High-performance liquid chromatography with fluorescence detection fingerprinting combined with chemometrics for nut classification and the detection and quantitation of almond-based product adulterations

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Abstract

Economically motivated food fraud has increased in recent years, with adulterations and substitutions of high-quality products being common practice. Moreover, this issue can affect food safety and pose a risk to human health by causing allergies through nut product adulterations. Therefore, in this study, high-performance liquid chromatography with fluorescence detection (HPLC-FLD) fingerprints were used for classification of ten types of nuts, using partial least squares regression-discriminant analysis (PLS-DA), as well as for the detection and quantitation of almond-based product (almond flour and almond custard cream) adulterations with hazelnut and peanut, using partial least squares regression (PLS). A satisfactory global nut classification was achieved with PLS-DA. Paired PLS-DA models of almonds in front of their adulterants were also evaluated, producing a classification rate of 100%. Moreover, PLS regression produced low prediction errors (below 6.1%) for the studied adulterant levels, with no significant matrix effect observed.

Keywords: Nuts; Almond; HPLC-FLD; Fingerprinting; Chemometrics; Food Authentication; Food Safety

1. INTRODUCTION

Food fraud, which costs the global food industry approximately 30 billion euros a year, has increased because of the complex nature of the globalised world, where many individuals participate in the food chain between production and consumption. In the European Union (EU), the number of requests concerning fraud suspicions sent to the EU Administrative Assistance and Cooperation (AAC) system had increased by 49% from 2016 to 2018 (*European Comission, 2018*). There are different ways of perpetuating food fraud, such as deception during manufacturing, use of illicit supply chains, duplication, misrepresentation, and manipulation of the food product (e.g., adulteration, addition, substitution, etc.) (Manning & Soon, 2019). Although it is generally economically motivated, the addition or replacement of certain substances can be extremely dangerous for human health, for example, by causing allergies, thereby turning a food authentication issue into a food safety one (Fritsche, 2018).

Nuts and seeds, which are widely consumed mainly due to their beneficial effects on human health (De Souza, Schincaglia, Pimente, & Mota, 2017), encompass a wide range of food products such as almonds, Brazil nuts, cashew nuts, hazelnuts, macadamia nuts, peanuts, pecans, pine nuts, pistachios, pumpkin seeds, sunflower seeds, and walnuts. Some of them are at medium or high risk for food fraud (*Food Fraud Risk Information, 2019*), being susceptible to adulterations, replacements or substitutions with cheaper and lower-quality products, as well as to their characteristics being misrepresented (e.g., origin, year of the stock or organic production). For instance, almonds, which are one of the most expensive internationally produced nuts (more than 2 million tonnes produced in 2017, with USA the main producer (*Food and Agriculture Organization of the United Nations, 2019*)), as well as their byproducts (snacks, baked goods and pastry), can be partly or totally replaced with peanut or

hazelnut, constituting not only an economic deception, but also a threat to human health by causing allergies (Mustafa et al., 2019). Therefore, there is an increasing need to develop new analytical methodologies to guarantee the authenticity and safety of almond and almond-based products.

To date, most of the analytical methods described in the literature for almond authentication deal with its agricultural origin, with only a few focusing on its adulteration. For instance, several analytical platforms based on thermal analysis (Beltrán-Sanahuja, Grané-Teruel, Martín-Carratalá, & Garrigós-Selva, 2011), gas chromatography coupled to mass spectrometry for the determination of 12 targeted volatile compounds (Beltrán-Sanahuja, Ramos-Santonja, Grané-Teruel, Martín-Carratalá, & Garrigós-Selva, 2011), high-performance liquid chromatography with an evaporative light-scattering detector (HPLC-ELSD) for triacylglycerol profiling (Barreira et al., 2012), and approaches combining more than one technique (Čolić et al., 2017; García, Beltrán Sanahuja, & Garrigós Selva, 2013), have been successfully employed when combined with chemometric techniques for origin classification. However, to the best of our knowledge, there are very few studies investigating the adulteration of almond-based products. Multi-elemental profiling by inductively coupled plasma-optical emission spectrometry (ICP-OES) has been used to detect and quantitate the adulteration of almond powder with peanut (Esteki, Vander Heyden, Farajmand, & Kolahderazi, 2017), while fatty acid profiles obtained with gas chromatography with flame-ionisation detection (GC-FID) have been employed to study apricot kernel as an adulterant (Esteki, Farajmand, Kolahderazi, & Simal-Gandara, 2017). In both cases, multivariate data analysis was also used to quantify the adulterant level in the studied samples.

While most of the methods described in the literature for almond authentication are based on targeted profiling (a given group of known chemical compounds are determined), chromatographic fingerprinting involving non-targeted instrumental signals has emerged as a promising strategy in the food authentication field since it does not need specific biomarkers. This approach has already been proven in some studies on complex food matrices (Cuadros-Rodríguez, Ruiz-Samblás, Valverde-Som, Pérez-Castaño, & González-Casado, 2016). In fact, high-performance liquid chromatography with ultraviolet detection (HPLC-UV) fingerprinting has been demonstrated to be able to completely distinguish almond samples from peanut and hazelnut ones, although it could not discriminate the whole types of the studied nuts (Campmajó et al., 2019).

Therefore, this study aimed to classify nuts according to their typology, independently of their processing thermal treatment (natural, toasted or fried), by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) fingerprinting, which is a more selective technique than HPLC-UV, and partial least squares regression-discriminant analysis (PLS-DA). Moreover, the chromatographic fingerprints were also used to detect and quantitate hazelnut and peanut adulterations of almond and almond-based products by partial least squares (PLS) regression.

2. MATERIALS AND METHODS

2.1 Reagents and solutions

Unless otherwise stated, all the reagents were of analytical grade. Purified water was obtained using an Elix® 3 coupled to a Milli-Q® system (Millipore Corporation, Bedford, MA, USA) and filtered through a 0.22-µm nylon membrane. Acetone, hexane and formic acid (96%) were obtained from Sigma-Aldrich (St. Louis, MO, USA), whereas UHPLC-gradient grade methanol was from Panreac (Barcelona, Spain).

2.2 Instrumentation

The chromatographic system consisted of an Agilent 1100 Series HPLC instrument equipped with a binary pump (G1312A), a degasser (G1379A), an automatic injection system (G1329B), a fluorescence detector (G1321A) and a computer with the Agilent ChemStation software, all from Agilent Technologies (Waldbronn, Germany). The HPLC-FLD fingerprints were obtained by employing a Kinetex C18 column(100 mm × 4.6 mm id., 2.6 µm particle size), which was purchased from Phenomenex (Torrance, CA, USA), and a previously developed gradient elution mode with 0.1% (*v/v*) formic acid aqueous solution (solvent A) and methanol (solvent B) constituting the components of the mobile phase (Campmajó et al., 2019). The flow rate was 0.4 $mL \cdot min^{-1}$ and the injection volume 5 µL. For fluorescence acquisition, 280 nm and 350 nm were chosen as the excitation and emission wavelengths, respectively.

2.3 Samples and sample treatment

For nut classification, 149 nut samples obtained from Barcelona markets, belonging to various classes and some of them processed with different thermal treatments, were analysed (sample details are described in Table 1). Method repeatability and the robustness of the chemometric results were controlled by using a quality control (QC) sample, which was a mix prepared with 50 µL of each nut sample extract.

Hazelnuts and peanuts were studied as potential adulterants of almonds and almond-based products. Thus, they were added in proportions from 0 to 100%, as shown in Table 2, to two different almond matrices: natural almond flour and almond custard cream. The cream was made from hen eggs, milk, sugar, and corn flour. Afterwards, the almond custard cream and its adulterated samples were obtained by adding the adulterants as described above. Five replicates of each percentage of adulteration were

prepared, giving a total of 105 samples for each studied almond-based product. In this study, an additional 50% adulterated sample was prepared for use as the QC sample.

A simple two-step sample treatment was performed following a previously described method (Campmajó et al., 2019) based on an extraction with acetone:water (70:30 *v/v*) followed by a defatting step with hexane. Briefly, 0.125 g of the nut product were extracted by stirring in a Vortex (Stuart, Stone, United Kingdom) and sonication (5510 Branson ultrasonic bath, Hampton, NH, USA) in 3 mL of the extracting solvent. Then, centrifugation was performed for 30 min at 3,400 rpm (ROTANTA 460 RS Centrifuge, Hettich, Germany). the resulting supernatant extract was defatted with 3 mL of hexane, also by stirring in a Vortex followed by centrifugation for 15 min. After filtering the sample extract with a 0.22-µm nylon filter (Scharlab, Sentmenat, Spain), it was stored at -18°C in a 2-mL glass injection vial until HPLC-FLD analysis.

To avoid and control for systematic errors and cross-contamination during sample sequences, a QC sample and an extracting solvent blank were injected at the beginning and after every ten sample injections.

2.4 Data analysis

Depending on the aim of the multivariate data analysis, principal component analysis (PCA), PLS-DA or PLS regression was carried out by using the Solo 8.6 chemometrics software from Eigenvector Research (Manson, WA, USA) (Eigenvector Research Incorporated, 2019). Details of the theoretical background of these statistical methodologies are addressed elsewhere (Massart et al., 1997).

For the chemometric study, the construction of different data matrices was required. Thus, indistinctly of the chemometric method used, the X-data matrices of responses consisted of the HPLC-FLD chromatographic fingerprints acquired. Furthermore, PLS-

DA Y-data matrices defined each sample class, whereas PLS ones defined each percentage of adulteration.

HPLC-FLD fingerprints were smoothed, baseline-corrected, aligned, and autoscaled before building the chemometric model to improve data quality by reducing noise interferences, baseline drifts and peak shifting. Afterwards, the most appropriate number of principal components (PCs) in PCA, and latent variables (LVs) in the PLS-DA and PLS was established at the first significant minimum point of the venetian blind cross validation (CV) error.

Moreover, the applicability of the built chemometric models was tested through their validation. For instance, the PLS-DA models were validated by using 70% of a sample group as the calibration set, and the remaining 30% as the validation set. In the case of the PLS models, Table 2 shows the percentages of adulteration used in the calibration and validation sets.

3. RESULTS AND DISCUSSION

3.1 Nut classification

Several types of nuts are vulnerable to food fraud practices such as being substituted with cheaper adulterants. Therefore, analytical methodologies capable of classifying nut samples according to their type are required. Although a previous study demonstrated that HPLC-UV fingerprints were good chemical descriptors for classifying certain types of nuts, they could not achieve complete nut classification (Campmajó et al., 2019). Thus, in this work, HPLC-FLD fingerprints were used as an alternative to obtain better descriptors.

3.1.1 HPLC-FLD fingerprints

As previously mentioned in Section 2.3, a wide variety of nut samples were assessed by HPLC-FLD for classification. As can be seen in Figure S1 (Supplementary Material) showing the chromatographic fingerprints acquired for a selected sample, there were noteworthy differences in the abundance of the compounds detected (considering the retention time), as well as in the peak intensity. Moreover, since these features were reproducible among samples belonging to the same type of nut, these chemical descriptors were evaluated to classify nut types through a multivariate chemometric approach.

3.1.2 Chemometrics for classification

First, a preliminary exploratory chemometric PCA was performed to study QC sample behaviour. Therefore, a 164 × 4,863 (samples × variables) dimension data matrix, with the emitted fluorescence intensity at 350 nm a function of time for the analysed nut and QC samples, was examined. As shown in Figure S2, QC samples formed a compact group in the central part of the scores plot of PC1 *vs.* PC2 (two PCs were chosen for the PCA), indicating the absence of systematic errors during the sample injection sequence and demonstrating the validity of the chemometric results.

The supervised chemometric analysis for classification was conducted with PLS-DA. While the X-data matrix (149 × 4,863) consisted of the same information as that used in the PCA without the QC samples, the Y-data matrix (149 × 2) indicated the membership of each nut sample. Due to the large number of nut classes under study, a total of ten LVs were required for the construction of the PLS-DA model, which clearly enabled the discrimination of some of them. For instance, the scores plot of LV1 *vs.* LV2 (Figure 1A) shows a clear separation of walnuts and macadamia nuts, which are on the right side of the plot displaying positive LV1 values, whereas pine nuts are at the bottom of

the plot with negative LV2 values. Although the combination of other LVs and the use of 3D plots also enabled the classification of peanuts (Figure S3A) and sunflower seeds (Figure S3B), LV construction was mainly influenced by these classes of nuts, with the scores plots not visually discriminating between the remaining five classes. For that reason, a new PLS-DA model for almond, cashew nut, hazelnut, pistachio, and pumpkin seed samples was built with four LVs. This resulted in better classification, especially for sunflower seeds, as can be seen in the corresponding scores plot of LV1 *vs.* LV2 in Figure 1B.

As this work focused on the study of almond adulterations, whichcommonly constitute its substitution with cheaper nuts such as hazelnuts or peanuts, paired PLS-DA models with almond in front of hazelnut and peanut samples were constructed. As previously detailed in Section 2.4, 70% of the samples were used in the calibration set, whereas the remaining 30% were used in the validation set. Figure 2 presents these classification plots, the red dashed line indicating the classification boundary. The calibration and validation samples are located on the left and right side of the plot, respectively. A classification rate of 100% was obtained when studying almonds in front of their most common adulterating nuts, [9, 0; 0, 6] being the confusion matrix for both almond *vs.* hazelnut and almond *vs.* peanut validations.

Although UV fingerprints at 280 nm are much richer in peak features than the FLD counterparts, results presented in this paper demonstrate the better descriptive performance of HPLC-FLD data compared with HPLC-UV (Campmajó et al., 2019), with higher classification rates and lower prediction errors for some of the systems under study. The selectivity of UV spectroscopy at 280 nm is poor and a wide range of compounds are detected, mainly consisting of phenolic acids (and flavonoids with lower sensitivity), which are components occurring in all kinds of samples. As a result, the nut

discrimination is then based on cross selectivities (i.e., differences in concentration levels among classes), while more specific markers have not been encountered. In contrast, FLD fingerprints generally contain a fewer number of peaks since the selection of excitation and emission conditions provides more selective data (Bakhytkyzy, Nuñez, & Saurina, 2018). Moreover, signals from hydroxycinnamic acids, stilbenoids and various types of flavonoids are negligible; only hydroxybenzoic acids and flavanols are reasonably detectable under these conditions. In particular, the detection of flavanols is especially favored, thus achieving a great sensitivity for catechin, epicatechin, and related species. Therefore, despite having simpler chromatograms from FLD in terms of the number of features, the more selective detection of highly relevant descriptors may lead to better predictive figures.

3.2 Almond-based product adulterations

Following the satisfactory classification obtained with the PLS-DA models, HPLC-FLD fingerprints were also used for the detection and quantitation of adulterations in two types of almond-based matrices: natural almond flour and almond custard cream. PLS was applied as the most suitable chemometric approach to study them.

3.2.1 HPLC-FLD fingerprints

A set of almond-based product (natural almond flour and almond custard cream) samples, which were obtained by adding different percentages of the adulterant as specified in Section 2.3 and detailed in Table 2, were analysed with HPLC-FLD.

As shown in Figure S1, both the pure hazelnut and peanut fingerprints showed significant differences compared to the almond ones in terms of the number of compounds detected, abundance, and intensity. For instance, the peanut and hazelnut

samples presented a higher number of chromatographic peaks than the almond samples. In fact, an increase in the number of peaks could be seen when transitioning from pure almond to adulterated samples. Therefore, as the HPLC-FLD fingerprints seemed to vary according to the adulterant percentage, they were proposed as chemical descriptors to detect and quantitate adulterations, using PLS.

3.2.2 Chemometric detection and quantitation of adulterations

The ability of the HPLC-FLD fingerprints to detect and quantify almond adulterations with peanut or hazelnut was evaluated by PLS. Table 3 summarises the LVs used in each calibration PLS model, as well as the calibration and prediction error obtained in all the adulteration cases studied. The calibration models built were good, as indicated by the low calibration errors ($\leq$ 4.7%), bias values tending towards zero and good linearity with $R^2 \geq 0.982$. When focusing on a specific matrix, similar prediction errors were obtained independently of the adulterant used. As can be seen in Figure 3, the results achieved when predicting peanut levels in almond flour (Figure 3A) and almond custard cream (Figure 3B) were excellent, with no significant differences between the matrices (PLS results for the adulteration with hazelnut are shown in Figure S4). Hence, although almond custard cream is a fatter matrix than almond flour, no interfering matrix effect was observed in the results.

4. CONCLUSIONS

HPLC-FLD chromatographic fingerprints, using an excitation wavelength of 280 nm and an emission wavelength of 350 nm, were suitable chemical descriptors for nut classification and authentication. Satisfactory discrimination of nut samples according to their type was achieved by PLS-DA. Moreover, when focusing on the specific

adulteration of almond-based products with peanut or hazelnut, paired PLS-DA models showed complete sample distinction (classification rate of 100%), while PLS models produced low prediction errors below 6.1% for both matrices when predicting the percentages of adulteration. Thus, the HPLC-FLD fingerprinting method described in this study can classify nut samples according to their type, as well as detect and quantitate the levels of peanut or hazelnut adulteration of almond-based products. Therefore, it can be used as a simple and reliable method to prevent food fraud and guarantee food product safety.

Conflict of Interest

There are no conflicts of interest to declare.

Funding

The authors are grateful for the financial support received from the Spanish Ministry of Science, Innovation and Universities, through the grant PGC2018-095013-B-I00, and from the Agency for Administration of University and Research Grants (Generalitat de Catalunya), through the grants 2017SGR-171 and 2017SGR-310.

Acknowledgements

G. Campmajó thanks the University of Barcelona for the APIF grant.

Supporting Information: Figure S1. HPLC-FLD fingerprints (acquired with an excitation and emission wavelength of 280 and 350 nm, respectively) for a selected sample for each nut type under study; **Figure S2.** PCA scores plot of PC1 *vs.* PC2 showing the correct behaviour of QC samples; **Figure S3.** PLS-DA scores plot of (A) LV1 *vs.* LV3 and (B) LV1 *vs.* LV4, using the HPLC-FLD fingerprints acquired for all the nut samples assessed; **Figure S4.** PLS results of (A) almond flour and (B) almond custard cream adulterated with hazelnut.

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Figure legends

Figure 1. (A) PLS-DA scores plot of LV1 *vs.* LV2, using the HPLC-FLD fingerprints acquired for all the nut samples tested. (B) PLS-DA scores plot of LV1 *vs.* LV2, using only the almond, cashew nut, hazelnut, pistachio, and pumpkin seed HPLC-FLD fingerprints.

Figure 2. Classification plot depicting Sample *vs.* Y predicted 1 score plot for (A) almond *vs.* hazelnut samples and (B) almond *vs.* peanut samples. Solid symbols, calibration samples; empty symbols, validation samples.

Figure 3. Scatter plot of measured *vs.* predicted percentages of adulteration, using PLS. Results are shown for (A) almond flour and (B) almond custard cream adulterated with peanut.

Figure 1

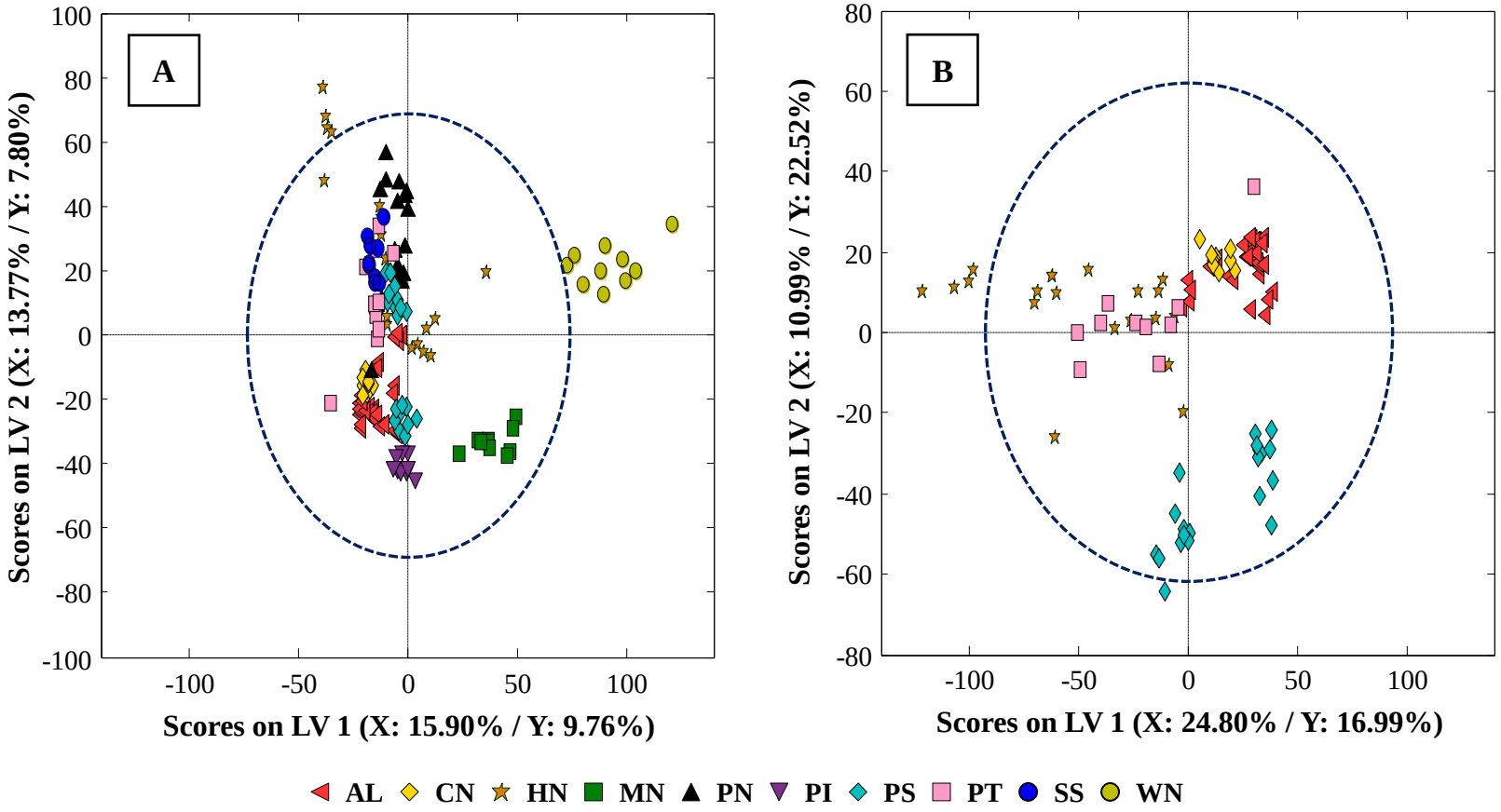
A
B
Scores on LV 2 (X: 13.77% / Y: 7.80%)
Scores on LV 1 (X: 15.90% / Y: 9.76%)
Scores on LV 2 (X: 10.99% / Y: 22.52%)
Scores on LV 1 (X: 24.80% / Y: 16.99%)
AL CN HN MN PN PI PS PT SS WN

Figure 2

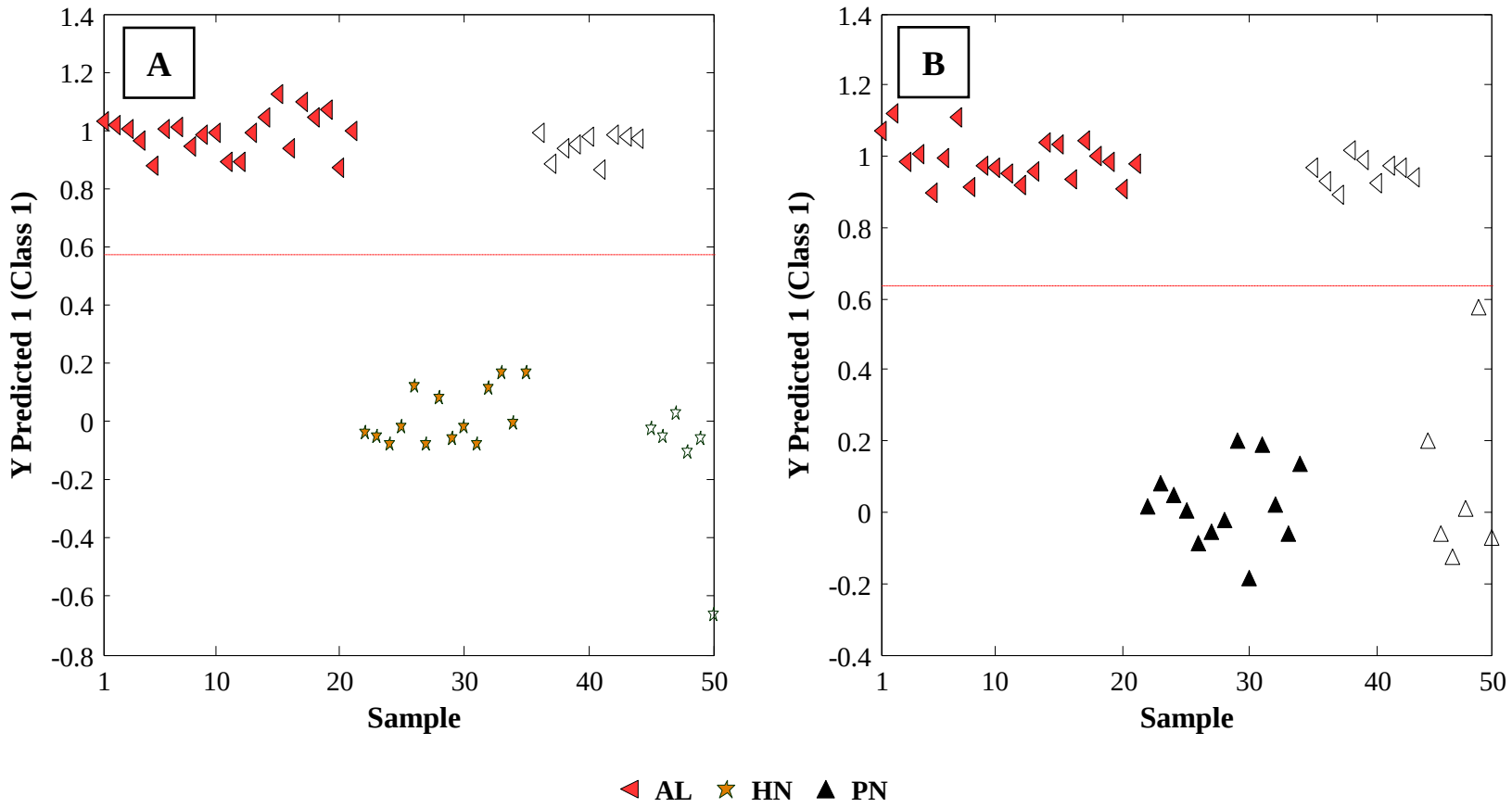

Figure 3

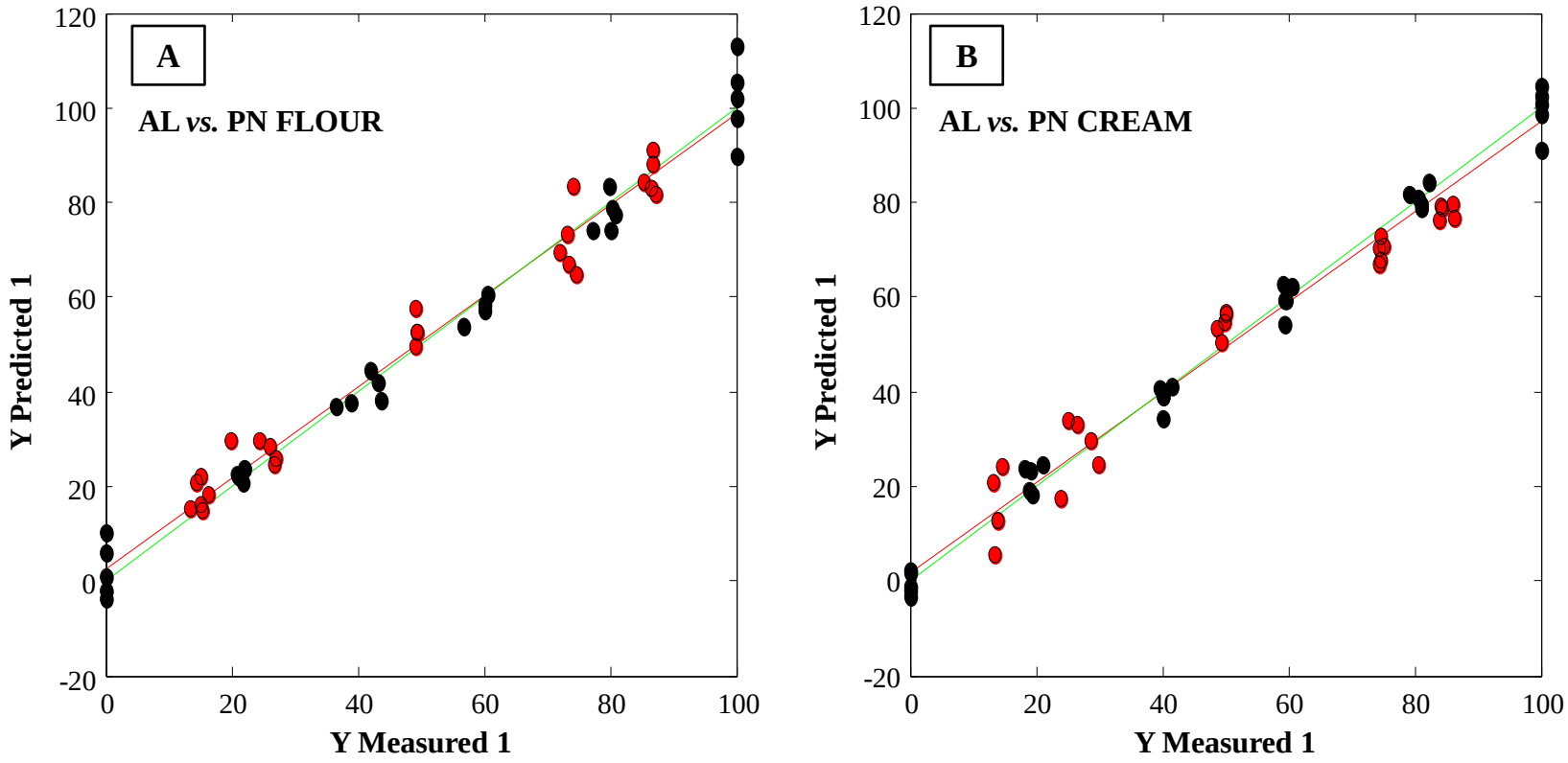
A
AL vs. PN FLOUR
Y Predicted 1
Y Measured 1
120
100
80
60
40
20
0
-20
0
20
40
60
80
100
B
AL vs. PN CREAM
Y Predicted 1
Y Measured 1

Table 1. Description of the samples analysed in the nut classification study.

NUT TYPE	ABBREVIATION	NUMBER OF SAMPLES		
		Natural	Fried	Toasted
Almonds	AL	10	10	10
Cashew Nuts	CN	-	10	-
Hazelnuts	HN	10	-	10
Macadamia Nuts	MN	10	-	-
Peanuts	PN	-	10	10
Pine Nuts	PI	10	-	-
Pistachios	PT	-	-	9
Pumpkin seeds	PS	-	10	10
Sunflower seeds	SS	-	-	9
Walnuts	WN	10	-	-

Table 2. Samples used in the PLS adulteration studies as calibration or validation set. Hazelnut and peanut were proposed as adulterants of a natural almond flour and an almond custard cream.

	ALMOND, %	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

Table 3. Overall results for the evaluation of the adulteration of almond flour and almond custard cream with hazelnut and peanut by PLS. LVs, number to build each PLS mode; Cal. Error, error in the calibration step; Pred. Error, error in the prediction step.

	ALMOND FLOUR			**ALMOND CUSTARD CREAM**		
	LVs	**Cal. Error (%)**	**Pred. error (%)**	**LVs**	**Cal. Error (%)**	**Pred. error (%)**
HAZELNUT	5	2.6	5.6	4	3.5	6.1
PEANUT	3	4.7	5.0	4	3.1	6.1

Supplementary Material

High-performance liquid chromatography with fluorescence detection fingerprinting combined with chemometrics for nut classification and the detection and quantitation of almond-based product adulterations

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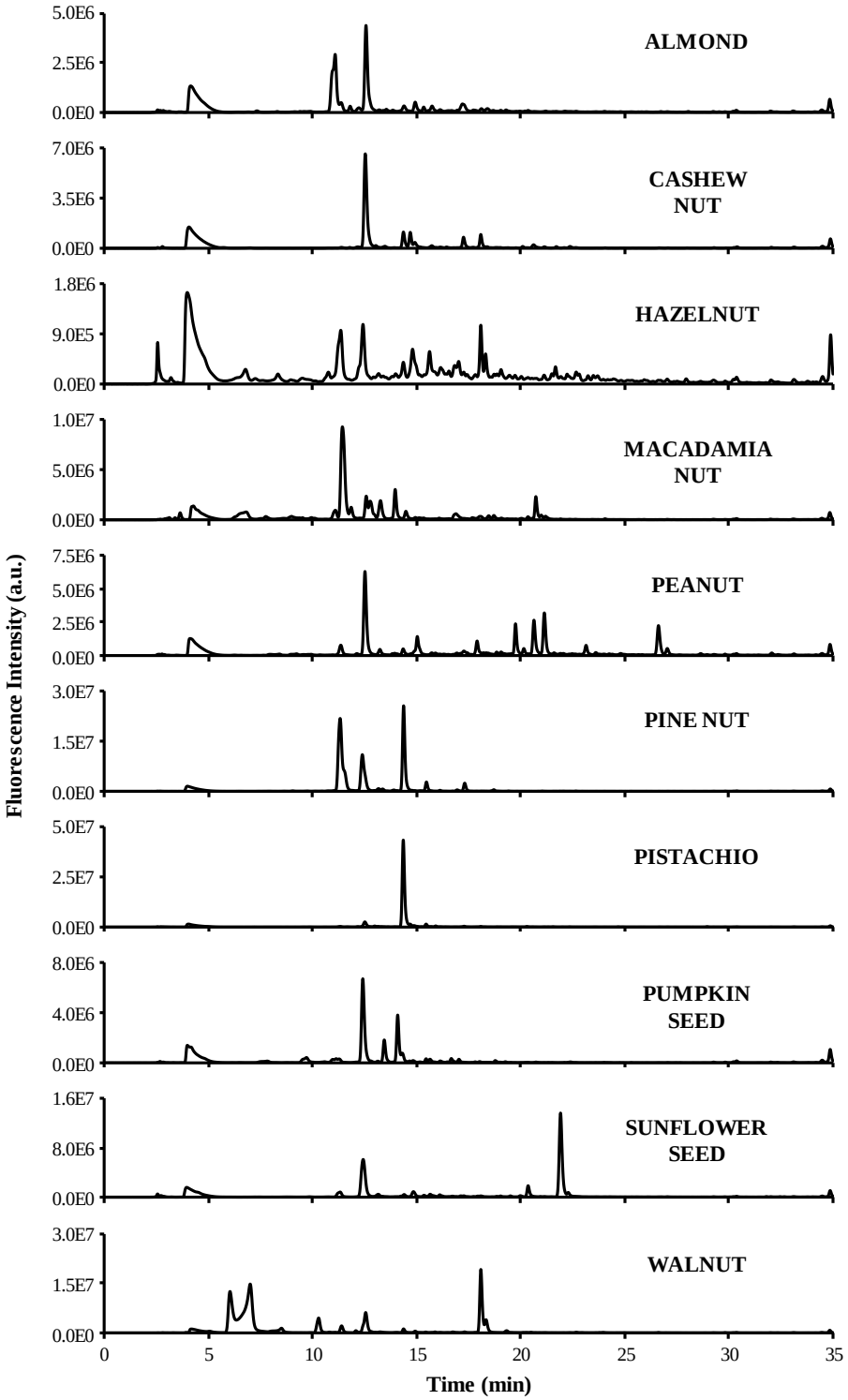

Figure S1. HPLC-FLD fingerprints (acquired with an excitation and emission wavelength of 280 and 350 nm, respectively) for a selected sample for each nut type under study.

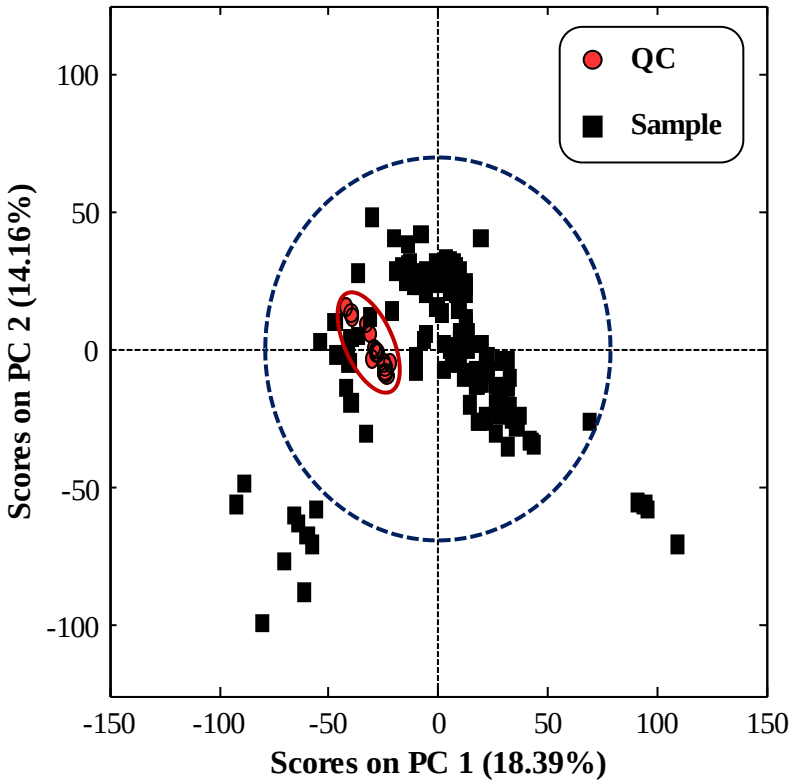

Figure S2. PCA scores plot of PC1 vs. PC2 showing the correct behaviour of QC samples.

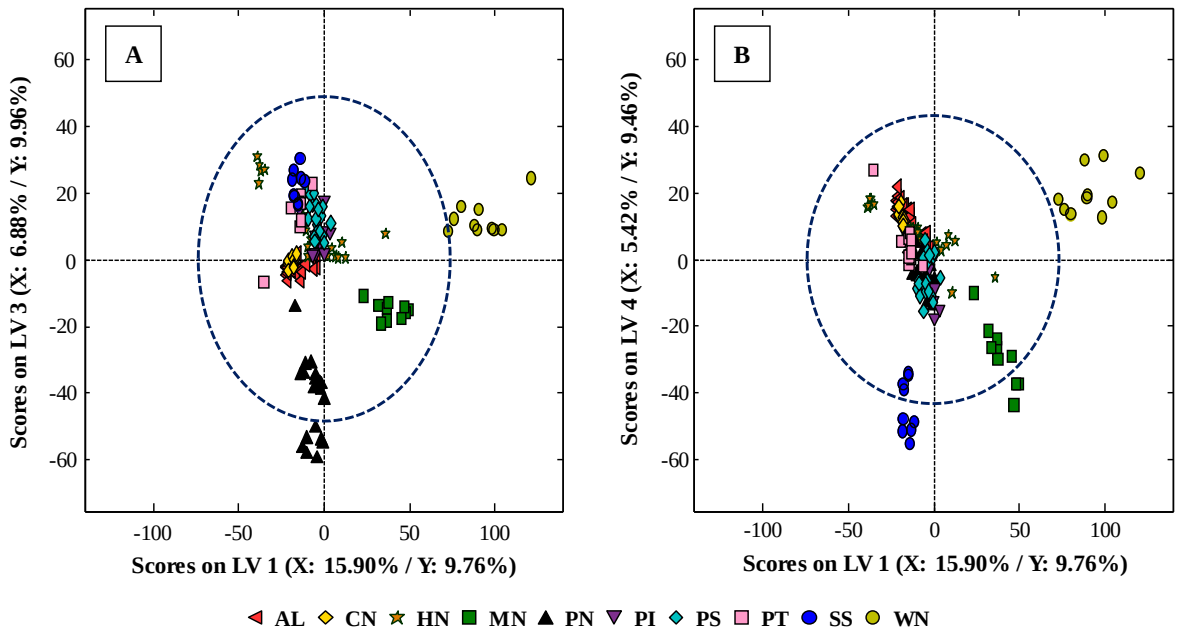

Figure S3. PLS-DA scores plot of (A) LV1 vs. LV3 and (B) LV1 vs. LV4, using the HPLC-FLD fingerprints acquired for all the nut samples assessed.

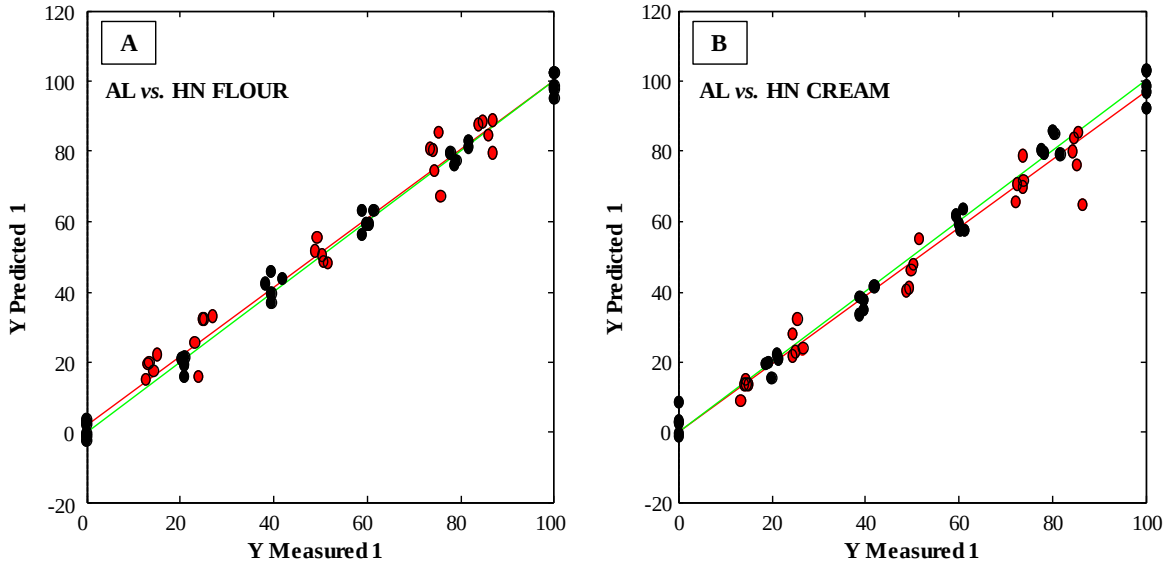

Figure S4. PLS results of (A) almond flour and (B) almond custard cream adulterated with hazelnut.