Non-targeted HPLC-FLD fingerprinting for the detection and quantitation of adulterated coffee samples by chemometrics

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Abstract

Coffee is today one of the most popular beverages in the world and the determination of its authenticity is an important issue considering the increase of adulteration cases in the last years. In this work, a simple and efficient non-targeted HPLC-FLD fingerprinting method was employed to detect and quantify adulteration levels in coffee samples by partial least squares (PLS) regression to guarantee food integrity and authenticity. For that purpose, different adulteration cases, involving both coffee production region and variety, were evaluated by pairs (Colombia-Ethiopia, Colombia-Nicaragua, India-Indonesia, Vietnam Arabica-Vietnam Robusta, Vietnam Arabica-Cambodia, and Vietnam Robusta-Cambodia adulteration cases). Overall, the proposed non-targeted HPLC-FLD fingerprinting strategy showed very good results with PLS cross-validation and prediction errors below 3.4% and 7.5%, respectively, for adulteration levels below 15%. Therefore, non-targeted HPLC-FLD fingerprints demonstrated to be suitable to assess coffee integrity and authenticity in the control and prevention of frauds.

Keywords: Coffees; HPLC-FLD; Fingerprinting; Chemometrics; Food Authentication; Food Safety

1. INTRODUCTION

Coffee, an infusion of ground roasted coffee beans, is one of the most popular beverages in the world. The coffee plant belong to *Coffea genus* from *Rubiaceae* family with more than 70 species being *Arabica Coffea* (Arabica) and *Canephora Coffea* (Robusta) the only ones that have an economic and commercial importance (Esquivel & Jiménez, 2012; Naranjo, Vélez, Benjamín, & Iii, 2011; Thorburn Burns, Tweed, & Walker, 2017). Intake of coffee is associated with a reduced risk of several diseases probably due to its antioxidant activity, known for its beneficial effects in human health. Thus, the content of bioactive substances depends on the coffee species (Arabica or Robusta), the production region, and the roasting degree, among other parameters (Crozier, Ahihara, & Tomás-Barbéran, 2012; Esquivel & Jiménez, 2012; Naranjo et al., 2011; Thorburn Burns et al., 2017).

Unfortunately, coffee is a drink with one of the highest number of fraud cases reported because it can be very easily adulterated through practices that include supplementation with flavours or aromas, and the use of unspecified additives to increase its volume, among others (Kamiloglu, 2019; Thorburn Burns et al., 2017). These practices are illegal worldwide and not only has economic consequences, but could imply a danger to the consumer health because only the food handler knows how the product has been modified being the only one with the information, but not necessarily with the experience to evaluate if such manipulation poses any risk for the consumer (G. Campmajó, Núñez, & Núñez, 2019; Gonzalvez, Armenta, & Guardia, 2009; Kamiloglu, 2019; Moore, Spink, & Lipp, 2012). Considering the complexity of the food chain, where many players are involved from the production to the consumption of ground coffee, it is practically impossible to know the origin of all the components that may conform the final commercial product. Consequently, adulteration cases in coffee for financial gain are

increasing. For that reason, analytical methodologies to guarantee food integrity and quality, as well as food safety, by assessing its authenticity are really necessary (G. Campmajó et al., 2019; Gonzalvez et al., 2009; Kamiloglu, 2019; Moore et al., 2012).

From the point of view of the development of analytical methodologies and strategies for the characterization, classification, and authentication of food products, two main analytical approaches, targeted and non-targeted, can be considered (G. Campmajó et al., 2019). Regarding coffee, several targeted methodologies have been described in the literature for the quantification of selected substances, some of them aiming to assess the discrimination and classification of different types of coffee. For instance, liquid chromatography with ultraviolet detection (LC-UV) was employed for the quantification of eight biogenic amines (BAs) to discriminate different coffee brewing procedures (Restuccia, Spizzirri, Parisi, Cirillo, & Picci, 2015). In another work, liquid chromatography with fluorescence detection (LC-FLD) was used for the identification and quantitation of Ochratoxin A, a toxic and carcinogenic substance, in green coffee (Moez et al., 2020). Lately, liquid chromatography coupled with mass spectrometry (LC-MS) have also been applied to coffee authentication (Mohd Yusop, Xiao, & Fu, 2019), by determining phosphodiesterase 5 inhibitors in instant coffee. Apart of liquid chromatography, other techniques such as gas chromatography (Ongo, Montevecchi, Antonelli, Sberveglieri, & Sevilla, 2020) and direct analysis in real-time ionization (Danhelova et al., 2012) have also been employed coupled to mass spectrometry for coffee analysis.

The use of non-targeted approaches, by registering instrumental signals associated to known or unknown compounds detected in the samples (fingerprinting approaches) is increasing in the last years. In this sense, the mass spectrometric data obtained from different separation techniques such as liquid chromatography (Mehari et al., 2016;

Pérez-míguez, Sánchez-lópez, Plaza, Castro-puyana, & Marina, 2018; Xu et al., 2019), gas chromatography (Mehari et al., 2019; Ongo et al., 2020; Putri, Irifune, Yusianto, & Fukusaki, 2019), and capillary electrophoresis (Pérez-Míguez, Sánchez-López, Plaza, Marina, & Castro-Puyana, 2019), and employing chemometric methods for data comparison, is among the most popular strategies to address the characterization and classification of coffee samples. Although mass spectrometry fingerprinting is excellent to achieve coffee authentication, other less expensive chromatographic fingerprinting strategies such as LC-UV (Núñez, Collado, Martínez, Saurina, & Núñez, 2020) or LC-FLD (Núñez, Martínez, Saurina, & Núñez, 2021) have been recently proposed to classify coffee samples according to the production region, coffee variety and roasting degree, with remarkable results. The fingerprinting volatilome analysis by employing an electronic nose was also recently applied to characterize and authenticate roasted coffee arabica beans from different countries (Marek et al., 2020).

Even though most of the studies described in literature focus on the analysis of original coffee samples, some of them work on coffee adulteration cases with of coffees of inferior quality (other coffee types, varieties, production region, etc.) or even different products such as chicory, corn, barley, brown sugar, soybean, wheat (Daniel, Lopes, Santos, & do Lago, 2018; de Morais, Rodrigues, de Carvalho Polari Souto, & Lemos, 2019; Song, Jang, Debnath, & Lee, 2019; Souto et al., 2015; Thorburn Burns et al., 2017; Winkler-Moser et al., 2015). For instance, LC-UV (Núñez et al., 2020; Song et al., 2019), capillary electrophoresis coupled to mass spectrometry (Daniel et al., 2018), nuclear magnetic resonance (NMR) (Ciampa, Renzi, Taglienti, Sequi, & Valentini, 2010; Milani et al., 2020), laser induced breakdown (LIB) (Sezer, Apaydin, Bilge, & Boyaci, 2018), and infra-red (IR) (Pizarro, Esteban-Díez, & González-Sáiz, 2007) spectroscopies, electronic

tongues (de Morais et al., 2019), and digital images (Souto et al., 2015) have been proposed to investigate different adulterants in coffee.

In a previous study, an HPLC-UV fingerprinting method was developed to deal with the classification and characterization of coffee samples from different regions of origin and varieties, achieving a satisfactory discrimination between the analyzed samples (Núñez et al., 2020). The method was also employed to study adulteration cases. Alternatively, a HPLC-FLD fingerprinting method was established to deal with similar purposes, in that case achieving a better discrimination between samples than that by HPLC-UV fingerprinting (Núñez et al., 2021). Because of the good results previosyly obtained, this work aims to evaluate the feasibility of non-targeted HPLC-FLD fingerprinting method to provide sample chemical descriptors to detect and quantify adulteration levels by partial least squares (PLS) regression in fraudulent coffee samples, involving production region and coffee variety adulterations.

2. MATERIALS AND METHODS

2.1 Chemicals

The mobile phase was composed of methanol from PanReac AppliChem (HPLC grade, Barcelona, Spain), formic acid (≥98%) from Sigma-Aldrich (St Louis, MO, USA), and Milli-Q water. An Elix 3 coupled to a Milli-Q system from Millipore Corporation (Millipore, Bedford, MA, USA) was used to purify the water, filtering it through a 0.22 µm nylon membrane integrated into Milli-Q system. Mineral water obtained from Eroski (Barcelona, Spain) was used for coffee brewing to keep constant any water influence on the obtained results.

153 **2.2 Instrumentation**

154 Chromatographic separation and chromatographic fingerprints were obtained on a HPLC
155 instrument from Agilent HPLC 1100 Series (Waldbronn, Germany) equipped with a
156 G1312A binary pump, a WPALS G1367A automatic sample injector, a G1321A
157 fluorescence detector, and a PC with the Agilent Chemstation software. The HPLC-FLD
158 fingerprints were generated with a Kinetex® C18 reversed-phase column (100 × 4.6 mm
159 i.d., 2.6 µm particle size) provided by Phenomenex (Torrance, California, USA) under
160 gradient elution conditions employing 0.1% formic acid in water (*v/v*) (solvent A) and
161 methanol (solvent B) as mobile phase components. The elution program applied consisted
162 of a linear gradient by increasing methanol percentage from 3 to 75% in 30 min. After
163 that, there was an isocratic step of 2 min. Then, methanol increased from 75% to 95% in
164 2 min. Finally, the elution program came back to mobile phase initial conditions in 0.2
165 min and, finally, there was an isocratic step of 5.8 min at 3% methanol to guarantee
166 column re-equilibration. The flow-rate was 0.4 mL/min and the injection volume was 5
167 µL. The FLD acquisition was carried out at 310 nm for excitation and 410 nm for
168 emission.

169 **2.3 Samples**

170 Master Origin Colombia, Ethiopia, India, Indonesia, and Nicaragua Nespresso® coffees,
171 all of them of *Coffea arabica*, were obtained from supermarkets in Barcelona (Spain).
172 Commercially available Vietnamese (both Arabica and Robusta varieties) and
173 Cambodian (unknown variety) coffee samples were obtained from supermarkets in
174 Vietnam and Cambodia, respectively. Available information regarding the employed
175 coffee samples is summarized in Table 1.

176 Six different coffee adulteration cases were studied involving different production
177 regions: (i) Colombian coffee adulterated with Ethiopian coffee, (ii) Colombian coffee

adulterated with Nicaraguan coffee, and (iii) Indian coffee adulterated with Indonesian one. Adulteration of coffees of different species and produced in close countries were also evaluated as follows: (i) an Arabica coffee adulterated with a Robusta coffee, both of them grown in Vietnam, (ii) a Vietnamese Arabica coffee adulterated with a Cambodian coffee, and (iii) a Vietnamese Robusta coffee adulterated with a Cambodian one. In order to achieve the quantification of the adulterant percentages by PLS, a calibration set and a validation set of samples were prepared as indicated in Table 2. The calibration set included the 20, 40, 60 and 80% adulteration levels, as well as the 100% pure coffee samples. For the validation set, 15, 25, 50, 75 and 85% adulteration levels were used. Besides, an additional quality control (QC) solution was prepared at a 50% of adulteration level to evaluate the repeatability of the method and the robustness of the chemometric results. Five replicates were prepared for each adulteration level, obtaining a total of 55 sample extracts to be analyzed in each one of the adulteration cases studied. Similar calibration/validation designs were used elsewhere for predicting adulteration rates by PLS with successful results (Guillem Campmajó, Saez-Vigo, Saurina, & Núñez, 2020; Núñez et al., 2020).

2.4 Data analysis

All the sample extracts were analyzed randomly with the proposed HPLC-FLD method, and injecting a QC after each ten samples. The obtained chromatograms were then exported to create different fingerprinting data matrices. These matrices were analyzed by PLS-DA and PLS methods using SOLO 8.6 chemometric software from Eigenvector Research (Manson, WA, USA). Details of the theoretical background of these chemometric methods are addressed elsewhere (Massart et al., 1997). For both, PLS-DA and PLS, the X-data matrix of responses consisted of the acquired HPLC-FLD chromatographic fingerprints. In contrast, Y-data matrix defines each sample class in

PLS-DA, whereas defines each adulterant percentage in PLS. To provide the same weight to each variable by suppressing differences in their magnitude and amplitude scales, HPLC-FLD fingerprints were autoscaled. The most appropriate number of latent variables (LVs) was established at the first significant minimum point of the cross-validation (CV) error from a Venetian blind approach.

3. RESULTS AND DISCUSSION

In a recently published work, we demonstrated the suitability of non-targeted HPLC-FLD fingerprints to be used as sample chemical descriptors for the classification of coffee samples according to the growing region (country of production) as well as the coffee species (Arabica vs. Robusta) by PLS-DA (Núñez et al., 2021). In views of the great classification rates (100% in all the cases studied), this work aims to evaluate the applicability of non-targeted HPLC-FLD fingerprints to detect coffee frauds and to quantify the adulteration levels. As described in section 2.3, six coffee adulteration cases were studied, involving both adulterations with coffees grown in different countries, as well as coffees of different species.

3.1. Non-targeted HPLC-FLD fingerprints of pure and adulterated coffee samples

Coffee adulterations were prepared for both calibration and validation sets as described in Table 2. Samples were brewed with mineral water, and the extracts analyzed with the proposed HPLC-FLD method to obtain the corresponding chromatographic fingerprints. These non-targeted fingerprints are based on the instrumental response (fluorescence intensity signal) registered as a function of the chromatographic retention time, but without assuming any information regarding the chemicals responsible for the signals.

As an example, Figure 1 shows some HPLC-FLD fingerprints of a Vietnamese Robusta coffee adulterated with a Cambodian coffee. As can be seen, similar non-targeted HPLC-FLD fingerprints were obtained regarding the number of detected signals and their

distribution from the analyzed coffee extracts. However, differences regarding their relative abundances are observed, as 100% pure Vietnamese Robusta coffee seems to be richer in bioactive components than the Cambodian one, as a general trend. For example, all sample extracts present intense signal peaks in the chromatographic range from 13 to 27 min. Some of these detected signals are more abundant in the original coffee sample (Vietnamese Robusta) than in the coffee used as adulterant (labelled with an asterisk in Figure 1), and consequently their signal is decreasing with the adulterant percentage. However, the relative signal of other peaks seems to remain constant independently of the adulterant level (labelled with a dark point in Figure 1), while other are increasing (labelled with an arrow in Figure 1) as they are more abundant in the Cambodian sample. This behavior was also observed with the other adulteration cases under study, with HPLC-FLD fingerprints progressively changing from one pure coffee sample to the other. Besides, the obtained HPLC-FLD fingerprints were reproducible among adulterated samples belonging to the same adulteration level, so they were used as sample chemicals descriptors to quantify the coffee adulterant levels in the analyzed samples by PLS. The other adulterations cases studied showed similar tendencies, highlighting the intensity differences according the adulterant level of the coffee sample.

3.2. Detection and quantitation of adulteration by PLS

The capacity of non-targeted HPLC-FLD fingerprints to quantify coffee adulterations by PLS regression was evaluated in the six adulteration under study. First, the obtained fingerprints were subjected to PLS-DA to see the distribution of all the adulteration levels in the space of LV1 vs. LV2 for both calibration and validation sets. Results obtained for two of the studied adulteration cases, Colombian coffee adulterated with Ethiopian one (both of them of Arabica variety) and Vietnamese Arabica coffee adulterated with Vietnamese Robusta one, are shown in Figures 2a and 2b, respectively. As can be seen,

samples tend to be distributed through the plot of scores according to the adulteration content, with the pure 100% coffee (considered as the original sample, 0% adulterant) located at the left of the plot, and the 100% pure adulterant coffee at the right. In between, samples are distributed according to the adulterant percentage from left to right, showing the predominant of LV1 in the adulteration factor. The sample distribution will be clearly related to differences on the regional origin and on the coffee variety attributes in Figure 2a and 2b, respectively. Then, PLS multivariate calibration models were obtained, and the set of validation samples quantified. The PLS models are also shown in Figures 2a and 2b for the same adulteration cases previously described. As can be seen, the performance of the PLS calibration models was satisfactory, showing good linearity and very acceptable calibration and prediction errors (see values depicted in Table 3). The number of LVs to be used in each PLS is also given in Table 3. As can be seen, overall very good results were achieved in all the adulteration cases studied, with high correlation among actual and predicted adulteration percentages ($R^2 \geq 0.988$), excellent calibration errors with values below 3.4%, as well as prediction errors ranging from 3.5% to 7.5%, thus demonstrating the applicability of non-targeted HPLC-FLD fingerprints as sample chemical descriptors for the detection and quantitation of coffee frauds. Besides, when comparing the obtained PLS results with those previously reported by HPLC-UV (Núñez et al., 2020), a considerable improvent was observed. While similar calibration errors are obtained with both HPLC-UV and HPLC-FLD fingerprints, in general, much better prediction errors were observed with HPLC-FLD fingerprints, especially in the case of Colombian coffee adulterated with the Nicaraguan Coffee, with prediction errors decreasing from 18.3% to 6.1% when using HPLC-UV or HPLC-FLD fingerprints, respectively. This improvement is probably due to the higher number of bioactive

substances detected from the analyzed samples and the superior selectivity of fluorescence detection.

4. CONCLUSIONS

In this work, non-targeted HPLC-FLD chromatographic fingerprints acquired at 310 nm and 410 nm for excitation and emission, respectively, have proved to be suitable sample chemical descriptors for the authentication and quantification the adulterant concentration levels in fraudulent coffee samples. Multivariate calibration by PLS was applied to six adulteration cases involving coffee origin and variety to evaluate the capability of the proposed HPLC-FLD method to detect and quantify coffee frauds, even with adulterant levels below 15%. Excellent calibration and prediction errors were obtained, with values lower than 3.4% and 7.5%, respectively, thus improving considerable the method performance with respect to the results previously published based on HPLC-UV fingerprints. Therefore, the proposed non-targeted HPLC-FLD fingerprinting methodology resulted to be an excellent, simple, and relatively economic approach to address coffee authentication, in special to prevent coffee frauds in developing coffee production countries.

Conflict of Interest

There are no conflicts of interest to declare.

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Supporting Information description:

There is no supporting information.

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Figure captions

Figure 1. Non-targeted HPLC-FLD fingerprints obtained for the calibration set employed in the adulteration study of a Vietnamese Robusta coffee adulterated with a Cambodian coffee. Adulteration levels (Vietnamese Robusta): (a) 0% (pure Vietnamese Robusta coffee), (b) 20%, (c) 40%, (d) 60%, (e) 80%, and (f) 100% pure Cambodian coffee. Peaks labelled with asterisk, dark circle and arrow represent signals that decrease, remain constant, or increase with the adultareant concentration level.

Figure 2. PLS-DA (LV1 vs. LV2) and PLS results of (a) Colombian coffee adulterated with Ethiopian coffee and (b) Vietnamese Arabica coffee adulterated with Vietnamese Robusta coffee. Left plots: PLS-DA scatter plots showing the distribution of both calibration and prediction samples according to the adulterant level. Right plots: scatter plots of measured vs. predicted percentages of adulterant.

464 Table 1. Description of the employed commercially available coffee samples.

Commercial Name	Coffee variety	Origin Region	Roasting degree
Master Origin Colombia	Arabica	Colombia	3/5
Master Origin Ethiopia	Arabica	Ethiopia	2/5
Master Origin India	Arabica-Robusta Mixture	India	5/5
Master Origin Indonesia	Arabica	Indonesia	4/5
Master Origin Nicaragua	Arabica	Nicaragua	2/5
Vietnamese Coffee	Arabica	Vietnam	Unknown
Vietnamese Coffee	Robusta	Vietnam	Unknown
Cambodian Coffee	Unknown	Cambodia	Unknown

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Table 2. Coffee concentration levels employed in both calibration and validation sets for every adulteration case were X was the original coffee sample and Y was the coffee sample used as adulterant.

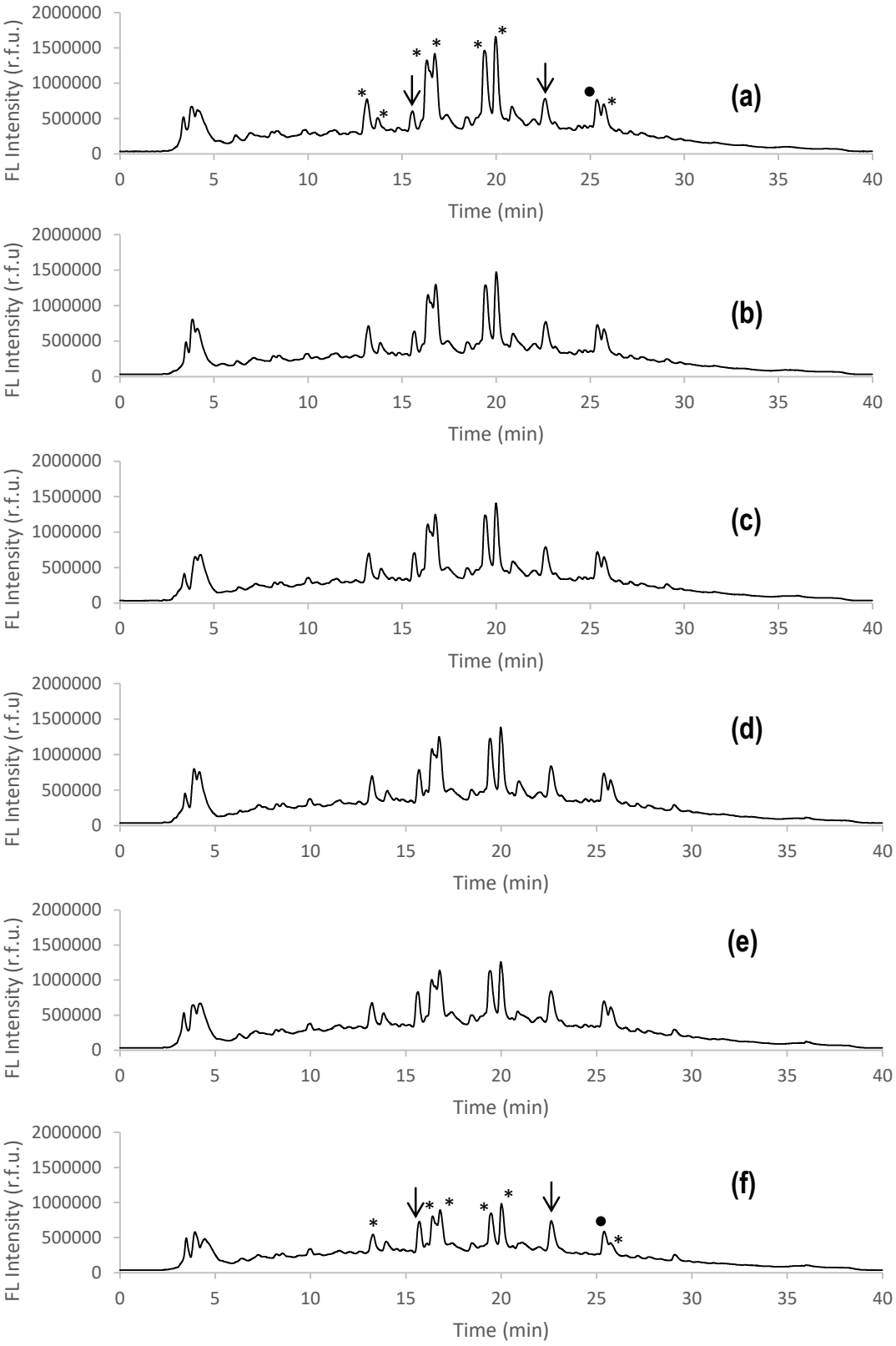
	Calibration set						Validation set				
X%	100	80	60	40	20	0	15	25	50	75	85
Y%	0	20	40	60	80	100	85	75	50	25	15

Table 3. Results for the evaluation of the adulteration cases using HPLC-FLD fingerprints as chemical descriptors for PLSR.

Original coffee	Coffee used as adulterant	LVs	Linearity (R^2)	Calibration error, (%)	Prediction error, (%)
Colombian	Ethiopian	5	0.997	1.7 (2.0)[a]	3.8 (6.7)[a]
Colombian	Nicaraguan	5	0.988	3.4 (2.9)[a]	6.1 (18.3)[a]
Indian	Indonesian	4	0.994	2.4 (2.3)[a]	7.5 (7.3)[a]
Vietnamese Arabica	Vietnamese Robusta	4	0.997	1.8 (1.7)[a]	5.7 (9.2)[a]
Vietnamese Arabica	Cambodian	5	0.996	2.0 (1.5)[a]	3.5 (2.9)[a]
Vietnamese Robusta	Cambodian	4	0.992	2.8 (1.5)[a]	5.3 (4.5)[a]

[a]Calibration and prediction errors previously reported by employing HPLC-UV fingerprints as chemical descriptors for PLSR (Núñez, Collado, Martínez, Saurina, & Núñez, 2020).

515 Figure 1



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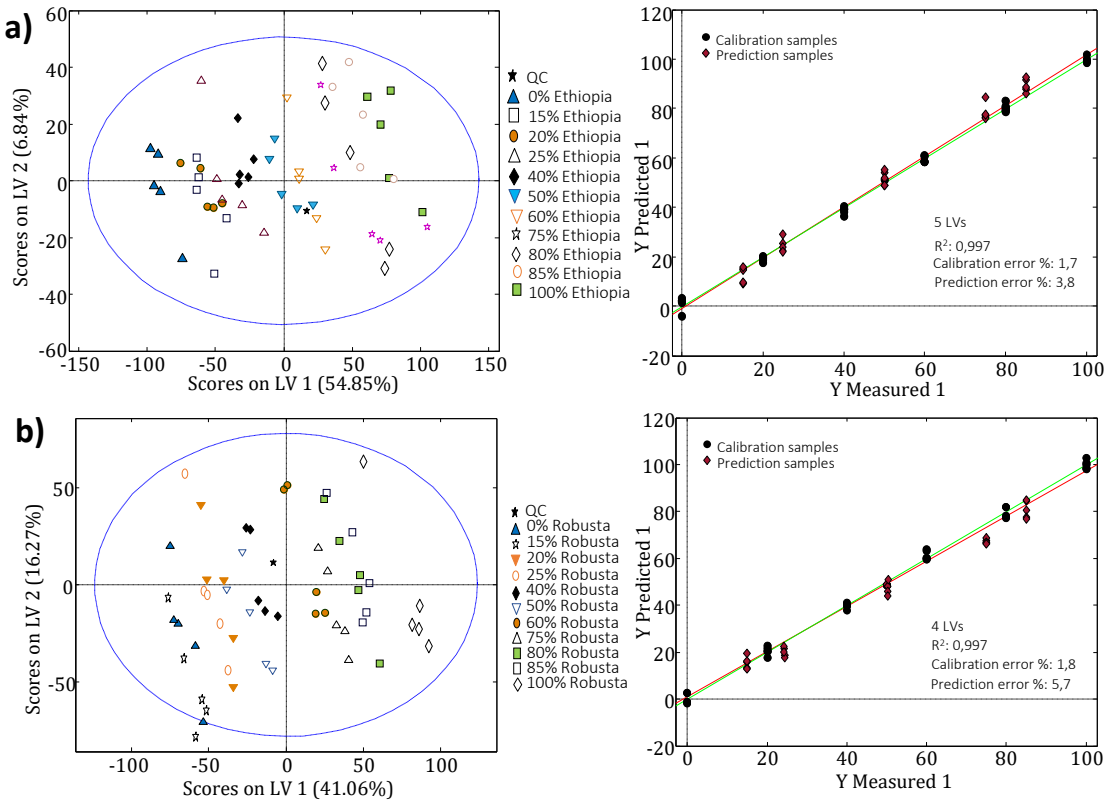
518 Figure 2



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