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Grau de Química

Treball Final de Grau

Determination of almond adulterations with hazelnut and peanut by HPLC-UV and HPLC-FL fingerprinting, and multivariate calibration methods.

Determinació d'adulteracions d'ametlla amb avellana i cacauet mitjançant empremtes HPLC-UV i HPLC-FL, i mètodes de calibratge multivariant.

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I want to say thanks:

To my family, for their love, for their help, and for their support in every step that allowed me to be here today. To Dr. Oscar Núñez, for his amiability, and for his effort in helping me make the best of this work. And to all the members of the CECEM research group, for showing me around, for helping me, and for making me feel at home.

Vull donar les gràcies:

A la meva família, per la seva estima, per la seva ajuda, i pel seu suport en cada pas que m'ha permès estar avui aquí. Al Dr. Oscar Núñez, per la seva amabilitat i pel seu esforç en ajudar-me a treure el millor d'aquest treball. I a tots els membres del grup d'investigació CECEM, per guiar-me, per ajudar-me i per fer-me sentir com a casa.

REPORT

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1. SUMMARY

Nowadays, society is worried more than ever about the quality of the food that they consume. Recent incidents regarding food frauds, like the melamine case in dairy products, may have acted as a trigger point for the arousal of concerns about consumer product safety. One of the most common food frauds is the adulteration of a product by replacing an expensive component with other cheaper and/or lower health beneficial components. Nuts are sometimes the target of this kind of practices, usually in bakery products where manufacturers adulterate almond flours with peanut, hazelnut or other cheaper nuts, which does not only imply and economic fraud, but also a health issue, as the unspecified ingredient may cause allergies in the consumer.

In this work, HPLC-UV chromatographic fingerprints were recorded, following a previously developed method in the research group used for polyphenol analysis, in order to achieve identification and quantification of adulteration levels in almond and almond custard cream samples adulterated with peanut or hazelnut, using chemometric methods such as principal component analysis (PCA) and partial least squares (PLS). Moreover, HPLC-FL fingerprinting was also evaluated to see if fluorescence detection could offer better chemical descriptors to achieve sample classification and authentication according to the different nut types than the one obtained by ultraviolet-visible detection, as well as a better quantification of adulteration levels in the same adulterated almond and almond custard cream samples.

In the end, HPLC-FL fingerprinting combined with chemometrics was proposed as the best option and as a suitable strategy to address nut product classification, as well as identification and quantification of nut frauds by means of adulteration.

Keywords: High Performance Liquid Chromatography, Fingerprinting, Chemometrics, Principal Component Analysis, Partial Least Squares, Food Fraud, Nuts.

2. RESUM

Avui dia, la societat està més preocupada que mai per la qualitat dels aliments que consumeix. Alguns incidents recents relacionats amb el frau d'aliments, com pot ser el cas de l'addició de melamina en productes làctics, poden haver augmentat la consciència sobre la seguretat dels consumidors. Un dels fraus més comuns en la industria alimentària és l'adulteració d'un aliment mitjançant la substitució d'un component amb un altre més barat i/o menys beneficiós per a la salut. Entre aquests tipus de pràctiques es troben els fruits secs, normalment en productes de pastisseria on els productors adulteren farines d'ametlla amb avellana, cacauet o altres fruits secs de menys cost. Això no només implica un frau econòmic, sinó un problema de salut, ja que l'addició d'aquest altre ingredient no especificat pot provocar al·lèrgies al consumidor.

En aquest treball s'ha aconseguit identificar i quantificar el nivell d'adulteració en ametlles i cremes d'ametlles adulterades amb cacauet i avellana, mitjançant la cromatografia líquida d'alta eficàcia amb perfil d'empremtes dactilars de detecció ultraviolada (empremtes HPLC-UV) combinada amb mètodes quimiomètrics com l'anàlisi de components principals (PCA) i la regressió de mínims quadrats parcials (PLS), tot seguint un mètode prèviament desenvolupat pel grup de recerca per a l'anàlisi de polifenols. A més, també s'ha utilitzat HPLC amb detecció de fluorescència (HPLC-FL) per avaluar si ofereix millors descriptors químics, tant per a la classificació de fruits secs, com per a la identificació i quantificació del nivell d'adulteració en ametlles i cremes d'ametlles amb els mateixos adulterants.

En conclusió, HPLC-FL en combinació amb quimiometria, ha demostrat ser la millor opció i un molt bon mètode per a la classificació de fruits secs, i per a la identificació i quantificació d'adulteracions en fruits secs.

Paraules clau: Cromatografia Líquida d'Alta Eficiència (HPLC), Empremtes, Quimiometria, Anàlisi de Components Principals (PCA), Regressió de Mínims Quadrats Parcials (PLS), Frau alimentari, Fruits Secs.

3. INTRODUCTION

3.1 FOOD INTEGRITY AND AUTHENTICITY

Food is often one of the hardest targets for chemists to analyse due to its complex matrix, and yet it is at the top of possible causes for health issues. More than 2500 chemical substances are intentionally added to foods to modify their flavour, colour, stability, texture or cost. In addition, an estimated of 12000 substances from food-packaging materials, processing aids, pesticide residues, and drugs given to animals may unintentionally enter the food supply.[1]

Nowadays, food manufacturers, retailers, consumers and society, in general, have become very interested in the quality of food products. The arousal of concerns about food quality and consumer product safety now include not only the nutritional aspect, but also many different points of view, like the presence of substances that are beneficial to our health, possible contaminants and even the geographical origin. This may be related with the recent alarms regarding consumer product safety incidents (see Figure 1). The consequences of these incidents ranged from lost sales and bankruptcies to adverse health issues and even fatalities.[2]

One of the most common food frauds is adulteration. According to the European Committee for Standardization Workshop Agreement (CWA), it is: "A type of food fraud which includes the intentional addition of a foreign or inferior substance or element; specially to prepare for sale by replacing more valuable with less valuable or inert ingredients."[3]

The deliberate adulteration of food products has a long history. It involved commodities such as bread flour, which was commonly adulterated with sand, sawdust or mustard flour. White flour, at the time was considered a luxury and sellers demanded premium price, and a whiter appearance could be achieved by adding ground animal bones to flour. Other common adulterations included tea that was adulterated using dried beech leaves, milk using the simple addition of water and coffee that could be bulked up with maize and other cereal grains.[4]

As the food supply chain is now global, it is exceedingly complex and there are many players involved between production and consumption, so it is in fact much easier to conduct fraud without being easily detected. Food adulteration has become increasingly sophisticated, often being

specifically designed to avoid detection through routine analysis. As the deliberate adulteration of food and its misrepresentation to deceive final consumers is illegal worldwide,[5] it is extremely important the development of analytical methodologies that are able to detect these kind of food frauds.

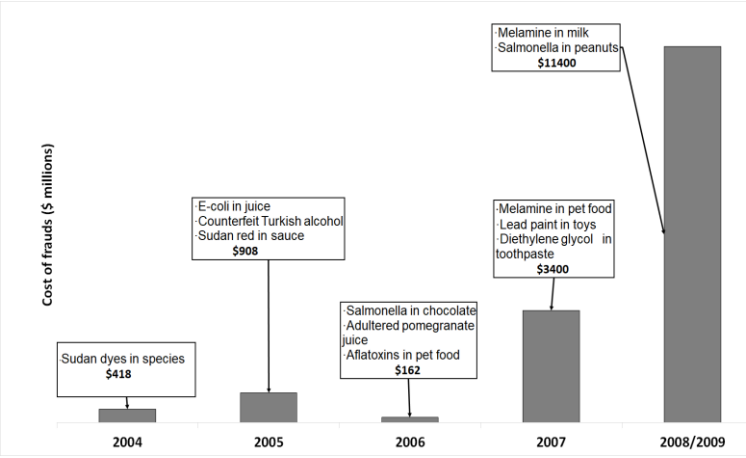

Figure 1. Timeline of major consumer product safety incidents.[2]

3.2 NUTS

A nut is a fruit composed of an inedible hard shell and a seed, which is generally edible. They are a source of energy, unsaturated fatty acids and oils, fibre, proteins, vitamins and minerals, including bioactive compounds such as polyphenols.[6–9] As healthy snacks they appeal to people working out of home, instead of sweets and biscuits. Their production has been increasing in the last years, produced mainly in the USA, followed by China, Turkey, Iran and India.[10]

Among the compounds found in nuts, polyphenols are antioxidant bioactive compounds known for having significant benefits in human health, as these have been investigated for potential effects in the prevention of cancer, heart disease and other diseases.[11,12] Their content can vary depending on the type of nut: walnuts are known for having the highest amount of bioactive compounds, followed by pistachios, peanuts, almonds and, with less content, cashews and hazelnuts.[13]

Unfortunately, the problem of food fraud is also present in nuts, either as a country of origin fraud or as an adulteration with another species, usually due to the huge differences in prizes. 4% of the food fraudulent practices reported in the European Union in 2016 were related to nuts and

seeds.[14] As an example, almonds are, in general, twice more expensive than hazelnuts. This sometimes leads to the adulteration of almonds with peanuts or hazelnuts, usually in bakery products. Some manufacturers adulterate almond flours with hazelnut or peanut, which does not only imply an economic fraud, but also a food safety issue involving possible allergies. It must be considered that tree nut allergy prevalence is estimated to be, when oral allergy syndrome (OAS) is included, around 8-11.4 % with individual tree nut allergies variances by region: hazelnut is the most common tree nut allergy in Europe, walnut and cashew in the USA and almond is most commonly reported in the UK.[15] Therefore, the deliberate nut adulteration can be really harmful to those affected with these allergies.

3.3 ANALYTICAL TECHNIQUES

To enforce regulations on authenticity and adulteration there are "targeted" and "untargeted" analytical methodologies. Targeted methods are used to detect and quantify a known substance, or family of substances, used for adulteration. In contrast, untargeted methods are primarily used to "fingerprint" foods by measuring several different variables and looking for characteristic patterns employing statistical techniques that use multivariate analysis (chemometrics).

Fingerprinting methods describe a variety of methods that provide analytical signals related to the composition of foodstuffs in a non-selective way, such as by collecting a spectrum or a chromatogram. Chromatography is today one of the most important separation techniques used for food characterization. Among them, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the most used techniques.[16,17] The versatility of the chromatographic methods allows the analyst to interact on both the separation and measurement steps in order to acquire an analytical signal with the maximum useful information, as well as to select the data to be treated. Nut authentication has been previously addressed in the literature, many times using chromatographic techniques in combination with chemometrics. For example, a gas chromatography-mass spectrometry (GC-MS) method for the detection and identification of extra virgin olive oil adulteration with four types of oils coming from different seeds, including corn, peanut, rapeseed, and sunflower oils, was proposed in 2013.[18] Partial least squares-linear discriminant analysis (PLS-LDA) was also used in that work for the detection of adulteration with a 1% detection limit and 90% prediction ability. Another example is the adulteration of almond powder samples with apricot kernel. It was studied by GC fatty acid fingerprinting combined with principal components analysis (PCA), principal components analysis-linear discriminant analysis

(PCA-LDA) and partial least squares (PLS).[19] Of most importance for this project was the study on nut classification and authentication by HPLC-UV fingerprinting combined with chemometric methods,[20] which lead to the proposal of this research work.

As it has been demonstrated, these methods are excellent candidates of getting proper fingerprints for food identification as well as for food authentication.

3.4 CHEMOMETRICS

Chemometrics is known for being chemical discipline that uses mathematical, statistical, and other methods employing formal logic to design or select optimal measurement procedures and experiments, and to provide maximum relevant chemical information by analysing large amounts of chemical data.

3.4.1. Principal component analysis (PCA)

PCA is a multivariate statistical technique used when dealing with large amounts of data. It can extract the most important information and reduce the dimensionality of the data. It works by choosing a new set of coordinate axes called principal components (PCs), orthogonal between them, which contain the most valuable information. The first principal component (PC1) collects the highest variance in the data set and the second principal component (PC2), orthogonal to PC1, collects the new highest variance in that direction. The next PCs (PC3, PC4…) successively retain less variance and, therefore, grant less valuable information.

The first two PCs, which provide the most important information, are usually displayed in a two-dimension diagram named Score plot. Score plot displays similar samples forming clusters that share different properties, and the scatter between them as they increase their differences. This allows the user the detection of trends, patterns and outliers in the data set.

Moreover, another two-dimension diagram called Loadings plot can also be obtained as a result of displaying the variables in PCs space. It shows how the variables contribute to create the component, which ones are correlated, and which ones are independent.

3.4.2. Partial least squares (PLS) and partial least squares-discriminant analysis (PLS-DA)

Partial least squares (PLS) is a statistical method that bears some relation to PCA. It uses an orthogonal transformation to convert a set of possible correlated variables into a set of linearly uncorrelated variables called latent variables (LVs) in order to obtain the most important

information about the data. It is a method that tries to find fundamental relationships between two matrices (X and Y) for constructing models and making predictions. The emphasis is usually on predicting the responses and not necessarily on trying to understand the underlying relationship between the variables. When prediction is the goal and there is no practical need to limit the number of measured variables, PLS can be a useful tool.

A subtle variation of PLS is PLS-DA, which is used for classification. When Y matrix is categorical, PLS-DA is used instead. It is performed in order to sharpen the separation between groups of samples, by rotating LVs such that a maximum separation among classes is obtained, and to understand which variables carry the class separating information.

4. OBJECTIVES

As the previous work developed in the research group had successfully accomplished the characterization and classification of nuts with a HPLC-UV fingerprinting method, the objective of this work was focused on the identification and quantification of nut adulterations. Thus, the aim was to see if we could tell if a nut product was adulterated and, eventually, to quantify the level of adulterant in the fraudulent sample. Among nuts, almonds are normally used in the preparation of bakery products, but the fact that the production of almonds is usually twice more expensive than the production of other common nuts leads the manufacturers to adulterate almond flours with hazelnut or peanut.

In order to achieve the aim of this work, we can differentiate two parts:

- The application of the previously developed HPLC-UV fingerprinting method for the identification and quantification of frauds in almonds adulterated with hazelnut or peanut.
- The evaluation with the same proposed method of a more real case scenario by means of analysing an almond custard cream adulterated with hazelnut or peanut custard creams.

In addition, in this work another method using HPLC-Fluorescence (HPLC-FL) fingerprinting was developed to evaluate if the previous results on nut classification could be improved. As fluorescence is a more selective technique, it was thought that it should improve the differentiation of the nuts, and for the same reason it was also used in the adulteration study with both the individual nuts and also with the almond custard cream. So two more objectives emerged:

- The development of a new of HPLC-FL fingerprinting method for the characterization, classification and authentication of nut samples.
- The application of the new HPLC-FL fingerprinting method for the identification and quantification of frauds in almonds adulterated with hazelnut and peanut, as well as in almond custard cream adulterated with hazelnut or peanut custard cream.

5. EXPERIMENTAL SECTION

5.1 CHEMICAL AND STANDARDS

The extraction solutions employed in the sample treatment were prepared with the following reagents:

- Water (H_2O LC-MS Chromasolv, from Fluka, Sigma-Aldrich, Switzerland)
- Acetone (from Fluka, Sigma-Aldrich, Switzerland)
- Hexane (Sigma-Aldrich, Germany)

The mobile phase employed for HPLC separation was prepared with the following reagents:

- Methanol (99,9% from Panreac, Barcelona)
- Formic acid(96% from Sigma-Aldrich, Germany)
- Milli-Q water. Water was purified using an Elix 3 coupled to a Milli-Q System (Millipore, Beleford, MA, USA) and filtered through a 0.22µm filter integrated into the Milli-Q System.

5.2 INSTRUMENTATION AND METHODS

An Agilent 1100 Series HPLC instrument equipped with a quaternary pump (G1311A), a degasser (G1322A), an autosampler (G1324A), a diode array detector (G1315B), a fluorescence detector (G1321A) and a computer with the Agilent Chemstation Software (Rev. A 10.02), all from Agilent Technologies (Waldbronn, Germany), were employed to obtain the HPLC-UV and HPLC-FL chromatographic fingerprints. Chromatographic separation performed by reversed-phase mode using a Kinetex C18 (100x4.6 mm I.D., particle size of 2.6 µm) column obtained from Phenomenex (Torrance, California, USA). The reversed-phase HPLC method employed in this work was previously developed in the research group.[20] Briefly, separation was carried out using gradient elution with 0.1% (v/v) formic acid aqueous solution (solvent A) and methanol (solvent B) as mobile phase components. The mobile phase flow rate was selected at 0.4 mL/min and the

chosen injection volume was 5 µL. The gradient elution program employed is indicated in Table 1. Chromatograms were recorded at wavelengths of 250, 280, 310, 370 and 550 nm for UV-Vis. In the case of fluorescence detection, 280 nm was used as excitation wavelength and 320, 350, 380, 410 nm as emission wavelengths.

Table 1. Chromatographic gradient elution program.

TIME [min]	SOLVENT B [%]	ELUTION MODE
0 - 30	5 → 75	Linear gradient
30 - 32.5	75 → 95	Linear gradient
32.5 - 35	95	Isocratic
35 - 35.10	95 → 5	Linear gradient
35.10 - 40	5	Isocratic

5.3 SAMPLE AND SAMPLE TREATMENT

For the adulteration studies of almonds and almond custard cream, three nut samples obtained from Barcelona markets were employed. Table 2 shows the type of nut employed, and some characteristics regarding the nut samples.

Table 2. Description of the nut samples used for the adulteration study.

Sample	Abbreviation	Brand	Packaging	Origin
Almond	AL	Frit Ravitch	Bag	USA
Peanut	PE	Capravo	Box	-
Hazelnut	HN	Eroski	Box	Turkey

Adulteration studies of almond

Two adulterants were used in this study, peanut and hazelnut. The adulteration study was carried out by preparing a total of 105 samples in which almond was adulterated with either hazelnut or peanut in proportions from 0% to 100% as indicated in Table 3. The samples were then split into calibration/validation sets in order to fit them for the PLS analysis. One additional sample of 50:50 almond:adulterant was prepared and used as quality control (QC) to evaluate the repeatability and robustness of the results.

Table 3. Set of samples used for each adulteration study. Adulterant: peanut and hazelnut.

Almond [%]	Adulterant [%]	Number of Samples	Calibration/Validation [C/V]
100	0	5	C
85	15	5	V
80	20	5	C
75	25	5	V
60	40	5	C
50	50	6	V
40	60	5	C
25	75	5	V
20	80	5	C
15	85	5	V
0	100	5	C

Adulteration studies of almond custard cream

An almond custard cream was prepared and adulterated with peanut or hazelnut custard creams. The custard cream was cooked using eggs, milk, sugar and cornflour. For that purpose, milk was heated (without boiling). Meanwhile, eggs were whisked and mixed with the sugar, the cornflour was dissolved in cold milk, and finally mixed with the eggs. Then, this mixture was added to the warmed milk, and all the mixture was heated, with stirring until the cream thickens. Nuts where then added to the custard cream in a final proportion of 50%.

Another 105 adulterated samples were prepared by adulterating almond custard cream with hazelnut or peanut custard creams in the same proportions as described before (Table 3), and again split into calibration/validation sets. QCs were also prepared following the same criteria (50:50 proportion).

Nut samples for HPLC-FL classification studies.

The same 149 samples used for the previous HPLC-UV fingerprinting classification work [20] were employed in this study. Table 4 shows the type of nut, the abbreviation used, the number of samples, the thermal processing treatment and the country of origin.

In this case, 16 QCs were prepared by mixing 50 µL of each sample.

Table 4. Set of samples used for the classification studies.

Sample	Abbreviation	Number of Samples	Characteristics	Origin
SUNFLOWER SEEDS	SF	9	All Toasted	Spain
MACADAMIA	MA	10	All Natural	South Africa
CASHEW NUTS	CH	10	All fried	Brazil
PINIONS	PN	10	All natural	Spain/China
WALNUTS	WN	10	8 Natural with shell/ 2 Natural without shell	USA/Chile
PISTACHIO	PT	10	All toasted	Germany/Spain/Iran
PUMPKIN SEEDS	PS	20	10 Natural/ 10 Toasted	Austria/China
HAZELNUT	HN	20	10 Natural/10 Toasted	Spain/Turkey
PEANUTS	PE	20	10 Fried / 10 Toasted	USA/ Brazil / China/ Spain
ALMONDS	AL	30	10 Natural / 10 Toasted / 10 Fried	Spain / USA

Sample treatment

Sample extraction treatment was performed following the method described by Campmajó et al. (2019).[20] Briefly, all samples were weighted (around 0.125 g for nuts and 0.250 g for custard creams) and extracted with 3 mL of an acetone:water (70:30 *v/v*) solution by shaking vigorously for 1 min in a vortex (Stuart, Stone, United kingdom), followed by ultrasonic extraction for 15 min (Branson 5510). The solutions were then centrifuged for 30 min at 3400 rpm and 4 ºC (Rotanta 460 RS, Andreas Hettich Gmb & Co, 2002, Germany).

Then defatting was performed with 3 mL of hexane, shaking vigorously for 1 min in a vortex and centrifuging for 15 min at 3400 rpm. Finally, the extracts were filtered through a 0.22 µm nylon filter and transferred into 2 mL injection vials to be analysed with the proposed HPLC-UV and HPLC-FL methods.

5.4 DATA ANALYSIS

Raw data was extracted from the Chemstation software from Agilent and exported with UniChrom from New Analytical Systems Ltd. SOLO from Eigenvector Research was used for calculations with PCA, PLS and PLS-DA regressions. X data matrices to be treated by PCA, PLS and PLS-DA consists on the HPLC-UV or HPLC-FL fingerprinting signal. The dimension of the data matrices employed were the number of samples x 6001 absorbance signal variables for UV and x 5001 for FL. Y data Matrices for PLS consists on the adulteration proportion (%). Y data for PLS-DA consists on the type of nut.

6. RESULTS AND DISCUSSION

6.1 HPLC-UV FINGERPRINTING FOR THE IDENTIFICATION AND QUANTIFICATION OF FRAUDS IN ALMOND

As early mentioned, in a previous work developed in the research group the characterization and classification of nuts was studied. The developed method by HPLC-UV fingerprinting was adequate to establish a good separation of bioactive substances in nuts and a successful classification and authentication according to the type of nut.[20] Seeing that it showed good results, it was proposed as a possible strategy to detect frauds involving any of the studied nut samples. Following that study, the identification and quantification of the frauds was the aim of this research.

Almond was chosen in order to study the frauds, as it is usually the target in bakery flours for the addition of cheaper nuts such as peanut and hazelnut, which we chose as adulterants. Almond samples, adulterated with different proportions of peanut and hazelnut samples, as already explained in the experimental section, were extracted with acetone/water (70:30 *v/v*), and analysed with the proposed HPLC-UV method to obtain the HPLC-UV fingerprinting profiles. Wavelength was selected at 280 nm due to the fact that it was the one providing more signals and with the highest intensity in the classification study.[20] SOLO was used to analyse the chromatographic fingerprints of the adulterated nuts. As an example, Figure 2 shows the raw chromatographic fingerprints of pure and adulterated nut samples. Chromatograms were cut to remove solvent peak and column-preconditioning steps.[20]

As can be seen in the figure, different HPLC-UV fingerprints are obtained depending on the nut type. Peanut samples have much bigger absorbances than almond and hazelnut, and the decrease in signal intensity can be appreciated when transitioning to the adulterated sample. Hazelnut also has a very different fingerprint than almond, although with not that different intensities, and the transition from one to another can also be appreciated with the adulterated sample. This level of transition is what will allow us to quantify the percentage of adulteration.

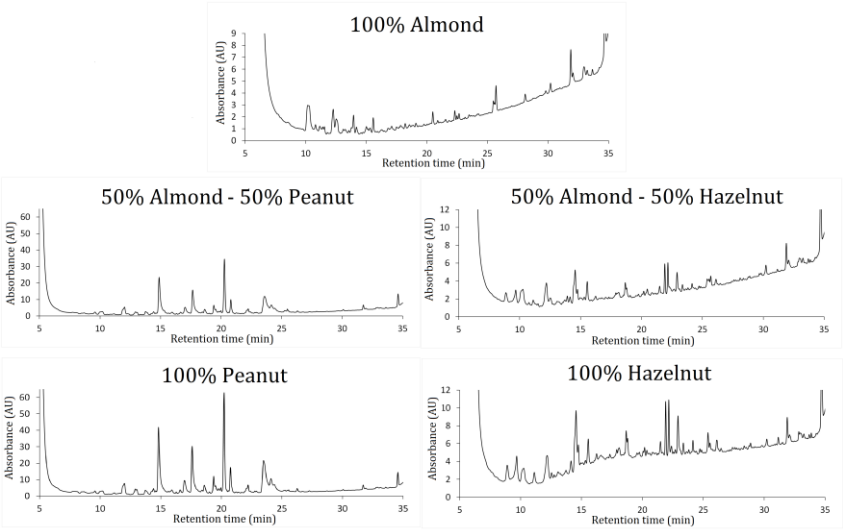

Figure 2. Raw chromatograms of pure and adulterated almond, peanut and hazelnut samples.

As a first approach, HPLC-UV fingerprints of all the almond adulterated samples (Table 3) were evaluated with PCA in order to have a general look and also evaluate the robustness of the method. Figure 3A shows the score plot of PC1 *vs* PC2 for the almond-peanut adulteration. QCs were a very important factor because, as they are the same sample injected several times, they must appear altogether as a clustered group after the PCA analysis. As this was not the case, raw chromatograms of all the QCs were plotted and they showed some variance in the retention times and an increase in the baseline. These variances were probably affecting the results, but fortunately this was easily corrected using some built in pre-process such as *variable alignment* and *Baseline (Automatic Weighted Least Squares)*. Other pre-process like *autoscale, normalize* and *mean centre* were also tried and used in some cases to remove variances between samples and improve the results. Figure 3B shows the score plot of PC1 *vs* PC2 for the almond-peanut adulteration after corrections were performed. As QCs now appear all in the same place, chemometric results can be trusted.

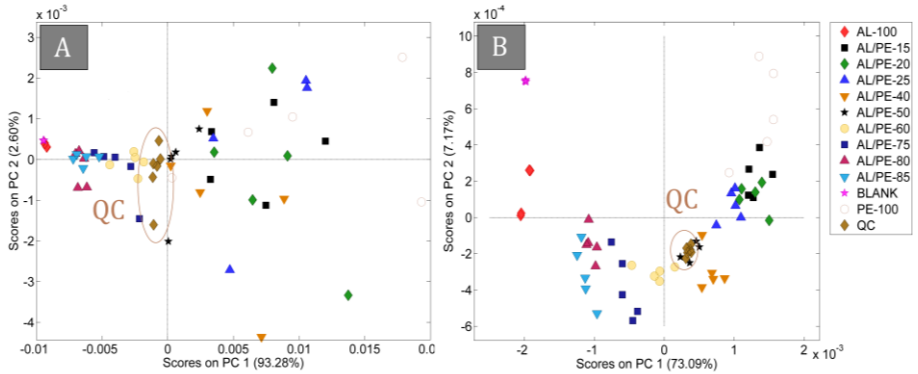

Figure 3. PCA score plot of almond-peanut samples, before (A) and after (B) correcting with QCs.

Once we were able to see, evaluate and confirm with the PCA that the repeatability and robustness of the method was good, blanks and QCs were removed from the previous matrix and the remaining data was split into calibration/test sets and submitted for the PLS study.

X calibration and X prediction matrices consisted in the number of samples of each set x 6000 variables. Y calibration and Y prediction matrices consisted in the real adulteration percentage based on each weighted sample. Before prediction was made, the built-in algorithm of *variable selection* based on *Variable Importance on Projection* (VIP) score was used in order to select the best variables. This procedure is very useful when dealing with continuous variables hence we decided to use it.

Figure 4 shows the Y measured *vs* Y predicted plot for the almond-peanut and almond-hazelnut adulteration.

As can be seen in the figure, both cases correctly follow a linear trend. The determination coefficient (R^2) of cross validation obtained for the fit was 0.998 for the almond-peanut adulteration and 0.985 for the almond-hazelnut adulteration, with a root mean square error of cross validation (RMSECV) of 5.3 and 6.9 respectively. R^2s of predictions were 0.957 and 0.968, and RMSEs of prediction (RMSEP) were 6.7 and 5.4. The fact that the adulterated samples are distributed in the linear trend following the increasing ratio of adulteration with these relatively low levels of error means that the HPLC-UV fingerprinting method is suitable for the identification and quantification of frauds in almond samples adulterated with peanuts or hazelnuts.

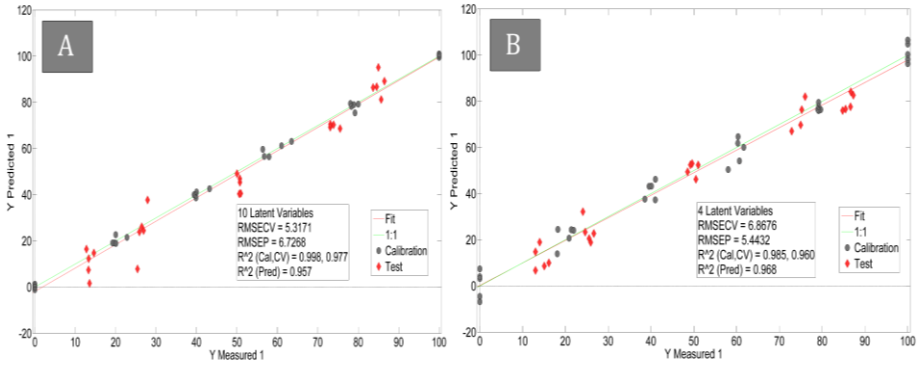

Figure 4. Y mesured *vs* Y predicted PLS plot of the (A) almond - peanut and (B) almond - hazelnut adulteration, using HPLC-UV fingerprinting.

6.2 HPLC-UV FINGERPRINTING FOR THE IDENTIFICATION AND QUANTIFICATION OF FRAUDS IN ALMOND CUSTARD CREAM

Previous results on the almond adulteration showed that identification and quantification of frauds in nuts can be studied with a simple method as HPLC-UV fingerprinting. However, if we look where these frauds are normally being made in real products, things may not be as easy as it looks at a first glance. As early commented, these nut frauds are often being made in bakery products[14] like nut flours or custard creams which may cause difficulties when analysed by HPLC. These products usually have a high content in protein and even higher content of fats (especially the case of custard creams), which may interfere and cover up the nut fingerprint profile. In order to address it to a more real case scenario, an almond custard cream was prepared and its adulteration with peanut and hazelnut was studied.

New samples following the same ratio of adulteration where prepared and pre-treated with the same explained method and HPLC-UV chromatograms were recorded to obtain the new fingerprinting profiles. As an example, Figure 5 shows a comparison of chromatograms of a pure almond nut sample and pure almond custard cream sample.

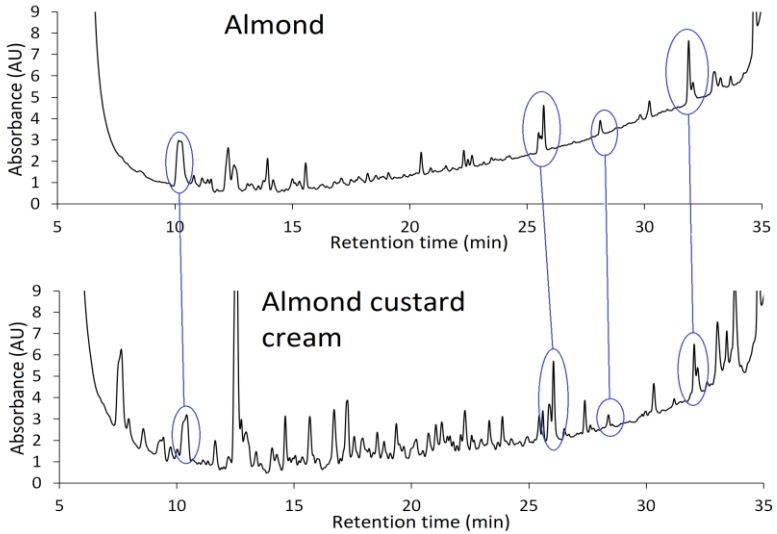

Figure 5. Raw HPLC-UV chromatograms of almond nut and almond custard cream recorded at 280 nm.

At first sight it can easly be observed that the almond custard cream chromatographic fingerprint is richer in extracted and detected bioactive components than the almond cream sample. That is to be expected, because despite a defating proces was performed, still some proteins, some fats and more components are now being extrated, separated and detected from the custard cream matrix (which includes milk, sugar, corn flour and egg). If we look closer, most of the new peaks that are detected are located in the middle of the chromatogram, at about 15-25 min. Since some of the almond nut peaks can still be observed (four of them are marked in the figure, as example), and the components extracted from the custard cream matrix will be the same in all analyzed samples, we expect that chemometrics should be able to "ignore" the peaks coming from the custard cream and still provide resonable results in the quantification of the adulteration levels.

For this study, PCA was also performed to acquire an overall look and make corrections to adjust QCs, and samples were again split into calibration/prediction sets and submitted for the PLS study. Obtained results are depicted in Figure 6.

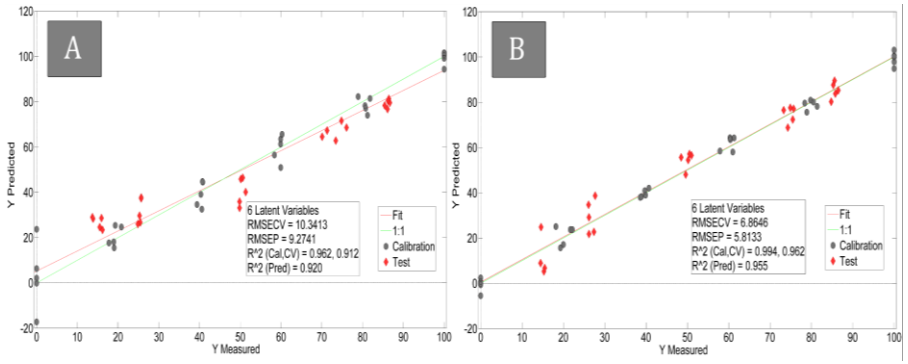

Figure 6. Y mesured vs Y predicted PLS plot of the (A) almond - peanut custard cream and (B) almond - hazelnut custard cream adulterations, using HPLC-UV fingerprinting.

As can be seen, both adulterations again follow a linear trend. In the case of the almond - hazelnut custard cream adulteration (Figure 6B), the regression shows a similar error than the one observed for the nuts adulteration studies (Figure 4B), with a RMSECV of 6.9 and RMSEP of 5.8, meaning that the HPLC-UV fingerprinting method is suitable for fraud identification and quantification in almond custard creams adulterated with hazelnut custard creams.

However, in the case of the almond - peanut custard cream adulterations (Figure 6A) the error obtained has almost doubled, with a RMSECV of 10.3 and a RMSEP of 9.2. Despite performing a defating step in the pre-treatment, the complexity of the custard cream matrix made the error of the regresion bigger, specially at low levels of adulteration. Identification of the fraud levels is still possible but some changes need to be made in order to have more precise quantification of the results. The employement of a more selective method such as HPLC-FL fingerprinting will be evaluated for that purpose.

6.3 HPLC-FL FINGERPRINTING FOR THE AUTHENTICATION AND CLASSIFICATION OF NUTS

Fluorescence detection is known to have a higher specificity and selectivity than UV-Visible detection, since the number molecules with fluorescence properties is lower. For this reason, HPLC-FL was proposed as a good strategy to improve the results obtained in the almond custard cream adulteration study. Before that, we intended to evaluate if HPLC-FL fingerprints could be used as good discriminant chemical descriptors to achieve sample classification and authentication according to the different type of nut. For this, the same samples of almond,

sunflower seeds, macadamia, cashew nuts, pinions, walnuts, pistachios, pumpkin seeds, hazelnuts, and peanuts analysed by HPLC-UV fingerprinting in a previous work[20] were used in this study, and the HPLC-FL fingerprinting profiles were obtained. Excitation wavelength was chosen at 280 nm and emission wavelength at 350 nm. As an example, HPLC-FL chromatographic fingerprints of all the different nuts analysed are displayed in Figure 7. As can be seen, very different HPLC-FL chromatographic fingerprints were obtained among the nut samples analysed, differing in both peak intensities as well as detected compound profiles. A priori, from these differences, we expect that HPLC-FL chromatographic fingerprints can be useful chemical descriptors to address sample classification by chemometrics.

Thus, a data matrix was constructed using the HPLC-FL fingerprints and submitted to exploratory PCA. The dimensions of the matrix employed were 165 samples x 5005 variables, and the same pre-process explained previously were employed in order to correct scatter between samples.

For comparison, Figure 8A shows the obtained score plot of PC1 *vs* PC2 when using HPLC-FL fingerprints as chemical descriptors in comparison with the PC1 *vs* PC2 score plot (Figure 8B) when using HPLC-UV fingerprints as chemical descriptors.[20] As it can be seen, the discrimination between nut types has significantly increased with the HPLC-FL method. The proposed method is able to perfectly separate walnuts, which are located in the top centre of the plot with highest PC1 and PC2 scores, possibly due to the fact that they have the highest polyphenol content, as already explained in section 3.2, with high intensity peaks and easy differentiable chromatograms. Macadamia samples, located in the top left, are now very well separated from the other samples which wasn't accomplished at all with the HPLC-UV fingerprints, and overall, all nut samples are now better grouped according to the type of nut than the previous results, as well as better discriminated among them. Consequently, HPLC-FL fingerprinting method seems to provide better chemical descriptors than HPLC-UV in order to achieve nut characterization. However, as PCA is not a classification method, PLS-DA will also be employed to evaluate the HPLC-FL fingerprints.

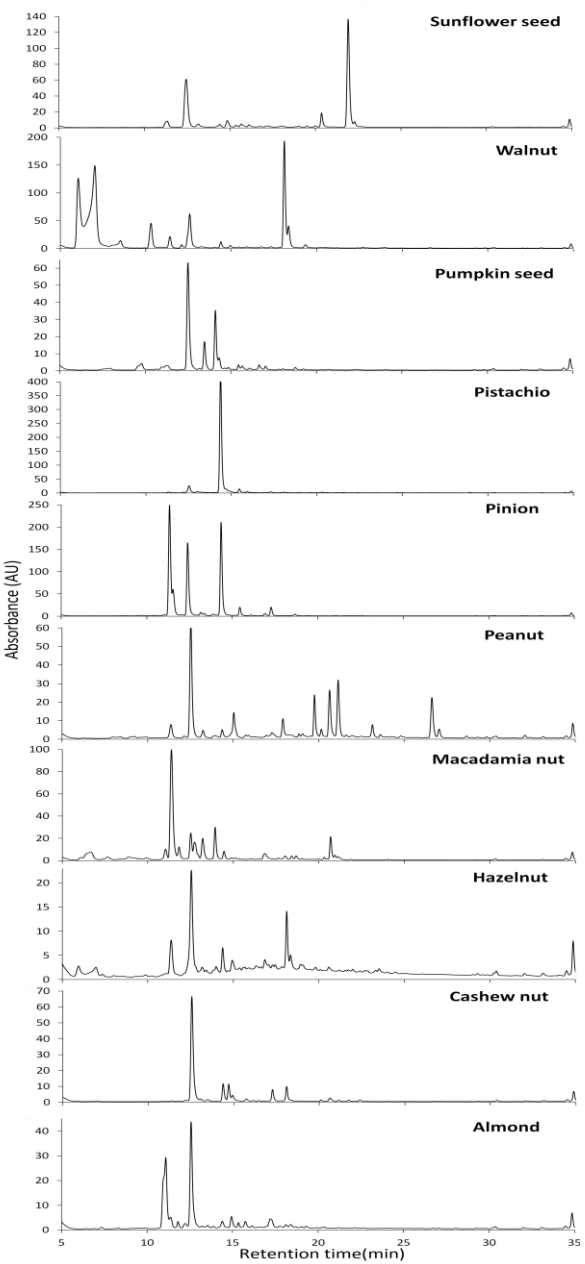

Figure 7. HPLC-FL chromatographic fingerprints of the diferent analysed nut samples.

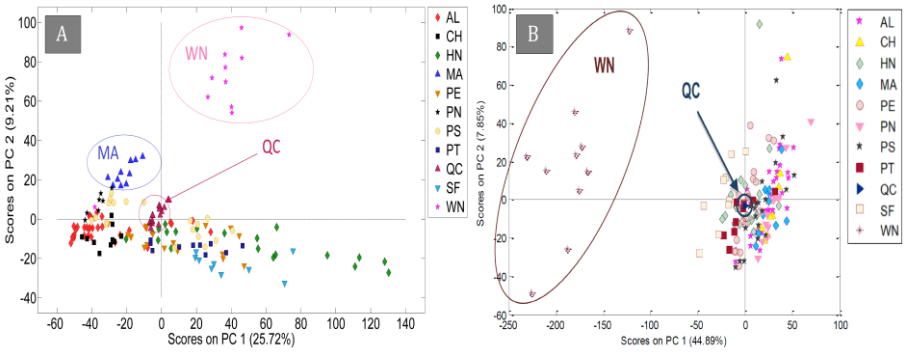

Figure 8. (A) PC1 *vs* PC2 score plots of nut samples when using HPLC-FL fingerprints as chemical descriptors for PCA. (B) PC1 *vs* PC2 scores plots in the same samples when using HPLC-UV fingerprints as chemical descriptors for PCA, reproduced from Campmajó et al. (2019)[20].

For the PLS-DA analysis, QCs were removed from the PCA X matrix and Y matrix was built containing the type of nut for each sample. Results of the classification analysis are displayed as LV1 *vs* LV2 score plot in Figure 9A, and LV1 *vs* LV2 *vs* LV3 score plot in Figure 9B. As it can be seen, a very acceptable classification is obtained. Samples are well distributed according to nut type: walnuts are again clearly discriminated being the only ones with negative scores on LV1 and LV2 at the bottom left corner, macadamia are separated at the bottom right corner, and sunflower seeds at the top. Moreover, if the 3D score plot containing also LV3 is displayed, a much better classification can be observed: peanuts with the higher LV3 score at the top, and pinions at the right side. Although it cannot be clearly seen in the depicted Figure 9B, if axis are rotated all the nut sample groups are perfectly separated.

The separation achieved with HPLC-FL is clearly superior than the obtained with HPLC-UV,[20] meaning that non-targeted HPLC-FL fingerprinting is a very good method to address nut sample classification and authentication, and a very promising method for nut frauds by means of adulteration. Therefore, HPLC-FL fingerprinting will also be evaluated to achieve the identification and quantification of frauds in adulterated almond and almond custard cream samples.

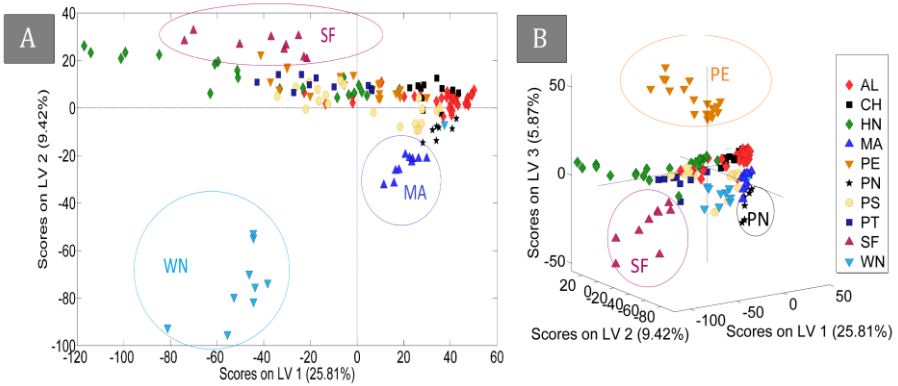

Figure 9. (A) 2D and (B) 3D PLS-DA score plots of all the nut samples analysed when using HPLC-FL fingerprints as chemical descriptors.

6.4 HPLC-FL FINGERPRINTING FOR THE IDENTIFICATION AND QUANTIFICATION OF FRAUDS IN ALMOND

As the HPLC-FL fingerprinting method turned out to be a much better method than HPLC-UV fingerprinting for the classification of nuts, the identification and quantification of adulterated almond products should, in theory, provide better results as well. In order to evaluate that, the same samples used in section 6.1 were employed, and the same pre-treatment was used for this study. HPLC-FL chromatograms were now recorded at wavelengths of excitation of 280 nm and emission of 350 nm. Same SOLO matrices than the ones used in section 6.1 were constructed for exploratory PCA and PLS analysis.

Figure 10 shows the results of the PLS analysis for the adulteration of almond with peanut (A) and hazelnut (B).

As can be seen in the figure, the obtained RMSEP, 4.1 for almond-peanut and also 4.1 for almond-hazelnut case, are quite lower than the RMSEP of the HPLC-UV method (Figure 4), by more than 2 units in the case of the almond-peanut adulteration, and by more than 1 unit for the almond-hazelnut adulteration. Just as it was predicted, the higher specificity and selectivity of the fluorescence detection allows for a more accurate quantification of the level of adulteration of the almond samples, meaning that HPLC-FL fingerprinting method is better than the HPLC-UV method for the identification and quantification frauds when almonds are adulterated with peanuts

and hazelnuts. This is very promising for the adulteration study of almond custard creams, as the results obtained with the HPLC-UV method were not that good, especially for the case of almond-peanut custard cream adulteration.

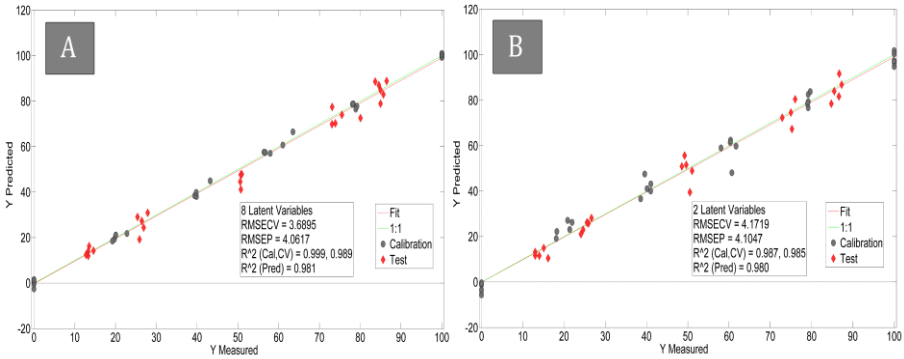

Figure 10. Y mesured vs Y predicted PLS plot of the (A) almond-peanut and (B) almond-hazelnut adulteration, using HPLC-FL fingerprinting.

6.5 HPLC-FL FINGERPRINTING FOR THE IDENTIFICATION AND QUANTIFICATION OF FRAUDS IN ALMOND CUSTARD CREAM

The HPLC-UV fingerprinting method employed in section 6.2 showed that it was possible to identify and quantify the fraud of adulteration in almond custard cream. However, the custard cream matrix complexity significantly increased the error in the regression, and mainly in the almond-peanut custard cream adulteration case. As the HPLC-FL fingerprinting method showed very good results in classification of nuts (section 6.3), as well as in the quantification of adulterated almonds (section 6.4) it was also employed in the identification and quantification of frauds when using almond custard creams. For this purpose, the same samples of almond custard creams than the ones used in section 6.2 were analysed by the proposed HPLC-FL method. Same pre-treatment was performed, and HPLC-FL chromatographic fingerprints were again recorded at an excitation wavelength of 280 nm and at an emission wavelength of 350 nm. The same SOLO matrices were constructed and submitted to exploratory PCA and PLS analysis.

Figure 11 shows the results obtained for the almond custard cream adulterated with peanut and hazelnut custard cream. As can be seen, the errors of calibration and prediction (CV/Peanut:

5.3; CV/Hazelnut: 5.4; P/Peanut: 4.9; P/Hazelnut: 4.5) are significantly lower than the ones obtained with the HPLC-UV method in both adulteration cases that were evaluated. The regression line is very well accomplished with low errors of prediction, and therefore, we can say that the proposed HPLC-FL fingerprinting method employing a liquid-solid extraction with acetone:H_2O (70:30 *v/v*) and a defatting step with hexane provides suitable chemical descriptors to achieve a correct identification and quantification of almond custard cream adulterated with peanut or hazelnut custard creams.

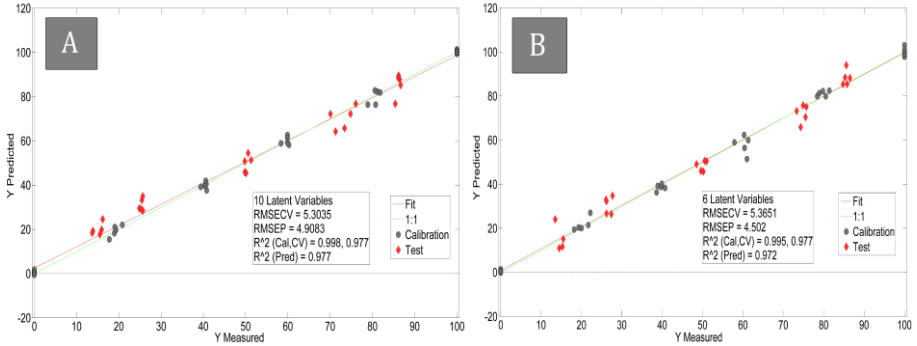

Figure 11. Y mesured *vs* Y predicted PLS plot of (A) almond-peanut custard cream and (B) almond-hazelnut custard cream adulterations, using HPLC-FL fingerprints.

10. CONCLUSIONS

In this work, non-targeted HPLC-UV chromatographic fingerprints were recorded and used as chemical descriptors to achieve the identification and quantification of adulterated almond and almond custard cream samples by chemometric methods. HPLC-FL fingerprints were also recorded to evaluate if fluorescence detection could offer better discriminant chemical descriptors to achieve sample classification and authentication according to the different nut types than ultraviolet-visible detection, as well as a better quantification of adulteration level in almond and almond custard cream samples. At the end, several conclusions can be extracted:

- HPLC-UV fingerprinting method provides suitable chemical descriptors to achieve identification and quantification of adulteration levels of almond samples with peanut and hazelnut. However, the study of the almond custard cream samples showed higher errors of prediction, especially with the almond custard cream adulterated with peanut case, and other methodologies are suggested instead.
- HPLC-FL fingerprinting method provides a superior separation and classification than HPLC-UV fingerprinting of different nut samples according to the nut varieties. Furthermore, HPLC-FL shows lower errors of prediction of the adulteration level in almond and almond custard cream samples adulterated with peanut and hazelnut.

Therefore, HPLC-FL fingerprinting combined with chemometrics is a suitable method to address nut product authentication and it is suggested, instead of HPLC-UV fingerprinting, as a much proper methodology to achieve classification of nut samples, and identification as well as quantification of nut food frauds by means of adulteration.

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12. ACRONYMS

HPLC: High performance liquid chromatography

LC-MS: Liquid chromatography - Mass spectroscopy

UV: Ultraviolet

FL: Fluorescence

GC: Gas chromatography

PCA: Principal component analysis

PLS: Partial least squares

PLS-LDA: Partial least squares - linear discriminant analysis

PLS-DA: Partial least squares - discriminant analysis

RMSECV: Root mean square error of cross validation

RMSEP: Root mean square error of prediction

VIP: Variable importance on projection

QC: Quality controls

CV: Calibration

OAS: Oral allergy syndrome

CWA: European committee for standardization workshop agreement

V: Validation

P: Prediction

PC: Principal components

LV: Latent variable

WN: Walnut

PS: Pumpkin seed

PT: Pistachio

PN: Pinions

SF: Sunflower seed

AL: Almond

PE: Peanut

HN: Hazelnut

CH: Cashew nut

MA: Macadamia

H_2O: Water

MeOH: Methanol

µL: microlitre

nm: Nanometre

min: Minute

mL: Millilitre

v/v: Percent concentration volume/volume

rpm: Revolutions per minute