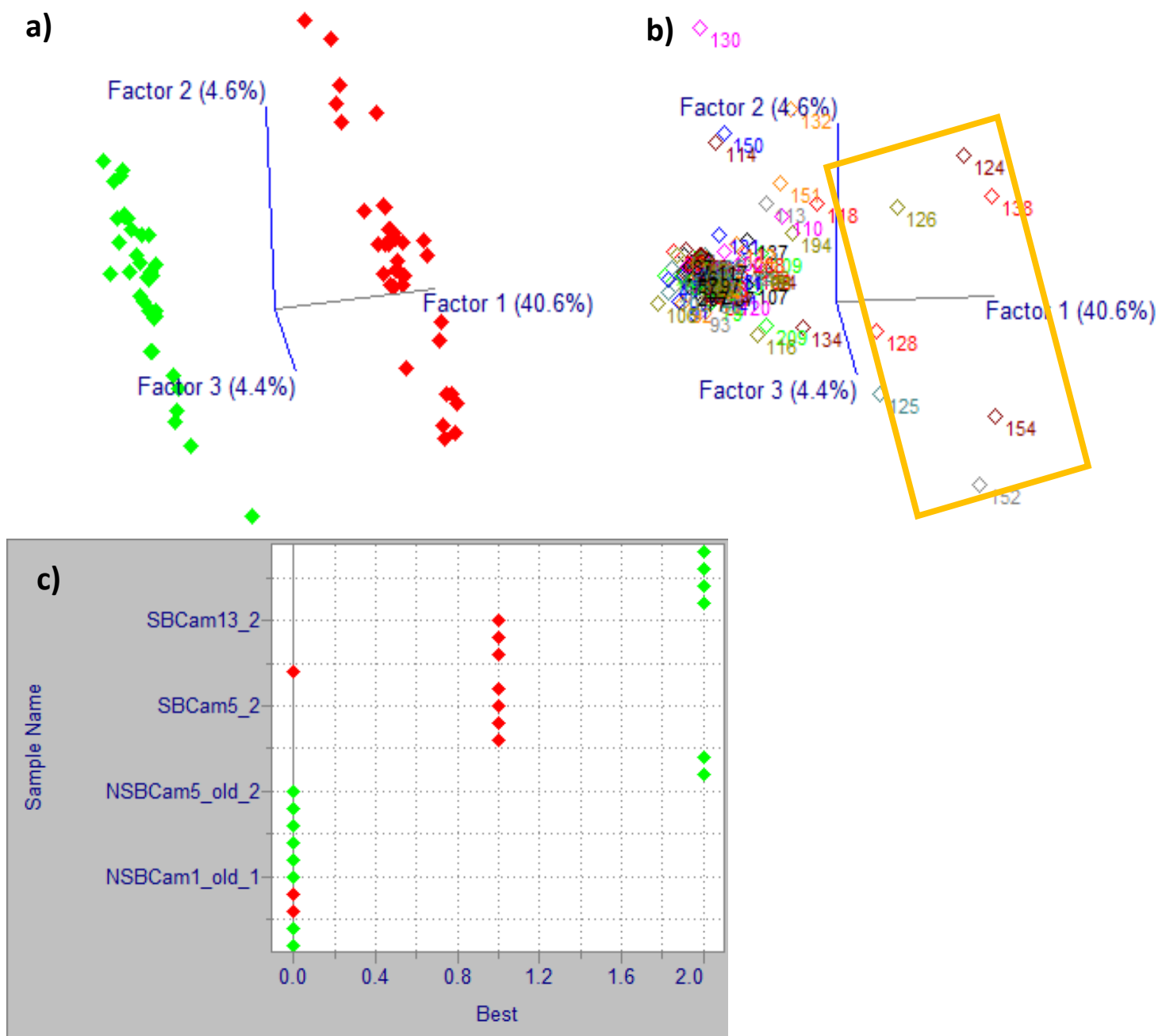


Figure 3

a)

Training set misclassification: SIMCA

	PredCS1@5	PredCS2@5	No match
ActualCS1	21	0	0
ActualCS2	0	20	0
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	100	100	100%
CS2	100	100	

Training set misclassification: SIMCA 94.5

	PredCS1@5	PredCS2@5	No match
ActualCS1	27	1	0
ActualCS2	9	146	4
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	96.4	100	94.5%
CS2	91.8	99	

Training set misclassification: PLS-DA

	PredCS1@5	PredCS2@5	No match
ActualCS1	19	0	1
ActualCS2	0	20	1
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	95.0	100	95.1%
CS2	95.2	100	

Training set misclassification: PLS-DA

	Pred1@7	Pred2@7	No match
ActualCS1	23	4	1
ActualCS2	0	151	8
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	82.1	100	
CS2	94.9	97.0	97.7%



b)

Test set misclassification: SIMCA

	PredCS1@5	PredCS2@5	No match
ActualCS1	9	0	1
ActualCS2	0	13	1
Unmodeled	0	0	0
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	90.0	100	91.6%
CS2	92.8	100	

Test set misclassification: SIMCA

	PredCS1@5	PredCS2@5	No match
ActualCS1	8	0	0
ActualCS2	1	31	0
Unmodeled	0	0	0
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	100	89	97.5
CS2	96.8	100	

Test set misclassification: PLS-DA

	PredCS1@5	PredCS2@5	No match
ActualCS1	8	2	0
ActualCS2	1	12	1
Unmodeled	0	0	0
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	80.0	89	83.3
CS2	85.7	86	

Test set misclassification: PLS-DA

	PredCS1@6	PredCS2@6	No match
ActualCS1	6	2	0
ActualCS2	1	31	0
Unmodeled	0	0	0
	<i>Sensitivity</i>	<i>Specificity</i>	Total
CS1	75.0	86.0	92.5
CS2	96.8	94.0	



Figure 4

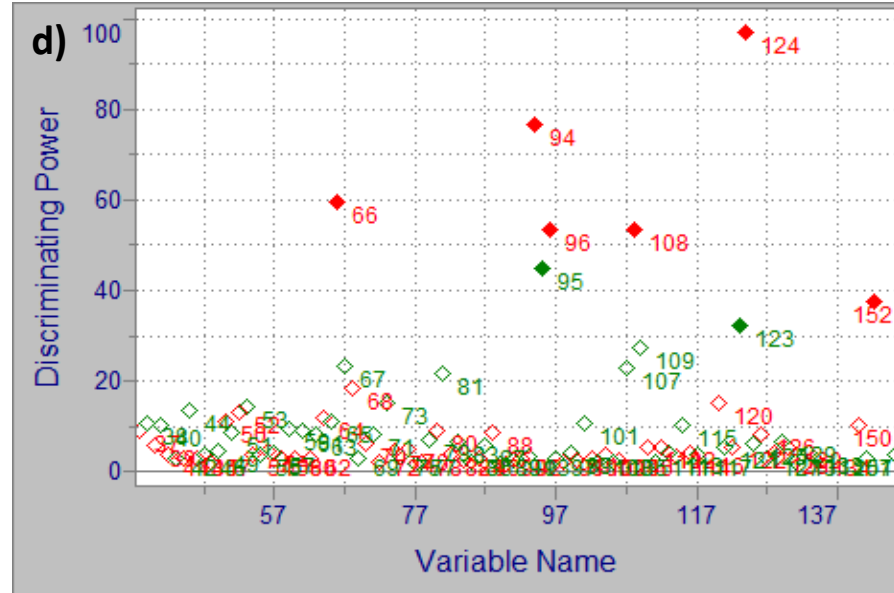
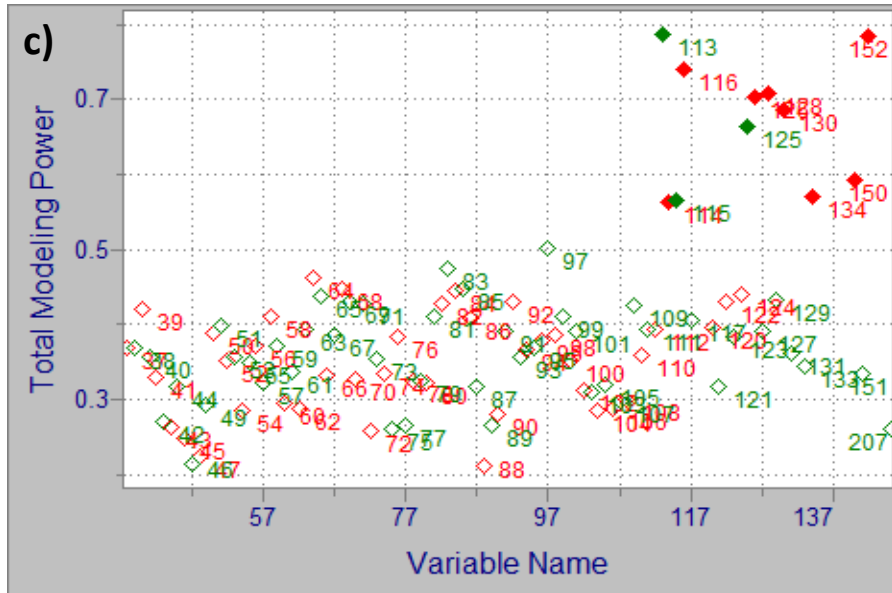
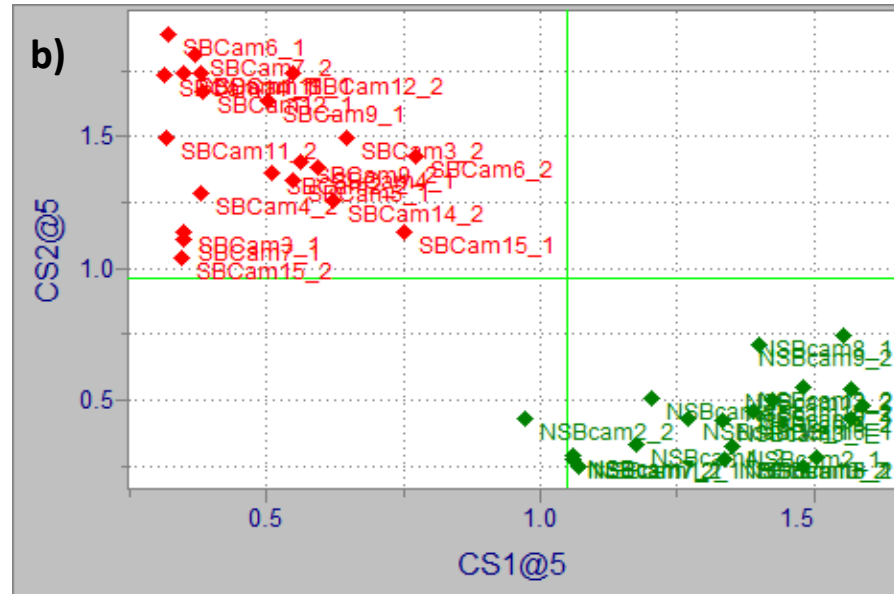
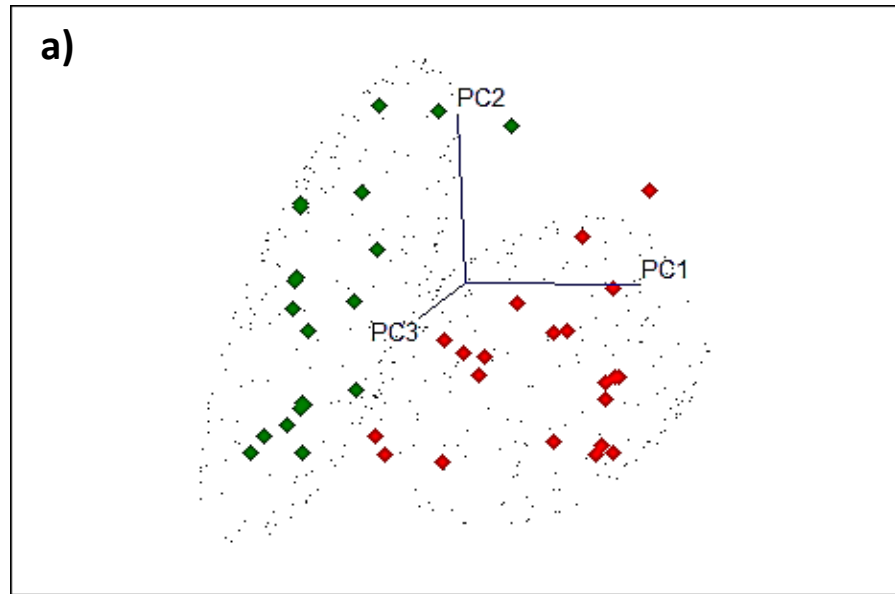
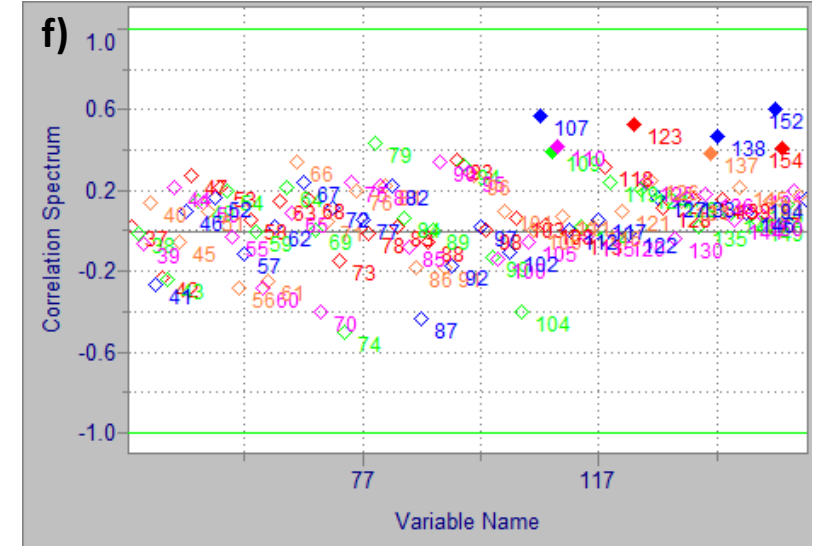
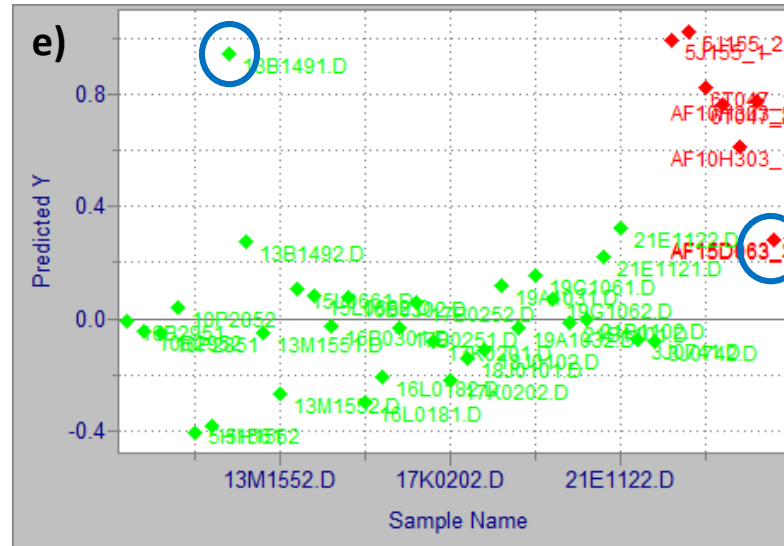
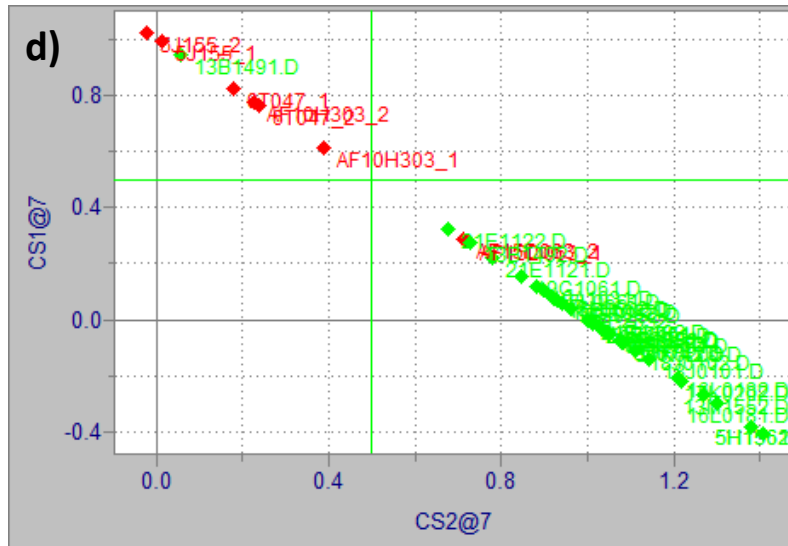
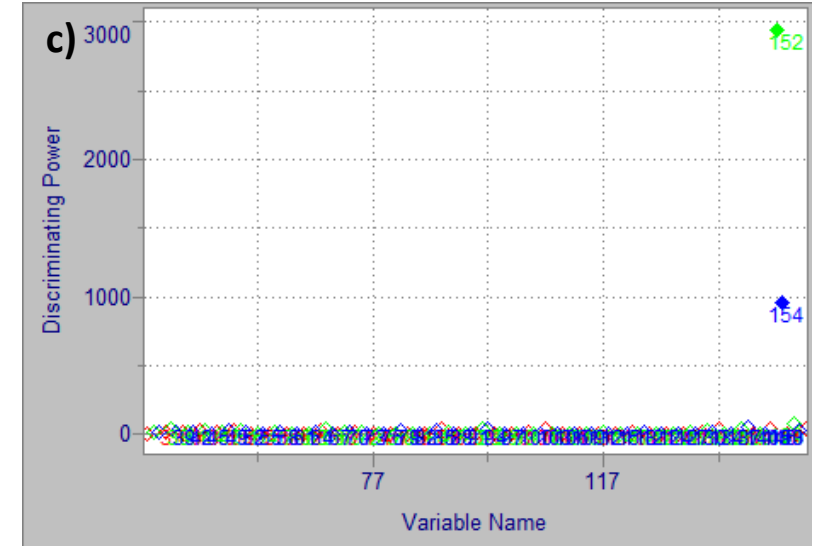
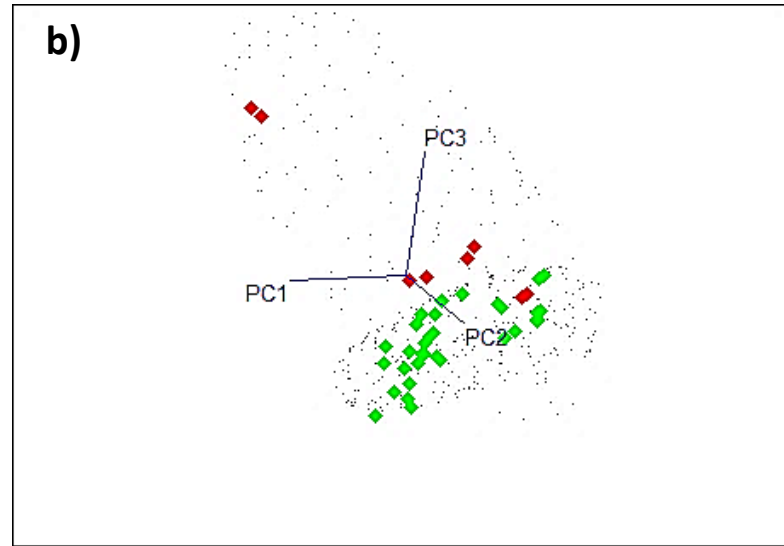
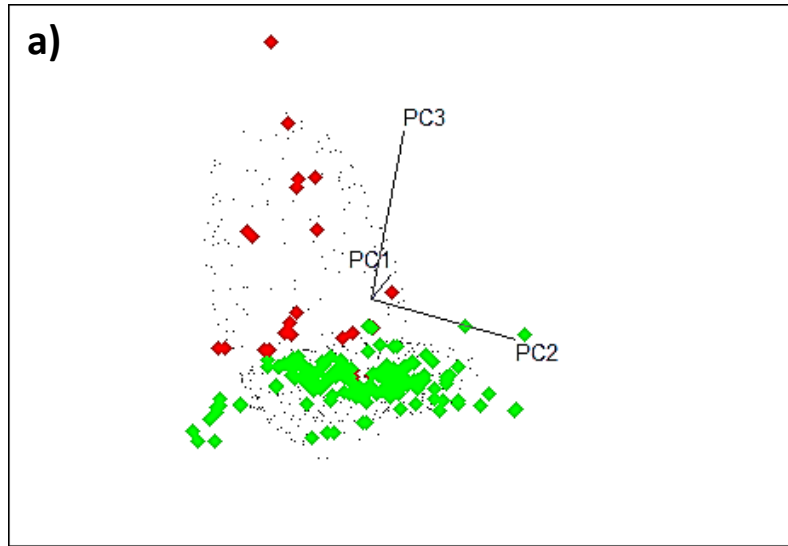


Figure 5



Cocoa smoky off-flavour: a MS-based analytical decision maker for routine controls

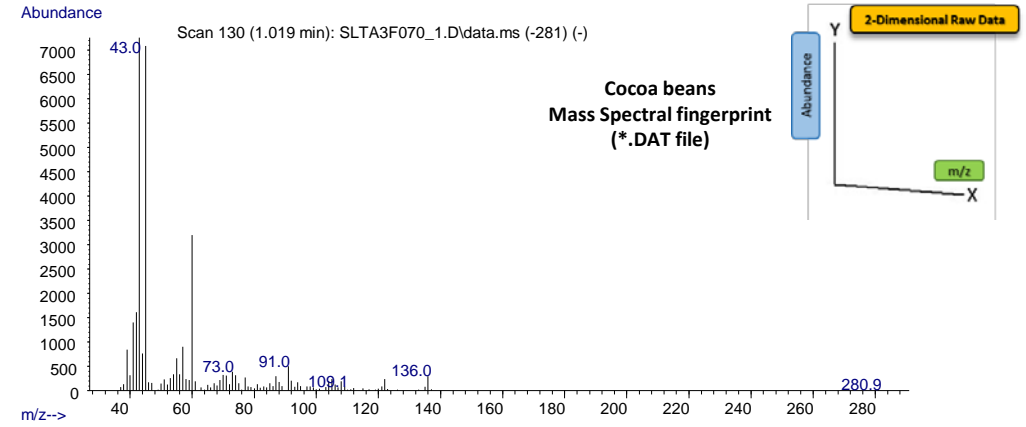
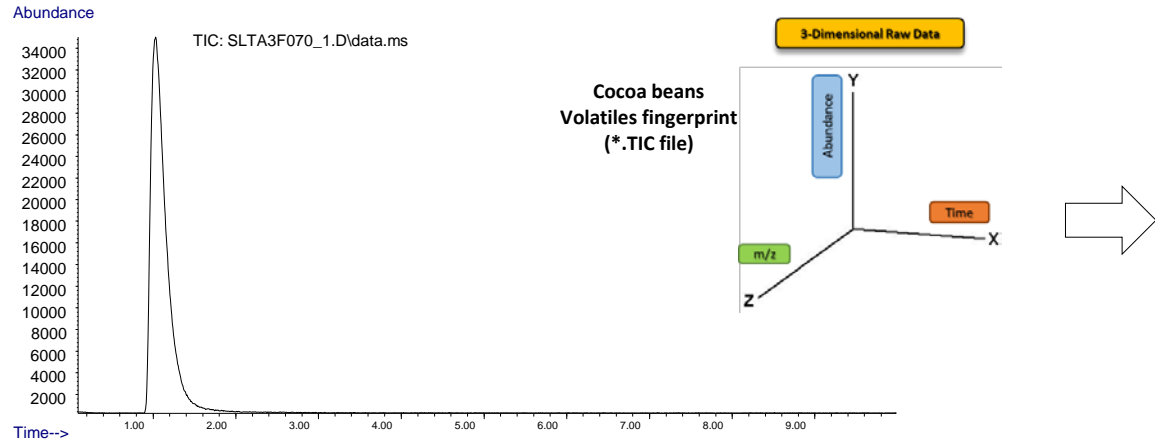
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Figure 1 SM



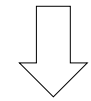
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		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
11	NSL_5_G	3781.0000	595.0000	5448.0000	9466.0000	4120.0000	8650.0000	10388.0000	26222.0000	17094.0000	44245.0000	9886.0000	11918.0000	81603.0000	5546.0000	935.0000	1865.0000	525.0000	4718.0000
12	NSL_5_G	3762.0000	588.0000	5203.0000	9003.0000	3800.0000	7763.0000	8339.0000	23891.0000	14608.0000	39338.0000	8668.0000	6391.0000	77301.0000	5542.0000	828.0000	1877.0000	385.0000	4246.0000
13	NSL_10_P	3510.0000	670.0000	5854.0000	10518.0000	5117.0000	10427.0000	16394.0000	24610.0000	17106.0000	44501.0000	9444.0000	8382.0000	63729.0000	4078.0000	592.0000	1365.0000	541.0000	3130.0000
14	NSL_10_P	3630.0000	879.0000	5889.0000	10389.0000	4911.0000	9905.0000	15533.0000	22924.0000	14685.0000	40509.0000	9159.0000	6419.0000	64161.0000	4012.0000	716.0000	1411.0000	478.0000	2974.0000
15	NSL_5_Z	3824.0000	786.0000	5115.0000	9197.0000	4319.0000	9240.0000	12379.0000	22507.0000	13710.0000	37594.0000	8271.0000	8046.0000	63857.0000	3856.0000	608.0000	1167.0000	430.0000	3006.0000
16	NSL_5_Z	3894.0000	755.0000	4952.0000	8828.0000	4146.0000	8847.0000	11739.0000	20794.0000	11740.0000	33137.0000	7995.0000	6445.0000	62258.0000	3577.0000	599.0000	1325.0000	490.0000	2766.0000
17	NSL_5_B	3828.0000	767.0000	6308.0000	11066.0000	5072.0000	10244.0000	15920.0000	22939.0000	12670.0000	34982.0000	10191.0000	6761.0000	64365.0000	4291.0000	756.0000	1708.0000	536.0000	3425.0000
18	NSL_5_B	3644.0000	728.0000	5970.0000	10827.0000	4967.0000	9982.0000	15422.0000	23224.0000	13605.0000	36526.0000	10159.0000	8103.0000	64058.0000	4324.0000	672.0000	1658.0000	525.0000	3406.0000
19	SL_H_046	7979.0000	421.0000	4695.0000	8053.0000	4723.0000	10403.0000	17617.0000	20400.0000	10141.0000	32516.0000	5910.0000	7262.0000	157681.0000	6548.0000	1059.0000	1611.0000	456.0000	3784.0000
20	SL_H_046	7769.0000	314.0000	4684.0000	8100.0000	4727.0000	10402.0000	17166.0000	19740.0000	9961.0000	31594.0000	5716.0000	6984.0000	153641.0000	6457.0000	996.0000	1577.0000	486.0000	3734.0000
21	SL_E_154	5650.0000	453.0000	5746.0000	9917.0000	5064.0000	9975.0000	13845.0000	22861.0000	11957.0000	34573.0000	9419.0000	6671.0000	84903.0000	4617.0000	899.0000	1854.0000	575.0000	4146.0000
22	SL_E_154	5634.0000	538.0000	5672.0000	9937.0000	5002.0000	10108.0000	14484.0000	22698.0000	11936.0000	34493.0000	9424.0000	7362.0000	85610.0000	4751.0000	863.0000	1879.0000	633.0000	4074.0000
23	SI_N_155	6259.0000	569.0000	5083.0000	8741.0000	3940.0000	8710.0000	9143.0000	19138.0000	8066.0000	26543.0000	7099.0000	6272.0000	56706.0000	3578.0000	898.0000	1966.0000	552.0000	4574.0000



n samples acquired

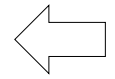
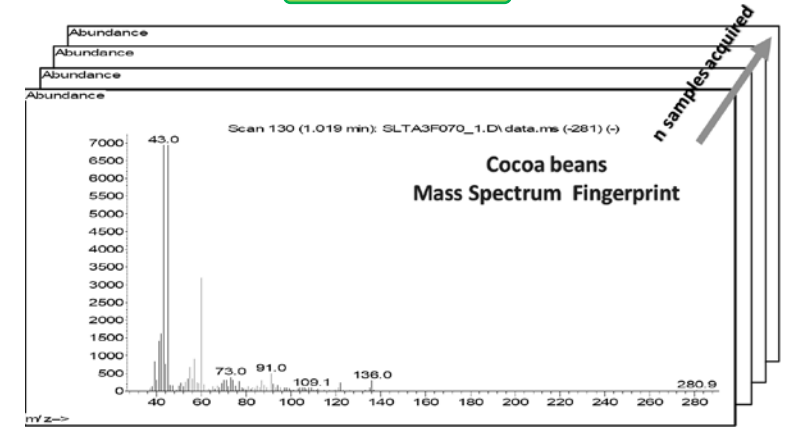


Figure 2 SM

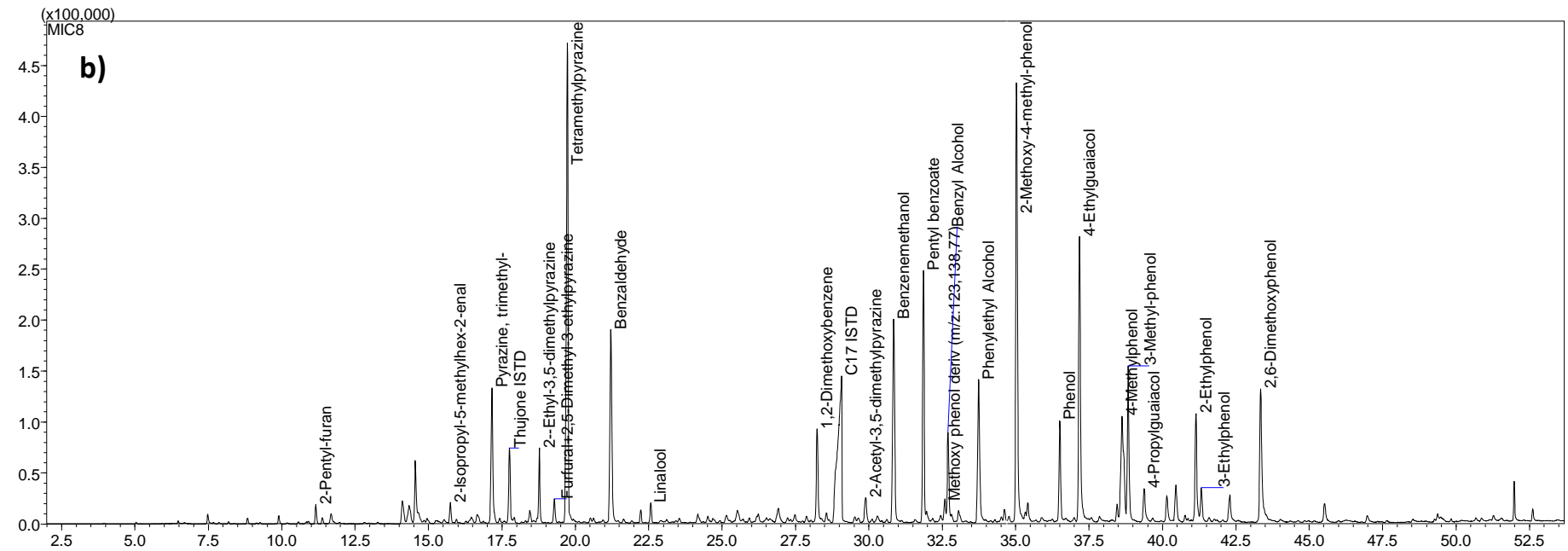
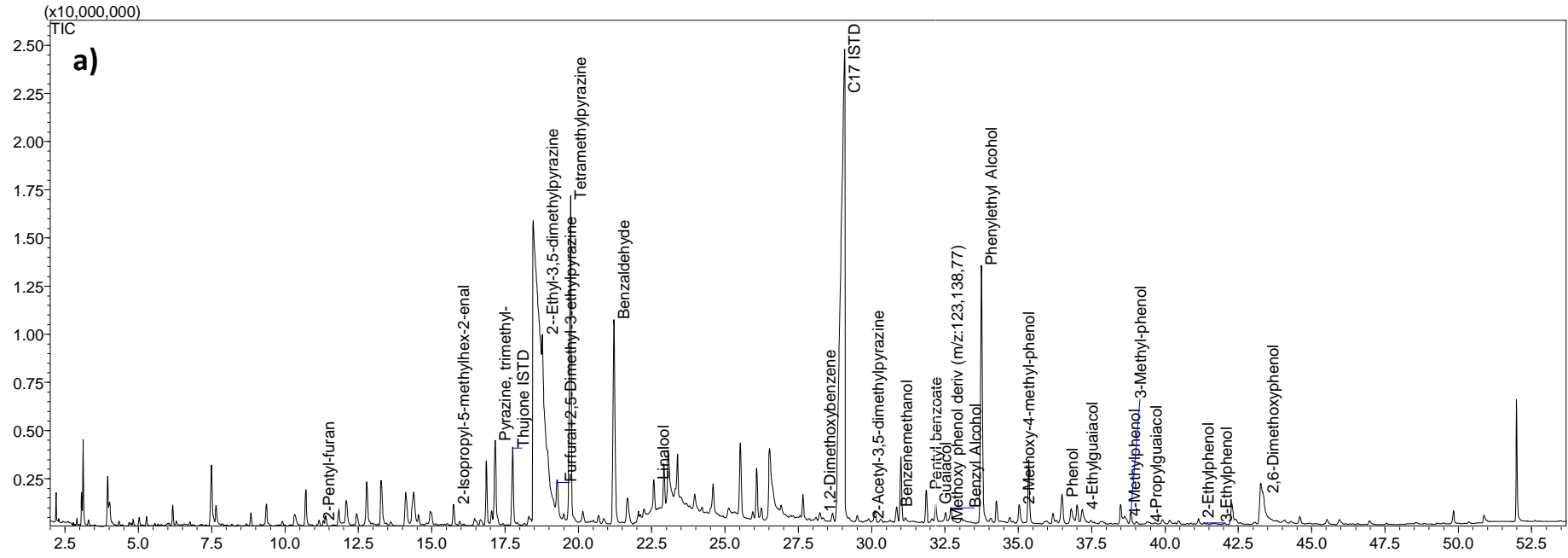
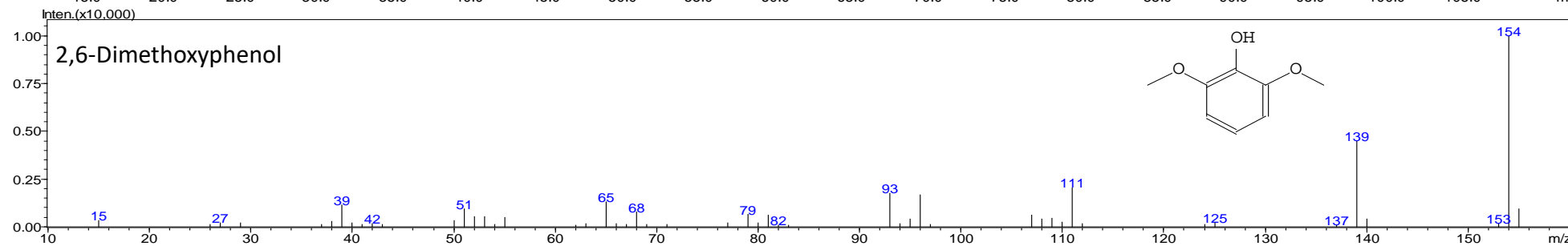
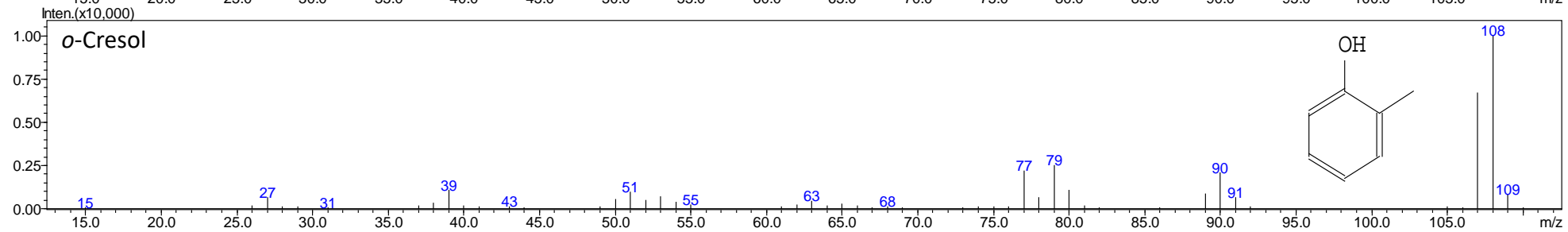
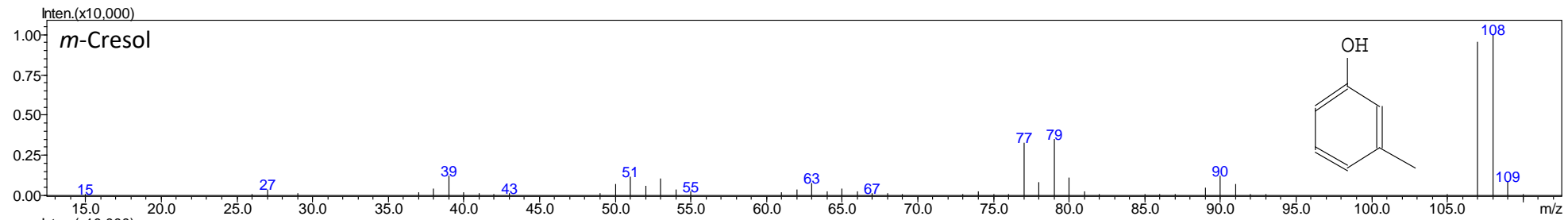
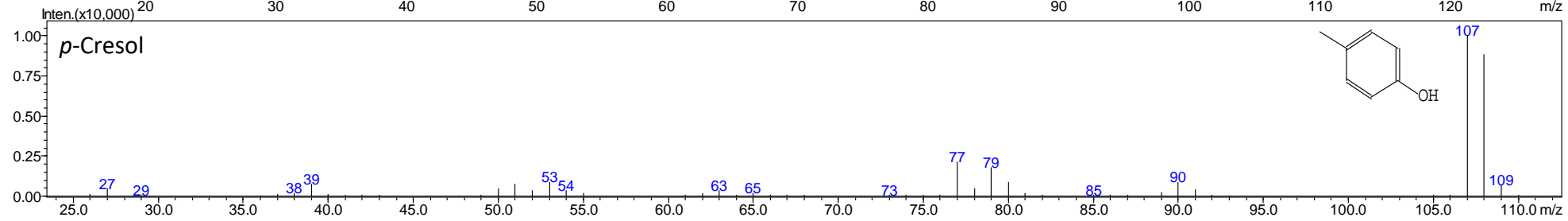
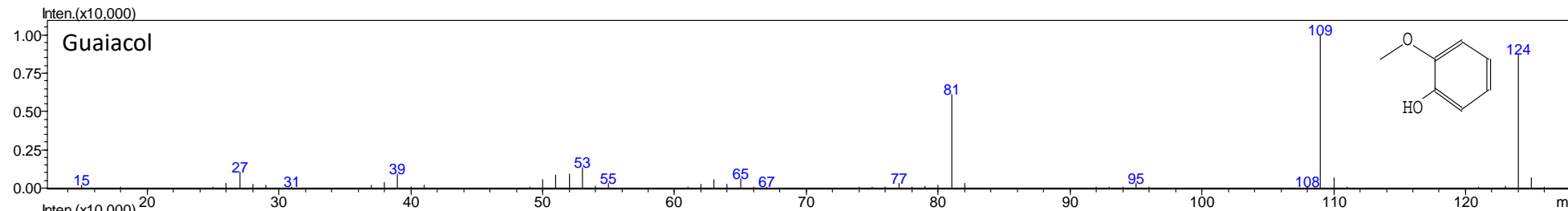


Figure 3SM



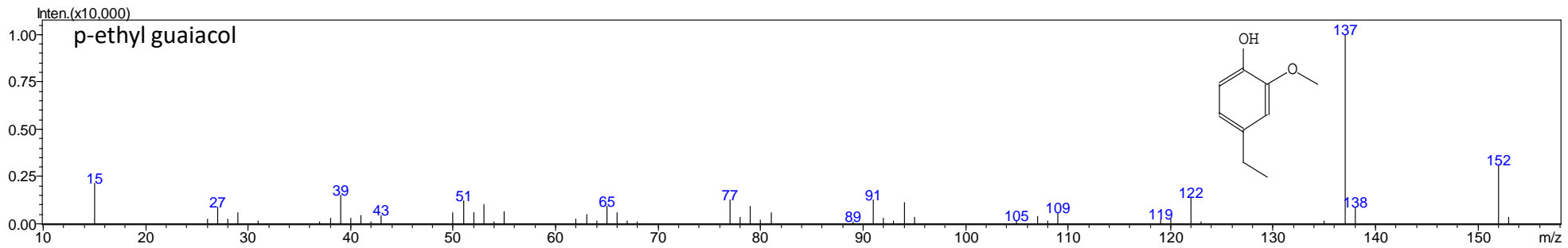
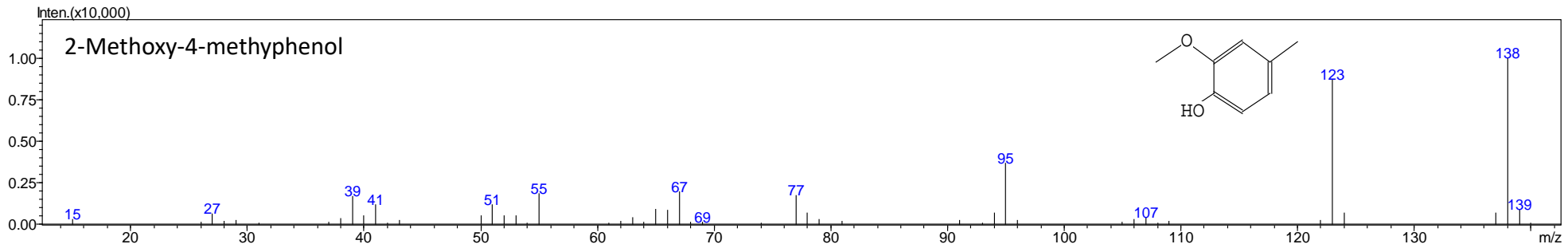
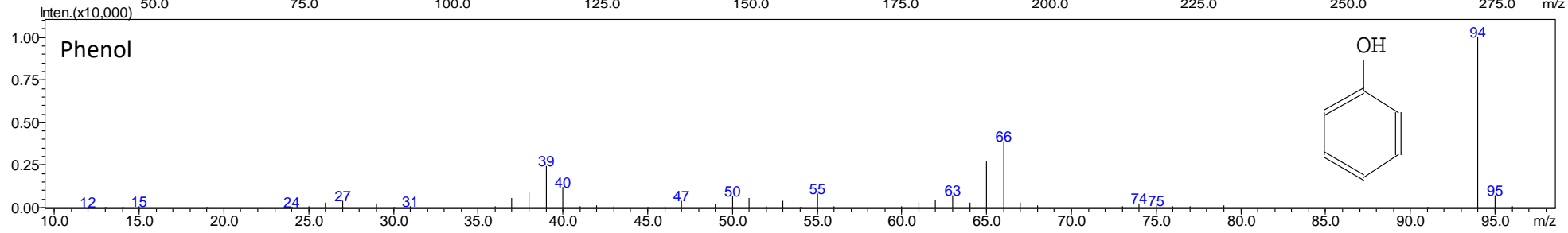
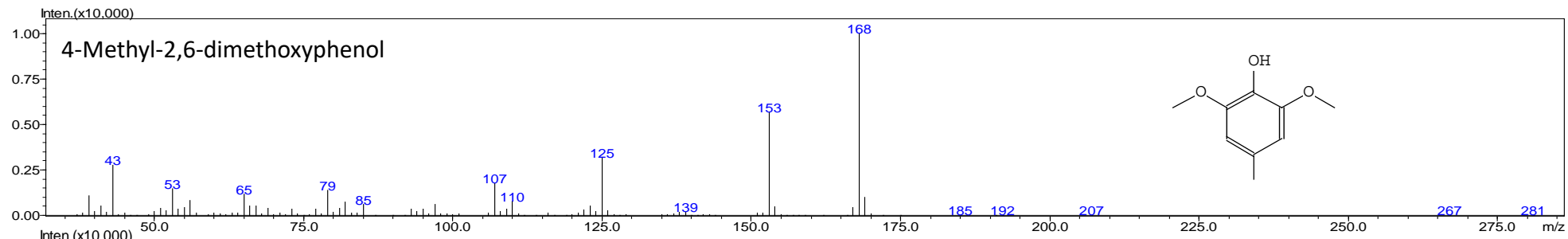
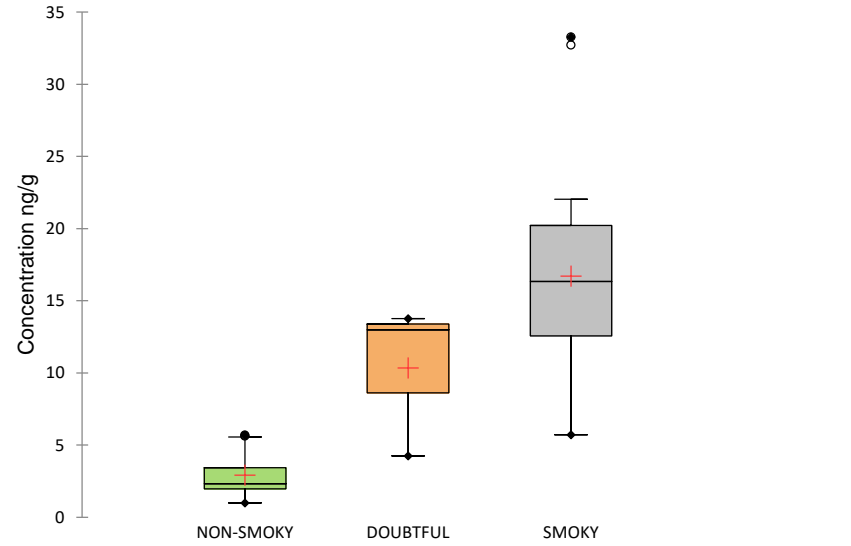
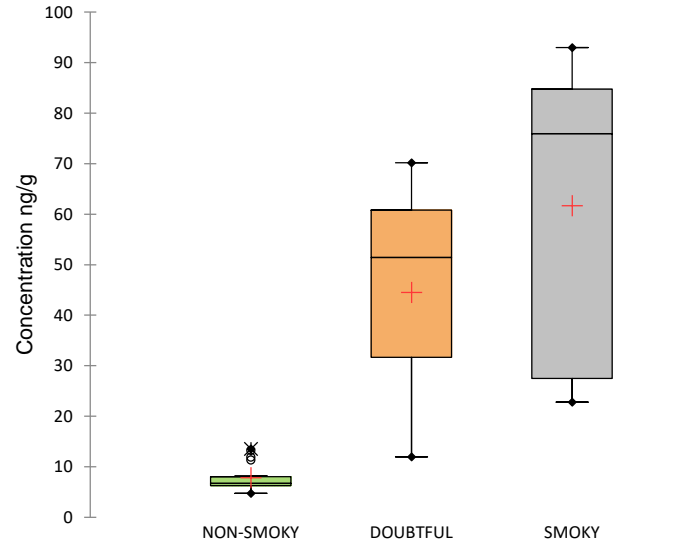


Figure 4 SM

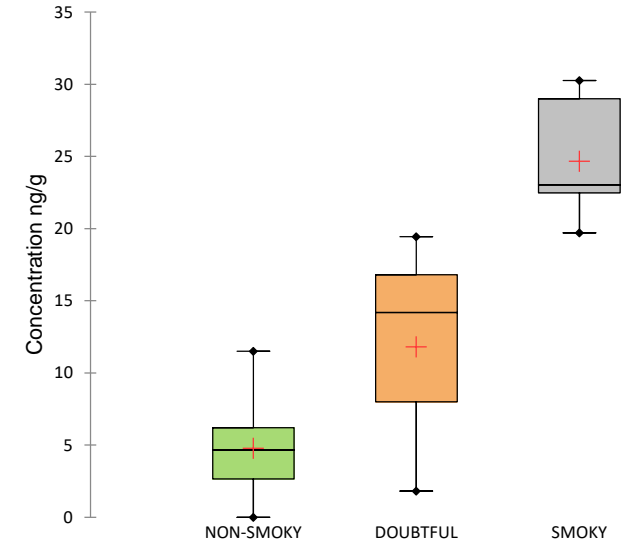
Naphthalene



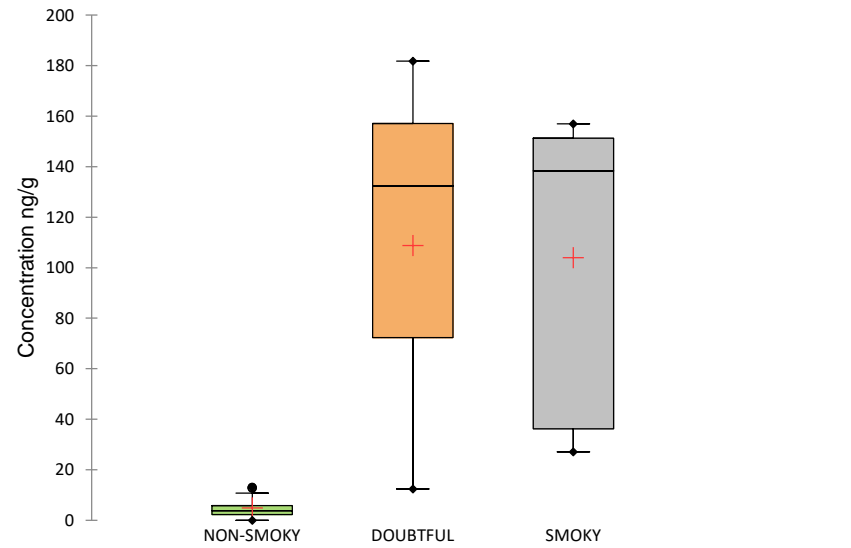
Guaiacol



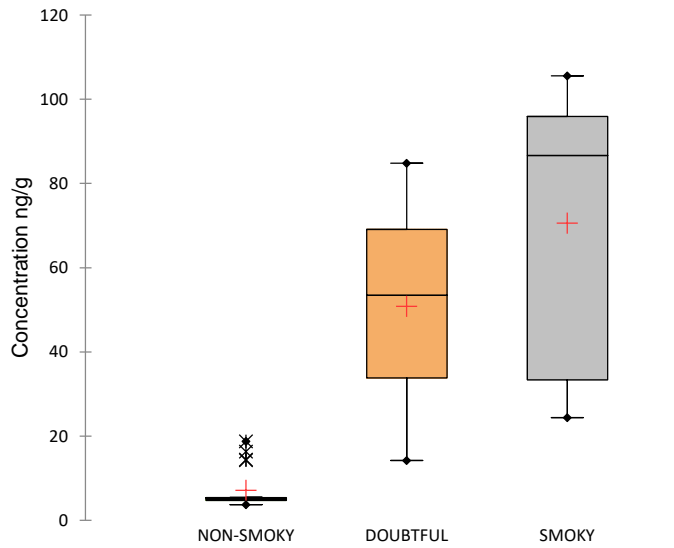
2-Methoxy-4-methylphenol



Phenol



p-Ethylguaiacol



p-Cresol

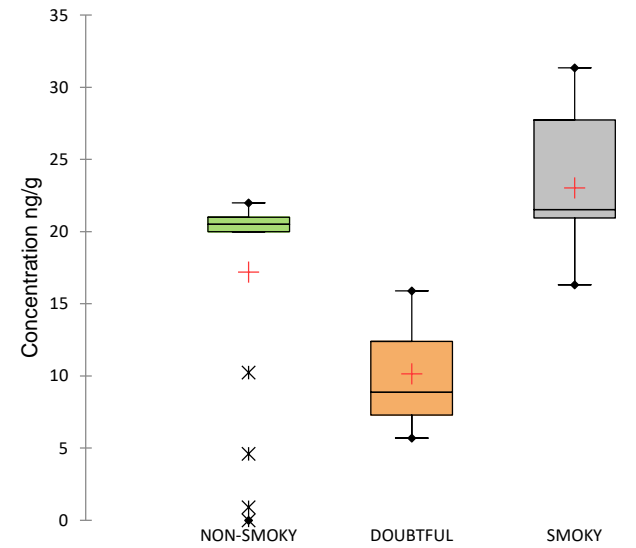


Figure 5 SM

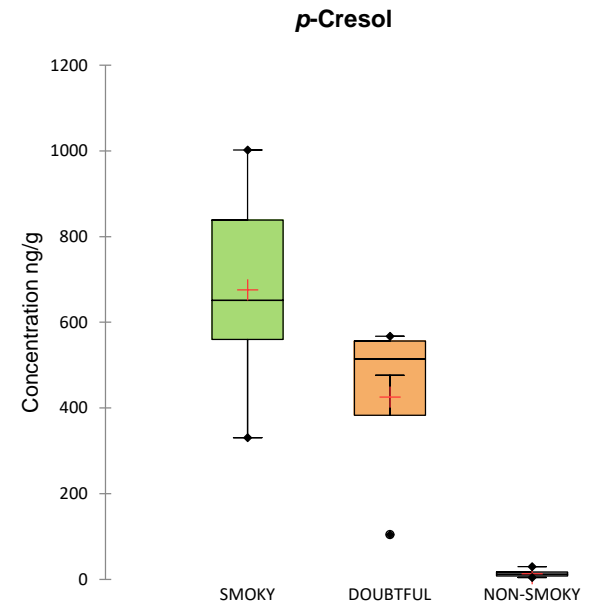
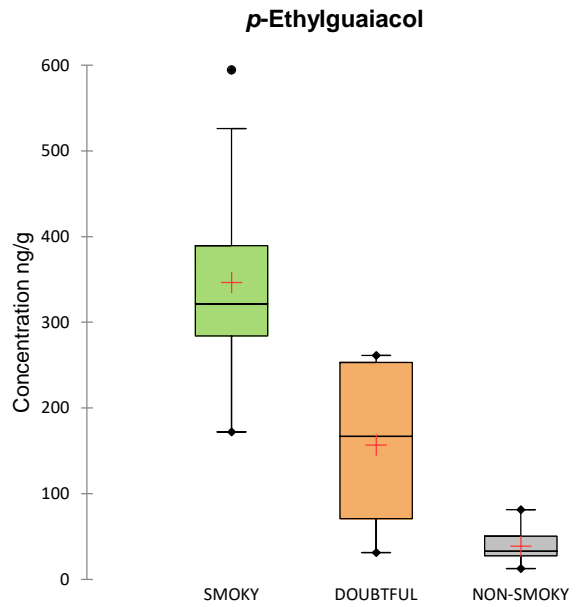
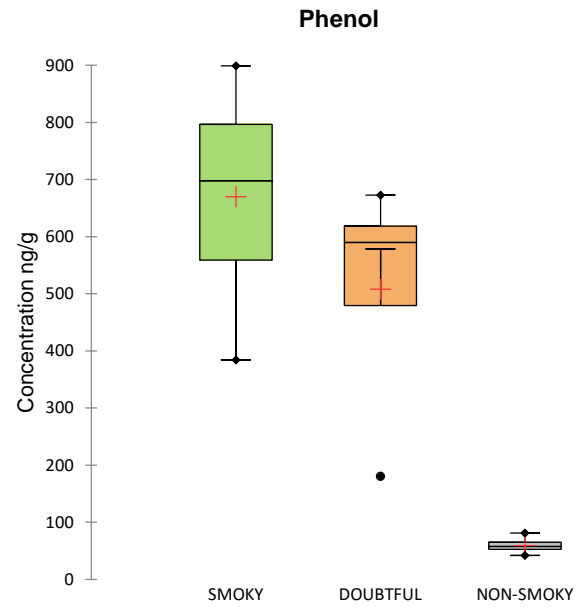
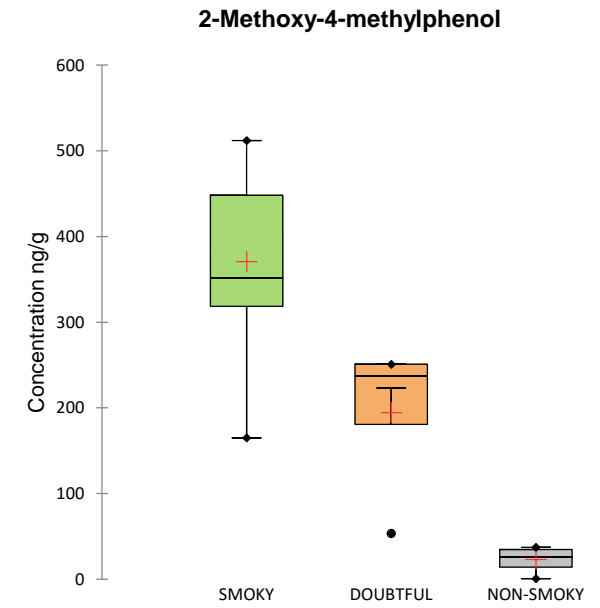
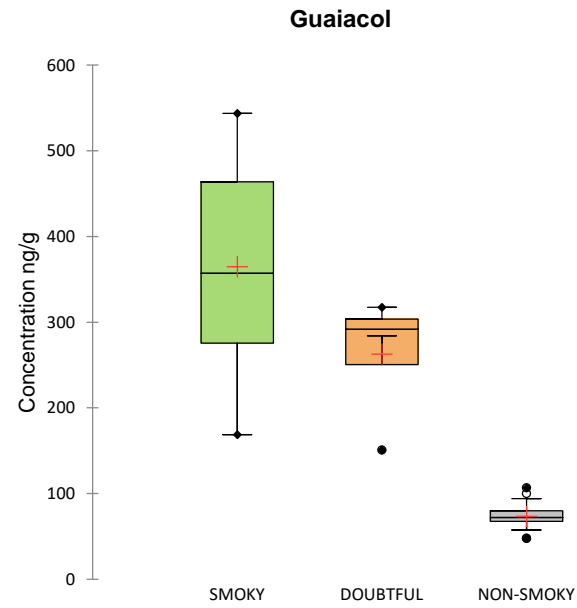
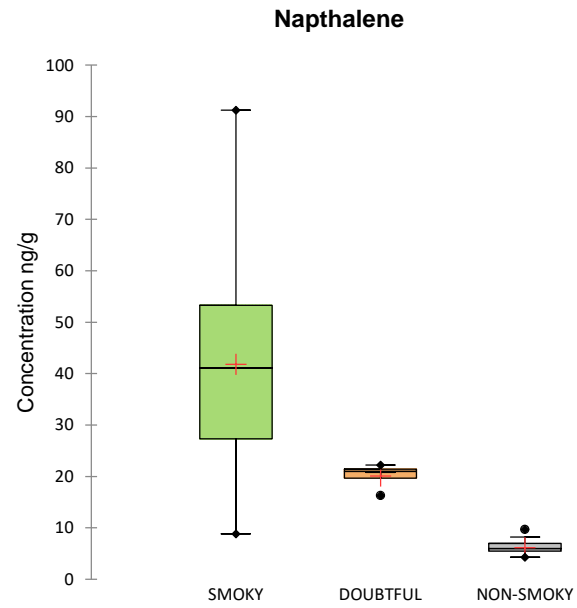


Figure 6 SM

