Non-targeted high-performance liquid chromatography with ultraviolet and fluorescence detection fingerprinting for the classification, authentication, and fraud quantitation of instant coffee and chicory by multivariate chemometric methods.

Nerea Núñez[a], Josep Pons[a], Javier Saurina[a,b], Oscar Núñez[a,b,c,*]

[a] Department of Chemical Engineering and Analytical Chemistry, University of Barcelona, Barcelona, Spain

[b] Research Institute in Food Nutrition and Food Safety, University of Barcelona, Santa Coloma de Gramenet, Spain

[c] Serra Húnter Program, Generalitat de Catalunya, Barcelona, Spain

* Corresponding author: Oscar Núñez

Department of Chemical Engineering and Analytical Chemistry. University of Barcelona. Martí i Franquès 1-11, E-08028, Barcelona, Spain.

Tel.: +34-93-403-9116

E-mail: oscar.nunez@ub.edu

ORCID:	Nerea Núñez	(0000−0002−6088−0965)
	Josep Pons	(0000−0002−7043−538X)
	Javier Saurina	(0000−0002−4892−9535)
	Oscar Núñez	(0000−0001−5850−8972)

1 **Abstract**

2 Non-targeted strategies based on high-performance liquid chromatography with
3 ultraviolet detection (HPLC-UV) and fluorescence detection (HPLC-FLD) fingerprints
4 were evaluated to accomplish the classification and authentication of instant coffee (40
5 samples), instant decaf coffee (26 samples), and chicory (22 samples, including both
6 ground and instant), as well as to detect and quantify frauds based on chicory adulteration
7 by multivariate chemometric methods. HPLC-UV and HPLC-FLD fingerprints were
8 simultaneously obtained with a HPLC-UV-FLD instrument, and they proved to be
9 excellent chemical descriptors for the classification of coffee and decaf coffee against
10 chicory samples by partial least squares regression-discriminant analysis (PLS-DA). In
11 contrast, HPLC-UV fingerprints improved the classification results when addressing
12 coffee against decaf coffee samples (94.4% classification rate in comparison to 83.3% for
13 HPLC-FLD fingerprints). Besides, the proposed methodologies resulted to be excellent
14 to detect and quantify fraud levels in coffee and decaf coffee samples adulterated with
15 chicory by using partial least squares (PLS) regression, exhibiting good calibration
16 linearities, calibration errors, and prediction errors. In this case, improved capabilities
17 were observed with HPLC-FLD fingerprints, providing better PLS calibration linearities
18 (R^2>0.999), lower calibration errors (≤0.8%), and similar to better prediction errors (2.9-
19 3.2%) in comparison to HPLC-UV fingerprints.

20

21 **Keywords:** HPLC-UV-FLD fingerprints; Coffee; Chicory; Food authentication;
22 Chemometrics.

23

24

25 ## 1. Introduction

26 Coffee is one of the most popular beverages around the world, which is consumed as
27 an infusion of ground roasting coffee beans with a characteristic taste and flavor, with
28 social aspects related to the provision of hospitality. Coffee drinks have been widely
29 employed as a stimulant, a property mainly attributed to the presence of caffeine, probably
30 the most frequently ingested pharmacologically active substance worldwide. Coffee also
31 contains a high number of bioactive substances such as polyphenols and phenolic acids,
32 being the main source of, for instance, caffeic and chlorogenic acids, providing a high
33 antioxidant capacity. Its intake has been related to the improvement of some prevalent
34 and serious diseases such as type II diabetes, cancer, liver cirrhosis, and cardiovascular
35 diseases (Dam, Willett, Manson, & Hu, 2006; Heath, Brahmbhatt, Tahan, Ibdah, &
36 Tahan, 2017; Rodríguez-Artalejo & López-García, 2018; Zhao et al., 2020). It should be
37 noted that the content and distribution of those bioactive substances and, consequently,
38 the antioxidant and healthy properties can vary depending on the different coffee
39 varieties, origin, roasting degree, and climate conditions, among many other factors (Król,
40 Gantner, Tatarak, & Hallmann, 2020; Seninde & Chambers IV, 2020). Besides, these
41 varietal, geographical and processing features strongly influence on taste and flavor
42 characteristics of coffees.

43 Among the more than 120 species of coffee, those having the major economic and
44 commercial importance are produced by the *Coffea arabica* (Arabica) and *Coffea*
45 *canephora* (Robusta). The higher sensorial properties of the Arabica species make it the
46 most appreciated by consumers and even it is usually considered better than the Robusta
47 one. Hence, Arabica coffee usually has a higher price in the international market (ICO,
48 2020), and consequently, it is important to authenticate Arabica and Robusta commercial
49 blends to detect possible commercial frauds.

50 Coffee adulteration is a common practice aimed at obtaining an economic profit by
51 making the final product prize less expensive (Aline Theodoro Toci, Farah, Pezza, &
52 Pezza, 2016). The most common coffee adulteration cases are those related to the Arabica
53 and Robusta blends (Badmos, Lee, & Kuhnert, 2019; Núñez, Martínez, Saurina, &
54 Núñez, 2021), but fraudulent practices may also be related to the quality of the coffee
55 beans (the different species, geographical origin, and use of defective beans) (Bosmali et
56 al., 2020; Núñez, Collado, Martínez, Saurina, & Núñez, 2020; Núñez, Martínez, et al.,
57 2021; Aline T. Toci & Farah, 2014), as well as the addition of other substances such as
58 coffee husks and stems, maize, brown sugar, or soybean to the coffee blends (Pauli et al.,
59 2014; Aline Theodoro Toci et al., 2016). Among them, chicory roots (*Chicorium intybus*
60 var. *sativum*) are baked, ground, and used as a coffee substitute (with a similar bitter taste
61 but without caffeine), but on many occasions is also used as a non-declared coffee
62 adulterant, especially in instant coffee (Briandet, Kemsley, & Wilson, 1996; Charlton,
63 Farrington, & Brereton, 2002; Ferreira et al., 2016; Martins et al., 2018), for fraudulent
64 economical profit as it is a low-cost raw material in comparison to coffee.

65 Targeted analytical methodologies, in which specific chemicals or groups of
66 chemicals belonging to the same family are monitored (profiling strategies), are
67 frequently employed in food authentication. Regarding coffee, 3,4- dimethoxycinnamic
68 acid (Andrade, Leitão, Seabra, Oliveira, & Ferreira, 1998), sterols (Carrera, León-
69 Camacho, Pablos, & González, 1998), tocopherols and triglycerides (González, Pablos,
70 Martín, León-Camacho, & Valdenebro, 2001), phenolics and methylxanthine (Alonso-
71 Salces, Serra, Remero, & Heberger, 2009), chlorogenic acids (Badmos et al., 2019),
72 triacylglycerols (Cossignani, Montesano, Simonetti, & Blasi, 2016), 16-O-methylcafestol
73 and kahweol (Finotello et al., 2017), as well as multi-elemental and stable isotope
74 profiling (Peng et al., 2019), are among the target analytes under study for its

75 characterization and authentication. The concentrations of these targeted compounds (or
76 their related instrumental signals) are then used as food features (markers) to address
77 coffee authentication, requiring in most cases the use of commercially available
78 standards, but also quantitative approaches which sometimes are a difficult task. In
79 contrast, non-targeted analytical methodologies, based on the analysis of instrumental
80 responses without assuming any previous knowledge of relevant or irrelevant coffee
81 chemical components (fingerprinting strategies), appear as faster and simpler alternatives
82 to accomplish coffee authentication. In this sense, spectroscopic techniques based on
83 near-infrared (NIR) (Bona et al., 2017; Monteiro et al., 2018), ultraviolet-visible (UV-
84 vis) (Yulia & Suhandy, 2019), fluorescence (FLD) (Suhandy & Yulia, 2018), and nuclear
85 magnetic resonance (NMR) (Milani et al., 2020), fingerprinting have been widely
86 employed to address the authenticity of coffee. Volatile fingerprinting obtained by gas
87 chromatography with a flame-ionization detector (GC-FID) or coupled with mass
88 spectrometry (GC-MS), and liquid chromatographic fingerprinting with different
89 detection systems (UV-vis and FLD), or coupled with low- (LRMS) or high-resolution
90 mass spectrometry (HRMS), or direct sample analysis by mass spectrometric techniques,
91 have also been described for that purpose (Aquino et al., 2014; Jumhawan, Putri,
92 Yusianto, Bamba, & Fukusaki, 2015; Núñez et al., 2020; Núñez, Martínez, et al., 2021;
93 Núñez, Saurina, & Núñez, 2021; Aline T. Toci & Farah, 2014). Obviously, in most of the
94 previously commented methodologies, the use of chemometrics becomes essential for
95 data comparison to assess coffee classification and authentication.

96 In the present work, the applicability of non-targeted high-performance liquid
97 chromatography with ultraviolet and fluorescence detection fingerprinting to assess the
98 classification and authentication of instant coffee, decaf coffee and chicory samples will
99 be evaluated. Under this approach, remarkable conclusions can be drawn without the need

100 to quantify or identify the chemical species responsible for the different sample patterns.
101 For that purpose, the obtained HPLC-UV and HPLC-FLD fingerprints will be employed
102 as sample chemical descriptors and submitted to supervised PLS-DA chemometric
103 method for classification. In addition, the application of the proposed methodology for
104 fraud detection and quantitation of adulterated instant coffees with chicory by partial least
105 squares regression will also be studied.

106 2. Experimental

107 *2.1. Reagents and materials*

108 Methanol (HPLC grade) was obtained from PanReac AppliChem (Barcelona, Spain)
109 and formic acid (≥98%) from Sigma-Aldrich (St. Louis, MO, USA). Water was obtained
110 from an Elix 3 coupled to a Milli-Q system from Millipore Corporation (Burlington, MA,
111 USA), which was filtered through an integrated 0.22 µm nylon membrane. Commercial
112 mineral water obtained from Eroski (Barcelona, Spain), with a chemical composition of
113 403 mg/L dry residue at 180 ºC, 326 mg/L bicarbonate, 44 mg/L chloride, 85 mg/L
114 calcium, 28 mg/L magnesium, 18 mg/L sodium, and 8 mg/L silica, was employed for
115 coffee brewing to keep constant any water influence on the results.

116 *2.2. Samples and sample treatment*

117 The proposed HPLC-UV and HPLC-FLD fingerprinting methods were applied to the
118 analysis of 88 commercially available samples grouped as instant coffee (40 samples),
119 instant decaf coffee (26 samples), and both instant and ground chicory (22 samples),
120 which were purchased from supermarkets in Barcelona (Spain). Description of the
121 analyzed samples (commercial name, number of samples, varieties, etc.) is summarized
122 in Table 1.

123 Instant coffee (normal and decaf) and chicory samples were prepared by dissolving
124 0.5 g of sample in 25 mL of mineral water heated at boiling point with a water heater
125 (Moulinex, Barcelona, Spain). Conventional chicory samples were brewed by employing
126 a Moka coffee maker. For that purpose, 35 g of sample and 400 mL of mineral water were
127 employed for the chicory brewing, with the help of a Bunsen burner to carry out the
128 chicory lixiviation. All samples were then filtered with 0.45 µm nylon filters
129 (Phenomenex) into 2 mL glass vials and stored at -4 ºC until HPLC analysis. The first
130 fraction of filtrate (ca. 1 mL) was discarded and the following mL was collected in the
131 injection vial for further analysis. Under these circumstances, possible losses of
132 compounds by adsorption in the membrane were minimized since the filter reached the
133 saturation. Besides, a quality control (QC) solution used to evaluate the repeatability of
134 the methods and the robustness of the chemometric results was prepared by mixing 50
135 µL of each sample extract. The QC was analyzed every ten samples. All samples were
136 analyzed randomly with the proposed HPLC-UV-FLD method to minimize the influence
137 of instrumental drifts in the chemometric models.

138 Two adulteration cases, an instant coffee (Nescafé Origins sample) and an instant
139 decaf coffee (Marcilla Crème sample), both adulterated with instant chicory (Chicorée
140 Biocop sample), were studied by partial least squares (PLS) regression. For each
141 adulteration cases, two sets of samples were created for calibration (to build the PLS
142 model) and validation (prediction purposes). Amounts of chicory adulterations in each
143 set of samples are summarized in Table 2. Each adulteration was prepared in
144 quintuplicate. Besides, an additional 50:50 adulteration mixture was used as QC in each
145 case and analyzed every ten samples. All samples of the adulteration studies were also
146 analyzed randomly with the proposed HPLC-UV-FLD methodology.

147 *2.3. Instrumentation*

148 HPLC-UV and HPLC-FLD fingerprints were obtained with an instrument from
149 Agilent 1100 HPLC Series (Waldbronn, Germany), which was equipped with a binary
150 pump (G1312A), an automatic sample injector (WPALS G1367A), and a UV-vis diode-
151 array detector (G1315B) and a fluorescence detector (G1321A) connected in series. The
152 instrument was controlled with the Agilent Chemstation software (Waldbronn,
153 Germany). Reversed-phase (RP) chromatographic separation in a Kinetex® C18 (100 ×
154 4.6 mm i.d., 2.6 µm partially porous particle size) column from Phenomenex (Torrance,
155 CA, USA) was employed under gradient elution using 0.1% formic acid acidified water
156 and methanol as mobile phase components (flow-rate at 400 µL min^{-1}). The elution
157 program used was as follows: from 0 to 30 min, linear gradient increase from 3 to 75%
158 methanol; from 30 to 32 min, linear gradient increase up to 95% methanol; from 32-34
159 min, isocratic elution at 95% methanol; from 34 to 34.2 min, back to initial conditions at
160 3% methanol; and from 34.2 to 40 min, isocratic elution at initial conditions for column
161 re-equilibration. The injection volume was 5 µL. HPLC analyses were performed at room
162 temperature. HPLC-UV fingerprints at 280 nm and HPLC-FLD fingerprints at 310 nm
163 (λexc) and 410 nm (λem) were chosen for authentication purposes.

164 *2.4. Data analysis*

165 HPLC-UV and HPLC-FLD fingerprints were used as sample chemical descriptors to
166 assess the characterization, classification, and authentication of the analyzed samples by
167 chemometrics. Principal component analysis (PCA), partial least squares regression-
168 discriminant analysis (PLS-DA), and PLS were used under Stand Alone Chemometrics
169 Software (SOLO) v. 8.6 from Eigenvector Research (Manson, WA, USA). More details
170 about the theoretical background of these chemometric methods are addressed elsewhere
171 (Massart et al., 1997). In all the chemometric models, data were autoscaled to provide the

172 same weight to each variable by suppressing differences in both their magnitude and
173 amplitude of scales.

174 PCA was employed as an exploratory method to evaluate the performance of the QCs
175 and to ensure the robustness of the chemometric. In contrast, PLS-DA was used as the
176 classification method, building the models from a training set composed of well-known
177 samples belonging to the different classes to be assessed (coffee, decaf coffee, and
178 chicory). PLS was employed as a multivariate calibration method to quantify the
179 percentage of chicory in the adulteration cases described in the previous section.

180 Independently of the chemometric method employed, X-data matrices consisted of
181 the acquired HPLC-UV or HPLC-FLD fingerprints (UV-vis absorbance and fluorescence
182 emitted signal, respectively, as a function of chromatographic time). Instead, Y-data
183 matrices defined each sample class in PLS-DA, whereas for PLS the adulteration
184 percentages were employed. The most appropriate number of latent variables (LVs) in
185 PLS-DA and PLS were established at the first significant minimum point of the cross-
186 validation (CV) error based on Venetian blind approach. The applicability of the built
187 PLS-DA models was evaluated by external validation. For that purpose, 70% of a sample
188 group (randomly selected) was used as the training set (data set used for model generation
189 and optimization), and the remaining 30% of the samples, used as unknown samples,
190 constituted an independent validation set (used for model prediction). In the case of the
191 PLS studies, models were validated by using the prediction sets as defined in Table 2.

192 3. **Results and discussion**

193 As previously commented in the introduction section, non-targeted chromatographic
194 fingerprinting approaches are emerging as simple and feasible strategies to assess the
195 classification and authentication of food products. In previous works, we demonstrated
196 that HPLC-UV and HPLC-FLD fingerprints were excellent chemical descriptors to

classify coffee samples according to their variety (Arabica vs. Robusta), and could also be considered appropriate for tentative discrimination of the samples according to their production region and roasting degrees (Núñez et al., 2020; Núñez, Martínez, et al., 2021). The present work aims at evaluating the applicability of non-targeted HPLC-UV and HPLC-FLD chromatographic fingerprints as sample chemical descriptors to assess the authenticity of instant coffee and decaf coffee to prevent fraudulent practices, especially regarding their adulteration with instant chicory, a low-cost raw material described as a common adulterant in coffee.

3.1. HPLC-UV and HPLC-FLD chromatographic fingerprints

In the present work, non-targeted HPLC-UV and HPLC-FLD chromatographic fingerprints were obtained simultaneously by connecting in series both UV-vis and fluorescence detectors (HPLC-UV-FLD method). Fig. 1 shows the HPLC-UV (a) and HPLC-FLD (b) chromatographic fingerprints obtained for a representative sample of each group (instant coffee, instant decaf coffee, and ground chicory). As can be seen, HPLC-FLD fingerprints are in all cases richer regarding the number of signals (bioactive substances) detected in comparison to HPLC-UV fingerprints. When comparing the different groups of samples, coffee and decaf coffee samples seem to provide similar fingerprints for both HPLC-UV and HPLC-FLD from the point of view of signal distributions, but differences are observed in the relative abundances of each signal depending on the sample. In contrast, chicory fingerprints are clearly different from those observed with coffee samples, not only from the point of view of peak signal distributions but also regarding their relative abundances. This is clearly noticeable with HPLC-UV fingerprints (Fig. 1a) in the case of chicory, with lower number of detected compounds in comparison to coffee samples, but with important differences in their ultraviolet profiles.

222 Noteworthy differences were then observed in the obtained fingerprints among the
223 three groups of analyzed samples, and since these features were reproducible among the
224 samples belonging to the same group, these chemical descriptors were then evaluated to
225 classify the samples through multivariate chemometric approaches.

226 *3.2. Exploratory study by PCA*

227 The repeatability of the proposed fingerprinting methods and the robustness of the
228 obtained chemometric results were first evaluated by PCA from the study of the
229 performance of the QCs. Fig. 2 depicts the PCA score plots of PC1 vs. PC2 when HPLC-
230 UV fingerprints (a) and HPLC-FLD fingerprints (b) were employed. As observed, QCs
231 appeared perfectly grouped when using HPLC-UV fingerprints (Fig. 2a). In contrast,
232 when HPLC-FLD fingerprints were utilized, the grouping of QCs was more disperse, and
233 a tendency was observed related to the duration of the instrumental sample sequence (see
234 the distribution from the first injected QC at the bottom of the plot to the last one at the
235 top of the plot in Fig. 2b). As both chromatographic fingerprints were obtained
236 simultaneously for each analyzed sample (HPLC-UV-FLD method), the differentiation
237 on the repeatability of the obtained results cannot be attributed to reproducibility
238 problems during injection and chromatographic separation but to the decay of the
239 fluorescence intensity throughout the sequence of analysis time (probably because of the
240 drift in the Xe lamp intensity), in comparison to the UV-vis detector. Therefore, HPLD-
241 FLD fingerprinting data need to be corrected to guarantee a good interpretation of the
242 chemometric results when performing classification and authentication studies. As the
243 QC is the same extract solution injected at different moments during the sample sequence,
244 to perform this correction, each sample fingerprinting was divided by the equivalent one
245 in the closest QC injected in the sequence, while each QC signal was divided by itself
246 (resulting in fingerprinting variables defined by the value 1). The PCA score plot of PC1

247 vs. PC2 when using corrected HPLC-FLD fingerprints is provided in Fig. 2c. As
248 observed, QCs appeared all in the same position, and the samples distribution changes in
249 comparison to the non-corrected PCA plot (Fig. 2b), increasing the grouping of samples.

250 Regarding the distribution of the analyzed samples by PCA, chicory samples are
251 perfectly discriminated from the other two groups in all cases (Fig. 2). In contrast,
252 exploratory PCA is not able to separate coffee and decaf coffee in two groups with none
253 of the HPLC fingerprints employed, although it should be mention that PCA is not a
254 classificatory chemometric method.

255 Once established that the performance of the non-targeted HPLC-UV and HPLC-
256 FLD fingerprinting strategies and the chemometric evaluation is good enough from the
257 point of view of QCs, the chemical descriptors were subjected to classificatory studies by
258 PLS-DA.

259 *3.3. Sample classification by PLS-DA*

260 The characterization and classification of the analyzed coffee, decaf coffee and
261 chicory samples was evaluated by the supervised PLS-DA method. Fig. 3 shows the PLS-
262 DA score plots of LV1 vs. LV2 when HPLC-UV (a) and HPLC-FLD (b) fingerprints
263 were employed as sample chemical descriptors. As can be seen, samples tend to be clearly
264 classified and grouped according to the sample type. Perfect discrimination among
265 chicory samples with respect the coffee ones was achieved with both HPLC-UV and
266 HPLC-FLD fingerprints, being an interesting result as chicory is employed as adulterant
267 of coffee. Chicory samples are exhibiting negative LV1 values while coffee samples tend
268 to exhibit positive LV1 values. In contrast, full discrimination among coffee and decaf
269 coffee samples was not accomplished, although certain separation was achieved
270 according to LV2.

271 To ensure the applicability of the proposed non-targeted HPLC fingerprinting
272 methodologies, the classification rate was studied for paired PLS-DA models: (i) coffee
273 vs. decaf coffee, (ii) coffee vs. chicory, and (iii) decaf coffee vs. chicory, being the last
274 two models the more interesting ones regarding fraudulent practices. With this aim, the
275 paired PLS-DA models were established by using 70% of the samples (randomly
276 selected) for each group as the calibration set, and the remaining 30% of the samples for
277 each group were "unknown samples" for prediction and validation purposes. As an
278 example, Fig. 4 shows the validation results of paired PLS-DA models when HPLC-UV
279 fingerprints (a, c, and e) or HPLC-FLD fingerprints (b, d, and f) were used as sample
280 chemical descriptors. Regarding the calibration models, all of them achieved a 100%
281 classification rate into their corresponding groups. When addressing the prediction of
282 coffee versus decaf coffee samples, few of them were not correctly classified depending
283 on the model. For example, when using HPLC-UV fingerprints (Fig. 4a) one coffee
284 sample was incorrectly classified as a decaf one (providing a 94.4% classification rate).
285 Three samples (1 coffee and 2 decaf coffees) were not assigned to their correct group
286 when using HPLC-FLD fingerprints (Fig. 4b), which represented an 83.3% classification
287 rate. This could be expected as these two groups of samples were grouped in closest areas
288 by PLS-DA as shown in Fig. 3. However, when addressing the prediction of both coffee
289 and decaf coffee against chicory samples (which is the probable adulterant when
290 fraudulent practices are present), all the samples used as "unknown" for prediction and
291 validation purposes were correctly classified in their corresponding group (100%
292 classification rate) independently of the type of fingerprints employed as chemical
293 descriptors. The classification capabilities of the proposed fingerprinting strategies for
294 instant coffee and chicory samples are comparable or even better than the one described
295 by employing IR and chemometrics (Briandet et al., 1996). In addition, the observed

296 classification performance is in agreement with results found using the same
297 methodology when addressing the classification of coffee samples according to their
298 region of origin, variety and roasting degree (Núñez et al., 2020; Núñez, Martínez, et al.,
299 2021). Because of these good classification rate results with chicory samples, PLS
300 regression was then employed to build multivariate calibration models for the quantitation
301 of chicory levels in the adulteration of both coffee and decaf coffee samples.

302 *3.4. Detection and quantitation of coffee adulterations by chemometrics*

303 The feasibility of HPLC-UV and HPLC-FLD fingerprints to detect and quantify
304 instant coffee adulterations with chicory was evaluated by PLS. Two adulteration cases
305 were used: (i) an instant coffee (Nescafé Origins sample) and (ii) an instant decaf coffee
306 (Marcilla Crème sample), both adulterated with different amounts of an instant chicory
307 (Chicorée Biocop sample). The LVs employed and the PLS calibration and prediction
308 results in the quantitation of chicory percentages of adulteration are summarized in Table
309 3. Fig. 5 depicts, as examples, the PLS-DA plots showing the distribution of both
310 calibration and prediction samples (top plots), and the scatter plots of measured vs.
311 predicted percentages of chicory adulteration (bottom plots), when an instant decaf coffee
312 sample was adulterated with chicory, and by employing HPLC-UV (a) and HPLC-FLD
313 (b) fingerprints as sample chemical descriptors. As can be seen, the PLS-DA plots clearly
314 show the distribution of samples according to the adulterant percentage, with pure decaf
315 coffee and chicory samples located at the left and right side of the plot, respectively. In
316 between, samples tend to be located according to the chicory percentage, showing the
317 predominant of LV1 in the adulteration factor. Besides, the obtained PLS calibration
318 models (Fig. 5, bottom plots) were very good, as indicated by the low calibration errors
319 ($\leq$2.1%), bias values tending towards zero, and good linearities ($R^2 \geq 0.996$) (Table 3),
320 independently of the fingerprints employed. Excellent prediction performance was also

321 accomplished, with prediction errors lower than 3.5% in all cases. Although very good
322 results were achieved with both HPLC-UV and HPLC-FLD fingerprints, overall HPLC-
323 FLD seemed to provide better calibration (≤0.8%), and similar to better prediction errors
324 than HPLC-UV, probably due to the superior selectivity of fluorescence detection. This
325 behavior was also observed when employing the same non-targeted HPLC fingerprinting
326 methodologies to address coffee fraud adulterations based on production region and
327 coffee varieties, although exceptionally better results were obtained with the chicory
328 adulteration cases under study (Núñez et al., 2020; Núñez, Saurina, et al., 2021).
329 Regarding the authentication of instant coffee samples adulterated with chicory, the
330 proposed methodology showed both better classification and fraud authentication
331 capabilities than previously described methodologies (Briandet et al., 1996; Charlton et
332 al., 2002). Moreover, the advantage of the proposed methodology is that not only
333 adulteration cases with chicory can be identified but also the chicory adulterant
334 concentration level was quantified with low prediction errors.

335 **4. Conclusions**

336 Non-targeted HPLC-UV and HPLC-FLD fingerprints have proved to be good sample
337 chemical descriptors to accomplish the characterization, classification, and authentication
338 of instant coffee (normal and decaf ones) and chicory samples. The discrimination
339 capabilities achieved by both fingerprinting methods by PLS-DA were similar when
340 addressing the classification of coffee or decaf coffee against chicory samples, with a
341 100% classification rate. In contrast, when classifying coffee against decaf coffee, HPLC-
342 UV fingerprinting provided better classificatory results, with a classification rate of
343 94.4% in comparison to the 83.3% classification rate observed with HPLC-FLD
344 fingerprints.

345 The capability of the proposed HPLC fingerprints to detect and quantify coffee frauds
346 (down to 15% adulterant levels) using PLS multivariate calibration was established by
347 evaluating two adulteration cases, instant coffee and instant decaf coffee both adulterated
348 with instant chicory. Excellent results were accomplished with both non-targeted HPLC
349 fingerprinting strategies, with overall PLS calibration and prediction errors below 2.1%
350 and 3.5%, respectively. In this study, HPLC-FLD fingerprinting showed better
351 performance in comparison to HPLC-UV fingerprinting, providing better PLS calibration
352 linearities, lower calibration errors, and similar prediction errors.

353 Therefore, both non-targeted HPLC-UV and HPLC-FLD fingerprinting strategies
354 resulted to be feasible, simple, and relatively cheap methodologies to address instant
355 coffee authentication and to detect and quantify frauds based on chicory adulteration.
356 Nevertheless, the analysis of a large number of samples belonging to different producers
357 will be required to better assess with confidence the capabilities of the proposed
358 methodology for authentication purposes

359

360 **Acknowledgments**

361 This work was supported by the Agency for Administration of University and
362 Research Grants (Generalitat de Catalunya, Spain) under the projects 2017SGR-171 and
363 2017SGR-310.

364 **References**

365 Alonso-Salces, R. M., Serra, F., Remero, F., & Heberger, K. (2009). Botanical and
366 geographical characterization of green coffee (Coffea arabica and Coffea
367 canephora): Chemometric evaluation of phenolic and methylxanthine contents.
368 *Journal of Agricultural and Food Chemistry*, *57*(10), 4224–4235.

369 https://doi.org/10.1021/jf8037117

370 Andrade, P. B., Leitão, R., Seabra, R. M., Oliveira, M. B., & Ferreira, M. A. (1998).
371 3,4-Dimethoxycinnamic acid levels as a tool for differentiation of Coffea
372 canephora var. robusta and Coffea arabica. *Food Chemistry*, *61*(4), 511–514.
373 https://doi.org/10.1016/S0308-8146(97)00067-8

374 Aquino, F. J. T., Augusti, R., Alves, J. de O., Diniz, M. E. R., Morais, S. A. L., Alves,
375 B. H. P., … Sabino, A. A. (2014). Direct infusion electrospray ionization mass
376 spectrometry applied to the detection of forgeries: Roasted coffees adulterated with
377 their husks. *Microchemical Journal*, *117*, 127–132.
378 https://doi.org/10.1016/j.microc.2014.06.016

379 Badmos, S., Lee, S. H., & Kuhnert, N. (2019). Comparison and quantification of
380 chlorogenic acids for differentiation of green Robusta and Arabica coffee beans.
381 *Food Research International*, *126*(April).
382 https://doi.org/10.1016/j.foodres.2019.108544

383 Bona, E., Marquetti, I., Link, J. V., Makimori, G. Y. F., da Costa Arca, V., Guimarães
384 Lemes, A. L., … Poppi, R. J. (2017). Support vector machines in tandem with
385 infrared spectroscopy for geographical classification of green arabica coffee. *LWT*
386 *- Food Science and Technology*, *76*, 330–336.
387 https://doi.org/10.1016/j.lwt.2016.04.048

388 Bosmali, I., Lagiotis, G., Stavridou, E., Haider, N., Osathanunkul, M., Pasentsis, K., &
389 Madesis, P. (2020). Novel authentication approach for coffee beans and the brewed
390 beverage using a nuclear-based species-specific marker coupled with high
391 resolution melting analysis. *Lwt*, (May), 110336.
392 https://doi.org/10.1016/j.lwt.2020.110336

393 Briandet, R., Kemsley, E. K., & Wilson, R. H. (1996). Approaches to adulteration

394 detection in instant coffees using infrared spectroscopy and chemometrics. *Journal*
395 *of the Science of Food and Agriculture*, *71*(3), 359–366.
396 https://doi.org/10.1002/(sici)1097-0010(199607)71:3<359::aid-jsfa593>3.0.co;2-d
397 Carrera, F., León-Camacho, M., Pablos, F., & González, A. G. (1998). Authentication
398 of green coffee varieties according to their sterolic profile. *Analytica Chimica Acta*,
399 *370*(2–3), 131–139. https://doi.org/10.1016/S0003-2670(98)00303-1
400 Charlton, A. J., Farrington, W. H. H., & Brereton, P. (2002). Application of 1H NMR
401 and multivariate statistics for screening complex mixtures: Quality control and
402 authenticity of instant coffee. *Journal of Agricultural and Food Chemistry*, *50*(11),
403 3098–3103. https://doi.org/10.1021/jf011539z
404 Cossignani, L., Montesano, D., Simonetti, M. S., & Blasi, F. (2016). Authentication of
405 Coffea arabica according to Triacylglycerol Stereospecific Composition. *Journal*
406 *of Analytical Methods in Chemistry*, *2016*. https://doi.org/10.1155/2016/7482620
407 Dam, R. M. Van, Willett, W. C., Manson, J. E., & Hu, F. B. (2006). Coffee, Caffeine,
408 and Risk of Type 2 Diabetes. *Diabetes Care*, *29*(2), 398–403.
409 https://doi.org/10.1093/occmed/kqh123
410 Ferreira, T., Farah, A., Oliveira, T. C., Lima, I. S., Vitório, F., & Oliveira, E. M. M.
411 (2016). Using Real-Time PCR as a tool for monitoring the authenticity of
412 commercial coffees. *Food Chemistry*, *199*, 433–438.
413 https://doi.org/10.1016/j.foodchem.2015.12.045
414 Finotello, C., Forzato, C., Gasparini, A., Mammi, S., Navarini, L., & Schievano, E.
415 (2017). NMR quantification of 16-O-methylcafestol and kahweol in Coffea
416 canephora var. robusta beans from different geographical origins. *Food Control*,
417 *75*, 62–69. https://doi.org/10.1016/j.foodcont.2016.12.019
418 González, A. G., Pablos, F., Martín, M. J., León-Camacho, M., & Valdenebro, M. S.

419 (2001). HPLC analysis of tocopherols and triglycerides in coffee and their use as
420 authentication parameters. *Food Chemistry*, *73*(1), 93–101.
421 https://doi.org/10.1016/S0308-8146(00)00282-X
422 Heath, R. D., Brahmbhatt, M., Tahan, A. C., Ibdah, J. A., & Tahan, V. (2017). Coffee:
423 The magical bean for liver diseases. *World Journal of Hepatology*, *9*(15), 689–696.
424 https://doi.org/10.4254/wjh.v9.i15.689
425 ICO. (2020). *ICO Internacional Coffee Organization, ICO Daily Coffee Prices.*
426 *Available at http://www.ico.org/coffee_prices.asp (Accessed on 15th November*
427 *2020).*
428 Jumhawan, U., Putri, S. P., Yusianto, Bamba, T., & Fukusaki, E. (2015). Application of
429 gas chromatography/flame ionization detector-based metabolite fingerprinting for
430 authentication of Asian palm civet coffee (Kopi Luwak). *Journal of Bioscience*
431 *and Bioengineering*, *120*(5), 555–561. https://doi.org/10.1016/j.jbiosc.2015.03.005
432 Król, K., Gantner, M., Tatarak, A., & Hallmann, E. (2020). The content of polyphenols
433 in coffee beans as roasting, origin and storage effect. *European Food Research and*
434 *Technology*, *246*(1), 33–39. https://doi.org/10.1007/s00217-019-03388-9
435 Martins, V. D. C., Godoy, R. L. D. O., Gouveâ, A. C. M. S., Santiago, M. C. P. D. A.,
436 Borguini, R. G., Braga, E. C. D. O., … Nascimento, L. D. S. D. M. Do. (2018).
437 Fraud investigation in commercial coffee by chromatography. *Food Quality and*
438 *Safety*, *2*(3), 121–133. https://doi.org/10.1093/fqsafe/fyy017
439 Massart, D. L., Vandeginste, B. G. M., Buydens, L. M. C., de Jong, S., Lewi, P. J., &
440 Smeyers-Verbeke, J. (1997). *Handbook of chemometrics and qualimetrics*.
441 Amsterdam, The Netherlands: Elsevier.
442 Milani, M. I., Rossini, E. L., Catelani, T. A., Pezza, L., Toci, A. T., & Pezza, H. R.
443 (2020). Authentication of roasted and ground coffee samples containing multiple

444 adulterants using NMR and a chemometric approach. *Food Control*,
445 *112*(November 2019), 107104. https://doi.org/10.1016/j.foodcont.2020.107104
446 Monteiro, P. I., Santos, J. S., Alvarenga Brizola, V. R., Pasini Deolindo, C. T., Koot, A.,
447 Boerrigter-Eenling, R., … Granato, D. (2018). Comparison between proton
448 transfer reaction mass spectrometry and near infrared spectroscopy for the
449 authentication of Brazilian coffee: A preliminary chemometric study. *Food*
450 *Control*, *91*(10), 276–283. https://doi.org/10.1016/j.foodcont.2018.04.009
451 Núñez, N., Collado, X., Martínez, C., Saurina, J., & Núñez, O. (2020). Authentication
452 of the Origin, Variety and Roasting Degree of Coffee Samples by Non-Targeted
453 HPLC-UV Fingerprinting and Chemometrics. Application to the Detection and
454 Quantitation of Adulterated Coffee Samples. *Foods*, *9*(3), 378.
455 https://doi.org/https://doi.org/10.3390/foods9030378
456 Núñez, N., Martínez, C., Saurina, J., & Núñez, O. (2021). High-performance liquid
457 chromatography with fluorescence detection fingerprints as chemical descriptors to
458 authenticate the origin, variety and roasting degree of coffee by multivariate
459 chemometric methods. *Journal of the Science of Food and Agriculture*, *101*(June
460 2020), 65–73. https://doi.org/10.1002/jsfa.10615
461 Núñez, N., Saurina, J., & Núñez, O. (2021). Non-targeted HPLC-FLD fingerprinting for
462 the detection and quantitation of adulterated coffee samples by chemometrics.
463 *Food Control*, *124*(December 2020), 107912.
464 https://doi.org/10.1016/j.foodcont.2021.107912
465 Pauli, E. D., Barbieri, F., Garcia, P. S., Madeira, T. B., Acquaro, V. R., Scarminio, I. S.,
466 … Nixdorf, S. L. (2014). Detection of ground roasted coffee adulteration with
467 roasted soybean and wheat. *Food Research International*, *61*, 112–119.
468 https://doi.org/10.1016/j.foodres.2014.02.032

469 Peng, C. yi, Zhang, Y. ling, Song, W., Cai, H. mei, Wang, Y., & Granato, D. (2019).
470 Characterization of Brazilian coffee based on isotope ratio mass spectrometry
471 (δ13C, δ18O, δ2H, and δ15N) and supervised chemometrics. *Food Chemistry*,
472 *297*(January), 124963. https://doi.org/10.1016/j.foodchem.2019.124963
473 Rodríguez-Artalejo, F., & López-García, E. (2018). Coffee Consumption and
474 Cardiovascular Disease: A Condensed Review of Epidemiological Evidence and
475 Mechanisms. *Journal of Agricultural and Food Chemistry*, *66*(21), 5257–5263.
476 https://doi.org/10.1021/acs.jafc.7b04506
477 Seninde, D. R., & Chambers IV, E. (2020). Coffee Flavor : A Review. *Beverages*, *6*(3),
478 44. https://doi.org/https://doi.org/10.3390/beverages6030044
479 Suhandy, D., & Yulia, M. (2018). Discrimination of several Indonesian specialty
480 coffees using Fluorescence Spectroscopy combined with SIMCA method. *IOP*
481 *Conference Series: Materials Science and Engineering*, *334*(1).
482 https://doi.org/10.1088/1757-899X/334/1/012059
483 Toci, Aline T., & Farah, A. (2014). Volatile fingerprint of Brazilian defective coffee
484 seeds: Corroboration of potential marker compounds and identification of new low
485 quality indicators. *Food Chemistry*, *153*, 298–314.
486 https://doi.org/10.1016/j.foodchem.2013.12.040
487 Toci, Aline Theodoro, Farah, A., Pezza, H. R., & Pezza, L. (2016). Coffee Adulteration:
488 More than Two Decades of Research. *Critical Reviews in Analytical Chemistry*,
489 *46*(2), 83–92. https://doi.org/10.1080/10408347.2014.966185
490 Yulia, M., & Suhandy, D. (2019). Authentication of organic Lampung robusta ground
491 roasted coffee by UV-visible spectroscopy and PLS-DA method. *Journal of*
492 *Physics: Conference Series*, *1341*(2). https://doi.org/10.1088/1742-
493 6596/1341/2/022006

494 Zhao, L. G., Li, Z. Y., Feng, G. S., Ji, X. W., Tan, Y. T., Li, H. L., … Xiang, Y. B.
495 (2020). Coffee drinking and cancer risk: An umbrella review of meta-analyses of
496 observational studies. *BMC Cancer*, *20*(1), 1–12. https://doi.org/10.1186/s12885-
497 020-6561-9
498
499
500

501 ***Figure Captions***

502 **Fig. 1** Non-targeted HPLC-UV at 280 nm (a) and HPLC-FLD at 310 nm (excitation)
503 and 410 nm (emission) (b) chromatographic fingerprints obtained for a
504 representative sample of each group: instant coffee (Nescafé Classic Crème
505 sample), instant decaf coffee (Marcilla Classic sample), and ground chicory
506 (Chicorée Lima Original sample).

507 **Fig. 2** PCA score plots of PC1 vs. PC2 to evaluate QC performance when (a) HPLC-
508 UV fingerprints, (b) HPLC-FLD fingerprints, and (c) HPLC-FLD
509 fingerprints corrected with the QC signals were used as sample chemical
510 descriptors.

511 **Fig. 3** PLS-DA score plots of LV1 vs. LV2 for the classification of the analyzed
512 samples when using (a) HPLC-UV and (b) HPLC-FLD fingerprints as sample
513 chemical descriptors. The number of LVs employed to build the PLS-DA
514 models for (a) and (b) were 7 and 6, respectively.

515 **Fig. 4** Validation of the paired PLS-DA models when using HPLC-UV fingerprints
516 (a, c, and e) and HPLC-FLD fingerprints (b, d, and f) as sample chemical
517 descriptors.

518 **Fig. 5** PLS-DA and PLS results for the instant decaf coffee adulterated with chicory
519 case when HPLC-UV (a) and HPLC-FLD (b) fingerprints were employed as
520 sample chemical descriptors. Top plots: PLS-DA scatter plots showing the
521 distribution of both calibration and prediction samples according to the
522 chicory adulterant level. Bottom plots: scatter plots of measured vs. predicted
523 percentages of chicory adulteration.

524

525 Table 1. Description of the coffee and chicory samples analyzed.

Sample	Commercial brand	Type	Number of samples*
Coffee	Marcilla Classic Natural	Instant	4
	Marcilla Crème Natural	Instant	4
	Nescafé Classic	Instant	6
	Nescafé Classic Crème	Instant	4
	Nescafé Black Roast	Instant	4
	Nescafé Gold	Instant	6
	Nescafé Origins	Instant	6
	Eroski	Instant	6
Decaf Coffee	Marcilla Classic	Instant	4
	Marcilla Crème	Instant	4
	Nescafé Classic	Instant	6
	Nescafé Gold	Instant	6
	Eroski	Instant	6
Chicory	Chicorée lima original	Ground	8
	Chicorée Biocop	Instant	8
	Chicorée La niña	Ground	6

526 *Number of samples collected from different containers

527

528

529

530

531

532

533

534 Table 2. Samples used as calibration and validation sets in the assessment of
535 adulteration studies by PLS.

	Coffee or decaf coffee (%)	Chicory as adulterant (%)
Calibration set	100	0
	80	20
	60	40
	40	60
	20	80
	0	100
Validation set	85	15
	75	25
	50	50
	25	75
	15	85

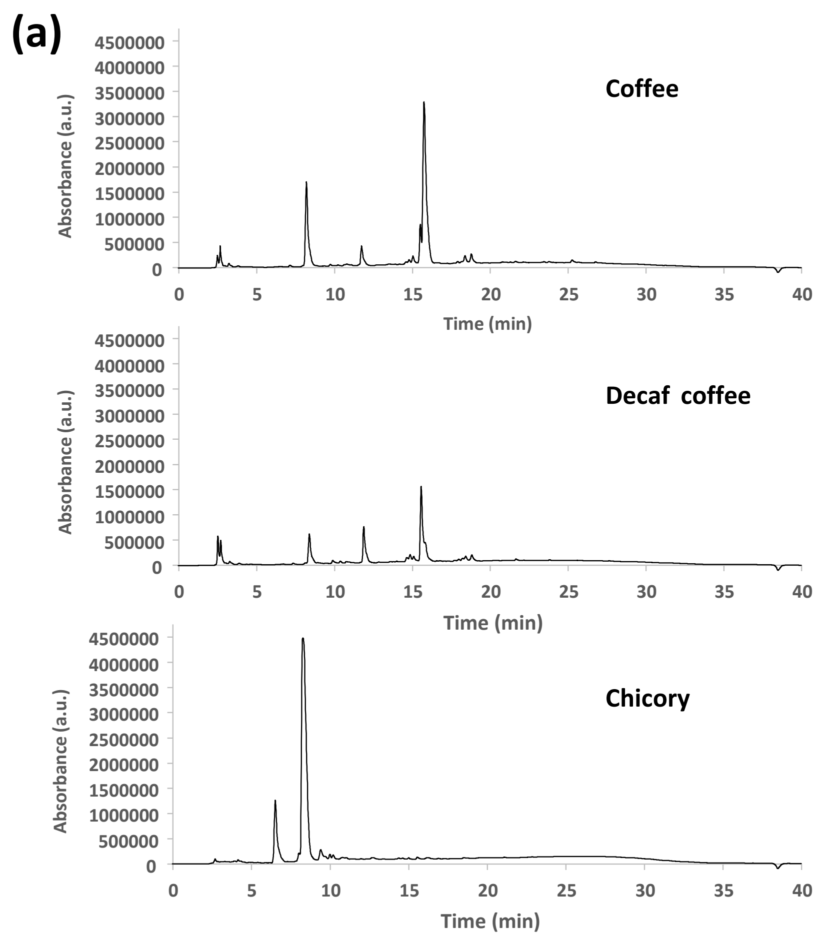
536
537
538
539
540
541
542
543
544
545
546

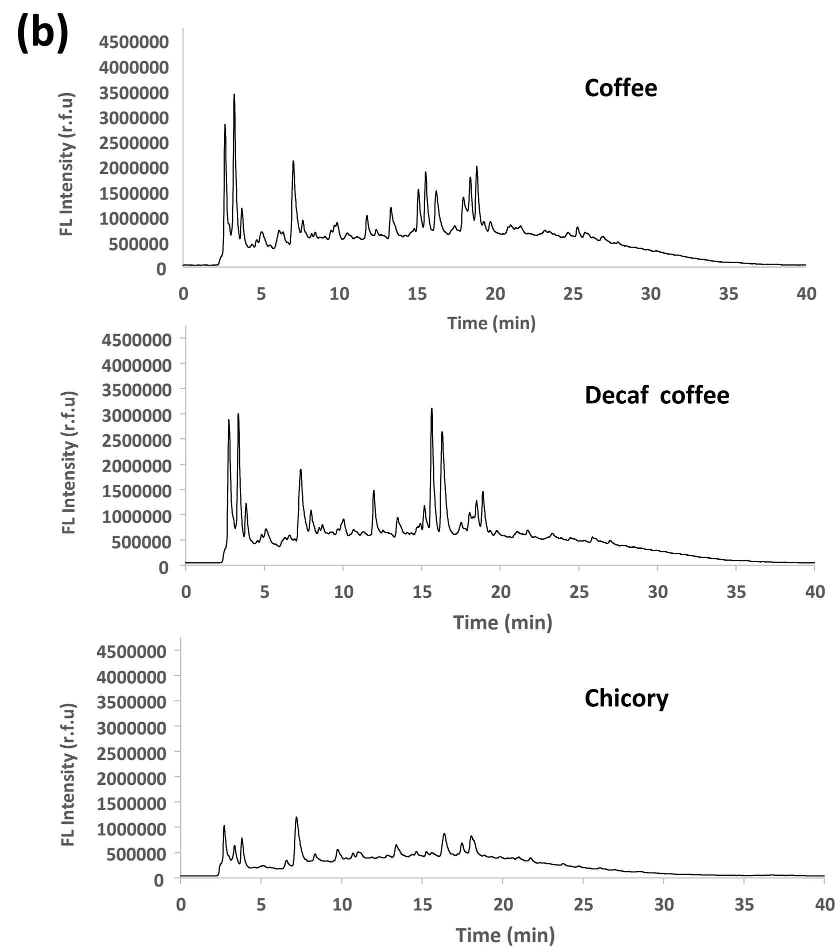
547 Table 3. Results for the evaluation of the adulteration of instant coffee and instant decaf coffee
548 with instant chicory using HPLC-UV and HPLC-FLD as chemical descriptors for PLS.

	Chicory adulteration							
	HPLC-UV fingerprints				**HPLC-FLD fingerprints**			
	LVs	**Linearity (R^2)**	**Calibration error (%)**	**Prediction error (%)**	**LVs**	**Linearity (R^2)**	**Calibration error (%)**	**Prediction error (%)**
Instant coffee	3	0.996	2.1	2.4	5	0.999	0.8	2.9
Instant decaf coffee	3	0.996	2.0	3.5	5	1.000	0.7	3.2

549
550
551
552
553
554
555
556
557
558
559
560
561
562
563

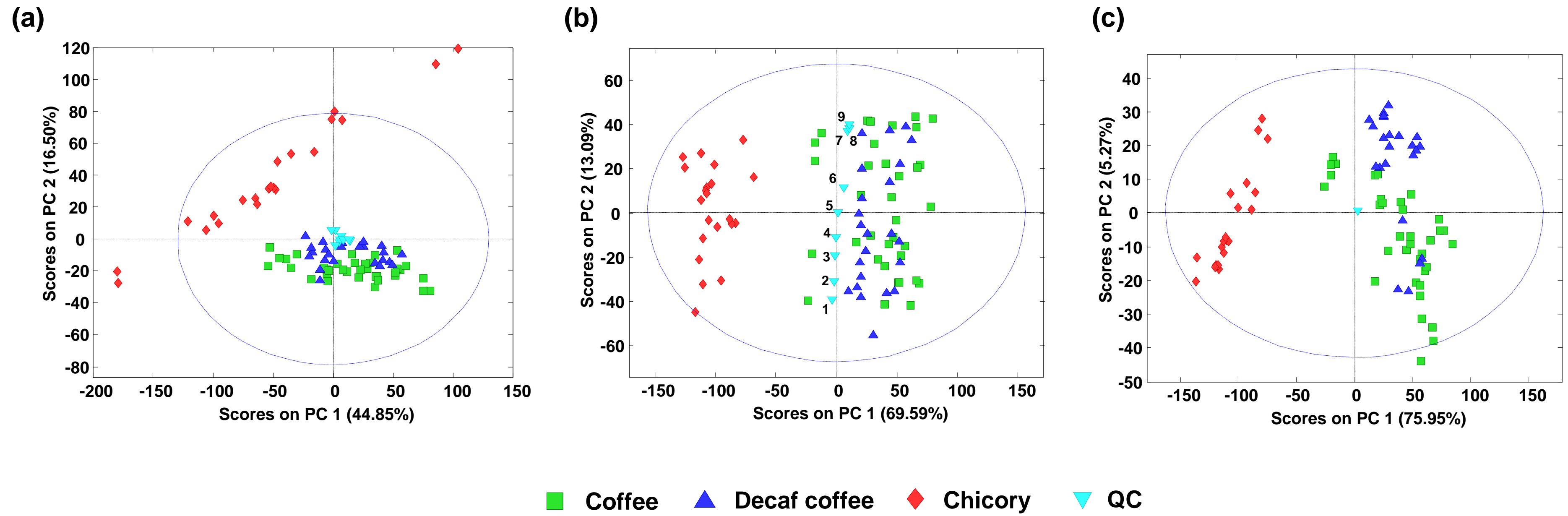
564 Figure 1





565

566 Figure 2



567

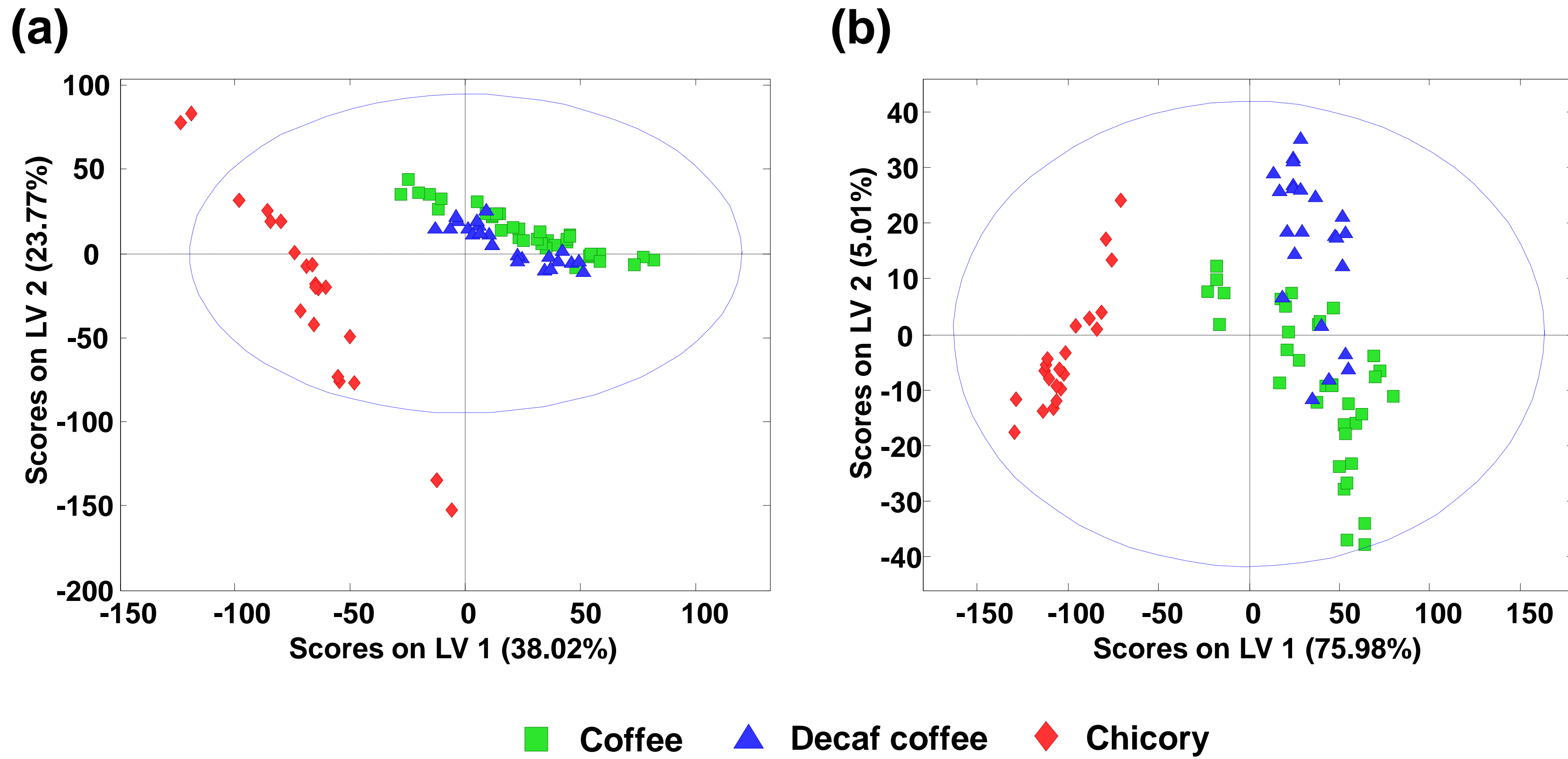
568

569

570

571

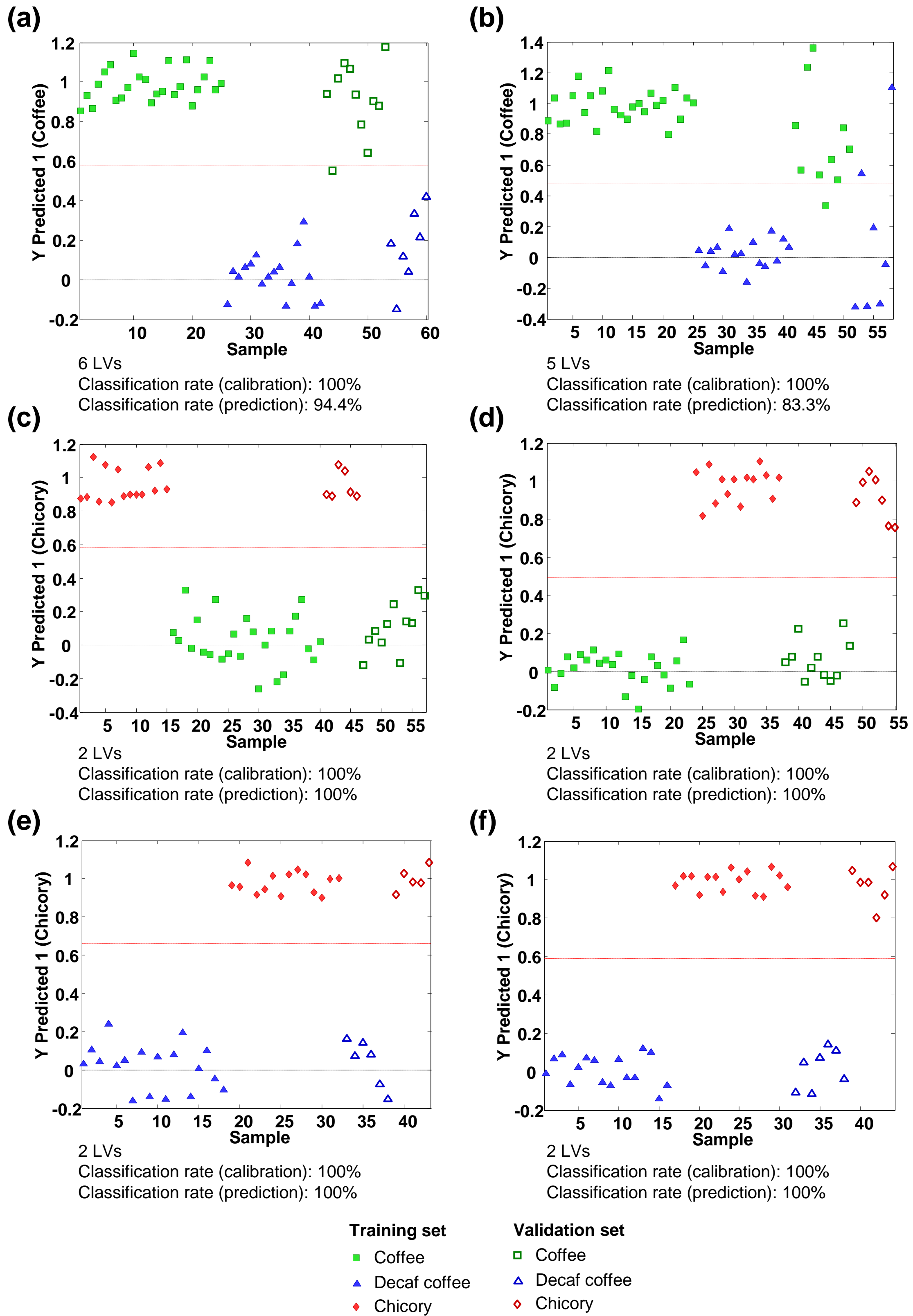
572 Figure 3



573

574

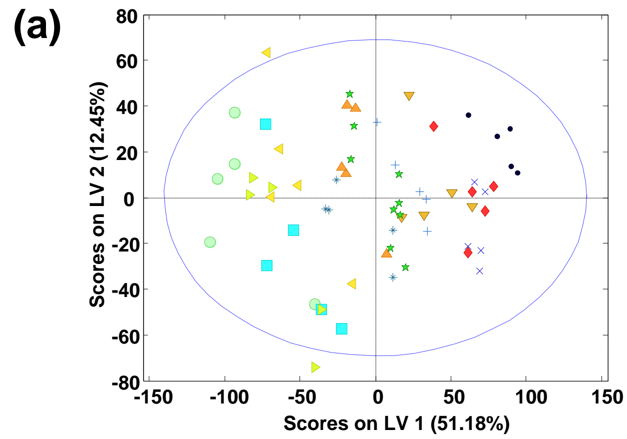
575 Figure 4

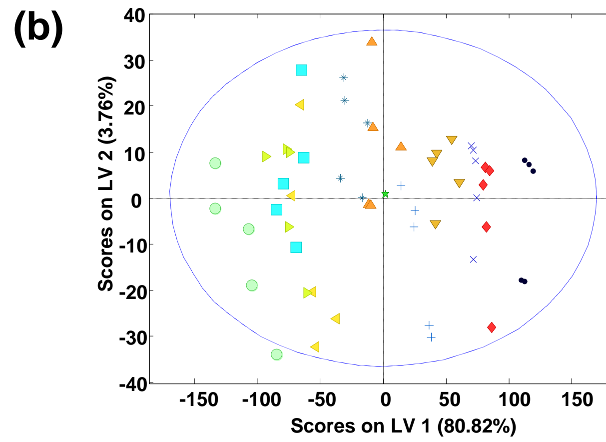


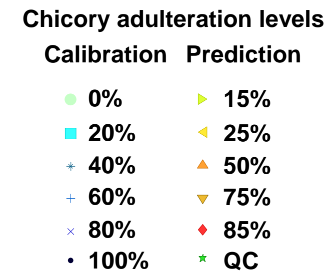
576

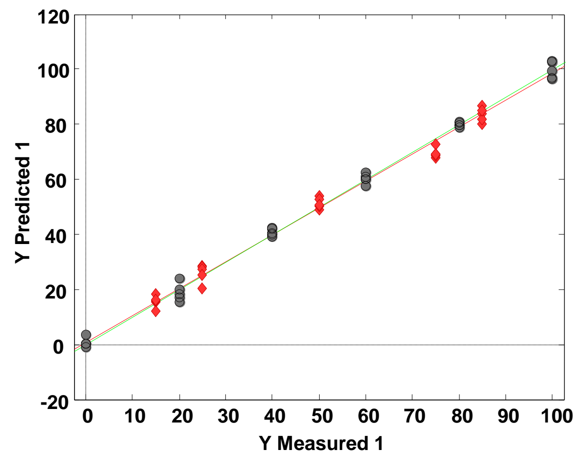
577

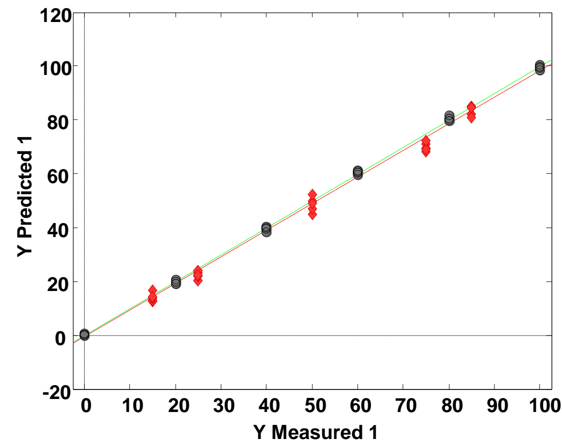
578 Figure 5











579