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Potential of high-throughput FIA-MS/MS and LC-MS/MS polyphenolic profiling to assess tea authenticity. Application to tea adulterations with chicory

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ABSTRACT

Tea can be found among the beverages more susceptible to fraudulent practices because of its high worldwide consumption and the increases on prices for some specific varieties due to climate change and geopolitical instability. Tea adulteration with other plants, such as chicory, is a common practice to gain an illicit profit. Polyphenols are abundant bioactive substances in tea, determining its quality and health function. In addition, they can be employed as markers to address authentication issues. The present contribution assesses the potential of polyphenolic profiling by high-throughput FIA-MS/MS and LC-MS/MS methodologies for tea authenticity. One hundred tea samples belonging to different varieties (green, black, red, oolong, and white teas) and 20 chicory samples were analyzed with both methodologies after a simple brewing process to profile fifty-five polyphenols belonging to different families. The resulting chemical descriptors were used to address tea classification and authentication by partial least-squares-discriminant analysis (PLS-DA). An excellent classification performance by PLS-DA was accomplished, with sensitivity and specificity values for FIA-MS/MS higher than 90% and 88.9%, respectively, and for LC-MS/MS higher than 85% and 86%, respectively. Good accuracy was also attained, with calibration errors below 10.5 and 14.5% for FIA-MS/MS and LC-MS/MS, respectively. Overall, FIA-MS/MS showed a better performance than LC-MS/MS, with the additional advantage of shorter analysis time as no chromatographic separation was required. The capability of phenolics to quantify tea adulterations with chicory was also assessed by partial least squares (PLS) regression, with prediction errors below 10.9 and 14.8% for FIA-MS/MS and LC-MS/MS, respectively, in the determination of adulterant levels. Thus, both methodologies demonstrated to be feasible for assessing tea authentication issues.

1. Introduction

Food fraudulent practices are rising and are considered nowadays one of the main issues within the agri-food chain. Although EU legislation does not provide a specific definition of fraud, Commission Regulation EU 2019/1715 [1] defines a "fraud notification" in the Rapid Alert System for Food and Feed (RASFF) and, therefore, indicates the key elements to consider. Thus, agri-food fraud is "a non-compliance concerning any suspected intentional action by businesses or individuals, for the purpose of deceiving purchasers and gaining undue advantage therefrom, in violation of the rules referred to in Article 1(2) of Regulation EU 2017/625 [2]". Although fraud has mainly financial consequences, these intentional infringements of the EU agri-food chain legislation may hinder the functioning of the EU Single Market and may also constitute a risk to human, animal, or plant health. Fraudulent practices can happen at any stage of production, processing, and trade, and the victim can be the final consumer as well as a business operator.

Tea is a worldwide consumed beverage made by pouring hot (or boiling) water over fresh or cured leaves of the plant *Camelia sinensis*, native to China and East Asia; nowadays, its production has expanded to Europe, among other regions. Drinking tea is appreciated by society because of its characteristic flavor and aroma, as well as its health-

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beneficial attributes such as antioxidant, anti-inflammatory, anti-hypertensive, antimicrobial, neuroprotective, and anticarcinogenic properties, among others [3–8]. Besides, tea contains a wide variety of bioactive substances, among them polyphenols, being one of the main sources of these secondary metabolites and the main responsible for tea antioxidant activity [9,10]. Different tea varieties are available that differ mainly in the fermentation processes involved. Among them, black and green teas are the most traditional ones, accounting for 78 % and 22 % of the world's production, respectively [11]. Black is a fully fermented and oxidized tea, on the contrary green tea is produced from dried tea leaves without any fermentation. Another no fermented tea is the white one. While green tea is steamed or pan-fired immediately after harvest to stop its oxidation, white tea is commonly packaged after drying with minimum pre-processing, being, for this reason, the most expensive and appreciated by consumers. Additionally, white tea is usually produced from the very first tips and buds of the tea plant. Red and oolong teas are also fermented tea varieties. A specific variant of the tea plant (*Camellia sinensis* var. assamica), which is produced only in China (Yunnan region), is employed for Red (Pu-erh) teas after being processed in humid conditions, allowing composting by bacteria activity. In contrast, oolong tea fermentation is controlled to limit its oxidation to 10–70 %.

Tea is one of the drinks more susceptible to fraudulent practices. This is mainly due to its high consumption and the rising prices, as for many other commodities, caused by geopolitical instability and the dramatic changes in climate conditions affecting tea grown in East Africa and India [12–14]. Tea authenticity involves several issues including geographical origin production and adulteration with other products such as leather flakes, dyes, coal tar, sand, cereal starch, legume husks, lower-quality or exhausted tea leaves, and other plant materials such as chicory [15–17]. Chicory (*Cichorium intybus*) is a perennial herbaceous plant worldwide cultivated as animal feed and supplement (if declared) in coffee and tea beverages. However, several authors have reported the use of chicory as an adulterant of coffee and tea [16,18]. Hence, its use in tea must be completely prohibited when non-declared due to possible adverse health effects [19]. It should be commented that, although initially, the appearance of chicory is quite different from that of tea, depending on the way of commercialization of tea samples, once tea and chicory are grinded and mixed together, it will become difficult, especially for consumers, to visually detect if a tea sample has been adulterated with chicory. This also happens with other common tea adulterants such as sand, cereal starch, legume husks, etc. This fact is even aggravated when the tea is commercialized, for example, in tea bags, where normally the users do not visualize directly the internal content.

Tea authentication issues have been addressed by both targeted and non-targeted approaches in combination with multivariate chemometric methods. Regarding targeted approaches, the elemental composition determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) or mass spectrometry (ICP-MS) [20–22], the volatilome (volatile profiling) obtained by gas chromatography (GC) [23], and the determination of polyphenols by high-performance liquid chromatography with ultraviolet (HPLC-UV) detection [24,25] have been reported for. For example, we recently proposed the determination of seventeen polyphenolic compounds by HPLC-UV to classify and authenticate tea and chicory samples [25]. Fingerprinting approaches, based on monitoring instrumental responses without focusing on any specific chemical, are also widely employed in tea authentication [26–31]. For instance, HPLC-UV and HPLC with fluorescence detection (HPLC-FLD) fingerprinting in combination with partial least squares-discriminant analysis (PLS-DA) was applied to the characterization and authentication of tea and chicory [30]. High-throughput flow injection analysis-mass spectrometry (FIA-MS) fingerprinting was also able to discriminate chicory from different tea varieties with acceptable classification rates, but the reported prediction errors for chicory adulterant determination were still too high when black and green tea samples were employed [31].

In this work, the potential of using polyphenolic profiling by high-throughput FIA-MS/MS and LC-MS/MS to assess tea authenticity was evaluated. For that purpose, fifty-five polyphenolic compounds belonging to different families were monitored in 100 tea samples (including black, green, red, oolong, and white varieties) and 20 chicory samples. The obtained polyphenolic profiles were then employed as chemical descriptors for sample discrimination and classification by PLS-DA. Finally, the capability of the proposed profiles to detect tea fraudulent practices based on chicory adulteration was also evaluated by partial least squares (PLS) regression. The proposed research aimed to evaluate the feasibility of FIA-MS/MS as a high-throughput screening methodology for the authentication and classification of tea/chicory samples, to identify potential suspected samples, and LC-MS/MS as a confirmatory analytical methodology for those suspected cases to be adulterated with chicory.

2. Experimental

2.1. Chemicals

Fifty-five polyphenolic compounds belonging to different families (phenolic acids, benzoic acids, cinnamic acids, phenolic aldehydes, phenolic terpenes, flavones, flavanols, proanthocyanidins, and stilbenes), all of them of analytical grade, were used (chemical structures, molecular formulas, molecular weights, and CAS numbers are indicated in Table S1 of supplementary material). Caffeic, gallic, ellagic, quinic, *p*-coumaric, *trans*-coutaric, sinapic, caftaric, vanillic, ferulic, syringic, 3,4-di-O-caffeoylquinic, 4,5-di-O-caffeoylquinic, 4-hydroxybenzoic, 2,5-dihydroxybenzoic, and 3,4-dihydroxybenzoic acids, ethyl gallate, (−)-epicatechin, astilbin, catechol, 3-methylcatechol, 4-methylcatechol, 4-ethylcatechol, polydatin, triacetin, galangin, pinobanksin, oleuropein, 4-vinylguaiacol, guaiacol, pyrogallol, resveratrol, and apigenin were purchased from Sigma-Aldrich (St. Louis, MO, USA); (+)-catechin, 3-hydroxytyrosol, myricetin, and rutin from TCI (Tokyo, Japan); diosmin from Alfa Aesar (Kandel, Germany); (−)-epigallocatechin, naringenin, and luteolin from Biosynth Carbosynth (Berkshire, United Kingdom); quercetin, chlorogenic acid, and chrysin from Merck (Darmstadt, Germany); hesperidin and hesperetin from Glentham (Wiltshire, United Kingdom); naringin from TargetMol (Boston, MA, USA); pinocembrin from Thermo Fisher Scientific (Waltham, Massachusetts, USA); and quercetin-3-glucoside, kaemferol, procyanidin A1, B2, and C1, *trans*-cinnamic acid, and vanillin from Fluka (Madrid, Spain). Stock standard solutions of all the studied polyphenolic compounds (ca. 1000 mg L^{-1}) were prepared in dimethyl sulfoxide (DMSO) or methanol (depending on the compound). Working solutions were then obtained by dilution from the stock standard solution with Milli-Q water.

Methanol (Chromosolv^{TM} for HPLC, ≥ 99.9 %), acetonitrile (UHPLC supergradient ACS quality), and DMSO (Reg. Ph. Eur. for analysis, ACS) were obtained from PanReac AppliChem (Barcelona, Spain); and formic acid (98–100 % purity) from Sigma-Aldrich. Water (Milli-Q) was purified with an Elix 3 coupled to a Milli-Q system from Millipore Corporation (Bedford, MA, USA), and filtered through a 0.22 μm nylon membrane.

For the preparation of tea and chicory extracts, a mineral water commercially available from Eroski (Elorrio, Spain) was employed.

2.2. Samples and sample treatment

One hundred tea samples consisting of white, black, green, red, and oolong varieties (20 samples/variety) and twenty chicory samples were employed. All samples were obtained from different local markets in Barcelona (Spain). Detailed information regarding the commercial brands, countries of production, and the number of lots for each tea and chicory is summarized in Table S2 (supplementary material).

Tea and chicory extracts were obtained as previously described [25]. Briefly, ca. 0.5 g of sample (tea, chicory, or blended samples) were weighed into a 50 mL PTFE centrifuge tube (Serviquimia, Barcelona, Spain), and bioactive substances were extracted with 25 mL of boiling mineral water using a vortex (Stuart, Stone, United Kingdom) by vigorously shaking for 1 min. The mixture was centrifuged for 5 min at 3,500 rpm with a Rotanta 460 RS centrifuge (Hettich, Tuttlingen, Germany) and the supernatant separated. Extracts were filtered through 0.45 μm syringe nylon membrane filters into 2 mL glass injection vials (discarding the first mL) and maintained at 4 °C in a refrigerator until analysis by FIA-MS/MS or LC-MS/MS.

A quality control (QC) sample was also prepared by mixing 50 μL of each aqueous sample extract for the evaluation of the methodology reproducibility as well as the robustness of the chemometric results.

2.3. Instrumentation

Flow-injection analysis-mass spectrometry/mass spectrometry (FIA-MS/MS) was performed by employing an Agilent 1100 Series HPLC instrument (Waldbronn, Germany) coupled to an AB Sciex 4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer (Framingham, MA, USA). 10 μL of sample extract were injected on a 1:1 (v/v) mixture of water acidified with 0.1 % formic acid (v/v) and acetonitrile, employed as the carrier and pumped at a flow rate of 0.15 mL min^{-1}. QTrap parameters were as follows: ion spray voltage: −2500 (negative polarity); source temperature: 400 °C; Curtain gas: N_2 at 10 arbitrary units (a.u.); ion source gas 1 and 2: N_2 at 50 a.u. Polyphenolic and phenolic acid compounds were analyzed in negative electrospray ionization (ESI), and acquisition was performed in multiple reaction monitoring (MRM) mode. The MS/MS parameters such as declustering potential (DP), collision energy (CE), and the collision cell exit potential (CXP), as well as the selected reaction monitoring (SRM) transitions (precursor and product ions) are summarized in Table S3 (supplementary material). The total analysis time for the FIA-MS/MS experiments was 1.5 min.

The same HPLC and mass spectrometry systems described above were employed for LC-MS/MS. The chromatographic separation was accomplished on a Kinetex® C18 porous-shell reversed-phase column (100 × 4.6 mm i.d., 2.6 μm partially porous particle size) from Phenomenex (Torrance, CA, USA) by gradient elution using 0.1 % (v/v) formic acid in water (solvent A) and acetonitrile (solvent B) as mobile phase components at a flow rate of 0.8 mL min^{-1}. The elution program applied was: 0–1 min, linear gradient from 5 % to 10 % solvent B; 1–4 min, linear gradient from 10 to 16 % solvent B; 4–8 min, isocratic elution at 16 % solvent B; 8–8.5 min, linear gradient from 16 % to 25 % solvent B; 8.5–13.5 min, linear gradient from 25 % to 60 % solvent B; 13.5–16 min, linear gradient from 60 % to 100 % solvent B; 16–16.5, isocratic elution at 100 % solvent B; 16.5–16.6, back to initial conditions at 5 % solvent B; and 16.6–22 min, isocratic elution at 5 % solvent B for column re-equilibration. The column was kept at room temperature, and the injection volume was 5 μL. LC-MS/MS experiments were performed in negative electrospray ionization mode and the acquisition in multiple reaction monitoring (MRM) mode. The same ion source and MS acquisition parameters as in FIA-MS/MS experiments were employed. The total LC-MS/MS analysis time was 22 min.

2.4. Data analysis

All the samples were analyzed randomly with the proposed methodologies. In the two cases, a QC, a polyphenolic standard mixture (ca. 10 mg L^{-1}), and a blank (mineral water) were injected every 10 samples. The peak areas in FIA-MS/MS and LC-MS/MS for all the detected polyphenolics (MRM transitions) in the analyzed samples were recorded for profiling. SOLO 8.6 software (Eigenvector Research, Manson, WA, USA) was used for PCA, PLS-DA, and PLS regression. The theoretical background of these methodologies can be found in reference [32]. In all cases, an X-data matrix of response variables was built with the peak area of the detected polyphenolic compounds. In addition, a Y-data matrix defined each sample class (tea variety or chicory) in PLS-DA and the chicory adulterant percentages in PLS regression. The X-data were autoscaled to provide the same weight to each variable, aiming to remove magnitude and amplitude scale differences. The number of latent variables (LVs) in PLS-DA and PLS was estimated from the first significant minimum point of the cross-validation (CV) error from a Venetian blind approach.

To validate the classification performance in PLS-DA, 60 % of the samples (randomly selected) were used for calibration while the remaining samples (the other 40 %) were employed for prediction purposes. In addition, the predictive performances of the classification models were evaluated with sensitivity (capacity to detect true positives), specificity (capacity to detect true negatives), and accuracy, all in percentage. Briefly, sensitivity was calculated as TP/(TP+FN), with TP being the number of positive samples correctly assigned to the corresponding class and FN the number of false negatives incorrectly assigned as not belonging to the class. Specificity was calculated as TN/(TN+FP), with TN being the number of negative samples correctly assigned (i.e., not belonging to the corresponding class) and FP the number of false positives incorrectly assigned to the corresponding class. Finally, accuracy (expressed as the classification error), was calculated as (TP+TN)/TS, being TS the total number of samples.

Five adulteration cases, consisting of each tea variety adulterated with chicory, were evaluated by PLS regression. With this aim, adulteration levels of 0 (pure tea), 20, 40, 60, 70, 80, and 100 % (pure chicory) were employed for calibration, and 15, 25, 50, 75, and 85 % for validation and prediction. All the adulteration levels were prepared in quintuplicate. It should be mentioned that five pooled tea samples were prepared for each tea variety by mixing 10 different samples (different origins) of the same variety, and then each pooled tea sample was adulterated with a different chicory sample. Therefore, the five replicates for each adulteration level were obtained using different combinations of tea/chicory samples, as indicated in Table S4 (supplementary material). Besides, an additional 50 % adulteration level was prepared as QC extract. Within each adulteration case, all the blended samples were randomly analyzed with the proposed methodologies, and the QC, a polyphenolic standard mixture, and a blank (mineral water) were injected every ten samples.

3. Results and discussion

3.1. FIA-MS/MS and LC-MS/MS polyphenolic profiles

In previous publications, we evaluated the suitability of fingerprinting strategies based on HPLC with spectroscopic detection (UV–vis and fluorescence) [30] and FIA-MS [31] for the characterization and authentication of tea and chicory samples. Although acceptable results were observed, full discrimination among the different tea varieties was not accomplished, and prediction errors were higher than 20 % in some cases. As polyphenols are among the most characteristic bioactive substances found in tea, the targeted polyphenolic profiling seems reasonable to assess tea and chicory characterization and authentication issues. In this sense, a method based on profiling seventeen polyphenolic compounds by HPLC-UV improved the results, but still relatively high classification errors by cross-validation (around 20 %) were reported for some tea varieties, especially for black tea [25]. In this work, the potential of FIA-MS/MS as a high-throughput screening methodology, and LC-MS/MS as a possible confirmatory methodology was evaluated for the characterization, classification, and authentication of tea and chicory samples. For that purpose, fifty-five polyphenolic compounds belonging to different families were targeted in MRM mode to take advantage of the sensitivity and selectivity performance of QTrap instruments.

Sample extracts were randomly analyzed with both methodologies,

and the polyphenolic peak areas were then employed as sample chemical descriptors to assess tea and chicory authentication. As an example, Fig. 1 shows the profile of a green tea sample by (a) FIA-MS/MS and (b) LC-MS/MS. The figure depicts all the detected MRM transitions, as well as the extracted SRM transition for two selected chemicals: quinic acid (191→84) and 4-hydroxybenzoic acid (137→93). In FIA, the direct sample injection (without separation) led to the coelution of all the chemicals detected and generated a broad peak of about 0.5 min. In this case, the characteristic electrospray ion suppression phenomenon may have a more relevant effect on the obtained signals. However, the vaporization efficiency of the TurboIonSpray source of the QTrap instrument is very efficient, which guarantees the perfect vaporization of the carrier solvent (1:1 0.1 % aqueous formic acid:acetonitrile at 0.15 mL min^{-1}) and reduces ion suppression. On the other hand, although sensitivity is obviously reduced, selectivity is not affected thanks to the MRM acquisition mode (Table S3). Chromatographic separation reduces the possibilities of ion suppression and increases selectivity (Fig. 1b). In addition, better sensitivity is accomplished as can be observed, for example, from the signals of the two chemicals depicted in Fig. 1 (please note that 5 μL and 10 μL were injected in LC-MS/MS and FIA-MS/MS, respectively). The main disadvantage of the LC-MS/MS method is the total run time per sample (22 min) compared to 1.5 min in FIA.

3.2. Exploratory PCA study

Peak areas obtained by FIA-MS/MS for the targeted polyphenols were employed to build the X-data matrix for exploratory PCA to assess the natural behavior patterns of the different sample types and the data repeatability and robustness through the examination of QCs behavior. The resulting data matrix had a dimension of 131 × 36 (tea + chicory + QC samples × detected polyphenolic band areas by FIA-MS/MS). The scores plot of PC1 vs. PC2, which retained 44.25 % of variance, is depicted in Fig. 2a. As can be seen, QCs were perfectly grouped close to the center of the plot, demonstrating that the proposed FIA-MS/MS method is reproducible and ensuring the robustness of the obtained chemometric results. Besides, chicory samples are located at the left-bottom (negative values for both PC1 and PC2), red tea samples more grouped with negative PC1 and positive PC2 values, oolong and black teas in the center, green teas primarily on the right with positive PC1 values, and white teas partly overlapping green teas, but more spread out over PC1. In any case, chicory samples were always clearly distinguished from all the tea varieties.

Regarding LC-MS/MS experiments, peak area of polyphenols were also employed to build the X-data matrix for PCA. From the list of 55 polyphenolic and phenolic acids explored, 46 were detected in the samples (ten more polyphenols than with FIA-MS/MS, including ethyl gallate, astilbin, caftaric acid, diosmin, hesperetin, naringin, catechol, procyanidin B2, 3-hydroxytyrosol, and pyrogallol). The score plot of PC1 vs. PC2 (retaining 46.07 % of variance) is shown in Fig. 2b. In this case, QCs are not perfectly clustered, probably due to sensitivity decay throughout the analysis produced by the fact that the ionization source gets dirty over time. This fact was not observed in FIA-MS/MS since the fast measurement process, ca. 15-fold shorter run analysis time per sample. As a consequence, the obtained data may be influenced by signal drifts through the sample sequence. In this case, sample data were corrected using QC data as a reference since it was injected repeatedly in the study (every 10 samples). For that purpose, signals were normalized by dividing the peak area obtained for each polyphenol on each sample by the corresponding to the closest injected QC (accordingly, the normalized signal of each compound in the QC was 1). The new data matrix was subjected to PCA, and the score plot of PC1 vs. PC2 is depicted in Fig. 2c. Again, chicory samples perfectly discriminated from tea extracts. Comparing tea classes, red tea samples are also perfectly discriminated from the other groups, being located at the top-left area of the plot (exhibiting negative PC1 and positive PC2 values), and showing a better clustering in comparison to the PCA model without QC correction (Fig. 2b). Regarding the other tea samples, oolong and green ones displayed mainly negative PC2 values, being located in the center (oolong) and right area (green) of the plot (positive PC1 values), in contrast to black teas mainly located at the top area of the plot; white teas are widely dispersed through the plot, overlapping with oolong, green and black samples.

3.3. PLS-DA study

X-data matrices (without considering the QCs) were subjected to supervised PLS-DA to assess sample classification. In this line, a Y-data matrix defining the sample classes (black, green, oolong, red, and white teas, and chicory) was used. In case of LC-MS/MS normalized X-data matrix is used.

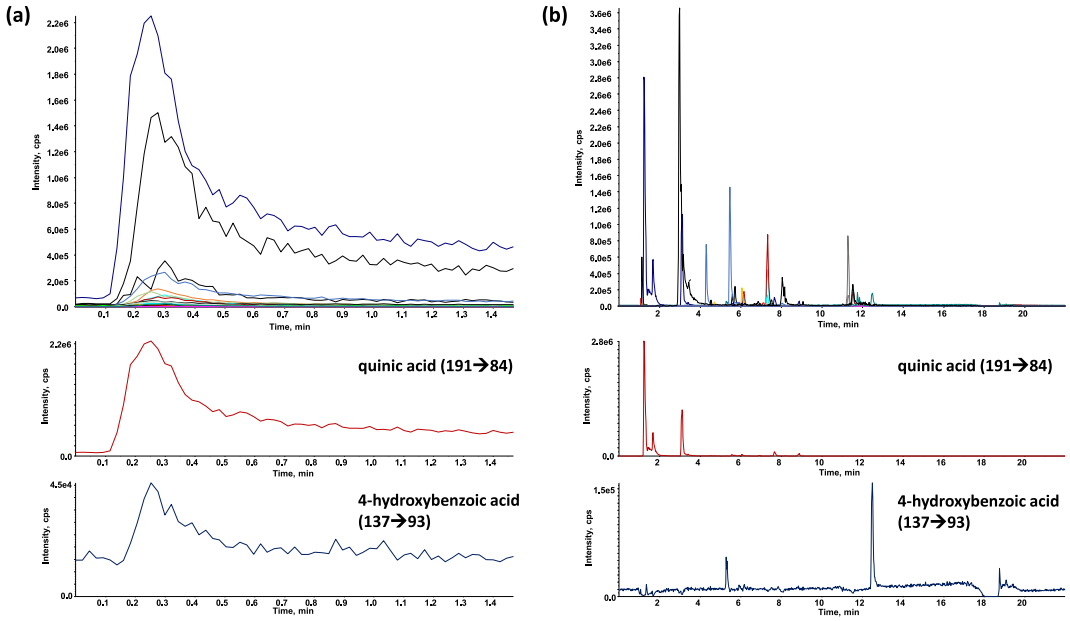

Fig. 1. Polyphenolic and phenolic acid profiles (showing all the detected MRM transitions), and the extracted MRM transitions for quinic acid (191→84) and 4-hydroxybenzoic acid (137→93), obtained by (a) FIA-MS/MS and (b) LC-MS/MS.

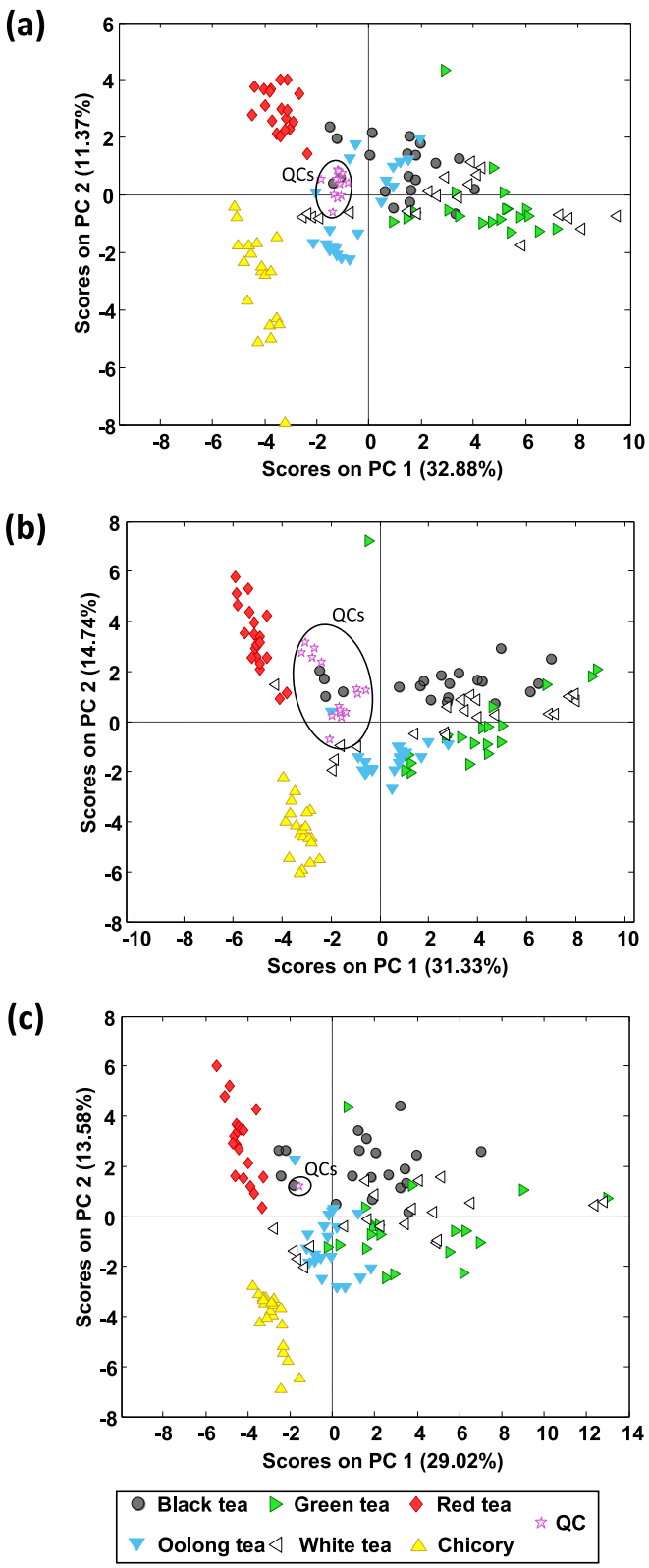

Fig. 2. Exploratory PCA scores plot of PC1 vs. PC2 employing polyphenolic profiles obtained by (a) FIA-MS/MS, (b) LC-MS/MS, and (c) LC-MS/MS (after QC correction).

Fig. 3 shows the obtained PLS-DA (a) scores and (b) loadings plots of LV1 vs. LV2 and LV1 vs. LV3 from the polyphenolic profiles obtained by FIA-MS/MS (6 LVs were employed to build the PLS-DA model). Besides, Table 1 summarizes the sensitivity, specificity, and accuracy (classification prediction error) for calibration and cross-validation obtained with the multiclass prediction PLS-DA model. As can be seen in the scores plots of Fig. 3a, samples are clustered according to their sample groups, with chicory and red tea samples perfectly discriminated from the other sample groups, and differentiated themselves by means of LV2. For LV1 vs. LV3, better discrimination for the other samples groups is observed, with oolong and green samples separated from the other groups, and differentiating themselves through LV1 (showing negative LV3 values), while black and white samples are overlapped at positive LV3 values. In any case, very good multiclass PLS-DA performance was attained by FIA-MS/MS polyphenolic profiling (Table 1), with sensitivity values higher than 95 % and 90 %, and specificity values higher than 90 % and 88.9 %, for calibration and cross-validation, respectively. Very good PLS-DA accuracy was also accomplished, with calibration and cross-validation classification errors below 7.0 % and 10.5 %, respectively.

The PLS-DA loading plots (Fig. 3b) reveal the polyphenols and phenolic acids contributing to the sample distribution and show markers of each particular sample group, among the 36 compounds annotated by FIA-MS/MS. For example, oleuropein, caffeic acid, and 3,4-di-O-caffeoylquinic acid are up-expressed in chicory samples. For red tea, syringic, 2,5-dihydroxybenzoic, 3,4-dihydroxybenzoic acids, 4-methylcatechol, and 4-ethycatechol seem to be more involved. Polydatin, tricetin, myricetin, and epigallocatechin are, in contrast, more remarkable for green tea extracts. Other compounds such as gallic, *trans*-cinnamic, chlorogenic, quinic, and 4-hydroxybenzoic acids, as well as hesperidin and rutin, tend to be more characteristic of both black and white tea samples.

The classification capability of the polyphenolic profiles by FIA-MS/MS to distinguish tea against chicory was evaluated by a paired PLS-DA model with 60 % of the samples (randomly selected) for training, and the remaining 40 % for validation and prediction. Results summarized in Fig. S1a (supplementary material) show that 100 % of the calibration samples were perfectly classified, and only 1 chicory sample was not correctly predicted, resulting in a classification rate of 97.9 %.

Regarding the LC-MS/MS experiments, Fig. 4 shows the PLS-DA score (a) and loading (b) plots of LV1 vs. LV2 using the corresponding polyphenolic profiles, and the multiclass PLS-DA performance (sensitivity, specificity, and accuracy) is summarized in Table 2. Very acceptable sample discrimination was also accomplished, with chicory samples grouped at the top-left area, perfectly discriminated from the other sample groups. Similarly, red tea samples are located at the bottom-left area of the plot and separated from the other classes. The other tea types appeared overlapped in the center to right area of the plot. In any case, the multiclass PLS-DA performance accomplished is also very acceptable (Table 2), with sensitivity values of 100 % (only 95 % for white tea) and higher than 85 % for calibration and cross-validation, respectively. Specificity values higher than 95 % and 86 % for calibration and cross-validation, respectively, were observed. The accuracy was also notable, with classification errors below 4.0 % for calibration and 7.5 % for cross-validation, with the only exception of white tea (classification error 14.5 %).

Regarding the polyphenolics and phenolic acids contributing on the sample discrimination, the loading plot study reveals that oleuropein, and vanillic, *trans*-coutaric, 3,4-di-O-caffeoylquinic, and caffeic acids can be considered as chicory markers, some of them also annotated by FIA-MS/MS. In contrast, syringic, 2,5-dihydroxybenzoic, and 3,4-dihydroxybenzoic acids, and epigallocatechin, hesperetin, and catechol are overexpressed in red tea, again some of them also annotated by FIA-MS/MS.

The classification of tea vs chicory based on LC-MS/MS polyphenolic profiles was also evaluated by a paired PLS-DA using 60 % of the samples (randomly selected) for training, and the remaining 40 % for prediction. In this case, all the samples were correctly classified for both calibration and prediction (see Fig. S1b in supplementary material), showing 100 % classification rates. This better performance compared to FIA-MS-MS is probably due to the reversed-phase chromatographic separation in LC-

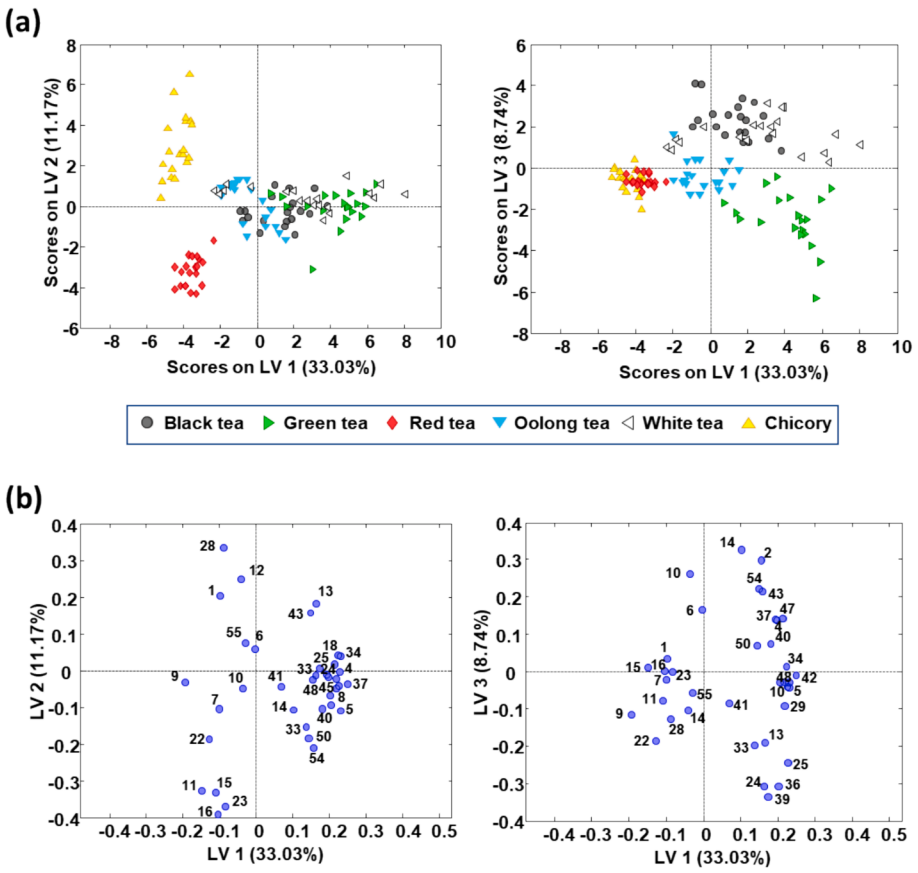

Fig. 3. PLS-DA scores (a) and loadings (b) plots of LV1 vs LV2 and LV1 vs. LV3 employing polyphenolic profiles obtained by FIA-MS/MS as sample chemical descriptors. For simplification, some phenolics are not indicated in the loading plots. Phenolics in (b) are number-labelled as in Table S1 (supplementary material).

Table 1
PLS-DA calibration and cross-validation multiclass prediction when employing polyphenolic profiles obtained by FIA-MS/MS as sample chemical descriptors.

Sample class	Sensitivity (%)		Specificity (%)		Accuracy(classification error, %)	
	Calibration	Cross-validation	Calibration	Cross-validation	Calibration	Cross-validation
Black tea	95	90	93.9	92.9	5.5	8.5
Green tea	100	100	99	96	0.5	2.0
Oolong tea	95	95	94.9	96	5.0	4.5
Red tea	100	100	99	98	0.5	1.0
White tea	95	90	90.9	88.9	7.0	10.5
Chicory	100	100	100	100	0	0

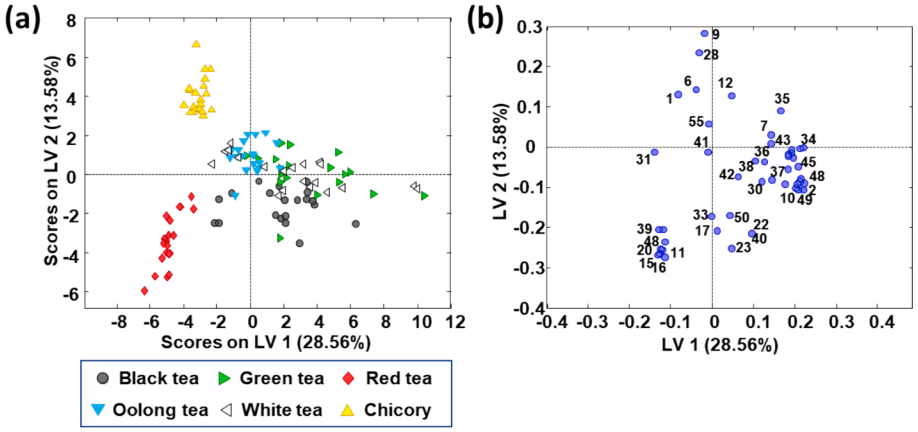

Fig. 4. PLS-DA scores plots (a) and loadings plots (b) of LV1 vs LV2 employing polyphenolic profiles obtained by LC-MS/MS as sample chemical descriptors. For simplification, some phenolics are not indicated in the loading plots. Phenolics in (b) are number-labelled as in Table S1 (supplementary material).

Table 2
PLS-DA calibration and cross-validation multiclass predictions when employing polyphenolic profiles obtained by LC-MS/MS as sample chemical descriptors.

Sample class	Sensitivity (%)		Specificity (%)		Accuracy(classification error, %)	
	Calibration	Cross-validation	Calibration	Cross-validation	Calibration	Cross-validation
Black tea	100	95	97	90	1.5	7.5
Green tea	100	95	98	91	1.0	7.0
Oolong tea	100	100	95	94	2.5	3.0
Red tea	100	100	98	98	1.0	1.0
White tea	95	85	97	86	4.0	14.5
Chicory	100	100	100	99	0	0.5

MS/MS,

Both, FIA-MS/MS and LC-MS/MS polyphenolic profiles have shown to be excellent sample chemical descriptors to assess the classification and authentication of different tea varieties and chicory. Excellent multiclass PLS-DA calibration and cross-validation performances were attained with both methodologies. For example, when focusing on the accuracy, FIA-MS/MS results are much better than those accomplished by LC-MS/MS, except for black and oolong tea with similar errors for the two methods (Table 1 and 2). This aspect, and the fact that the FIA is faster than LC, allows us to propose FIA-MS/MS as a great methodology for tea authentication. Furthermore, the results obtained with both targeted methodologies based on polyphenolic profiles are much better than those previously described using 17 polyphenolic compounds determined by HPLC-UV[25]. In that application, although similar accuracies to those reported in the present contribution were achieved for most of the analyzed classes, the classification errors by cross-validation for black tea were around 20 %. In any case, it must be highlighted that the proposed targeted polyphenolic methodologies clearly surpass the performances from non-targeted HPLC-UV-FLD, LC-MS, and FIA-MS fingerprinting methodologies [30,31]. Thus, both FIA-MS/MS and LC-MS/MS approaches can be proposed to address tea and chicory classification and authentication, with the advantage of FIA-MS/MS for high-throughput screening to reduce the number of suspicious adulterated samples to be submitted to confirmatory methods.

Although the aim of the present contribution was not to quantify the content of polyphenols in the tea and chicory analyzed samples but to classify and authenticate them based on the polyphenolic signal profiling obtained by either LC-MS/MS or FIA-MS/MS as sample chemical descriptors, these signals can also be employed to show the relative abundance of each detected polyphenolic compound on the different sample groups. For this purpose, Fig. S2 (supplementary material) shows the corresponding heatmap reporting the average levels of detected polyphenols by LC-MS/MS in the analyzed tea and chicory sample categories. As can be seen, gallic acid seems to be the compound showing the highest levels in the analyzed samples, with a higher contribution in black tea, followed by white, green, oolong, and red tea samples, and it was not detected in chicory. A similar trend was observed for quinic acid, being very abundant in black tea, followed by oolong, green, white, and red tea samples, and again not detected in chicory. Other polyphenols that also showed relatively high abundance in some of the tea sample groups are (+)-catechin, (−)-epicatechin, rutin, 4-hydroxybenzoic acid, chlorogenic acid, and 3,4-dihydroxybenzoic acid. In general, chicory samples are characterized by having few polyphenols, and occurring at low contents, compared to tea samples. Other polyphenolic compounds were also detected at low levels in some of the analyzed tea samples, such as ferulic acid, vanillic acid, ethyl gallate, myricetin, syringic acid, astilbin, diosmin, naringenin, vanillin, epigallocatechin, 4-ethylcatechol, 4-methylcatecol, 3-hydroxytyrosol, kaempferol, apigenin, polydatin, and sinapic acid, among others. However, despite the low levels of these polyphenolic compounds, their mere presence in some tea groups allows them to be discriminating compounds for the authentication of such samples, as has been previously described.

3.4. Detection and quantitation of tea frauds with chicory

The potential and effectiveness of the polyphenolic profiles to detect tea frauds and quantify tea adulteration levels with chicory was studied by PLS. Five adulteration cases, based on each tea variety being adulterated with chicory, were designed. For PLS regression, two sets of blended tea-chicory mixtures were prepared for training and validation/prediction. The training one included the adulteration levels of 0 % (pure tea), 20, 40, 60, 80, and 100 % (pure chicory). The second set, including the adulteration levels of 15, 25, 50, 75, and 85 %, was used for prediction and validation purposes. All the levels were prepared in quintuplicate using 5 different teas and 3 different chicory (see Table S4, supplementary material) to introduce sample variability on the design. Besides, an additional adulterated sample at 50 % was employed as QC to assess the reproducibility and robustness of the PLS predictions.

First, for each adulteration case, polyphenolic profiles by FIA-MS/MS and LC-MS/MS methodologies were subjected to PCA to evaluate the behavior of the QCs and to see the distribution of the adulteration levels evaluated in the PC1 versus PC2 plot. Then PLS regression was performed. As an example, the results obtained with the black tea adulteration case when employing FIA-MS/MS and LC-MS/MS polyphenolic profiles as sample chemical descriptors are shown in Fig. 5. PLS performance accomplished with the five tea adulteration cases is summarized in Table S5 and S6 (supplementary material) for FIA-MS/MS and LC-MS/MS, respectively. As can be seen in the PCA scores plots of Fig. 5, QCs appeared perfectly clustered showing good reproducibility and robustness of the PLS chemometric results, and similar results were also achieved with the other tea adulterations cases studied. Besides, samples tend to be distributed according to the level of chicory adulteration, with pure chicory and tea located in opposite areas of the plots through PC1. Excellent performance was accomplished for the detection and quantitation of tea frauds with chicory, with R^2 values higher than 0.932 and 0.979 for FIA-MS/MS and LC-MS/MS, respectively, for the scatter plots of measured vs. predicted. In the case of FIA-MS/MS, PLS calibration, cross-validation, and prediction errors were lower than 8.9 %, 15.6 %, and 10.9 %, respectively. In contrast, with LC-MS/MS polyphenolic profiles, errors below 5.0 %, 10.5 %, and 14.8 % for calibration, cross-validation and prediction errors, respectively, were obtained. With the exception of red and oolong tea, PLS figures of merit for FIA-MS/MS were much better than those attained by LC-MS/MS. Compared to other approaches, PLS results from FIA-MS/MS profiling surpass those previously obtained by FIA-MS fingerprinting (in both negative and positive ionization modes), where black and green tea varieties adulterated with chicory reached prediction errors in the range 7.8–16.4 % and 11.5–12.8 %, respectively [31].

Therefore, both FIA-MS/MS and LC-MS/MS polyphenolic profiles can be proposed as suitable methodologies to detect and quantify chicory adulterant levels from tea fraudulent practices.

4. Conclusions

High-throughput FIA-MS/MS profiling of 55 polyphenolic compounds have shown to be an excellent targeted methodology to assess the characterization, classification, and authentication of tea samples from different varieties, and their discrimination against chicory. In

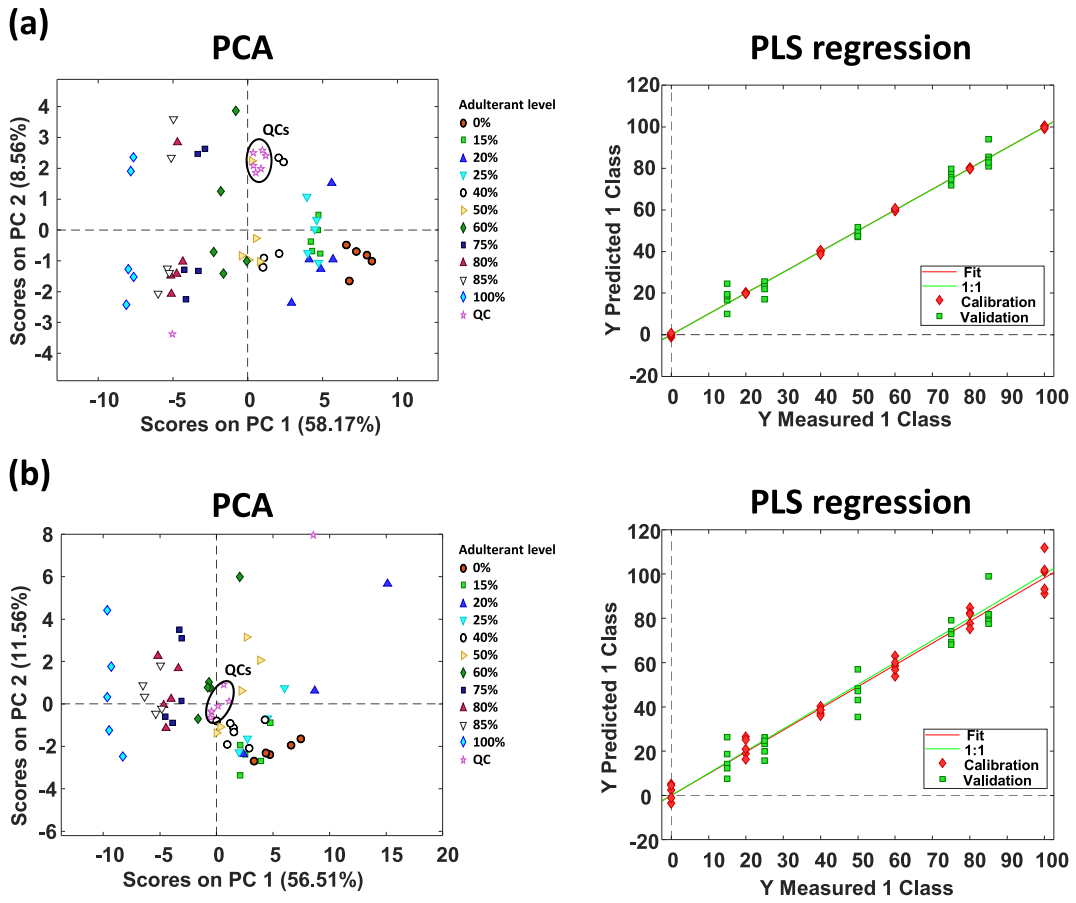

Fig. 5. Black tea adulteration with chicory case. PCA (PC1 vs. PC2) results showing the distribution of both calibration and prediction samples according to the chicory adulterant level and PLS results showing the scatter plot of measured vs. predicted percentages of chicory adulterant when (a) FIA-MS/MS and (b) LC-MS/MS polyphenolic profiles were employed as sample chemical descriptors.

general, FIA-MS/MS data provides better PLS-DA results than LC-MS/MS counterparts, with the additional advantage of fast analysis (run time approximately fifteen times lower). PLS-DA sensitivity and specificity from FIA-MS/MS are higher than 90.9 % and 88.9 % for both calibration and cross-validation, respectively, and the accuracy is excellent, with classification error below 7.0 % and 10.5 % for calibration and cross-validation, respectively. The overall performance is also better than the one previously reported with HPLC-UV, HPLC-FLD and FIA-MS fingerprinting methodologies.

PLS is applied to five adulteration cases based on teas of each variety adulterated with chicory. Quantification results are excellent, with overall calibration, cross-validation, and prediction errors below 8.9, 15.6 and 10.9 %, respectively. These values are better than those from previous reports with fingerprinting approaches, specially regarding prediction capabilities.

In conclusion, FIA-MS/MS consists of a high-throughput, simple, cost-effective, and reliable screening methodology. The resulting polyphenolic profiles have shown an excellent potential to be used as sample chemical descriptors to classify and authenticate tea and chicory samples, as well as to prevent fraudulent practices when using chicory as tea adulterant. FIA-MS/MS results may even be improved if FIA coupled to high-resolution mass spectrometry (FIA-HRMS) is employed, considering that the higher resolution and accurate mass measurements accomplished with HRMS instruments would complement the lack of chromatographic separation characteristic of FIA methodologies. However, this will clearly increase the cost of the analysis per sample, a fact that also needs to be considered depending on the food control laboratory capabilities.

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CRediT authorship contribution statement

Thom Romers: Writing – review & editing, Writing – original draft, Validation, Software, Investigation. **Sònia Sentellas:** Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. **Javier Saurina:** Writing – review & editing, Software, Investigation, Funding acquisition. **Oscar Núñez:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.111723.

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