Tutor/s

Dr. Oscar Núñez Burcio
Departament d'Enginyeria Química i Química Analítica

Dr. Javier Saurina Purroy
Departament d'Enginyeria Química i Química Analítica

Grau de Química

Treball Final de Grau

Non-targeted Fingerprinting Methodologies for the Authentication of Tea. Application to the Detection and Quantitation of Frauds in Adulterated Tea Samples with Chicory.

Metodologies d'Empremtes No-dirigides per a l'Autenticació de Té. Aplicació a la Detecció i Quantificació de Fraus en Mostres de Té Adulterades amb Xicoira.

Àlex Bedmar Chamarro

January, 2022

Universitat de Barcelona

B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

Els resultats que aconsegueixes seran directament proporcionals a l'esforç que apliques.

Denis Waitley

M'agradaria començar donant les gràcies als meus tutors, n'Oscar Núñez i en Javier Saurina, pel seu ajut durant el transcurs d'aquest treball, així com per la seva vocació educativa que m'ha fet aprendre i enriquir-me tant a nivell acadèmic com personal. A part d'ells, també vull fer especial menció a la Sonia Sentellas, que no ha dubtat en cap moment en donar-me un cop de mà quan ho he necessitat.

També agrair la col·laboració d'en Guillem, na Nerea i n'Aina, unes persones increïbles amb les que he compartit molt temps al laboratori i de les que he après moltíssim.

Finalment, mencionar a la meva família, parella i amics, ja que el suport fora de la universitat també ha sigut imprescindible per tirar endavant aquest projecte.

A tots vosaltres, gràcies de tot cor.

REPORT

CONTENTS

1. SUMMARY

In recent years, in an increasingly globalised world the food industry like so many others has undergone a paradigm shift. This increasing globalisation has generated high competition, and some industries have turned to fraudulent practices in order to reduce production costs. Food frauds are very diverse, ranging from the substitution and/or addition of components to fraud in product labelling. This malpractice for economic purposes becomes a serious problem for consumers by putting their health at risk. To avoid this kind of negligence, it is very important to develop new fraud detection methodologies, especially in a world where these practices are becoming increasingly sophisticated. This project focuses on tea adulteration with chicory.

For this purpose, two methodologies have been developed simultaneously for the authentication of different tea varieties (black, red, green, white and oolong). These are two non-targeted fingerprinting methods that use the chromatograms obtained by high performance liquid chromatography with ultraviolet (HPLC-UV) and fluorescence (HPLC-FLD) detection. The obtained data have been subjected to multivariate chemometric treatments, such as Principal Component Analysis (PCA) and Partial Least Squares regression (PLS). The good results obtained have shown that both methodologies are effective for the detection and quantification of tea adulteration with chicory, exhibiting good linear regressions ($R^2 > 0.998$) and calibration, cross-validation, and external validation errors below 1.4%, 6.4% and 3.7%, respectively. Moreover, very acceptable prediction errors have been obtained (less than 21.7%), except for white tea extracts which show higher errors due to the similarity of their fingerprints to those of chicory.

In addition to these two methods, two new studies have been started to further advance the authentication of teas. The first one is a non-targeted FIA-MS fast-screening method and the second one is a non-targeted method using the fingerprints obtained by liquid chromatography coupled to a mass spectrometer (LC-MS) as chemical descriptors. Data from both methodologies have also been chemometrically treated with PCA and Partial Least Squares-Discriminant Analysis (PLS-DA) and the results are very promising since both techniques are able to

distinguish between chicory and tea sample extracts (although further work is needed to optimise them).

The most interesting approach is the FIA-MS method, which represents a great advantage over previous methods, as it entails significant time saving in tea authentication analysis.

Keywords: HPLC-UV, HPLC-FLD, FIA-MS, LC-MS, Tea, Chicory, PCA, PLS, PLS-DA, Fingerprinting, Chemometrics, Food Fraud.

2. RESUM

En els darrers anys, en un món cada cop més globalitzat, la indústria alimentària, com tantes altres, ha viscut un canvi de paradigma. Aquesta globalització creixent ha generat una competència cada cop major, i algunes indústries han optat per realitzar pràctiques fraudulentes amb la finalitat de reduir els costos de producció. Els fraus alimentaris són molt diversos, i van des de la substitució i/o addició de components fins al frau en l'etiquetat dels productes. Aquesta mala praxi amb finalitats econòmiques esdevé un greu problema per al consumidor, posant fins i tot en risc la seva salut. Per evitar que se segueixin cometent aquestes negligències, és molt important el desenvolupament de noves metodologies de detecció de fraus, sobretot en un món en el que aquestes pràctiques són cada cop més sofisticades. Aquest treball centra la seva atenció en l'adulteració de te amb xicoira.

Amb aquesta finalitat, s'han desenvolupat simultàniament dues metodologies per a l'autentificació de diferents varietats de te (negre, vermell, verd, blanc i oolong). Es tracta de dos mètodes no dirigits que utilitzen com a perfil d'empremtes els cromatogrames obtinguts mitjançant cromatografia de líquids d'alta eficàcia amb detecció ultraviolat (HPLC-UV) i de fluorescència (HPLC-FLD). Les dades obtingudes han estat sotmeses a tractaments quimiomètrics multivariants, com l'anàlisi de components principals (PCA) i la regressió parcial per mínims quadrats (PLS). Els bons resultats obtinguts han demostrat que ambdues metodologies són efectives per a la detecció i quantificació de l'adulteració de tes amb xicoira, exhibint unes bones regressions lineals ($R^2 > 0.998$) i uns errors de calibració, validació creuada i validació externa inferiors a 1.4%, 6.4% i 3.7%, respectivament. A més, s'han obtingut uns errors de predicció molt acceptables (inferiors al 21.7%), excepte en el cas dels extractes de te blanc que mostren uns errors majors degut a la similitud entre els seus perfils d'empremta i els de la xicoira.

A més d'aquests dos mètodes, s'han iniciat dos nous estudis per tal de continuar avançant en l'autentificació dels tes. El primer d'ells és un mètode no dirigit de detecció ràpida FIA-MS i el segon és un mètode no dirigit que utilitza com a descriptors químics les empremtes obtingudes mitjançant cromatografia de líquids acoblada a un espectròmetre de masses (LC-MS). Les dades

d'aquestes dues metodologies també han estat tractades quimiomètricament amb PCA i anàlisi discriminant amb regressió de mínims quadrats (PLS-DA). Els resultats obtinguts són molt prometedors ja que ambdues tècniques són capaces de diferenciar entre la xicoira i els tes (tot i que cal continuar treballant en ells per optimitzar-los).

El que és més interessant és el mètode de FIA-MS, que suposa un gran avantatge respecte els mètodes anteriors, ja que comporta un important estalvi de temps en l'anàlisi d'autentificació de te.

Paraules clau: HPLC-UV, HPLC-FLD, FIA-MS, LC-MS, Té, Xicoira, PCA, PLS, PLS-DA, Perfil d'empremtes, Quimiometria, Frau Alimentari.

3. INTRODUCTION

3.1. FOOD AUTHENTICATION

In an increasingly globalized world, in which any product can reach our homes from anywhere in the world (something unimaginable a few years ago), large multinational companies struggle to obtain the highest possible profit in this new scenario [1]. In this eagerness to obtain large economical gains, food fraud has increased dangerously in recent years, due to the difficulty in detecting it. Although the reason for food fraud is clearly economic, the result is a real risk for the public health, growing in danger.

Nowadays, it is increasingly common for consumers to pay attention to characteristics that were not previously given much importance, such as the origin, processing, or properties of the products. The reputation of high-quality and unique products makes them highly priced in the market. It is for this reason that large multinationals, food producers, etc., have opted to commit different food frauds. Lying about the origin of a product (or its ingredients), or adulterating foods with different substances not specified on the label, are the most common frauds. Generally, adulterations are aimed at increasing the size of the product, masking the presence of lower quality components, or replacing some substances to reduce production costs. As well as these food frauds can result in financial penalties for companies, they represent a danger for consumers, as prohibited substances are sometimes added, or non-declared allergens are included [2].

For all the above reasons, governments and different regulatory bodies have established strict rules on food control and labeling. Nevertheless, traditional food safety approaches may not be the most effective option for detecting or deterring food fraud [3]. Therefore, an increasing number of organizations are working on the development of food authentication methods. These methods serve to confirm that product specifications are true and, if they are not, to detect and quantify alimentary frauds [4].

Among the food and beverages with the highest risk of fraud we can find tea products, commonly adulterated with other low-quality ingredients. In this work we will focus on the authentication of tea extracts.

3.2. TEA

Tea is, with coffee, one of the most widely consumed beverages in the world. It is an infusion prepared with hot or boiling water and the leaves or buds of the *Camellia sinesis* plant. This evergreen shrub is native to China and East Asia. Over the years, it has expanded to Europe and other continents, but this plant grows mainly in tropical and subtropical climates [5].

There are mainly five types of tea based on how it is processed (Figure 1). They differ basically in two aspects: fermentation and leaves treatment. Depending on the fermentation process, tea leaves can be unfermented (green, white, and red teas), partially fermented (oolong tea), and fully fermented (black tea). Green and black teas are the most consumed around the world [6].

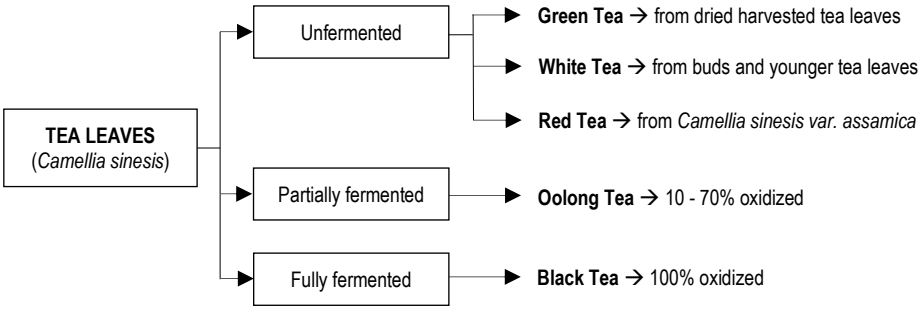


Figure 1. Tea classification.

In addition to its refreshing effects, tea's popularity is due to its positive health benefits. Numerous studies corroborate that these infusions confer social welfare, as well as antioxidant, anticarcinogenic, cardioprotective, cholesterol-lowering, antihistamine, and antimicrobial properties [7,8]. These properties mentioned above are due to the chemical composition of *Camellia sinesis* leaves, with a complex mixture of caffeine, polyphenols, polysaccharides, and nutrients like proteins, amino acids, lipids, and vitamins [9].

It is well known that the quality of tea is determined by the appearance, colour, aroma freshness and flavour. These properties are influenced by factors related to the origin of the

product, such as climate, soil, harvesting time, manufacturing process and storage method [10]. All these factors influence the standard parameters that provide a variation in the market value of tea. As a result, products with higher yields have higher prices and better properties.

For the reasons commented above and the difficulty to identify the origin of a tea by its appearance, such beverages are highly vulnerable to food fraud. The use of inferior ingredients, undeclared fillers and mislabelling of origin and processing methods are the most common frauds. In addition to the fact that these products violate consumer rights, they could suppose a health risk for them. For instance, adulterants can cause adverse health effects such as allergic reactions or, in the worst case, toxic effects [11].

Nowadays, the issue of adulteration of these products is receiving much attention from food analysts. Even so, there is always new ways to deceive consumers and, therefore, new challenges to detect food fraud and protect consumers.

3.3. TEA ADULTERANTS

In recent years, a huge number of counterfeiting and adulteration in herbal products has been reported. As it was mentioned before, some teas present a high value, which make them particularly susceptible to adulterations and frauds. The most common committed frauds are caused by the quality reduction of the ingredients and the addition of cheaper vegetable elements that reduce production costs [12].

The perception that herbal drugs are very safe and free from side effects is not the reality. Generally, plants have a lot of constituents, and some are very toxic. Taking the wrong herbal drugs could have adverse effects [13]. In addition, mislabelled teas could cost perjuries to human health via allergies, intolerances, and various offenses to the immunologic system. Therefore, it is necessary to develop accurate methods for the authentication of these plant-based beverages.

Tea has been found to be adulterated with different types of vegetable elements such as chicory, husk of pulses, cereal starch or used tea leaves. Sometimes other components are added, such as azo dyes, sand, or kaolin. All of them are non-permitted materials (to be employed as tea adulterants), used to reduce manufacturing costs and to deceive consumers [14,15]. In addition, in some cases, teas of different provenience are mixed and sold as a tea product produced in a specific region, lying on labelling.

This work will focus on the study of tea adulteration with chicory (*Chicorium intybus*), a perennial herbaceous plant of the Asteraceae family. This vegetable, native from Europe, is less sensible than tea plants to the climate conditions, allowing its cultivation throughout the world [16]. For this reason, adulteration with chicory is very affordable for companies.

In fact, it has been scientifically proven that chicory has positive effects on human health and its medicinal uses date back to Egyptian times. Used as a coffee substitute, chicory contains polyphenols, minerals and inulin, a carbohydrate that reduces the risk of gastrointestinal diseases [17]. Despite this, its use as an adulterant remains a food fraud for economic purposes and must be detected and reported.

3.4. ANALYTICAL METHODS

Traditionally, food authentication methods have been targeted towards single analytes, detecting only one compound at a time, and providing limited information. Many adulterants can potentially be added to a food product and these methods represent an inefficient response to a growing problem, unless one specific adulterant is suspected [18].

Globalization of food supplies, which makes them more complex, increases the potential for adulteration in many types of food. Conventional techniques are time consuming and cannot satisfy the requirements of speediness and high performance to analyse a large volume of food products. This scenario and the expansion in the use of herbal beverages worldwide, makes it necessary to develop more appropriate analytical methods [19]. These techniques need to be consistent because food characteristics from diverse origins can be dissimilar. Furthermore, traceability is also needed because it is an essential element for food safety.

There are two types of analytical methods, according to their nature: Targeted and non-targeted methods. Both are used in food authentication.

Most of the strategies proposed for fraud detection are targeted methods, focusing on the determination or quantification of a specific analyte or group of analytes (sometimes belonging to the same chemical family). These methodologies need analytical markers (commercially standards) that offer direct (primary makers) or indirect information (secondary markers) about the product authenticity. When considering some complex food samples, these methods severely limit the analysis, because they do not offer the opportunity to observe unexpected changes in a

global way. Hundreds of adulterants can be added to a food product, but these methods only focus on a limited group of analytes.

On the other hand, non-targeted methods are becoming increasingly popular in food authentication. They are based on the detection of instrumental responses (fingerprinting) without assuming any previous knowledge of the food components [20]. These techniques include both relevant and irrelevant components to discriminate an adulteration, generating a lot of information and, therefore, requiring some multivariable statistical software to recover the valuable data. Numerous analytical techniques including vibrational spectroscopic (such as Fourier Transform Infrared (FTIR), Near-Infrared (NIR) and Raman), nuclear magnetic resonance (NMR), spectrometry and chromatography-based technologies have been employed to develop non-targeted methodologies for food authentication [21].

For the analysis of tea samples, some analytical targeted and non-targeted methods have been used. NIR [5,9,12,22–24], FTIR [25], and Ultraviolet-Visible (UV-Vis) [25–27] spectroscopy fingerprinting strategies have been used to deal with food frauds. However, these techniques, especially non-targeted fingerprinting strategies, provide a huge amount of chemical data. This large quantity of information obtained will require some chemometric treatments to find relationships among chemical variables and samples features, as well as to assess the significance of the employed descriptors [28].

3.5. CHEMOMETRICS

Chemometrics is a chemistry discipline which applies mathematical and statistical models in gathering information from a chemical analysis. They are multivariate methods used to extract relevant qualitative or quantitative information from complex data, as well as represent and display this information. These methodologies allowed the evolution from the univariate to multivariate methods, leading to more complex analyses.

Nowadays, multivariate methods are employed in many disciplines such as biochemistry, chemistry, medicine, or chemical engineering. As far as food authentication is concerned, chemometrics is widely used.

The biggest application areas of chemometrics include calibration, validation, and significance testing; optimization of chemical measurements and experimental procedures; and the extraction of the maximum chemical information from analytical data [29].

Multivariate data for the chemometric treatments is commonly arranged in a matrix structure, in which each row corresponds to a given sample and each column to each experimental variable as can be seen in Figure 2.

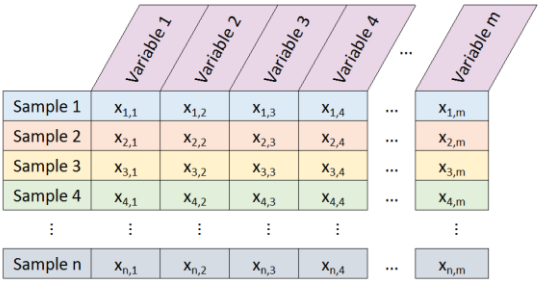


Figure 2. Chemometric data matrix.

Furthermore, the use of chemometrics makes possible to employ pre-treatments to improve the data quality for a more efficient work. Autoscale, Smoothing, Baseline and Variable Alignment pre-processing are used in this study.

As mentioned above, one of the most used techniques in chemometrics is the application of multivariate analysis methods to process all data. In the present work, Principal Component Analysis (PCA), Partial Least Squares (PLS) regression, and Partial Least Squares-Discriminant Analysis (PLS-DA) are employed.

3.5.1. Principal Component Analysis (PCA)

Principal Component Analysis (PCA) is a multivariate method used to extract the dominant patterns in the data matrix in terms of a complementary set of scores and loading plots. This technique forms the basis for multivariate data analysis, permitting the qualitative discrimination of class samples.

As an exploratory method, PCA estimates the correlation structure of the variables. It can be applied to any data matrix as an initial step of any multivariate analysis to obtain a first look at the structure of the data, helping to identify outliers, delineate classes, etc. Used with well selected set of samples and variables, this method permits building a model of how a chemical system behaves.

PCA can reduce the correlated variables of data matrix into a new set of independent variables, known as Principal Components (PCs) that contain the most valuable information. Each PC contains a determined variance of the original variables, the first of which (PC1) describes the maximum amount of data variance, and the following ones (PC2, PC3…) provide gradually less variance [29]. Thus, PCA generates a scores matrix (T) and a loadings matrix (P^T), which give information about the distribution of samples and variables, respectively (Figure 3).

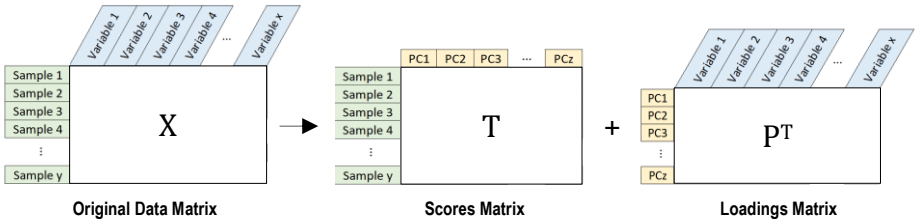


Figure 3. PCA decomposition.

In its exploratory function, the distribution of the samples is studied from the scatter plot of scores of two (or three) PCs. Samples with similar properties appear closely and the greater is the difference, the bigger is the separation. Furthermore, a projection of the variables on the PCs can be used to study the influence of each characteristic variable (loadings plot).

3.5.2. Partial Least Squares (PLS)

Partial Least Squares (PLS) is a multivariate regression method used to build a model able to predict sample properties such as concentration levels. It considers the information of an X-matrix (fingerprinting responses) and a Y-matrix (sample concentrations). This method works using latent variables (linear combinations of the original variables) and the optimal number of these variables is estimated by cross-validation (choosing the number of LVs that minimize the prediction error) [29].

The main result of this method is the prediction of the concentration of a given analyte in unknown samples. The performance of the prediction process can be assessed from a graphic that plots the predicted Y as a function of the calculated Y. As in other regression methods, an intercept close to 0, a slope close to 1, and a correlation coefficient close to 1 are indicative of a good overall prediction.

3.5.3. Partial Least Squares - Discriminant Analysis (PLS-DA)

Partial Least Squares - Discriminant Analysis (PLS-DA) is a supervised multivariate classification method that aims to find a mathematical model able to recognize the membership of each sample to its appropriate class. As its name suggests, this technique combines the features of PLS regression with the discrimination power of a classification method.

As a chemometric discrimination method, PLS-DA correlates the X-matrix (variables data) with Y-matrix (class assignment) to build a classification model with the minimum prediction error in assigning the samples into the corresponding classes. For this purpose, as in the PLS regression model, PLS-DA works with latent variables (LVs), and the optimum LV number is chosen by cross-validation [30].

4. OBJECTIVES

This work has two main objectives. The first goal is to assess the capability of non-targeted HPLC-UV and HPLC-FLD fingerprinting methods for the detection of tea frauds based on chicory adulterations, as well as for the quantitation of the chicory adulteration level by partial least squares regression. To achieve this objective, the next steps will be carried out:

1. Tea and chicory samples will be employed to prepare blended samples at different adulteration levels (chicory as adulterant), and their components extracted with hot mineral water.
2. The obtained blended sample extracts will be analysed by HPLC-UV and HPLC-FLD methods to obtain their characteristic chromatographic fingerprints which will be used as sample chemical descriptors.
3. Principal Component Analysis (PCA) will be employed as an exploratory method to evaluate the reproducibility of the proposed methodologies and the robustness of the obtained chemometric results.
4. Partial Least Squares (PLS) will be employed to quantify the chicory adulteration level in the analysed blended samples.

The second aim of this work is to start a new study based on developing a fast-screening non-targeted FIA-MS and a LC-MS fingerprinting methods for the characterisation and classification of tea and chicory samples. The next steps will be performed to achieve this objective:

1. Tea and chicory samples will be submitted to a simple sample treatment to extract their compounds.
2. The obtained sample extracts will be analysed by FIA-MS and LC-MS fingerprinting methods.
3. Principal Component Analysis (PCA) will also be employed as an exploratory method.
4. Partial Least Squares–Discriminant Analysis (PLS-DA) will be employed to study the classification of samples by type: Chicory, Black Tea, Green Tea, Oolong Tea, Red Tea or White Tea

5. EXPERIMENTAL SECTION

5.1. REAGENTS AND SOLUTIONS

The solvent employed for tea and chicory extraction was:

- Commercial mineral water obtained from Eroski (Barcelona, Spain), with a chemical composition of 402 mg L^{-1} dry residue at 180 °C, 326 mg L^{-1} bicarbonate, 44 mg L^{-1} chloride, 85 mg L^{-1} calcium, 28 mg L^{-1} magnesium, 18 mg L^{-1} sodium, and 8 mg L^{-1} silica.

For the chromatographic separation, the solutions used were:

- Methanol (Chromosolv™ for HPLC, ≥ 99.9%, from PanReac AppliChem, Barcelona).
- Formic Acid (≥ 98%, from Sigma-Aldrich, USA).
- Milli-Q Water. Water purified with an Elix 3 coupled to a Milli-Q system (Millipore, Bedford, MA, USA).

5.2. INSTRUMENTATION AND CONDITIONS

HPLC-UV-FLD method

An Agilent 1100 Series HPLC instrument (Waldbronn, Germany) equipped with a G1312A binary pump, a WPALS G1367A automatic sample injector, a G1315B diode-array detector and a G1321A fluorescence detector connected in series, and a PC with the Agilent Chemstation software was employed to obtain simultaneously the non-targeted HPLC-UV and HPLC-FLD chromatographic fingerprints. The absorbance wavelength for UV detection was 280 nm, while FLD acquisition was carried out at 280 and 350 nm as excitation and emission wavelengths, respectively.

A Kinetex® C18 reversed-phase column (100 × 4.6 mm i.d., 2.6 µm partially porous particle size) provided by Phenomenex (Torrance, California, USA), and working under gradient elution using 0.1% formic acid in Milli-Q water (solvent A) and methanol (solvent B) as mobile phase components was employed to obtain the chromatographic fingerprints. The gradient elution

conditions are shown in Table 1. First, methanol increases from 20 to 75% in 15 min. Then, methanol increases to 95% in 2 min. Next, there is an isocratic step of 2 min. Finally, the gradient elution come back to initial conditions in 0.2 min and there is an isocratic last step for column re-equilibration at initial conditions. The injection volume was 5 µL, and the mobile phase flow rate was 400 µL/min during the whole experiment.

Table 1. Gradient elution conditions to obtain the HPLC fingerprints.

Time [min]	Elution Mode	Methanol [%]
0 → 15	Linear Gradient	20 → 75
15 → 17	Linear Gradient	75 → 95
17 → 19	Isocratic	95
19 → 19.2	Linear Gradient	95 → 20
19.2 → 25	Isocratic	20

FIA-MS method

Flow injection analysis (FIA) was performed using the Agilent 1100 Series HPLC instrument (Waldbronn, Germany) coupled to an AB Sciex 4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer. The sample injection volume was 10 µL. The carrier consisted of a 50:50 (v/v) mixture, composed of water acidified with 0.1% formic acid (v/v) and methanol, and was pumped isocratically at 200 µL/min for 1.5 min. FIA-MS fingerprints were obtained in negative electrospray ionization (ESI) and acquired in full scan MS mode (*m/z* 100 – 550). The ion spray voltage was set at -2500 V, and the source temperature at 400 ^{o}C. Nitrogen was used as nebulizer and auxiliary gas wand was set at 10, 50, and 50 arbitrary units (a.u.) for the curtain gas, ion source gas 1, and ion source gas 2, respectively. A declustering potential (DP) of -80 V was employed.

LC-MS method

LC-MS fingerprinting was also performed using the Agilent 1100 Series HPLC instrument (Waldbronn, Germany) coupled to the AB Sciex 4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Framingham, MA, USA). Same chromatographic conditions

(column and gradient elution program) than the ones employed with the HPLC-UV-FLD method were used. LC-MS fingerprints were also acquired in negative ESI mode, in full scan MS (*m/z* 100-550) by using the same ion spray and acquisition conditions than the ones given for FIA-MS analysis.

5.3. SAMPLES AND SAMPLE TREATMENT

Sample treatment consisted of extracting 0.5 g of tea/chicory sample with 25 mL of hot mineral water in a 15 mL PTFE centrifuge tube (Serviquimia, Barcelona, Spain) by vigorously shaking for 1 min using a Vortex (Stuart, Stone, United Kingdom). Then, the extract was centrifuged (Rotanta 460 RS centrifuge, Hettich, Tuttlingen, Germany) at 3500 rpm for 5 min. Finally, the obtained aqueous extracts were filtered with 0.45 µm nylon filters (first mL was discarded) and were stored in a fridge at 4 °C until HPLC analysis.

Adulteration study by HPLC-UV-FLD fingerprinting

The detection and quantitation of adulterations in tea extracts by non-targeted HPLC-UV-FLD fingerprinting and PLS was carried out by studying 5 blended cases based on each class of tea (Black, Green, Oolong, Red and White Tea) adulterated with chicory. For each case, a total of 85 blended mixtures were prepared. These blended samples consisted of different mixtures of tea and chicory (as adulterant), both purchased from several supermarkets in Barcelona (Spain). Commercial brands and specifications of the tea and chicory samples of this study are summarised in Table 2. For each tea adulteration study, a quality control (QC) was prepared which consisted of a 50% adulteration mixture with chicory.

Table 2. Description of teas and chicory samples used in the adulteration study.

Sample	Commercial Brand	Commercial Name	Specification
Black Tea	Tea Shop	Golden Yunnan Finest Tippy	Black Tea from Yunnan, China
	Tea Shop	Formosa Tarry Lapsang Souchong	Smoked Lapsang Souchong Black Tea
Green Tea	Tea Shop	Organic Gunpowder	Pure organic Green Tea from China
	Tea Shop	Sencha	Sencha Green Tea from China
Oolong Tea	Tea Shop	Tie Kuan Yin	Oolong Tea from Fujian, China
	Tea Shop	Milky Oolong	Oolong Tea from China
Red Tea	Tea Shop	Pu Erh Original	Special fermented Pu Erh Red Tea

	Tea Shop	Pu Erh Imperial	Special fermented Pu Erh Red Tea
White Tea	Tea Shop	Silver Needles	White Tea from Yunnan, China
	Tea Shop	Pai Mu Tan	White Tea from Fujian, China
Chicory	Herbes del Molí	Chicory	-
	Valley Of Tea	Chicory Roots	-

Classification study by FIA-MS and LC-MS fingerprinting

For the tea and chicory extract classification studies by non-targeted FIA-MS and LC-MS fingerprinting, a total of 107 tea and chicory samples (Table 3) belonging to different classes and purchased from supermarkets in Barcelona (Spain) were analysed. A QC was prepared by mixing 50 µL of each sample extract.

Table 3. Summary of analysed samples in the classification study.

Sample Class	Sample Type	Number of Samples
Tea	Black Tea	35
	Green Tea	20
	Oolong Tea	10
	Red Tea	12
	White Tea	10
Chicory	Chicory	21

5.4. DATA ANALYSIS

Independently of the fingerprinting method, all the obtained sample aqueous extracts were analysed randomly. The corresponding QC and an instrumental blank (mineral water) were injected after each ten sample extracts. The QCs were used to evaluate the repeatability of the proposed methodologies and the robustness of the obtained chemometric results. Different data matrices were created with the experimental fingerprints to be subjected to PCA, PLS, and PLS-DA chemometric methods depending on the study case (adulteration or classification). Details of the theorical background of these chemometric methods are covered elsewhere [31].

SOLO 8.6 chemometric software from Eigenvector Research (Manson, WA, USA) was employed for chemometric calculations. PCA was used as an exploratory method to evaluate the

system behaviour and find out some sample and variable patterns. PLS-DA was employed as a classification method and PLS regression was applied to detect and quantify tea frauds based on chicory adulterations.

For the adulteration study by non-targeted HPLC-UV and HPLC-FLD chromatographic fingerprinting, the X-data matrix consisted of the acquired HPLC-UV (absorbance signal vs. retention time) and the HPLC-FLD (fluorescence intensity vs. retention time) chromatograms, respectively. The Y-data matrix defined the adulterant percentage. For this study, the models were built and validated by using calibration and validation sets consisting of different blending mixtures, as described in Table 4. External prediction was also performed by using tea and chicory samples different than the ones employed to build the PLS models, and by preparing mixtures at 15, 50 and 85 % of chicory as adulterant.

Table 4. Blended mixtures used in the adulteration studies by PLS (n=5 for each sample)

	Tea [%]	Chicory [%]		Tea [%]	Chicory [%]
Calibration Set	100	0	Validation Set	85	15
	80	20		75	25
	60	40		50	50
	40	60		25	75
	20	80		15	85
	0	100			

For the classification study by non-targeted FIA-MS and LC-MS fingerprinting, the X-data matrix for both PCA and PLS-DA consisted of the acquired FIA-MS (peak signal as a function of m/z values) and the LC-MS (peak signal as a function of m/z values and retention time) fingerprints, respectively. In contrast, the Y-data matrix defined each sample class. For both studies, different tea and chicory samples belonging to different classes were used, as shown in Table 3.

To obtain the FIA-MS fingerprints, raw data results were subjected to wavelet transform mass detection, shoulder peaks filter, chromatogram builder, and RANSAC aligner, using the mzMine 2.37 software [32]. First, the wavelet transform mass detection step generated mass lists for each

scan acquired in a sample, considering a noise level of 4.0×10^4. Secondly, the shoulder peaks filter removed false signals (Fourier transform residuals) by establishing a Gaussian peak model function. Then, the chromatogram builder constructed extracted ion chromatograms for masses that have been detected by mass spectrometry continuously over a certain duration of time, establishing a peak time range of 0.00 – 1.48 min, a *m/z* tolerance of 5000 ppm, an intensity threshold of 4.0×10^4, and a minimum highest intensity of 1.0×10^4. Finally, the RANSAC aligner allowed matching of the different masses detected across samples, establishing a *m/z* tolerance of 5000 ppm, a peak time tolerance of 0.3 min and 0.1 min after correction, an 80% of minimum number of points, and a threshold value of 0.15. At the end of this workflow, a data matrix was constructed containing FIA–MS fingerprints of the studied samples: samples × variables, where ion signal intensity values were provided as a function of *m/z*.

In the same way, the LC-MS fingerprints were also obtained with the mzMine 2.37 software. In this case, raw data results were submitted to wavelet transform mass detection, shoulder peaks filter, chromatogram builder, chromatogram deconvolution and join aligner. First, the wavelet transform mass detection step generated mass lists for each scan acquired in a sample, considering a noise level of 1.5×10^4. Secondly, the shoulder peaks filter removed false signals based on a Gaussian peak model function. Then, the chromatogram builder constructed the extracted ion chromatograms for the *m/z* detected over the time range of 0.00 – 20.00 min, with *m/z* tolerance of 5000 ppm, an intensity threshold of 1.5×10^4, and a minimum highest intensity of 1.0×10^4. Next, the chromatogram deconvolution removed the lowest part of the chromatograms below the baseline level specified. Finally, the join aligner allowed matching of the different *m/z* values detected across samples, establishing a m/z tolerance of 5000 ppm, a peak time tolerance of 2.0 min, 90% of weight for *m/z*, and 1% of weight for time. At the end of this workflow, a data matrix was constructed containing LC–MS fingerprints of the studied samples: samples × variables, where variables consisted of ion signal intensity values as a function of *m/z* and retention time.

In all the chemometric data treatments, the fingerprints were pre-processed by smoothing, baseline and variable alignment to improve data quality. Then, data was also autoscaled to achieve the same weight to each variable by supressing differences in their magnitude and amplitude scales.

6. RESULTS AND DISCUSSION

6.1. DETECTION AND QUANTITATION OF TEA ADULTERATION WITH CHICORY

6.1.1. HPLC-UV and HPLC-FLD chromatographic fingerprints

As mentioned in previous sections, one of the objectives of this study is to determine and quantify tea adulterations with chicory. For this purpose, non-targeted HPLC-UV and HPLC-FLD chromatographic fingerprinting methodologies were employed. The chromatographic fingerprints of tea and chicory mixtures were obtained simultaneously, connecting in series both UV-Vis and fluorescence detectors (HPLC-UV-FLD technique). As non-targeted fingerprinting methods, a huge amount of chemical data was generated, without focusing on any particular species. The chromatographic fingerprints were obtained by reversed-phase chromatography in a Kinetex® C18 column using 0,1% formic acid in water and methanol as mobile phase components, and under a gradient elution from 20% to 75% methanol in 15 min (Table 1). As examples, Figure 4 shows the HPLC-UV (a) and HPLC-FLD (b) chromatographic fingerprints for a red tea sample adulterated with chicory at different concentrations (0%, 20%, 40%, 60%, 80%, and 100%).

Some differences can be observed among the chromatograms. In the case of HPLC-UV fingerprints (Figure 4, a.1-a.6), there is one peak that clearly stands out from the others with a retention time close to 12 minutes. Although the peak distribution is maintained in all of them, their intensities vary significantly with the chicory adulteration. As the percentage of tea decreases, the intensity of the peaks decreases significantly. Only in the last fingerprint (100% chicory) a different distribution can be observed, as there is no tea. A different behaviour was obtained for HPLC-FLD fingerprints (Figure 4, b.1-b.6). In this case, a different peak distribution was found among the different chromatographic fingerprints. There are different characteristic tea peaks at a retention time close to 3 and 12 minutes which decrease their signal when the percentage of tea decreases. In addition, when the concentration of chicory increases, different peaks such as the peaks at a retention time around 10 and 11.5 minutes increase their signal, as they seem to be characteristic of chicory extracts.

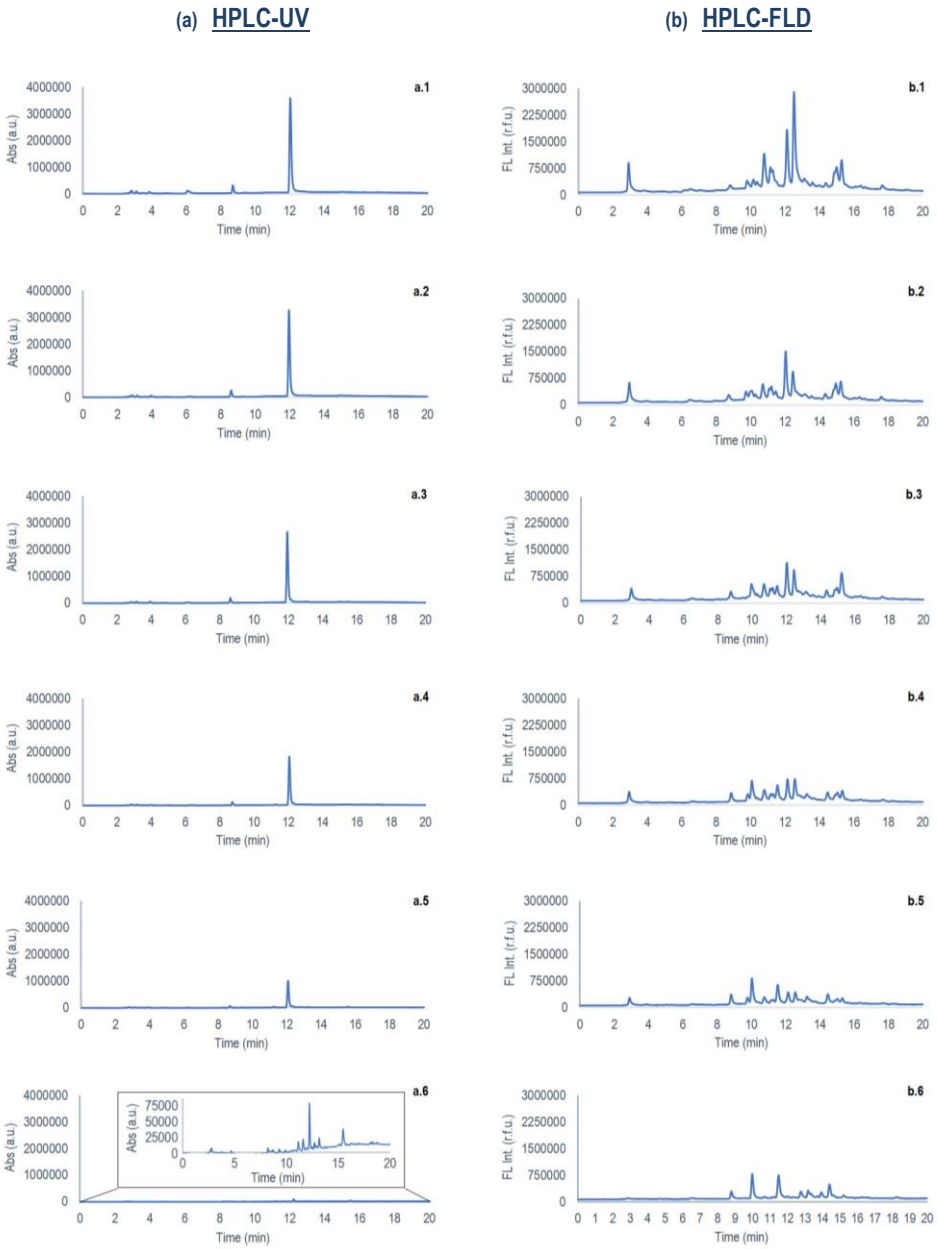


Figure 4. Non-targeted HPLC-UV (a) (at 280 nm) and HPLC-FLD (b) (at 280 nm (excitation) and 350 nm (emission)) chromatographic fingerprints obtained for a red tea sample adulterated with chicory at different concentrations: 0% (a1, b1), 20% (a2, b2), 40% (a3, b3), 60% (a4, b4), 80% (a5, b5), 100% (a6, b6).

As the differences among HPLC-UV and HPLC-FLD fingerprinting were significant between both methods and at different adulterant concentrations within the same method, these chromatographic fingerprints were used as chemical descriptors in subsequent multivariate chemometric techniques to obtain a method able to detect and quantify tea adulteration with chicory.

6.1.2. Principal Component Analysis

Principal Component Analysis was employed as an exploratory method to examine sample distribution according to the percentage of adulterant. In addition, the repeatability of the fingerprints and the robustness of the obtained chemometric results were evaluated with the performance of the QCs in order to detect possible irregularities during the analysis. In this way, calibration and validation samples for adulteration study were submitted to PCA. Thus, PCA scores plots of PC1 vs. PC2 when using HPLC-UV fingerprints (a) and HPLC-FLD fingerprints (b) as chemical descriptors in case of a red tea sample adulterated with chicory are shown in Figure 5.

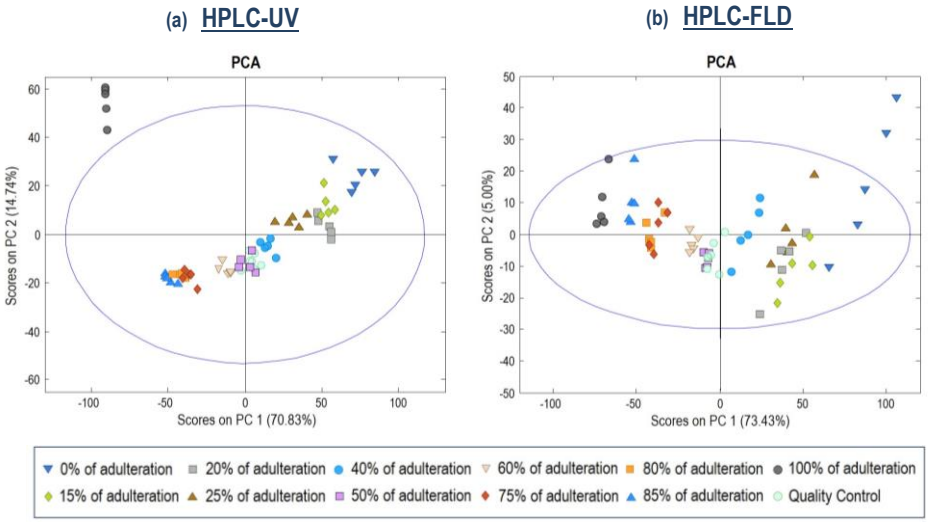


Figure 5. Exploratory Principal Component Analysis (PCA) scores plots on PC1 vs. PC2 of the analysed red tea and chicory blended mixtures using HPLC-UV fingerprints (a) and HPLC-FLD fingerprints (b) as chemical descriptors.

The first thing that can be observed is that QCs appear grouped together and centred on the plot, showing a good reproducibility and a suitable robustness of the obtained chemometric results. Furthermore, these score plots show, in both cases, a logical distribution of the samples, where pure red tea samples are placed on the right side of the plots and the other samples are distributed further to the left as the concentration of chicory (adulterant) increases. In any case, chicory samples are groped at the left part of the plots showing negative PC1 values, whereas as the tea percentage increases, the samples are distributed towards higher values of PC1. In the case of HPLC-UV chromatographic fingerprints, there is a big difference between the pure chicory samples (situated on the left side) and the other samples. The first ones appear completely differentiated from the others because they do not contain tea. In addition, samples tend to be better grouped in more compact groups according to the adulterant percentage with HPLC-UV fingerprints (Figure 5a) in comparison to HPLC-FLD fingerprints (Figure 5b).

After checking the good performance of the samples, HPLC-UV and HPLC-FLD fingerprints were also submitted to a multivariate calibration method such as PLS for the quantitation of the adulteration levels.

6.1.3. Quantitation of chicory adulteration in tea by Partial Least Squares

Partial Least Squares regression was employed to quantify the adulteration level of the analysed samples. For that reason, five tea blended scenarios were evaluated: Black, Green, Oolong, Red, and White teas, all of them adulterated with chicory. As mentioned in previous sections, the PLS models were built and validated by using calibration and validation sets (Table 4) and an external prediction using different tea and chicory samples than the ones employed in the PLS models was also performed. Each adulteration level was prepared in quintuplicate. The resulting plots show measured vs. predicted percentage levels of adulteration (chicory). As an example, Figure 6 shows these scatter plots in case of a red tea sample adulterated with chicory, using HPLC-UV (a) and HPLC-FLD (b) chromatographic fingerprints. For each method, a calibration was carried out with the same calibration set and an external validation PLS (with validation set) and a prediction PLS (with prediction set) were done.

As can be seen, in this red tea adulteration with chicory study, very good validation and prediction PLS results were obtained, with linearities higher than 0.999, slopes close to 1, and intercepts close to 0. Furthermore, in these plots we can see that predicted Y values practically

match to the measured ones. However, as it was expected, prediction errors were higher than external validation errors because prediction PLS was done with different sample extracts than the ones used in the calibration model. Table 5 summarizes the LVs employed, the linearity (R^2) and the PLS calibration, cross-validation, external validation, and prediction errors for each adulteration case under study (Black, Green, Oolong, Red, and White teas, adulterated with chicory).

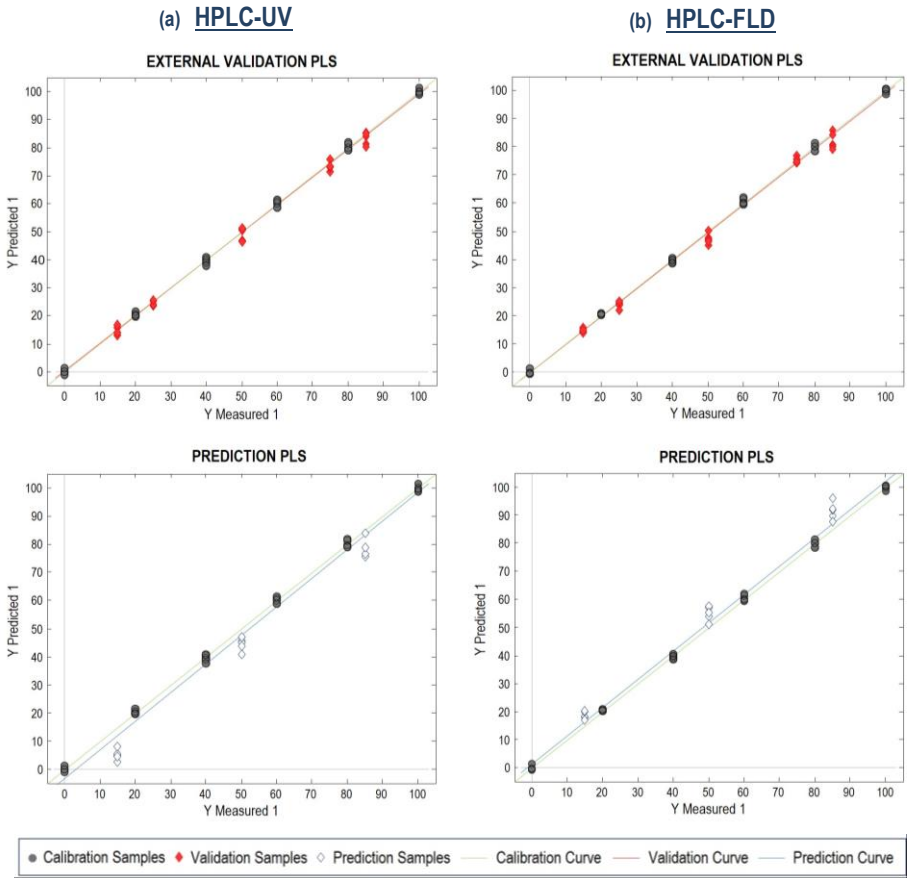


Figure 6. Scatter plots of measured vs. predicted percentages of adulterant by PLS when red tea was adulterated with chicory for external validation and prediction employing HPLC-UV (a) and HPLC-FLD (b) fingerprints.

Table 5. Results for the evaluation of adulteration of tea samples with chicory using HPLC-UV (a) and HPLC-FLD (b) fingerprints as chemical descriptors for PLS.

(a) **HPLC-UV chromatographic fingerprints**						
	LVs	Linearity [R^2]	Calibration Error [%]	Cross-Validation Error [%]	External Validation Error [%]	Prediction Error [%]
Black Tea	4	0.999	1.0	3.6	2.1	21.6
Green Tea	5	0.999	0.5	1.3	1.0	15.7
Oolong Tea	2	0.998	1.4	6.4	1.8	15.0
Red Tea	4	0.999	1.0	1.5	2.0	8.0
White Tea	3	0.999	1.3	2.3	2.2	80.7

(b) **HPLC-FLD chromatographic fingerprints**						
	LVs	Linearity [R^2]	Calibration Error [%]	Cross-Validation Error [%]	External Validation Error [%]	Prediction Error [%]
Black Tea	3	0.999	1.1	1.6	1.6	2.6
Green Tea	5	0.999	0.3	1.1	1.4	18.3
Oolong Tea	2	0.998	1.4	1.9	1.4	13.0
Red Tea	4	0.999	0.9	2.4	2.5	5.7
White Tea	3	0.999	0.8	2.3	3.7	132.9

As can be seen, in all cases, good linearity values ($R^2 > 0.998$) and calibration and cross-validation errors lower than 1.4% and 6.4%, respectively, were obtained. As the table shows, quite reasonable external validation errors, with values below 2.2% and 3.7% for HPLC-UV and HPLC-FLD fingerprints, respectively, were obtained. As mentioned above, PLS prediction was carried out with a test set prepared with different sample extracts than those used in the calibration and, for this reason, prediction errors were higher than external validation errors. In this case, different behaviours were observed, depending on the tea variety and the method used. In both fingerprinting methodologies and without considering the case of white tea, overall prediction errors were under 21,6% (HPLC-UV) and 18,3% (HPLC-FLD). Furthermore, the contribution to prediction errors came from the prediction of the lowest (15%) and the highest (85%) adulteration levels, as can be seen in the prediction PLS plots on Figure 6. These values can be considered acceptable due to the reality of the fraud under study, as tea adulteration with low chicory levels will not be expected (no economic gains) and high chicory adulteration levels would not make sense because they would be detectable to the naked eye. On the other hand, for white tea, high

prediction errors were obtained being the values 80,7% and 132,9% for HPLC-UV and HPLC-FLD fingerprints, respectively. These results are attributed to the similarity in the compositional fingerprints of white teas and chicory samples, being always grouped very close in both PCA and PLS-DA scores plots as observed in previous works [33].

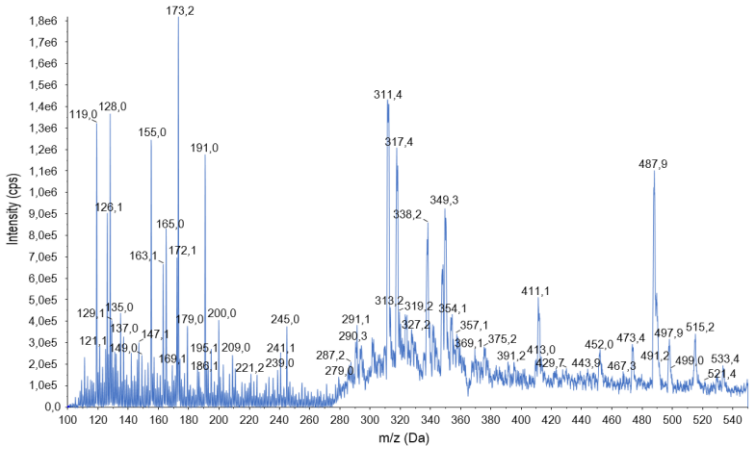
6.2. CHARACTERISATION AND CLASSIFICATION OF TEA AND CHICORY SAMPLES

6.2.1. FIA-MS and LC-MS fingerprints

As mentioned in the objectives section, another aim of this work was to begin a new study focus on developing a fast-screening method able to classify the different types of tea. For this purpose, a non-targeted FIA-MS fingerprinting methodology, where sample extracts are directly injected into a mass spectrometer without using a chromatographic separation, was evaluated. Under these conditions, sample data can be obtained in 1.5 min per sample, thus being a fast methodology. As examples, Figure 7 shows the spectral FIA-MS fingerprints for a black tea (a) and a chicory (b) samples.

As can be seen, important differences can be observed in the obtained fingerprints (mass spectral data), suggesting the feasibility of this methodology for the classification of the analysed samples.

(a) BLACK TEA



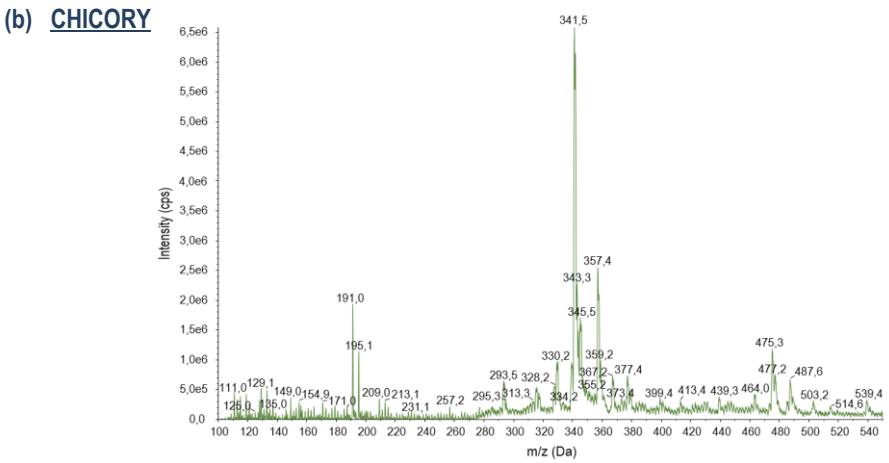


Figure 7. FIA-MS spectra obtained for a black tea (a) and chicory (b) samples (0.1 - 0.45 min range).

In addition, a LC-MS fingerprinting method was also tested as a classificatory method for tea samples. For this study, the same chromatographic conditions than the ones employed with the HPLC-UV-FLD method were used. This method will be employed for comparison purposes with the FIA-MS method using the same MS instrument. As examples, Figure 8 shows the LC-MS fingerprints (total ion chromatograms) for a black tea (a) and a chicory (b) samples.

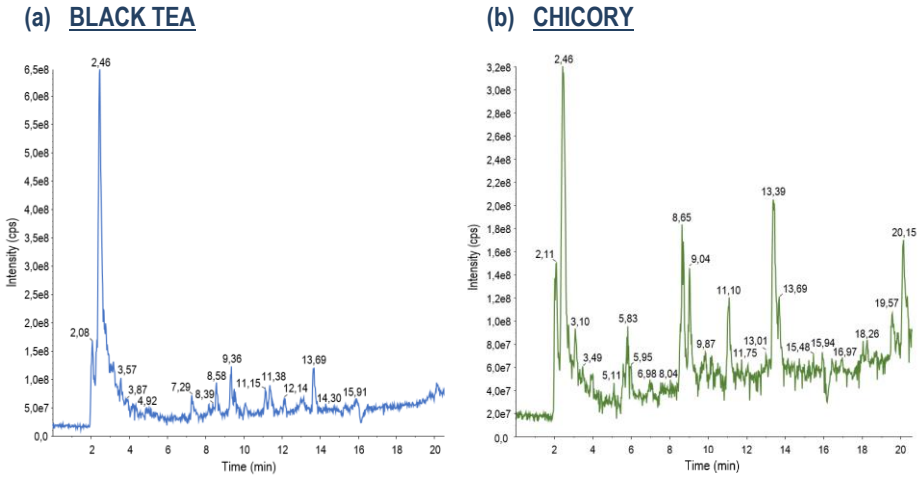


Figure 8. Non-targeted LC-MS fingerprints obtained for a black tea (a) and chicory (b) samples.

These figures show the total ion chromatograms, where the intensity signal for each retention time, corresponds to the sum of the signals for all the ions detected. Thus, the final obtained LC-MS fingerprints once processed will include the signal intensity as a function of both *m/z* values and retention time. Again, important differences between tea and chicory samples are observed, suggesting the feasibility of LC-MS fingerprints for the classification of the analysed samples.

Next, both FIA-MS and LC-MS fingerprints were subjected to chemometrics for samples characterization and classification.

6.2.2. Principal Component Analysis for FIA-MS study

Principal Component Analysis was employed as an exploratory method to examine sample distribution according to sample type. Furthermore, the QCs were included in order to test the robustness of the metho, as well as to detect possible errors during the analysis. In this way, different tea and chicory samples (Table 3) were submitted to PCA. Thus, PCA scores plot of PC1 vs. PC2 when using FIA-MS fingerprints as chemical descriptors is shown in Figure 9.

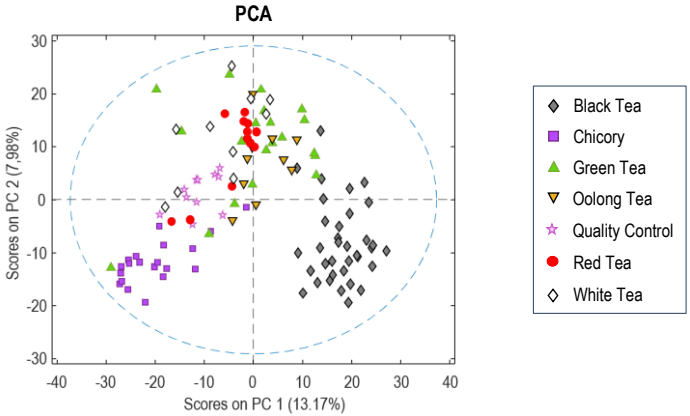


Figure 9. Exploratory Principal Component Analysis (PCA) scores plot on PC1 vs. PC2 of the analysed tea and chicory samples when FIA-MS fingerprints were used as chemical descriptors.

Firstly, it can be observed that QCs appear grouped together, showing a good reproducibility and robustness of the obtained chemometric results.

Secondly, although some tea samples appeared overlapping, with the exception of black tea (clearly differentiated), it can be observed that chicory samples could be distinguished, with some

minor exceptions, from tea samples. This is a good indicator for further work on a fast-screening method for the authentication of tea samples with possible adulterations with chicory.

After checking the good performance of the PCA model, FIA-MS fingerprints were also submitted to PLS-DA for classification according to the sample nature.

6.2.3. Classification of tea and chicory samples by PLS-DA

Classificatory PLS-DA chemometric method was employed in order to check if the FIA-MS fingerprinting chemical descriptors used in this study were able to discriminate the different samples according to the type of tea and chicory, and the obtained score plots results are depicted in Figure 10.

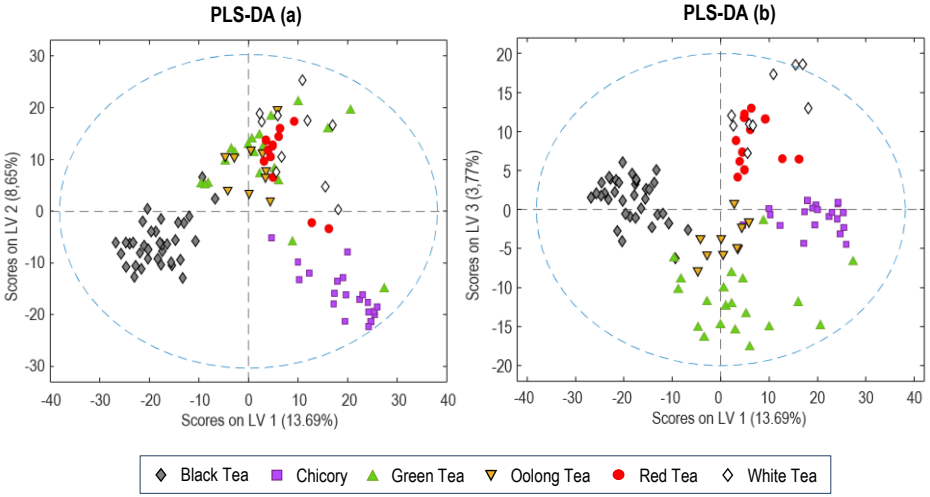


Figure 10. Scores plots of LV1 vs. LV2 (a) and LV1 vs. LV3 (b) by Partial Least Squares-Discriminant Analysis employing FIA-MS fingerprints.

As can be seen in Figure 10a, scores plot defined by LV1 vs. LV2 allowed to discriminate chicory samples (with some minor exceptions) from tea samples. In addition, this plot made it possible to differentiate black tea from the other tea samples.

As for Figure 10b, the score plot defined by LV1 vs. LV3 improves the previous obtained results. In addition to the discrimination between chicory and tea samples, this plot is able to

distinguish between the different types of tea (with the exception of red and white teas, which overlap).

So, as a first approach, it can be expected that this fingerprinting method would be able to classify the different tea and chicory samples, opening a new strategy much faster than those previously studied. For this reason, and considering the objectives of this study, it can be concluded that FIA-MS fingerprinting is a very good choice as a fast-screening method able to characterise and classify tea and chicory samples.

On the other hand, PLS-DA chemometric method was also employed with the LC-MS fingerprints, in order to test the feasibility of this methodology for the authentication of tea samples with possible adulterations with chicory. As shown in Figure 11 (scores plot defined by LV1 vs. LV3), it was possible to discriminate between chicory and tea samples. However, although the methodology was not able to distinguish between some types of tea that appear overlapping, the obtained results show that it can be a promising strategy for the authentication of tea if studied further.

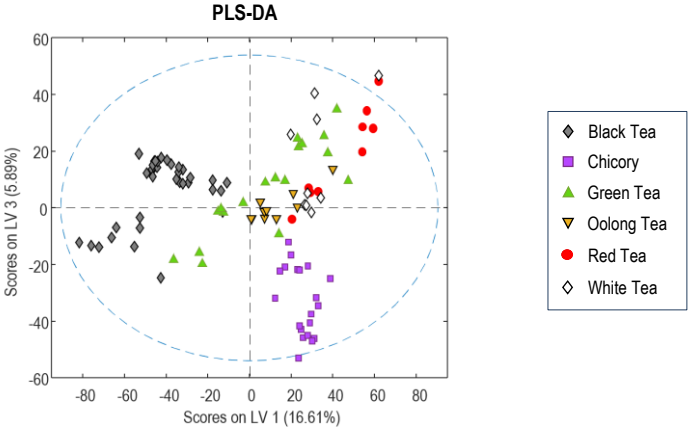


Figure 11. Scores plot of LV1 vs. LV3 by Partial Least Squares-Discriminant Analysis employing LC-MS fingerprints.

7. CONCLUSIONS

In this work, the feasibility of two simultaneous non-targeted HPLC-UV (λ_{abs}= 280 nm) and HPLC-FLD (λ_{exc}= 280 nm and λ_{em}= 350 nm) fingerprinting methods for the authentication of tea samples adulterated with chicory was assessed. For this purpose, PLS was used to detect and quantify tea adulteration with chicory by employing five different tea varieties (black, green, oolong, red and white).

Both methods gave very promising PLS results, such as calibration, cross-validation, and external validation errors below 1.4%, 6.4%, and 3.7%, respectively. Acceptable prediction errors using adulterated samples prepared with different extracts than the ones employed for PLS calibration (below 21.7%) were also obtained, except for white tea extracts which show higher errors due to the similarity of their fingerprints to those of chicory.

Based on these results, the proposed non-targeted HPLC-UV and HPLC-FLD fingerprinting methods represent appropriate methodologies for the assessment of tea adulteration with chicory, as way to help to prevent frauds on consumers.

On the other hand, two new methodologies were tested to characterise and classify chicory and the different tea varieties in order to see their potential as tea authentication methods.

Firstly, FIA-MS fingerprinting method was a very promising fast-screening methodology. Although some tea varieties appeared overlapping in PLS-DA models, this methodology was able to discriminate tea from chicory samples and this represents a great time saving in tea authentication, as only an analysis time of 1.5 min per sample was required.

Secondly, LC-MS fingerprinting method also showed promising results. As FIA-MS, this methodology was also able to distinguish between tea and chicory samples, even though PLS-DA models were not able to differentiate between all the studied tea varieties.

The latter two methods need further study, but this work demonstrates that they have a great analytical potential to deal with new tea authentication issues.

8. REFERENCES

1. Kennedy, G.; Nantel, G.; Shetty, P. *Globalization of food systems in developing countries: impact on food security and nutrition.*; 2004; Vol. 83; ISBN 92-5-105228-X.
2. Núñez, O. Current Legal Context in Food Integrity, Authenticity, and Frauds. In *Chromatographic and Related Separation Techniques in Food Integrity and Authenticity. Volume A: Advances in Chromatographic Techniques.*; Núñez, O., Campmajó, G., Eds.; World Scientific Publishing: London, UK, 2021; pp. 1–24 ISBN 978-1-78634-994-1.
3. Spink, J.; Moyer, D.C. Defining the Public Health Threat of Food Fraud. *J. Food Sci.* **2011**, *76*, doi:10.1111/j.1750-3841.2011.02417.x.
4. Cuadros-Rodríguez, L.; Ruiz-Samblás, C.; Valverde-Som, L.; Pérez-Castaño, E.; González-Casado, A. Chromatographic fingerprinting: An innovative approach for food "identitation" and food authentication - A tutorial. *Anal. Chim. Acta* **2016**, *909*, 9–23, doi:10.1016/j.aca.2015.12.042.
5. Budínová, G.; Vláčil, D.; Mestek, O.; Volka, K. Application of infrared spectroscopy to the assessment of authenticity of tea. *Talanta* **1998**, *47*, 255–260, doi:10.1016/S0039-9140(98)00055-1.
6. Cabrera, C.; Artacho, R.; Giménez, R. Beneficial Effects of Green Tea—A Review. *J. Am. Coll. Nutr.* **2006**, *25*, 79–99, doi:10.1080/07315724.2006.10719518.
7. Lagiotis, G.; Stavridou, E.; Bosmali, I.; Osathanunkul, M.; Haider, N.; Madesis, P. Detection and quantification of cashew in commercial tea products using High Resolution Melting (HRM) analysis. *J. Food Sci.* **2020**, *85*, 1629–1634, doi:10.1111/1750-3841.15138.
8. Liu, W.; Chen, Y.; Liao, R.; Zhao, J.; Yang, H.; Wang, F. Authentication of the geographical origin of Guizhou green tea using stable isotope and mineral element signatures combined with chemometric analysis. *Food Control* **2021**, *125*, 107954, doi:10.1016/j.foodcont.2021.107954.
9. Mishra, P.; Nordon, A.; Tschannerl, J.; Lian, G.; Redfern, S.; Marshall, S. Near-infrared hyperspectral imaging for non-destructive classification of commercial tea products. *J. Food Eng.* **2018**, *238*, 70–77, doi:10.1016/j.jfoodeng.2018.06.015.
10. Peng, T.Q.; Yin, X.L.; Gu, H.W.; Sun, W.; Ding, B.; Hu, X.C.; Ma, L.A.; Wei, S.D.; Liu, Z.; Ye, S.Y. HPLC-DAD fingerprints combined with chemometric techniques for the authentication of plucking seasons of Laoshan green tea. *Food Chem.* **2021**, *347*, 1–8, doi:10.1016/j.foodchem.2020.128959.
11. Uncu, A.T.; Uncu, A.O.; Frary, A.; Doganlar, S. Authentication of Botanical Origin in Herbal Teas by Plastid Noncoding DNA Length Polymorphisms. *J. Agric. Food Chem.* **2015**, *63*, 5920–5929, doi:10.1021/acs.jafc.5b01255.

12. Firmani, P.; De Luca, S.; Bucci, R.; Marini, F.; Biancolillo, A. Near infrared (NIR) spectroscopy-based classification for the authentication of Darjeeling black tea. *Food Control* **2019**, *100*, 292–299, doi:10.1016/j.foodcont.2019.02.006.

13. Osathanunkul, M. Bar-HRM for authenticating soursop (Annona muricata) tea. *Sci. Rep.* **2018**, *8*, 1–7, doi:10.1038/s41598-018-31127-9.

14. Sharma, K.; Singh, S.; Tanwar, K.; Chemistry Sem, S.; Mahila Mahavidyalaya, K.P.; Professor, A. Recognition and Evaluation of Authenticity of Tea and Coffee. *Int. J. Adv. Res. Sci. Commun. Technol.* **2020**, *11*, 2581–942, doi:563.112020/IJARSCT.

15. Pal, A.D.; Das, T. Analysis of adulteration in black tea. *Int. J. Biol. Res.* **2018**, *3*, 253–257.

16. Perović, J.; Tumbas Šaponjac, V.; Kojić, J.; Krulj, J.; Moreno, D.A.; García-Viguera, C.; Bodroža-Solarov, M.; Ilić, N. Chicory (Cichorium intybus L.) as a food ingredient – Nutritional composition, bioactivity, safety, and health claims: A review. *Food Chem.* **2021**, *336*, 127676, doi:10.1016/j.foodchem.2020.127676.

17. Nwafor, I.C.; Shale, K.; Achilonu, M.C. Chemical Composition and Nutritive Benefits of Chicory (Cichorium intybus) as an Ideal Complementary and/or Alternative Livestock Feed Supplement. *Sci. World J.* **2017**, *2017*, doi:10.1155/2017/7343928.

18. Ballin, N.Z.; Laursen, K.H. To target or not to target? Definitions and nomenclature for targeted versus non-targeted analytical food authentication. *Trends Food Sci. Technol.* **2019**, *86*, 537–543, doi:10.1016/j.tifs.2018.09.025.

19. Kucharska-Ambrożej, K.; Karpinska, J. The application of spectroscopic techniques in combination with chemometrics for detection adulteration of some herbs and spices. *Microchem. J.* **2020**, *153*, 104278, doi:10.1016/j.microc.2019.104278.

20. Campmajó, G.; Núñez, N.; Núñez, O. The Role of Liquid Chromatography-Mass Spectrometry in Food Integrity and Authenticity. *Mass Spectrom. - Futur. Perceptions Appl.* **2019**, 1–24, doi:10.5772/intechopen.85087.

21. McGrath, T.F.; Haughey, S.A.; Patterson, J.; Fauhl-Hassek, C.; Donarski, J.; Alewijn, M.; van Ruth, S.; Elliott, C.T. What are the scientific challenges in moving from targeted to non-targeted methods for food fraud testing and how can they be addressed? – Spectroscopy case study. *Trends Food Sci. Technol.* **2018**, *76*, 38–55, doi:10.1016/j.tifs.2018.04.001.

22. He, W.; Zhou, J.; Cheng, H.; Wang, L.; Wei, K.; Wang, W.; Li, X. Validation of origins of tea samples using partial least squares analysis and Euclidean distance method with near-infrared spectroscopy data. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2012**, *86*, 399–404, doi:10.1016/j.saa.2011.10.056.

23. Yan, S.M.; Liu, J.P.; Xu, L.; Fu, X.S.; Cui, H.F.; Yun, Z.Y.; Yu, X.P.; Ye, Z.H. Rapid discrimination of the geographical origins of an oolong tea (Anxi-Tieguanyin) by near-infrared spectroscopy and partial least squares discriminant analysis. *J. Anal. Methods Chem.* **2014**, *2014*, doi:10.1155/2014/704971.

24. Liu, P.; Wen, Y.; Huang, J.; Xiong, A.; Wen, J.; Li, H.; Huang, Y.; Zhu, X.; Ai, S.; Wu, R. A novel strategy of near-infrared spectroscopy dimensionality reduction for discrimination of grades, varieties and origins of green tea. *Vib. Spectrosc.* **2019**, *105*, 102984,

doi:10.1016/j.vibspec.2019.102984.

25. Aboulwafa, M.M.; Youssef, F.S.; Gad, H.A.; Sarker, S.D.; Nahar, L.; Al-Azizi, M.M.; Ashour, M.L. Authentication and discrimination of green tea samples using UV–vis, FTIR and HPLC techniques coupled with chemometrics analysis. *J. Pharm. Biomed. Anal.* **2019**, *164*, 653–658, doi:10.1016/j.jpba.2018.11.036.

26. Palacios-Morillo, A.; Alcázar, Á.; De Pablos, F.; Jurado, J.M. Differentiation of tea varieties using UV-Vis spectra and pattern recognition techniques. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2013**, *103*, 79–83, doi:10.1016/j.saa.2012.10.052.

27. Diniz, P.H.G.D.; Barbosa, M.F.; De Melo Milanez, K.D.T.; Pistonesi, M.F.; De Araújo, M.C.U. Using UV-Vis spectroscopy for simultaneous geographical and varietal classification of tea infusions simulating a home-made tea cup. *Food Chem.* **2016**, *192*, 374–379, doi:10.1016/j.foodchem.2015.07.022.

28. Sentellas, S.; Saurina, J. The Role of Chemometrics in Food Integrity and Authenticity. In *Chromatographic and Related Separation Techniques in Food Integrity and Authenticity. Volume A: Advances in Chromatographic Techniques*.; Núñez, O., Campmajó, G., Eds.; World Scientific Publishing: London, UK, 2021; pp. 167–200 ISBN 978-1-78634-994-1.

29. Granato, D.; Putnik, P.; Kovačević, D.B.; Santos, J.S.; Calado, V.; Rocha, R.S.; Cruz, A.G. Da; Jarvis, B.; Rodionova, O.Y.; Pomerantsev, A. Trends in Chemometrics: Food Authentication, Microbiology, and Effects of Processing. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 663–677, doi:10.1111/1541-4337.12341.

30. Ballabio, D.; Consonni, V. Classification tools in chemistry. Part 1: Linear models. PLS-DA. *Anal. Methods* **2013**, *5*, 3790–3798, doi:10.1039/c3ay40582f.

31. Bayne, C.K.; Haswell, S.J. *Practical Guide to Chemometrics*; 1995; Vol. 37; ISBN 9781574447835.

32. Pluskal, T.; Castillo, S.; Villar-Briones, A.; Orešič, M. MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* **2010**, *11*, doi:10.1186/1471-2105-11-395.

33. Pons, J.; Bedmar, À.; Núñez, N.; Saurina, J.; Núñez, O. Tea and chicory extract characterization, classification and authentication by non-targeted HPLC-UV-FLD fingerprinting and chemometrics. *Foods* **2021**, *10*, doi:10.3390/foods10122935.

9. ACRONYMS

a.u.: Arbitrary Units

c.p.s.: Counts Per Second

CV: Cross-Validation

DP: Declustering Potential

ESI: Electrospray Ionization

FIA: Flow Injection Analysis

FIA-MS: Flow Injection Analysis coupled to Mass Spectrometry

FLD: Fluorescence detection

FTIR: Fourier Transform Infrared

HPLC: High-Performance Liquid Chromatography

HPLC-FLD: High-Performance Liquid Chromatography with Fluorescence detection

HPLC-UV: High-Performance Liquid Chromatography with Ultraviolet detection

i.d.: Internal Diameter

LC: Liquid Chromatography

LC-MS: Liquid Chromatography coupled to Mass Spectrometry

LV: Latent Variable

MS: Mass Spectrometry

NIR: Near Infrared

NMR: Nuclear Magnetic Resonance

PC: Principal Component

PCA: Principal Component Analysis

PLS: Partial Least Squares

PLS-DA: Partial Least Squares-Discriminant Analysis

PTFE: Polytetrafluoroethylene

QC: Quality Control

r.f.u.: Relative Fluorescence Units

UV-Vis: Ultraviolet-Visible