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Grau de Química

Treball Final de Grau

FIA-MS Fingerprinting for the Characterization, Classification and Authentication of Tea.

Caracterització, Classificació i Autenticació de Te mitjançant empremtes de FIA-MS.

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Qui és autèntic, assumeix la responsabilitat per ser el que és i es reconeix lliure de ser el que és.

Jean Paul Sartre

En primer lloc, vull agrair als meus tutors, a l'Oscar i la Sònia, per tot el suport i l'ajuda que m'han proporcionat aquests mesos de treball intens, així com per tot el que m'han aportat especialment a nivell acadèmic, on dia rere dia he anat aprenent d'ells i adquirint nous coneixements.

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REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The Sustainable Development Goals (SDG) are a set of 17 global goals, grouped into five different areas, which were established in 2015 by the United Nations in order to protect the planet, eradicate poverty, and ensure prosperity and well-being for all people. These goals are part of a new sustainable development agenda, known as the 2030 Agenda [1].

The present work is based on the development of a fast and effective technique to deal with food fraud in tea. The fact that many daily foods and beverages, such as tea, contain unspecified ingredients that may be toxic or allergenic, can entail serious problems for public health. As a result, there has also been increased concern and uncertainty about the consumption of some foods, leading in some cases to the wastage of those foods that are not sold. Thus, it can be said that the two areas influenced by this work are *People* and *Planet*. In addition, this work provides a way of facing fraudulent practices that are carried out in tea, as well as providing a tool for society to know what they are really consuming, which is by obtaining an effective and fast method, such as flow injection analysis coupled to mass spectrometry (FIA-MS), to assess the authenticity of this beverage, since the sooner adulterations are detected, the sooner action can be taken, for example, by preventing an adulterated tea from being offered for sale and reaching consumers. The use of this method is, therefore, a form to fight to make tea production as transparent as possible and to ensure that tea, when marketed, complies with official standards and corresponds to what is written on the label. Furthermore, this fact is supported by the good results obtained after chemometric analysis of the data obtained, where it was demonstrated that FIA-MS is a technique capable of discriminating different tea varieties against an adulterant like chicory, as well as detecting and quantifying different levels of adulteration.

Thus, since ensuring healthy lives, promoting the well-being of all people of all ages, and increasing resources to address this food problem are very important aspects of sustainable development, the goals to which this work can be related are *Good Health and Well-Being,*

and *Responsible Consumption and Production*. Regarding the first goal, this work can be included in the following targets:

- 3.5. Strengthen the prevention and treatment of substance abuse, including narcotic drug abuse and harmful use of alcohol.
- 3.9. By 2030, substantially reduce the number of deaths and illnesses from hazardous chemicals and air, water and soil pollution and contamination.

However, in this case it would be more correct to focus these targets on reducing the number of deaths and illnesses due to the consumption of toxic and undeclared substances in tea, as well as on preventing the use of adulterants to improve the taste and flavour of this beverage.

On the other hand, regarding to the second goal, this work can also be included in the following targets:

- 12.3. By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses.
- 12.8. By 2030, ensure that people everywhere have the relevant information and awareness for sustainable development and lifestyles in harmony with nature.

Since, as mentioned before, not really knowing what you are consuming or knowing that there is a possibility that a certain food contains unspecified substances leads to a big waste of food, so it is very important to fight for the necessary knowledge of everything you want to consume.

CONTENTS

1. SUMMARY

Nowadays, many food products have been subjected to some kind of fraudulent practices, including incorrect labelling, adulteration or substitution of undeclared compounds, among others. The main purpose of these practices is mainly to obtain illegal economic benefits, although there is a great concern about their increase due to the problems that the presence of undeclared allergenic or toxic compounds may entail for public health.

Often, these frauds cannot be recognized visually or detected using simple methods, so the development of more advanced analytical techniques has become an urgent necessity. One of the techniques that is becoming increasingly important in this field is flow injection analysis coupled to mass spectrometry (FIA-MS) working in a non-targeted (fingerprinting) approach, which is characterized as a fast, simple, and effective technique for analyzing a large number of samples without the need to know the identity of their components.

This work has focused on evaluating the use of FIA-MS fingerprints as chemical descriptors to study the characterization, classification, and authentication of different tea varieties, as well as the detection and quantitation of one of the most common adulterants in this beverage, chicory. The data obtained have been subjected to multivariant chemometric methods, such as principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and partial least squares regression (PLS). The results obtained have been very promising, demonstrating that the proposed method is able to discriminate perfectly between different types of tea and chicory, as well as to quantify different levels of adulteration in two adulterated tea varieties (black and green tea); in fact, low calibration and cross-validation errors have been obtained (0.7-5.8% and 6.7-8.5%, respectively), and quite acceptable errors regarding to prediction (7.8-16.4%) have been obtained, demonstrating the good ability of this method to address tea authentication.

Keywords: Food fraud, FIA-MS, Fingerprinting, Tea, Chicory, Chemometrics, PCA, PLS-DA, PLS.

2. RESUM

Avui en dia, molts productes alimentaris han estat sotmesos a algun tipus de pràctiques fraudulentes que inclouen l'etiquetatge incorrecte, l'adulteració o la substitució de compostos no declarats, entre altres. La principal finalitat de dur a terme aquestes pràctiques és sobretot l'obtenció de beneficis econòmics il·lícits, tot i que també existeix una gran preocupació pel seu augment degut als problemes que pot comportar per a la salut pública la presència d'al·lèrgens o de compostos tòxics no declarats.

Sovint aquests fraus no es poden arribar a reconèixer visualment ni detectar emprant mètodes simples, de manera que el desenvolupament de tècniques analítiques més avançades ha esdevingut una necessitat urgent. Una de les tècniques que cada vegada està agafant més importància en aquest àmbit és l'anàlisi per injecció de flux acoblada a l'espectrometria de masses (FIA-MS) treballant en una aproximació no-dirigida (*fingerprinting)*, la qual es caracteritza per ser una tècnica ràpida, senzilla i eficaç per analitzar un gran nombre de mostres sense la necessitat de conèixer la identitat dels seus components.

Aquest treball s'ha centrat en avaluar l'ús de les empremtes de FIA-MS com a descriptors químics per estudiar la caracterització, classificació i autenticació de diferents varietats de te, així com la detecció i la quantificació d'un dels adulterants més comuns en aquesta beguda, la xicoira. Les dades obtingudes han estat sotmeses a mètodes quimiomètrics multivariants, com són l'anàlisi de components principals (PCA), l'anàlisi discriminant amb regressió de mínims quadrats parcials (PLS-DA) i la regressió de mínims quadrats parcials (PLS). Els resultats obtinguts han estat molt prometedors, demostrant que el mètode proposat és capaç de discriminar perfectament entre els diferents tipus de te i la xicoira així com per quantificar diferents nivells d'adulteració en dues varietats de te adulterades (te negre i verd); de fet, s'han obtingut baixos errors de calibratge i de validació creuada (0.7-5.8% i 6.7-8.5% respectivament) i errors també força acceptables pel què fa a la predicció (7.8-16.4%), demostrant així la bona capacitat d'aquest mètode per abordar l'autenticació del te.

Paraules clau: Frau alimentari, FIA-MS, *Fingerprinting*, Te, Xicoira, Quimiometria, PCA, PLS-DA, PLS.

3. INTRODUCTION

3.1. TEA

One of the most consumed and popular beverages worldwide is tea, an infusion of the leaves of a plant called *Camellia Sinensis*, which belongs to the Theaceae family [2]. The global area of tea plantations is about four million hectares, half of which are in China, the country where tea originated, but nowadays it is also cultivated in more than thirty countries [2,3].

The flavour and aroma of tea are very characteristic, which is why it has become a beverage that many people have incorporated into their daily lives. However, it has also been influenced by the fact that it is a healthy drink that provides many health benefits, in fact, some studies have shown that its components have antioxidant, anti-inflammatory, antimicrobial, anti-hypertensive, anticarcinogenic, neuroprotective, cholesterol-lowering, and thermogenic properties [4].

3.1.1. Tea composition

The main components of tea are catechins, polyphenols that belong to the flavan-3-ols class and usually represent 30-42% of the dry weight of tea leaves. The most important catechins found in significant amounts in tea leaves are (+)-catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, and (-)-epigallocatechin, with the last two being of great biological importance due to their antioxidant function [5].

Other important polyphenols also present in tea are gallic acid, flavonols, and flavones, which represent about 0.5-2.5% of the dry weight. Also predominant are proanthocyanidins, a group of flavan-3-ols abundant in many plants; non-protein amino acids, especially L-theanine and γ-aminobutyric acid (GABA); and some polyamines, such as methylxanthines. The main methylxanthine present is caffeine, although theobromine and theophylline (in smaller quantities) are also found. In addition, teas that have undergone a fermentation process (total or partial) contain specific derivatives of flavan-3-ols called theaflavins and thearubigins, which are formed in the oxidation process [3,6].

Even though tea has more components, the above mentioned are the major ones. It is important to keep in mind that their content is not the same in all teas, since it depends on whether fermentation has been carried out as well as on the type of tea and the growing, climatic, and horticultural conditions, among other parameters [7].

3.1.2. Tea varieties

Teas can be classified into five main varieties depending on the degree of fermentation and the manufacturing process:

Black Tea

Black tea is classified as a fully fermented tea. It is produced by grinding the tea leaves to promote fermentation and subsequent condensation of polyphenols, being in fact an enzymatic oxidation. It is characterized by containing less catechins than other teas, as their content decreases during fermentation. It also has high concentrations of gallic acid and, unlike non-fermented teas, contains theaflavins and thearubigins. It is the most consumed tea in the world, in fact, it represents 78% of the world tea production [3,8].

Red Tea

Also known as Pu-Erh, red tea is classified as a post-fermented tea, that is, it undergoes a total fermentation stage but, unlike black tea, this fermentation is achieved with microorganisms [7]. Its composition is quite similar to that of black tea because both are fermented, so it also contains theaflavins and thearubigins as well as a high content of gallic acid. As opposed to other varieties, this tea contains a large number of polysaccharides [9].

Oolong Tea

This type of tea is classified as semi-fermented due to, instead of total enzymatic oxidation, partial oxidation takes place before drying. In terms of its composition, this variety stands out for being an important source of catechins, especially (-)-epigallocatechin gallate. It also contains a mixture of monomeric polyphenols and, like black and red tea, it contains theaflavins and thearubigins too. It accounts for 2% of the world tea production [3,8].

Green Tea

Green tea, classified as non-fermented, is obtained from the drying and subsequent steaming of tea leaves by inactivating the enzymes responsible for the oxidation of polyphenols (polyphenols oxidases), so fermentation does not occur [3]. It is the variety richest in catechins (especially in (-)-epigallocatechin gallate) and flavan-3-ols. The composition of phenolic compounds is very similar to that of unprocessed tea leaves, and together with black tea, green tea is one of the most popular teas worldwide, accounting for 20% of global production [6,8].

White Tea

White tea is one of the least processed types of tea; in fact, it can be considered unfermented. However, a slight fermentation does happen as enzymatic deactivation does not occur in the manufacturing process. This type of tea also has high gallic acid content and, like green tea, is rich in catechins [8]. Unlike other teas, this one stands out as an important source of L-theanine and GABA [6]. Moreover, it is one of the most expensive varieties due to its good quality and special flavour, as well as for the health benefits it provides [10].

3.2. FOOD FRAUD

One of the main concerns in the world is the growth of food fraud, which can be considered as an intentional substitution, addition, or alteration of food products through false claims about their quality in order to reduce its price, increase its volume and, above all, obtain illegal economic benefits [11,12]. This supposes a serious risk to public health due to the possible presence of undeclared toxic substances or allergic compounds [13].

According to European Commission, the most common type of fraud is adulteration, which can be done by substituting nutrients that are less valued or do not conform to standards or official labelling, or by adding unknown and undeclared compounds to improve quality [12]. The increased vulnerability to fraud is determined by the ease of adulterating certain types of products as well as the general availability of knowledge and techniques to carry out these fraudulent practices. As a result, it is sometimes difficult to recognize at a glance if a product has been adulterated [14].

Beverages are one of the most commonly adulterated foods, with coffee, fruit juices, and alcoholic drinks being the highest adulterated [11]. Tea is no exception; it is also often adulterated. The most common adulterants used in tea are inorganic materials, such as leather flakes, sand,

dyes, and coal tar, or exhausted tea leaves or leaves of other species of lower quality. Other non-permitted materials that are also used are legume husks, borax, sodium carbonate, cereal starch, and chicory [15,16], the latter being the subject of study in this project.

Chicory (*Cichorium intybus*) is a perennial herbaceous plant of the genus *Cichorium* that belongs to the Asteraceae family. It is cultivated worldwide and its main use is in animal feed and in the food industry, for example, as a supplement (if declared) in coffee and tea, or as a source for the production of inulin, a starch-like polysaccharide [17]. It is also used to enhance the flavour of some beverages and is known for its nutritional, medicinal, and culinary qualities [18]. Recent studies have given importance to the benefits that chicory has on human health due to its bioactivity associated with its high content of polyphenols and minerals, as well as for reducing the risk of suffer gastrointestinal diseases [17]. However, it has also been studied how the presence of chicory extract in food and beverages can cause allergic reactions that trigger oral, cutaneous, and/or respiratory symptoms [19].

3.3. METHODS AND TECHNIQUES

Authenticity and traceability are very important tools for controlling food quality and safety, so finding fast and reliable analytical methods and techniques suitable for performing both authentication and classification/characterization of commercial products has become an urgent need [20].

3.3.1. Targeted and non-targeted approaches

There are two types of analytical approaches that are widely used in food fraud detection. On the one hand, there are the targeted methods, which are based on prior knowledge of the compounds to be determined, focusing on the detection of a few selected analytes. These methods provide quantitative information and are usually more selective and sensitive than non-targeted methods, although they are slow and laborious. On the other hand, there are the non-targeted methods (also known as fingerprinting). Unlike the previous ones, they are based on the simultaneous detection of sample instrumental responses without the need of knowing which compounds are responsible for these responses. A global fingerprint is obtained, which usually contains complete and valuable information; it is, therefore, a qualitative analysis. This type of method is advantageous when information about possible adulterants is not yet known or when the sample contains adulterants that are unlikely to be detected by targeted approaches. Another

difference is that they do not require complex and expensive extraction procedures, but a simple sample preparation is enough to detect a large number of compounds [21].

Nowadays, non-targeted methods are increasingly used and, together with targeted methods, are expected to play a major role in the detection of food fraud. In fact, some studies have already been initiated to test the effectiveness of some analytical techniques to differentiate tea varieties according to their geographic origin, or chemical composition, as well as to detect the presence of possible adulterants [10]. Some of these techniques are chromatographic techniques, such as high-performance liquid chromatography (HPLC) [13], or coupled to mass spectrometry, such as liquid/gas chromatography-mass spectrometry (LC/GC-MS) [22]. Inductively coupled plasma-mass spectrometry (ICP-MS) [23], capillary electrophoresis (CE) [24], spectroscopy techniques like nuclear magnetic resonance (RMN) and near infrared reflectance (NIR) [12], and even electrochemical techniques have also been employed [25]. All of these have been widely used to successfully perform non-targeted analysis, even though they have also been used for targeted analysis.

Among these, LC-MS has been widely used due to its high separation effectiveness and sensitivity, as well as for the easy detection of trace amounts of adulterants present in food. Nevertheless, it is an expensive and time-consuming technique, that does not make it very suitable for high-throughput analysis [20]. Mainly for this reason, faster and more cost-effective analytical techniques are now being developed that can perform the same function by obtaining fingerprints of a large number of samples. One such technique being developed and becoming important in this field is flow injection analysis coupled to mass spectrometry (FIA-MS).

3.3.2. Flow injection analysis-mass spectrometry (FIA-MS)

FIA-MS is an alternative approach to chromatographic techniques coupled to mass-spectrometry in which an analytical column is not available, so separation of compounds from the sample does not take place, but detection is performed within the MS instrument [26].

The advantages of this technique over LC-MS are mainly the speed of analysis of a large number of samples, the simplicity and high yield without compromising precision, the sensitivity and accuracy, and the use of any sample solvent (provided it is compatible with the MS ionisation mode employed). However, the main drawback is that it offers lower selectivity since, as mentioned above, the compounds are not separated [27]. Therefore, this technique is suitable for

applications where fast and reliable quantitative fingerprinting, identification of complex samples, and screening of chemical substances are required [20].

FIA-MS fingerprinting, like many other methods, is often combined with chemometric methods to perform qualitative and quantitative analysis, such as classification and authentication of a wide variety of compounds in samples with complex matrices.

3.4. CHEMOMETRICS

Chemometrics is a discipline in chemistry that uses mathematics, statistics, and formal logic to design and select optimal experimental procedures and provide the maximum relevant chemical information from the analysis of chemical data [28]. It is widely used to extract important analytical information from a big amount of data generated by many instrumental techniques, such as FIA-MS.

Chemometrics usually deals with classification, discrimination, and calibration problems, so it can predict both qualitative and quantitative properties. As for qualitative methods, these are mainly applied to classification problems (when studying whether individual samples belong to some group and to which one), as well as being applied in pattern recognition, i.e., to see if there is any kind of clustering or pattern in the structure of the data. These qualitative methods can be divided into two groups: on the one hand, there are the non-supervised methods, whose purpose is to reveal the structure of the data without prior knowledge of the pertinence of the samples to the groups, and on the other hand, there are the supervised methods, which are based on producing the best possible separation of the groups and, hence, maximizing the capacity of the method to predict to which defined class(es) the samples belong [29].

One of the most commonly used techniques in chemometrics is the application of multivariate analytical methods to process de data obtained in the analyses. In this study, the multivariate analytical methods used are briefly explained below.

3.4.1. Principal component analysis (PCA)

PCA is a non-supervised multivariate chemometric method based on exploratory analysis. It is characterized by reducing the dimensionality of a large number of interrelated variables while maintaining at all times the relevant information on the variation of the data. This reduction gives rise to new variables called principal components (PC), which are linear combinations of the original variables. PCs explain as much information as possible about the variability of the original

data without repeating information between them. PC1 is the most important, since it explains the greatest variability of the data, while the following PCs provide less and less new information [28].

In the PCA model, the experimental space is defined in relation to the samples, so that the data are structured in an X-matrix, where the rows represent the samples whereas the columns represent the variables. This X-matrix is decomposed into the scores matrix, which describes the samples in PC space, and the loadings matrix, which describes the original variables in PC space [28], as can be seen in Figure 3.1.

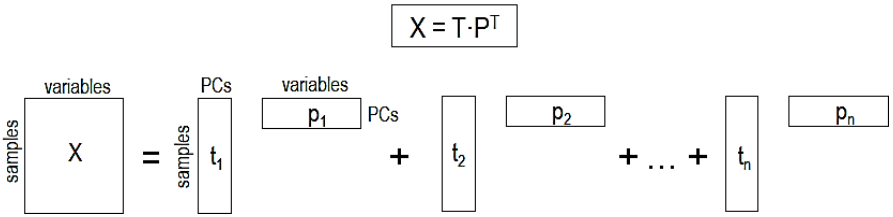


Figure 3.1. PCA decomposition where T is the scores matrix and P^T is the loadings matrix.

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

Unlike PCA, PLS-DA is a supervised multivariate classification method based on evaluating the pertinence of unknown samples to previously defined classes. Thus, it is used to optimize the separation between different groups of samples. This method correlates the X-matrix, which contains the variable data, with the Y-matrix, which encodes the pertinence to a given class. Like PCA, PLS-DA also reduces the dimensionality of the data, but instead of concentrating the most relevant information in the PCs, it does so in latent variables (LV), which perform the same function as the firsts [28,29].

3.4.3. Partial least squares (PLS)

PLS is a multivariate calibration method based on obtaining a model that correlates the X-matrix, which contains the variable data, in this case, the sample fingerprints, with the Y-matrix, which contains the different concentrations. The main objective of this method is to obtain the maximum covariance between both matrices to build a model that can be used to predict the concentration of a given analyte in unknown samples, as well as to quantify external samples. As in PLS-DA, the components needed to build this regression model are LVs, but the criterion for choosing the appropriate number of these is to choose those describing the X data that have a maximum covariance with the Y data [28,29].

4. OBJECTIVES

The main objective of this work is to evaluate the use of fingerprints obtained by a fast, simple, and effective method such as flow injection analysis-mass spectrometry (FIA-MS), both in negative and positive acquisition mode, as sample chemical descriptors to address the classification, characterization, and authentication of different tea samples of five varieties (black, green, oolong, red, and white tea) against chicory. In addition, the ability of the proposed method to detect and quantify adulteration levels in two tea varieties (black and green tea) using chicory as adulterant will also be assessed. To achieve this goal, different steps will be carried out:

1. A simple sample treatment will be performed in tea and chicory samples to extract, quantitatively, their compounds.
2. For the adulteration study, different blends of tea and chicory with different levels of adulteration will be prepared and extracted.
3. The obtained sample extracts will be analysed by FIA-MS fingerprinting.
4. The obtained fingerprints will be exploited as analytical data by chemometric methods:
    a. Principal component analysis (PCA) will be employed to evaluate the reproducibility of the proposed method and the robustness of the chemometric results.
    b. Partial least squares – discriminant analysis (PLS-DA) will be used to study the classification of tea and chicory samples according to their typology.
    c. Partial least squares (PLS) will be used in the adulteration study to quantify the levels of chicory adulteration in black and green tea samples.

5. EXPERIMENTAL SECTION

5.1. REAGENTS AND SOLUTIONS

The chemicals used in this work were Milli-Q water, which was purified with an Elix 3 coupled to a Milli-Q system (Millipore Corporation, Bedford, MA, USA) and was filtered through a 0.22 µm nylon membrane integrated into the Milli-Q system; Methanol (Chromosolv™ for HPLC, ≥ 99.9%), acquired from PanReac AppliChem (Barcelona, Spain), and formic acid (≥ 98%), obtained from Sigma Aldrich (St. Luis, MO, USA).

The solvent used for the tea and chicory extraction was a commercial and natural water with weak mineralization obtained from Eroski (Barcelona, Spain). This water is characterized by the following chemical composition: 402 mg L^{-1} of dry residue at 180 ºC, 326 mg L^{-1} of bicarbonate, 44 mg L^{-1} of chloride, 85 mg L^{-1} of calcium, 28 mg L^{-1} of magnesium, 18 mg L^{-1} of sodium, and 8 mg L^{-1} of silica.

5.2. SAMPLE PREPARATION

5.2.1. Tea and chicory classification and characterization

One hundred and one commercial tea samples of five different varieties and twenty chicory samples of four different types were selected for the classification and characterization study by non-targeted FIA-MS fingerprinting (Table 5.1). Accordingly, an identification code was assigned to each tea and chicory sample. Both chicory and teas were purchased from local supermarkets in Barcelona (Spain).

Table 5.1. Summary of tea and chicory samples used for the classification and characterization study.

Sample Class	Sample Type (Code)	Total Number of Samples
Tea	Black Tea (B)	39
	Green Tea (G)	20
	Oolong Tea (O)	10
	Red Tea (R)	12
	White Tea (W)	20
Chicory	Chicory (C)	20

The sample treatment was the same for all the teas and chicories and consisted of extracting around 0.5 g of tea/chicory sample with 25 mL of hot water in a 50 mL PTFE centrifuge tube (Serviquimia, Barcelona, Spain). The solution was shaken vigorously for one minute using a Vortex (Stuart, Stone, United Kingdom) to assure a quantitative extraction and then the extracts were centrifuged for five minutes at 3500 rpm (Rotanta 460 RS centrifuge, Hettich, Tuttlingen, Germany). After centrifugation, the obtained aqueous extracts were filtered with a syringe and 0.45 µm nylon filters (discarding the first mL) in glass injection vials and were kept at 4ºC until FIA-MS analysis.

In addition, a quality control (QC) solution was prepared by mixing 50 µL of all the obtained aqueous tea and chicory extracts. The purpose of the quality control was to evaluate the reproducibility of the proposed methodologies and the robustness of the chemometric results obtained.

5.2.2. Tea adulteration with chicory

The detection and quantitation of adulterations in tea extracts was evaluated by studying two blended cases based on black and green teas adulterated with chicory. For each adulteration case under study, different blended mixtures were prepared to build the corresponding PLS calibration and validation sets, as indicated in Table 5.2.

Table 5.2. Blended mixtures used in the adulteration studies of black and green tea with chicory (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 each level of adulteration five replicates were prepared, so fifty-five sample extracts were obtained for each one of the adulteration cases studied. Again, a QC sample was prepared, which in this case consisted of an additional blended tea and chicory sample at 50%.

In order to cover a wide range of tea samples within the same tea variety (black or green) as well as with the chicory samples, for the preparation of the blended mixtures indicated in Table 5.2, ten different tea samples of each variety (black or green) and four different chicory samples were employed. Thus, the five replicates of each blended level were done with different tea/chicory sample combinations (Appendix 1).

5.3. INSTRUMENTATION

Flow injection analysis-mass spectrometry (FIA-MS) was performed using an Agilent 1100 Series HPLC instrument (Waldbronn, Germany) coupled to an AB Sciex 4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer. FIA-MS fingerprints were acquired in full scan MS mode (*m/z* 100-550) and were obtained in negative and positive electrospray ionisation (ESI). The ion spray voltage was set at 2500V, and the source temperature was set at 400ºC. Furthermore, a declustering potential (DP) of 80V (in positive or negative value depending on the ionisation mode), was used. Nitrogen was used as a nebulizer, and for the curtain gas, ion source gas 1, and ion source gas 2, the auxiliary gas flow rate was set at 10, 50, and 50 arbitrary units (a.u.), respectively. The sample injection volume was 10 µL and the carrier, which consisted of a 50:50 (v/v) mixture of methanol and water acidified with 0,1% formic acid (v/v), was pumped isocratically with a flow rate of 150 µL min^{-1} for 1.5 min.

All analysed samples (characterization and adulteration studies) were injected randomly to minimize the effect of the instrumental drifts on the chemometric models. Moreover, the corresponding QC sample and an instrumental blank (mineral water) were injected at the beginning of the sequence and after every ten sample injections.

5.4. DATA PROCESSING

5.4.1. Data matrix construction

For both the classification/characterization and adulteration studies, raw data results obtained by FIA-MS were initially processed with the MSConvert free software to get them in mzML output format [30]. 32 bits as binary encoding precision and Threshold Peak Filter was applied for data simplification. Absolute intensity was defined as threshold type; most intense was fixed as orientation, and 10000 as value.

Once the data were obtained in the desired format, the next step was to obtain the data matrices with the FIA-MS fingerprints of the samples studied. The data matrices had the following dimension: samples x variables, where the ionic signal intensity values as a function of *m/z* were provided. For this purpose, mzMine 3 software was employed [30]. The first step was the wavelet transform mass detection, which generates mass lists for each of the scans acquired on a sample. Thus, the wavelet transform was selected as the mass detector since it is the most useful for low-resolution mass spectrometry. A peak time range of 0.00 – 1.48 min was set as well as the corresponding polarity, and a noise level of 4.0×10^4, a scale level of 3, and a wavelet window size of 30% were considered. The next step was to remove false signals with the FTMS shoulder peak filter, by setting a Gaussian peak model function and a mass resolution of 70.000. Then, to extract ion chromatograms for masses that were detected continuously for a given time, the ADAP chromatogram builder was used, defining a minimum scan group size of 5, a group intensity threshold of 4.0×10^4, a highest minimum intensity of 1.0×10^4, and an *m/z* tolerance of 5000 ppm, which is equivalent to 1 *m/z*. Lastly, the Join Aligner was performed to allow matching of the masses detected across the analysed samples, so an *m/z* tolerance of 5000 ppm, a weight for *m/z* of 80, a retention time tolerance of 2 minutes, and a weight for RT and a mobility weight of 1 were defined, thus obtaining the aligned feature list, that was finally exported as CSV format and ready for subsequent chemometric analysis.

5.4.2. Chemometric analysis

The data matrices obtained were then submitted to PCA, PLS-DA, and PLS using SOLO 8.6 chemometric software from Eigenvector Research (Manson, WA, USA). In any case, the X-data matrix consisted of the FIA-MS fingerprints (peak signal as a function of *m/z* values) obtained in negative or positive ionisation modes. Instead, the Y-data matrix defined each sample class in PLS-DA whereas defined each adulterant percentage in PLS.

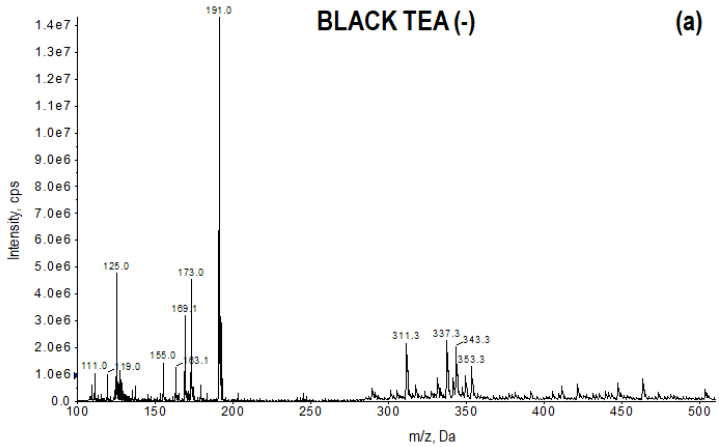
To build both the PCA and PLS-DA models, FIA-MS fingerprints were autoscaled to remove differences in their magnitude and amplitude scales. In this way, all the variables became equally weighted. Besides, in PLS-DA the VIP (Variable Importance in Projection) treatment was applied to achieve better separation. This treatment consisted of reducing the number of variables in the matrix by selecting only the most important for the separation, which were those found above 1. The most appropriate number of principal components/latent variables (PCs/LVs) in PCA/PLS models was established by considering the first significant minimum cross-validation (CV) error point, while the optimal number of latent variables (LVs) in PLS-DA model was established by considering the first significant minimum in the mean classification error. A Venetian Blind approach was used in both cases.

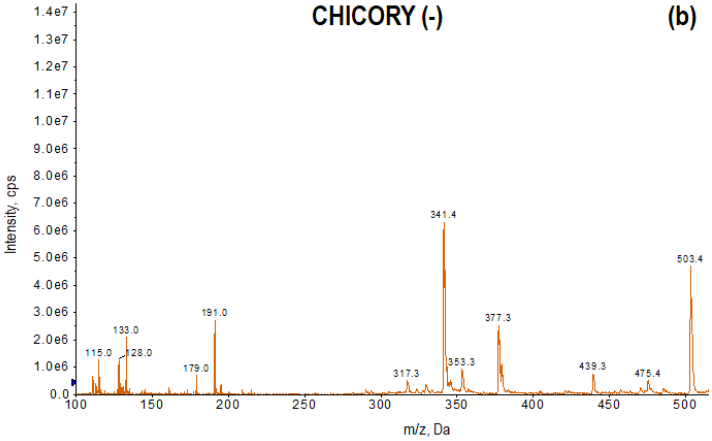
6. RESULTS AND DISCUSSION

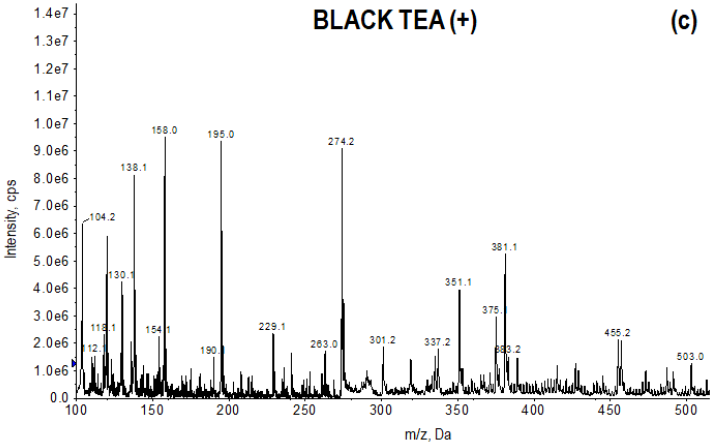
6.1. CLASSIFICATION AND CHARACTERIZATION OF TEA AND CHICORY SAMPLES

6.1.1. FIA-MS fingerprints

Before performing the corresponding chemometric analysis, the obtained FIA-MS fingerprints were visually inspected. As an example, the negative and positive ionisation FIA-MS spectra of a black tea sample and a chicory sample are shown in Figure 6.1.




CHICORY (-)
(b)
Intensity, cps
m/z, Da
341.4
503.4
191.0
377.3
133.0
115.0
128.0
179.0
317.3
353.3
439.3
475.4


BLACK TEA (+)
(c)
Intensity, cps
m/z, Da
158.0
195.0
274.2
138.1
104.2
381.1
130.1
351.1
229.1
455.2
503.0

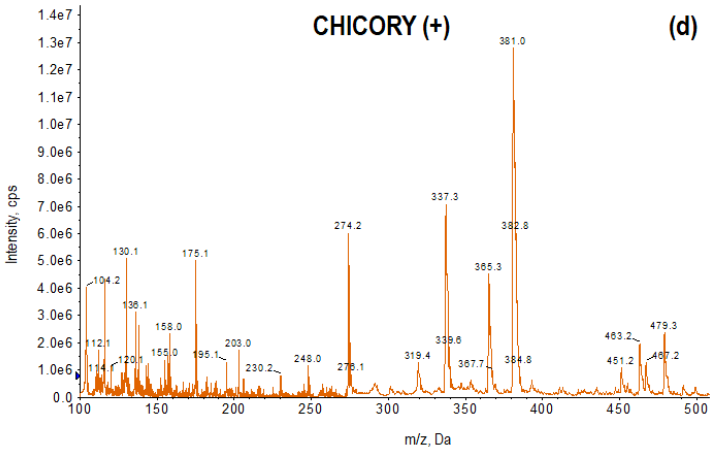


Figure 6.1. FIA-MS spectra obtained for a black tea (a) and a chicory (b) samples, both in negative acquisition mode, and for a black tea (c) and a chicory (d) samples, both in positive acquisition mode.

As can be seen, significant differences were obtained in terms of the profile of the spectra and the intensity of the peaks. Looking at the spectra obtained in both acquisition modes, the main difference observed is the abundance of more cationic than anionic compounds, whereas as for the comparison between the spectra of the black tea sample and the chicory sample, it is observed that they are very different since most of the peaks are not common. Therefore, these differences suggest that FIA-MS fingerprints may be good sample chemical descriptors to address the discrimination of these samples by chemometric methods for authentication purposes.

6.1.2. Sample exploration by PCA

To assess whether the obtained FIA-MS fingerprints had a good discriminatory ability and thus worked well as chemical descriptors, an initial exploration by principal component analysis (PCA) was carried out on the negative and positive ionisation data, with data matrices of dimensions of 139 x 320 and 139 x 339 (samples x variables), respectively. The main purpose of performing this analysis was to observe if the QC samples formed a compact group in the scores plot, but it also allowed a first exploration of sample groups and trends. For both negative and positive ionisation data, three principal components (PC) were used to build the PCA models. The variance explained with the PCA was 29.49% and 29.44% for the negative and positive acquisition modes, respectively.

Figure 6.2 shows the obtained PCA scores plots of PC1 vs. PC2 using FIA-MS fingerprints data in negative and positive ionisation modes (Figure 6.2a and 6.2b, respectively). As can be seen, the QC samples appear well clustered in the centre of both plots, indicating the absence of systematic errors during the sequence sample analysis. Moreover, the fact that these QC samples are grouped ensures the good reproducibility of the proposed method and the validity of the chemometric results, as well as the tendency of all samples to form different groups.

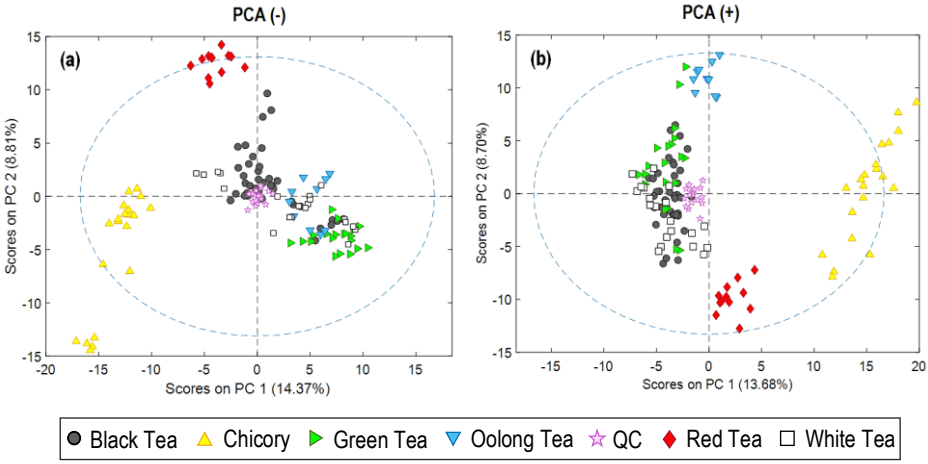


Figure 6.2. PCA scores plot of PC1 vs. PC2 obtained from tea and chicory samples using FIA-MS fingerprints in (a) negative acquisition mode and (b) positive acquisition mode.

As for the distribution of the samples, both plots show that they tend to be grouped according to their variety. Mainly, PC1 allows discriminating perfectly the chicory samples, which are situated on the left side of the plot in negative mode and on the right side in positive mode, from all the tea samples, whereas both PC1 and PC2 allow discriminating between some of the different tea varieties.

The discrimination between tea samples depends on their variety. This is especially observed in the case of the red tea samples (situated at the top of the plot in negative mode and at the bottom in positive mode), which are perfectly discriminated from the other tea varieties in both acquisition modes. However, the rest of the teas are a bit overlapped and seem to follow the same trend, although in the positive acquisition mode it is also possible to discriminate Oolong teas from the other tea varieties.

6.1.3. Sample classification by PLS-DA

After the exploratory analysis, FIA-MS fingerprints were subjected to a classificatory PLS-DA chemometric method. QC samples were excluded from the X-data matrices and the VIP treatment was applied, resulting in 121 x 113 and 121 x 128 data matrices for negative and positive ionisation data, respectively. Five latent variables (LVs) explaining 82.58% and 79.59% of the Y-variance for the negative and positive acquisition modes, respectively, were required to build the PLS-DA models. The resulting scores plots defined by LV1 vs. LV2 and LV1 vs. LV4 for both acquisition modes are shown in Figure 6.3.

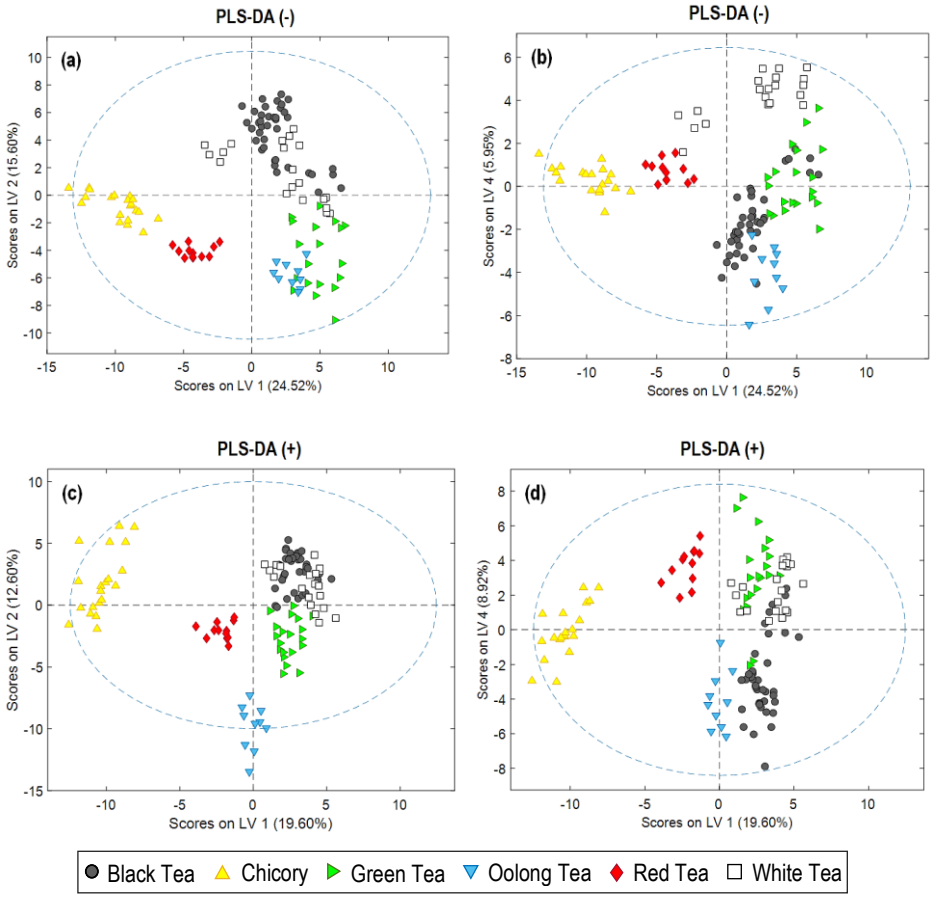


Figure 6.3. PLS-DA scores plot of (a, c) LV1 vs. LV2 and (b, d) LV1 vs. LV4 obtained from tea and chicory samples using FIA-MS fingerprints in (a, b) negative acquisition mode and (c, d) positive acquisition mode.

Once again, it can be seen that all samples tend to be grouped according to their variety; nevertheless, as expected, the discrimination between them improves compared to the results obtained previously by PCA.

Regarding the results obtained for the negative ionisation data (Figures 6.3a and 6.3b), it is observed that LV1 is the main latent variable that can perfectly discriminate chicory samples (located on the left side of the plot), from tea samples. Furthermore, depending on the second LV considered (LV2 or LV4), it is possible to discriminate between the different tea varieties, although both LVs allow clearly the discrimination between red tea from all the other teas. Thus, LV2 allows to differentiate two groups: black and white teas from green and Oolong teas, while LV4 manages to discriminate white tea a little from the others, besides improving the separation between black, green, and Oolong teas. On the other hand, as for the results obtained for the FIA-MS fingerprinting data in positive ionisation mode (Figures 6.3c and 6.3d), a similar behaviour is observed in comparison with the results previously commented, even though the separation seems to be better. Chicory samples are perfectly discriminated from tea samples, as well as the red tea samples. In this case, even LV2 can discriminate Oolong tea from the other teas. Likewise, LV2 can differentiate black and white tea from green tea, while LV4 allows a good separation between almost all tea varieties, although some overlaps are observed between black, white, and green tea samples.

So, these results demonstrate that FIA-MS fingerprints can be proposed as good chemical descriptors to approach the classification and characterization of tea and chicory samples. As observed, chicory samples are perfectly discriminated in both acquisition modes, so it can be stated that this method will be able to authenticate teas that have been adulterated with chicory. Furthermore, this method would also be suitable for authenticating teas adulterated with other tea varieties (especially in the positive ionisation mode), although this is out of the scope of this work.

Method validation

To demonstrate the applicability and validity of the proposed method, the classification rate of pair PLS-DA models (tea variety vs. chicory) was studied. This evaluation was performed by randomly selecting 60% of the samples from each class as the calibration set to build the model, and the remaining 40% of the samples were used as the ‘‘unknown’’ set for validation purposes. Figure 6.4 shows an example of the results obtained for the PLS-DA model validation of green tea vs. chicory samples when using FIA-MS fingerprinting data in both acquisition modes (filled

and empty symbols correspond to calibration and validation sets, respectively, and red lines represent the threshold separating one class from the other).

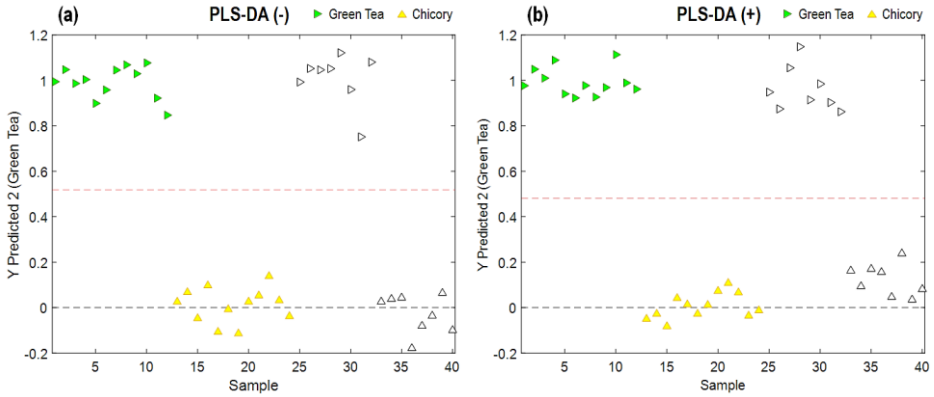


Figure 6.4. Validation of the paired PLS-DA models for green tea vs. chicory using FIA-MS fingerprinting data in (a) negative ionisation and (b) positive ionisation modes.

As can be seen in the figure above, and for all the paired PLS-DA models (tea vs. chicory) studied, the classification rate for calibration and validation was 100%, demonstrating the feasibility of the FIA-MS fingerprinting strategy to assess the classification and authentication of different tea varieties against chicory.

6.2. DETECTION AND QUANTITATION OF TEAS ADULTERATED WITH CHICORY

6.2.1. Exploratory PCA

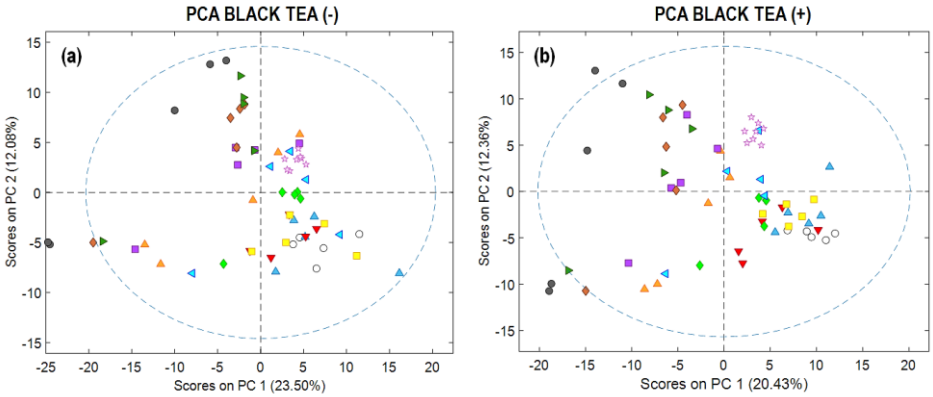
To evaluate the ability of FIA-MS fingerprints to detect frauds and to quantify tea extracts adulterated with chicory, the adulteration of black and green teas with chicory samples was studied by PLS. For that study, and in order to cover a wide range of tea samples within the same tea variety (black or green) as well as with the chicory samples, for the preparation of the blended mixtures ten different tea samples of each variety (black or green) and four different chicory samples were employed, as indicated in section 5.2.2.

First, an exploratory analysis by PCA of the FIA-MS fingerprinting data obtained in both negative and positive ionisation modes obtained for each case under study was performed. Again, the aim of this first exploration was mainly to see if the QC samples appear grouped together in

order to rule out the presence of possible errors during the sequence sample acquisition, but it was also useful to see the distribution of the adulterated samples depending on the percentage of chicory employed. A summary of the PCs required to build the PCA models of the negative and positive ionisation data obtained for the two cases studied, as well as the variances explained for each model and the dimension of the data matrices is given in Table 6.1, whereas the obtained PCA scores plots of PC1 vs PC2 for those same data are shown in Figure 6.5.

Table 6.1. PCs, % of variance explained, and dimension of data matrices for each PCA model.

Black tea adulterated with chicory			
Acquisition mode	PCs	Variance explained [%]	Data matrix dimension
Negative	2	35.58	63 x 276
Positive	3	39.35	63 x 314
Green tea adulterated with chicory			
Acquisition mode	PCs	Variance explained [%]	Data matrix dimension
Negative	3	43.02	63 x 286
Positive	3	37.52	63 x 300



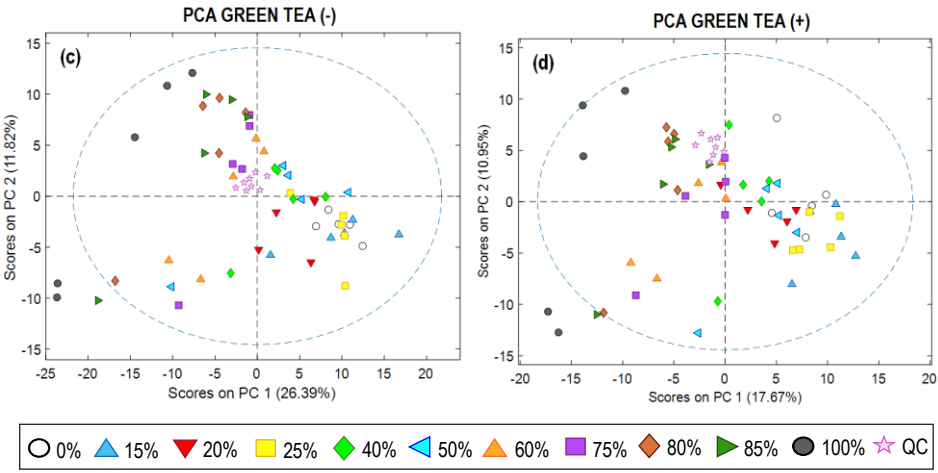


Figure 6.5. PCA scores plot of PC1 vs. PC2 obtained for adulterated black and green tea samples (a, c, respectively), using FIA-MS fingerprints in negative acquisition mode, and for adulterated black and green tea samples (b, d, respectively), using FIA-MS fingerprints in positive acquisition mode

As can be seen in the plots, the QC samples appeared grouped in the two adulteration cases under study and in both acquisition modes, showing a good reproducibility and suitable robustness of the obtained chemometric results.

The distribution of the adulterated samples depends both on the level of adulteration and on the type of tea and chicory used. For this reason, in both cases studied a perfect distribution of these samples according to the percentage of adulterant present is not observed, since the fact that each tea extract was not always adulterated with the same chicory makes the variability very high. However, although it is not very clear due to the overlaps between the adulterated samples, a certain gradation seems to be observed in which, as the concentration of chicory increases, the adulterated tea samples move towards negative PC1 values. Moreover, it is observed that, unlike the pure tea samples (situated at positive PC1 values), the pure chicory samples (situated on the left of the plot) can be discriminated from the blends.

6.2.2. Quantitation of adulteration levels by PLS

The quantitation of chicory adulteration levels in the black and green tea samples adulterated with chicory was carried out by PLS regression. The corresponding PLS models were built and then validated using the respective calibration and validation sets (see Table 5.2).

Figure 6.6 shows the scatter plots of Y measured vs. Y predicted obtained in the negative and positive acquisition modes for the two adulteration cases studied. The number of latent variables (LVs) required to build each model, the correlation coefficients (R^2), as well as the calibration error (RMSEC), the cross-validation error (RMSECV), and the prediction error (RMSEP) obtained for each PLS model are summarised in Table 6.2.

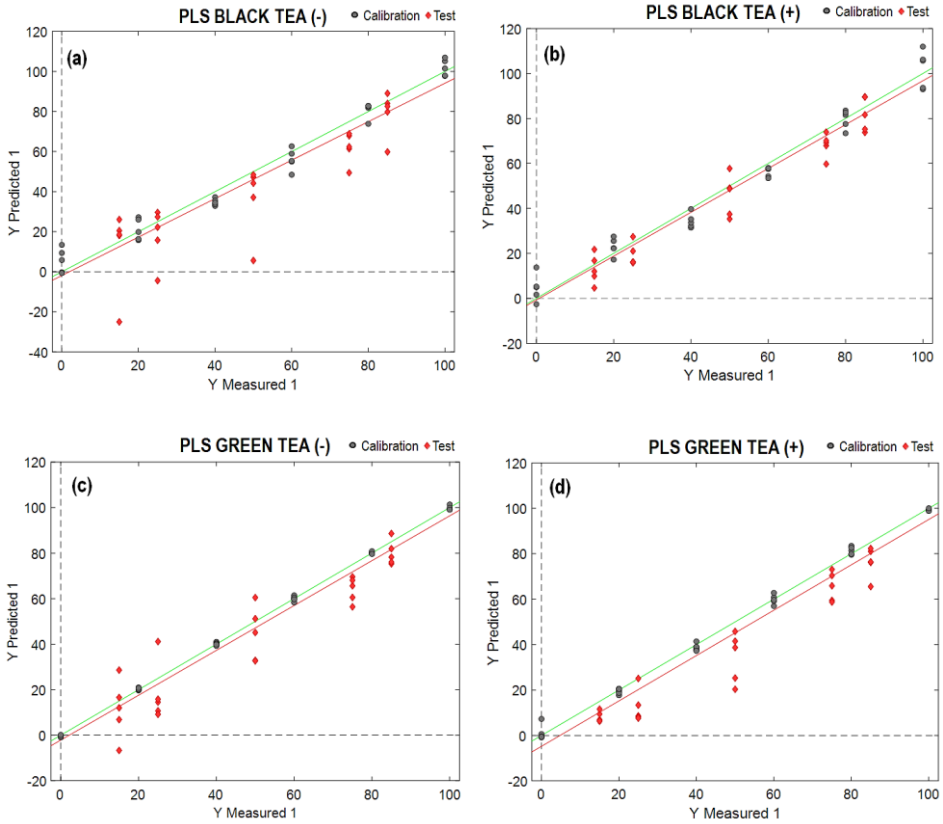


Figure 6.6. PLS regression scatter plots of measured vs. predicted percentages of chicory for adulterated black and green tea samples (a, c, respectively), using FIA-MS fingerprints in negative acquisition mode, and for adulterated black and green tea samples (b, d, respectively), using FIA-MS fingerprints in positive acquisition mode.

Table 6.2. Results obtained by PLS regression for the two adulterated cases under study.

Black tea adulterated with chicory							
Acquisition mode	LVs	Calibration R^2	Cross-Validation R^2	Prediction R^2	RMSEC [%]	RMSECV [%]	RMSEP [%]
Negative	2	0.974	0.949	0.770	5.5	7.9	16.4
Positive	2	0.971	0.944	0.946	5.8	8.5	7.8
Green tea adulterated with chicory							
Acquisition mode	LVs	Calibration R^2	Cross-Validation R^2	Prediction R^2	RMSEC [%]	RMSECV [%]	RMSEP [%]
Negative	4	1.000	0.965	0.881	0.7	6.7	11.5
Positive	4	0.997	0.960	0.935	2.0	7.2	12.8

As can be seen, the results obtained for both adulteration cases under study are quite satisfactory, even taking into account the great variability of samples employed within the study.

In the first case studied (black tea adulterated with chicory), the correlation coefficients obtained in both acquisition modes for the calibration, cross-validation, and prediction models are quite good ($R^2 \geq 0.944$), with the exception of the R^2 of the prediction model obtained in positive ionisation, where linearity is not so good (0.770). On the other hand, the calibration and cross-validation errors obtained are also quite good, with values below 5.8% and 8.5%, respectively, while the prediction errors obtained are quite acceptable, with values lower than 16.4%. Therefore, observing these results it can be said that the predictive ability of this proposed method to quantify adulteration levels in black tea adulterated with chicory is especially good using FIA-MS fingerprints in positive acquisition mode since the RMSEP value is practically half of that obtained in the negative mode.

In the second case studied (green tea adulterated with chicory), similar results to the previous case are observed. On the one hand, the R^2 values obtained are quite good ($R^2 \geq 0.935$), although the R^2 of the prediction obtained in negative acquisition mode is slightly lower (0.881). On the other hand, the calibration and cross-validation errors obtained are better than those of the previous study, with values below 2.0% and 7.2%, respectively. It should be noted that the

calibration errors in both ionisation modes are particularly low, as expected due to the good values presented by their R^2 values (1.000 and 0.997 in the negative and positive acquisition mode, respectively). As for the prediction, the errors obtained are also better than those of the previous adulteration study, with values lower than 12.8%. Therefore, unlike the previous case, as the RMSEP values obtained in the negative and positive acquisition modes are very similar, it can be said that FIA-MS fingerprints in both positive and negative acquisition modes are good to quantify adulteration levels in green tea samples adulterated with chicory, although slightly better results are obtained in the negative acquisition mode.

7. CONCLUSIONS

In the present project, FIA-MS fingerprints obtained in both negative and positive ionisation modes, and combined with chemometrics, has proven to be a good strategy and a good alternative to chromatographic techniques, due to its speed and simplicity, to address the classification, characterization, and authentication of five tea varieties (black, green, oolong, red and white tea) against an adulterant such as chicory. Chemometrics methods like PCA and PLS-DA have shown an excellent capacity to discriminate between chicory samples and the five tea varieties analysed in both acquisition modes and have even been able to differentiate between some varieties of tea.

Furthermore, very satisfactory results were obtained with respect to the validation of the paired tea versus chicory PLS-DA models, specifically 100% classification rates were obtained for both calibration and prediction of the five tea varieties studied against chicory independently of the ionisation mode employed, demonstrating the good ability of the proposed method to approach classification and authentication issues.

Finally, the capability of this method to detect and quantify chicory levels in adulterated black and green tea samples was also evaluated by PLS regression. The obtained results were quite promising in the two cases under study, especially in positive acquisition mode in the case of the adulterated black tea, obtaining prediction errors below 7.8%, and especially in negative acquisition mode (although very slightly) in the case of adulterated green tea, where prediction errors below 11.5% were obtained. Calibration and cross-validation errors in both cases and for both ionisation modes were also quite satisfactory.

Consequently, FIA-MS fingerprints can be proposed as good chemical descriptors for the classification and authentication of tea and chicory samples, as well as to detect tea adulterations with chicory.

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9. ACRONYMS

a.u.: Arbitrary Units

ADAP: Automated Data Analysis Pipeline

CE: Capillary Electrophoresis

CSV: Comma-Separated Values

DP: Declustering Potential

ESI: Electrospray Ionisation

FIA: Flow Injection Analysis

FIA-MS: Flow Injection Analysis coupled to Mass Spectrometry

FTMS: Fourier Transform Mass Spectrometry

GABA: γ-aminobutyric acid

GC: Gas Chromatography

GC-MS: Gas Chromatography coupled to Mass Spectrometry

HPLC: High-Performance Liquid Chromatography

i.e.: That Is

ICP-MS: Inductively Coupled Plasma coupled to Mass Spectrometry

LC: Liquid Chromatography

LC-MS: Liquid Chromatography coupled to Mass Spectrometry

LV: Latent Variable

MS: Mass Spectrometry

NIR: Near Infrared Reflectance

PC: Principal Component

PCA: Principal Component Analysis

PLS: Partial Least Squares

PLS-DA: Partial Least Squares-Discriminant Analysis

ppm: Parts Per Million

PTFE: Polytetrafluoroethylene

QC: Quality Control

RMN: Nuclear Magnetic Resonance

RMSEC: Root Mean Square Error of Calibration

RMSECV: Root Mean Square Error of Cross-Validation

RMSEP: Root Mean Square Error of Prediction

rpm: Revolutions Per Minute

RT: Retention Time

SDG: Sustainable Development Goals

VIP: Variable Importance in Projection

vs: Versus

APPENDICES

APPENDIX 1: MIXTURES USED FOR THE ADULTERATION STUDY

Table A1. Blends of black tea samples adulterated according to the percentage of chicory employed. The same procedure was applied to green tea samples.

Adulteration level [%]	Tea	Chicory	Adulteration level [%]	Tea	Chicory
0	B1		60	B1	C2
	B2			B2	C3
	B3			B3	C4
	B4			B4	C1
	B5			B5	C2
15	B6	C1	75	B6	C3
	B7	C2		B7	C4
	B8	C3		B8	C1
	B9	C4		B9	C2
	B10	C1		B10	C3
20	B1	C2	80	B1	C4
	B2	C3		B2	C1
	B3	C4		B3	C2
	B4	C1		B4	C3
	B5	C2		B5	C4
25	B6	C3	85	B6	C1
	B7	C4		B7	C2
	B8	C1		B8	C3
	B9	C2		B9	C4
	B10	C3		B10	C1

40	B1	C4	100	C2
	B2	C1		C3
	B3	C2		C4
	B4	C3		C1
	B5	C4		C2
50	B6	C1		
	B7	C2		
	B8	C3		
	B9	C4		
	B10	C1		