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Abstract

Powerful data pretreatment strategies inspired from the field of metabolomics were adapted to chemical food safety context to enable samples discrimination by multivariate methods based on low abundance ions. A highly automated workflow was produced. The open-source XCMS package was used and efficient data filtration strategies were set up. Data were treated using Independent Components Analysis, and data mining strategies developed to automatically detect and annotate ions of low abundance by coupling blind data exploration strategies with a broad scale database approach. Our method was efficient in discriminating tea samples based on their contamination levels (even at 10 $\mu g.kg^{-1}$) and detecting unexpected impurities in the spiking mix. Several "tracer" contaminants were considered, covering a broad range of physicochemical properties and structural diversity with overall 66% detected and annotated blindly. The methodology was successfully applied to a data set exhibiting only 3 "tracer" contaminants (at 50 $\mu g.kg^{-1}$) and more product diversity.

Keywords: Independent Components Analysis; XCMS; ToF; Chemical food safety; Non-targeted approaches; Unexpected contaminants

1. INTRODUCTION

In recent years, a new field of food research called "Foodomics", defined as "a discipline that studies the food and nutrition domains through the application of omics technologies", has emerged (Cifuentes, 2009). Even though foodomics was first intended for the study of major constituents of food products, related for instance to their authenticity (Ortea et al., 2012), its potential contribution to trace analysis for chemical food safety assessment has also been raised (Antignac et al., 2011). However, chemical food safety assessment faces several challenges since food matrices are highly complex, chemical contaminants are numerous and diverse (pesticides, mycotoxins, process-induced toxicants or migrants from packaging), and often found at trace levels (down to $\mu g.kg^{-1}$) (Antignac et al., 2011). Classical targeted analysis strategies are limited since unexpected or unknown contaminants remain non-detected (Tengstrand, Rosén, Hellenäs, & Åberg, 2013). Therefore, there is a rising interest (and demand) for developing untargeted (also referred as "non-targeted") analyzes of contaminants in food products using a relevant instrumental / analytical strategy combination, as pointed out by numerous reviews (Antignac et al., 2011; Castro-Puyana, Pérez-Míguez, Montero, & Herrero, 2017; Knolhoff & Croley, 2016; Lehotay, Sapozhnikova, & Mol, 2015). There is a consensus on the fact that hyphenated techniques (especially high resolution instrumentation operated in full-scan mode like Ultra-High Pressure Liquid Chromatography coupled to High Resolution Mass Spectrometry, UHPLC-HRMS) are key technologies for this application thanks to their sensitivity and broad analytical scope, as well as the formula information they provide on potential contaminants (Antignac et al., 2011; Castro-Puyana et al., 2017). Our previous study combining generic extraction and UHPLC-HRMS showed its efficiency on analysis of a wide variety of contaminants with a large range of physicochemical properties (Cladière, Delaporte, Le Roux, & Camel, 2018).

Among new analytical strategies proposed for global chemical food safety assessment, suspect-screening approaches (Gómez-Ramos, García-Valcárcel, Tadeo, Fernández-Alba, & Hernando, 2016; Gosetti, Mazzucco, Gennaro, & Marengo, 2016) and relevant chemical patterns detection using data mining tools (Cotton et al., 2014) show interesting performances in terms of sensitivity. They do not require any initial analysis of the non-contaminated food product but they both rely on *a priori* hypotheses on the chemical structures of contaminants. On the opposite, untargeted metabolomics-like strategies require the analysis of a reference food product (to compare signals between a control and a suspect group for differences detection),

without any *a priori* hypotheses on the structure of potential contaminants. Hence, only untargeted strategies based on tools from the field of metabolomics might enable the real "blind" detection of unknown or unexpected trace molecules in complex food samples. The main characteristic of such untargeted approaches lies in the generation of a very high number of signals (several thousand for a single sample). Therefore, powerful data analysis strategies must be set up to increase the probability to detect contaminated samples. Early results showed their potential in detecting unexpected compounds in food products (Inoue et al., 2015; Knolhoff, Zweigenbaum, & Croley, 2016; Kunzelmann, Winter, Åberg, Hellenäs, & Rosén, 2018; Tengstrand et al., 2013). However, food matrices studied remain relatively simple (orange juice, milk) with either high levels of contamination (near $mg.kg^{-1}$) (Tengstrand et al., 2013) or low molecular diversity of chemical contaminants (Inoue et al., 2015; Knolhoff et al., 2016). The latest published paper (Kunzelmann et al., 2018) shows promising results in terms of sensitivity (contamination detection down to 25 µg/kg) , but only focused on pesticides. More work is therefore needed to develop such untargeted strategies in the food safety field, especially considering even lower contamination levels (down to 10 $\mu g.kg^{-1}$ as frequently required by the European regulation) and a wider contaminants diversity (including migrants from packaging and process-induced toxicants). To that end, the method proposed here relies on the combination of three tools to take full advantage of UHPLC-HRMS data: (i) data filtration based on univariate statistics, (ii) separation of sample groups and highlighting of discriminating ions using Independent Components Analysis (ICA), an unsupervised multivariate method based on source signals decomposition (Rutledge & Jouan-Rimbaud Bouveresse, 2015), (iii) automated data mining-tools to help the annotation of discriminating ions. Thus, our data analysis strategy combines the use of XCMS open-source R package (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006) and ICA method: to the best of our knowledge, this combination for MS data analysis in untargeted food safety analysis is successfully performed for the very first time. Unless previous untargeted approaches reporting the use of either vendor (Knolhoff et al., 2016) or in-house tools (Tengstrand et al., 2013), that often work as "black boxes", our approach benefits from using a freely available package that exists for more than 10 years and is supported by a dynamic and worldwide scientific community, which made it become very versatile for MS data analysis. On top of that, it became user friendly thanks to the development of free web-based platforms like XCMS-Online (Tautenhahn, Patti, Rinehart, & Siuzdak, 2012) or Workflow4Metabolomics (Giacomoni et al., 2015).

Tea has been chosen as the development foodstuff, for it is the most consumed hot beverage in the world (Chang, 2015). Moreover, in its raw product form (tea leaves), it is classified as a difficult commodity by the European Commission (SANTE/EU, 2015) which makes it very interesting as a methodological development food sample. In addition, tea is frequently produced under remote areas where agricultural and production practices may be less controlled than in Europe. Therefore this food product is the subject of frequent alerts on the European Rapid Alert System for Food and Feed, relative to non-authorized pesticides or contaminant levels above regulated limits (i.e. minimum 10 $\mu g.kg^{-1}$ for most pesticides). Finally, recent metabolomics-like approaches have been reported on tea but they only focus on quality and authenticity issues (Fraser et al., 2013; Pongsuwan et al., 2008), i.e. on major constituent. The methodology that we propose here focuses on trace compounds, with specific analytical methods and data treatment strategies to be set up to achieve their detection.

2. MATERIAL AND METHODS

2.1 CHEMICALS AND REAGENTS

Acetonitrile (ACN) (HPLC plus gradient, LC/MS), water, methanol (MeOH) and formic acid (FA) (all LC/MS grade) were purchased from Carlo Erba. Ultrapure water (Milli-Q®) was produced by an Integral 3 water purification system from Millipore®. The compound used for ToF-MS calibration was Leucine Enkephalin (LC/MS grade), purchased from Waters®.

Analytical standards solutions (100 $\mu g.mL^{-1}$ in ACN or MeOH) for 21 pesticides, 4 mycotoxins, 2 process-induced toxicants and labelled compounds acrylamide-d3, dimethoate-d6 and malathion-d6 were purchased at CIL Cluzeau France. Ochratoxin-d5, bisphenols A, F and S, bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and bisphenol A-d14 (purity > 99%) were provided by Sigma Aldrich (Saint-Quentin Fallavier, France). Two pooled stock solutions containing respectively all non-labelled molecules (each at 1 $\mu g.mL^{-1}$), and all labelled molecules (each at 1 $\mu g.mL^{-1}$) were prepared in ACN and stored in the fridge. Regularly, target analyzes of these solutions were done to check for their stability.

2.2 SAMPLE COLLECTION AND STUDY SET-UP

The goal of this work is to assess the ability of a workflow based on UHPLC-HRMS and chemometrics methods (including multivariate analysis) to blindly detect an unexpected contamination in a food sample. To that end, a study was designed so that this workflow would face two very different situations: (i) a quite homogeneous product (i.e. samples from one brand) contaminated at several levels by a large number of molecules (development data set) (ii) a heterogeneous product (i.e. samples from two brands) contaminated at a single level by only few molecules (validation data set). Raw data sets have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gks1004. PubMed PMID: 23109552) with the respective identifiers MTBLS752 and MTBLS754 for data set n°1 (development) and n°2 (validation of the approach) (Haug et al., 2013). The complete data sets can be accessed at https://www.ebi.ac.uk/metabolights/MTBLS752 and https://www.ebi.ac.uk/metabolights/MTBLS754.

Green teas were purchased at local retailers (Paris, France) and crushed in our laboratory using a mortar and a pestle. Green tea n°1 is a Japanese Bancha tea, and green tea n°2 a Chinese tea. They were used to generate the development data set (green tea n°1) and the validation data set (green teas n°1 & 2).

Two spiking mixes were prepared. The first one (mix n°1), intended for the development data set, consists in a pool of 32 chemical contaminants (a detailed list of compounds used can be found in Supplementary data, Table S.1). These target molecules, called "tracers", were chosen to be representative of potential contaminants, both in terms of chemical structures, source types (mycotoxins, pesticides, process-induced toxicants and migrants from packaging) and analytical behavior (instrumental response, peak width, retention time and adduct / isotopic information). The second one (mix n°2) consists in selected three contaminants from the previous list, chosen for their chemical diversity, namely ochratoxin A (OTA), bisphenol S (BPS) and tolfenpyrad.

For the development data set, four samples were considered (each time three sub-samples were collected to obtain triplicates of preparation): three samples spiked with mix n°1 at 10, 50 or 100 $\mu g.kg^{-1}$, and a control sample (i.e. spiked only with the ACN solvent). Tea samples were initially analyzed using a classical multi-residue method in order to check for the absence of the "tracers" considered (Cladière et al., 2018).

For the validation data set, four samples were considered as well (again three sub-samples were collected each time to get triplicates of preparation): two control samples respectively made of unspiked green tea n°1 and unspiked green tea n°2, and two suspect samples respectively composed of green tea n°1 or green tea n°2 spiked at a level of 50 µg.kg^{-1} with mix n°2.

Spiking levels were chosen in accordance to EU regulation No 396/2005 and 1881/2006. Some of the least sensitive compounds (namely deoxynivalenol, bisphenols A and F) were spiked with a magnification factor of 5, and for the same reason acrylamide was spiked with a factor of 10. In addition, all sample groups were systematically spiked (at 40 µg.kg^{-1}) with the pool of labelled molecules for analytical quality control purpose. For spiking, samples of 1 g were weighted in centrifuge polypropylene tubes (Corning, New York, USA), and spiking was performed using the lowest possible volume of solution (maximum of 100 µL). After spiking, samples were homogenized using a vortex and allowed to equilibrate for 2 hours at room temperature.

The workflow employed (both for sample preparation and data treatment) is shown in **Figure 1**.

2.3 ANALYTICAL METHOD

The generic analytical method is based on previous work (Cladière et al., 2018). Tea samples were extracted using direct solvent extraction with 5 mL of an ACN/MeOH (90/10 v/v) mixture acidified with 0.1% FA, and tubes were agitated upside-down on an agitating plate during 1 h before centrifugation at 3,000 g for 10 min. The supernatant was then collected and an aliquot (2 mL) was evaporated to dryness under a gentle stream of nitrogen. The extract was further reconstituted in 0.2 mL of ACN acidified with 0.1% FA. Then 0.8 mL of ultrapure water with 0.1% FA was added in order to reconstitute 1 mL of final volume, and centrifuged at 12,000 g for 10 min. At the end, 0.5 mL of the final extract was sampled and filtered at 0.2 µm using a syringeless filter vial (mini-uniprep G2, Whatman) before analysis. A Quality Control (QC) sample for each data set was prepared by pooling together 0.2 mL of final extract from every sample of the set; an aliquot of 0.5 mL was then taken and filtrated at 0.2 µm using a syringeless filter vial.

Analyzes were performed on a Waters® Acquity UPLC® H-Class system, composed of a quaternary solvent manager pump, a refrigerated sample manager Flow-Through-Needle and a column oven, coupled to a Waters® high resolution Time-of-Flight mass spectrometer Xevo® G2-S ToF operated in centroid mode (UHPLC/HRMS-ToF) with a mass range from *m/*z 60 to 800. 10 µL of the final extract were injected and

separation was performed on a C18-PFP column (150×2.1 mm, 2 µm particles diameter, ACE supplied by AIT France). An electrospray ionization source was used in both positive (ESI^+) and negative (ESI^-) modes. ESI^+ and ESI^- modes were run separately. For ESI^+, the mobile phase was composed of water (A) and ACN (B), both acidified with 0.1% FA, and MeOH (C), flowing at 0.4 $mL.min^{-1}$. Gradient started at 100% A and reached 100% B in 10 min, being kept for 6 min before switching to 100% C to rinse the system in 1 min, being hold for 5 min, returning back to 100% A in 1 min and finally equilibrating for 3 min, with a total run duration of 26 min. For ESI^-, the mobile phase was composed of water buffered at pH 6.45 with 10 mM of ammonium formate (A) and MeOH (B) flowing at 0.3 $mL.min^{-1}$. The gradient started at 100% A and reached 100% B in 13 min, holding this condition for 7 min before turning back to 100% A in 1 min and finally equilibrating for 3 min, with a total run duration of 24 min. For both chromatographic methods the temperature of the column oven was kept at 30°C. Electrospray parameters have been fixed at the previously reported values (Cladière et al., 2018).

The analytical sequence started with injection of 10 mobile phase blanks in order to reach complete equilibrium of UHPLC-HRMS-ToF apparatus. Sample vials were randomized in the analytical sequence, and a blank as well as a QC sample were injected every 10 sample vials. For each ionization mode, all sub-samples were injected either in triplicate (development data set) or in quadruplicate (validation data set).

2.4 DATA TREATMENT

The data treatment workflow was set up to be as much automated as possible. Indeed, only few manual steps are remaining, the main one being the final curation of the automated annotation algorithm. Moreover, no information about the level or nature of contaminants are provided in the workflow, only the group information (ex: “vial n°1 belongs to group n°3”). The term “group” refers here to all injections related to the same sample (i.e. three replicates for sample preparation plus triplicates or quadruplicates of injections each time). In other word, for each sample several raw data are obtained, these being grouped together before data treatment.

Step 1: Building of data matrix from raw data

Vendors (Waters®) raw data files were first converted to the open-source format mzXML using ProteoWizard (Chambers et al., 2012) and then uploaded onto the Workflow4Metabolomics (W4M) platform (Giacomoni et

al., 2015). Data matrix building was then achieved using open-source XCMS package (Smith et al., 2006) on this platform.

XCMS builds the data matrix from raw data files using the following workflow. First, "xcmsSet" with CentWave method (Tautenhahn, Bottcher, & Neumann, 2008) extracts peaks from the data files. Peaks are then grouped across the samples and aligned using "group", "retcor" and then "group" functions again. The final step of the algorithm, "fillpeaks", identifies for each sample the peaks for which this sample has no value: for these peaks, the tool integrates the signal noise in this area to avoid missing values at the end. XCMS parameter values for each step of the workflow were chosen as suggested for UHPLC-Q-ToF instruments by Patti *et al*. (Patti, Tautenhahn, & Siuzdak, 2013), except for the "peak width" parameter in the "xcmsSet" step which was chosen less stringent (5-60 s instead of 5-20 s) to limit data loss. A complete list of XCMS parameters can be found in **Table S.2** of Supplementary material. The XCMS peak extractions were performed separately for ESI^+ and ESI^- sequences. Finally, the data matrix is a table gathering the different peak areas integrated by XCMS sorted by ions in row (combination of *m/z* and retention time) and by samples in column. Data matrix files (.txt) were then imported in Matlab using in-house scripts.

Step 2: Data filtration and reduction

Since the number of output ions generated by XCMS is very high (between 10,000 and 30,000), the data matrices needed to be filtrated to remove as many irrelevant ions as possible. Data cleaning by successive filtration steps is critical since an adequate filtration should enable to clean the data from irrelevant signals while avoiding or minimizing relevant chemical information loss. Data matrices were filtrated using only the group information (i.e. blind to the nature and levels of the spiked molecules). Therefore, ions that do not differ from the blanks or do not vary between samples were filtrated in order to keep only suspect ions and try to highlight a food contamination. This filtration strategy is commonly used in metabolomics approaches (Antignac et al., 2011) but applied here for the first time to non-targeted food safety analysis. It is generally based on statistical tests (t-test) designed to determine significant differences for each ion between samples at a commonly admitted p-value of 0.05 (Gika, Theodoridis, Plumb, & Wilson, 2014; Rubert et al., 2017; Thévenot, Roux, Xu, Ezan, & Junot, 2015). This strategy can be completed by using the fold change of each ion between samples: the common fold change value used is 2, but it is still under discussion for metabolomics purpose (Ortmayr, Charwat, Kasper, Hann, & Koellensperger, 2017).

Finally, filtration of data matrices composed initially of around 20,000 ions was done within three successive automated steps plus an automated pre-filtering step:

a) Pre-filtering step: unusable and unreliable variables that exhibit a poor stability, meaning relative standard deviation (%RSD) on peak area above 100% in every sample group were discarded (about 50 to 100 ions discarded).
b) First step of filtration: removal of ions that show no significant difference (peak area) between blank runs and any of the sample groups using pairwise t-tests results (blanks vs. sample groups, about 19,000 ions remaining).
c) Second step of filtration: removal of ions that show no significant difference (peak area) between any sample groups using pairwise t-tests results (sample groups vs. sample groups, about 10,000 ions remaining).
d) Third step of filtration: removal of ions that show a low fold change among sample groups, to select only ions exhibiting high contrast between sample groups. For each ion, the median value of peak areas of each sample groups (n=9, extraction triplicates, each analyzed in triplicate) was considered. The fold change is then calculated by dividing the highest median value by the lowest one (assumed to be the most concentrated sample divided by the least concentrated one, about 1,000 ions remaining after this step).

Step 3: Normalization and scaling

Missing values and algorithm artifacts such as zero, infinite and negative values in the data matrix were managed according to the guidelines given by Wherens et al.(Wehrens et al., 2016). Briefly, for each ion (named as "*m/z* – retention time" combination), any irrelevant value was replaced by the lowest value of this ion (blank excluded).

The data matrix was then log- and pareto- scaled (Antignac et al., 2011), and normalized using a median-based Probabilistic Quotient Normalization (PQN) (Dieterle, Ross, Schlotterbeck, & Senn, 2006) using the QC samples. Briefly, the median of each ion in QC samples is computed. Then, for each ion, values in samples are divided by a reference value (here the median of the measurement for the ion in QC samples), leading to a ratio matrix. Then, for each sample, the median of the ratios is computed, and the initial values are divided by the ratio median.

Step 4: Multivariate data analysis

Principal Component Analysis (PCA) and Independent Component Analysis (ICA) were tested in order to discriminate contaminated samples. ICA showed better performance than PCA to resolve complex signal mixtures as already demonstrated for metabolomics data (Liu et al., 2016). Therefore, ICA was used to interpret and visualize data with respect to their source signals, and then try to evidence potential discrimination between sample groups. Indeed, ICA is a blind source separation method, which aims at extracting from mixed signals their original source signals as well as the weights in which they are mixed. Among the different few algorithms enabling to compute ICA models, the JADE algorithm was used here (Rutledge & Jouan-Rimbaud Bouveresse, 2015).

The determination of optimal number of Independent Components (ICs) to use is the key step during the building of an ICA model. This optimal number was determined using the random ICA method (Kassouf, Jouan-Rimbaud Bouveresse, & Rutledge, 2018), briefly summarized as follows: the data set is randomly split into two equivalent groups. ICA models with 1 to F components (here, $F = 20$) are calculated in each subset. For each model (i.e., each investigated number of ICs), correlations between all ICs from one subset and all ICs from the other subset are calculated. The idea underlying this procedure is that if an IC is significant, it should be extracted in each subset and therefore, strong correlations should be observed between ICs from each subset. Hence, one looks for the highest number of ICs for which each IC of one subset is highly correlated with one IC of the other subset. However, the repartition of samples into the two subsets being random, there is a possibility that the subsets are not representative, in which case a significant IC might be extracted from one subset only. This is the reason why this procedure has to be repeated, here, 50 times.

Step 5: Annotation and interpretation

For automated annotation, an in-house broad-scale database was built combining data from several databases, namely the Toxin and Toxin-Target Database (T3DB, http://www.t3db.ca/ (Wishart et al., 2015)), the literature (Gallart-Ayala, Núñez, & Lucci, 2013; Nielsen & Smedsgaard, 2003), and to a lesser extent, the Pesticides Properties Database (Lewis, Tzilivakis, Warner, & Green, 2016).

After evidencing a discrimination along one component of the ICA model, the signal matrix, which gives the weight of each ion ("*m/z* – retention time" pair) in the component, was analyzed: ions were sorted by

descending contribution value along the components explaining the group separation, and the annotation was performed according to the following strategy:

a) In-house automated tools were developed for isotopic pattern detection (inspired from work by Cotton et al. [14]) and then for in-house toxicant database annotation to highlight suspect ions;
b) Manual curation of the annotation results was then performed for discriminating ions using information provided by step a) as well as online databases [such as Metlin (https://metlin.scripps.edu/), HMDB (http://www.hmdb.ca/), mzCloud (https://www.mzcloud.org/) and T3DB] and raw data visualization when necessary.

With mass spectrometry, and especially electrospray ionization, a single molecule usually produces several observed signals, either fragments, adducts or isotopic peaks. All ions assumed as coming from the same compound (i.e. retention time, correlation, known $\Delta m/z$: M+1, M+2 with relevant intensity ratio) were grouped in "features" during step a), each one representing a single compound. During the same step, adducts were annotated with the database search. Annotation levels nomenclature used is based on guidelines proposed by Sumner et. al. (Sumner et al., 2007).

3. RESULTS AND DISCUSSION

3.1 MULTIVARIATE ANALYSIS OF DEVELOPMENT DATA SET

All data treatments were developed and performed blindly, meaning without optimizing the parameters for our tracers. The objective of this approach is to evaluate the efficiency of a generic blind untargeted analysis based on multivariate tools to discriminate contaminated samples and annotate ions of potential contaminants.

A multivariate exploration of the data was first tried without any (pre)filtration, but it remained unsuccessful since no clear group separation could be observed (see **Figure 2**). The detailed filtration process was thus developed and applied. It appears (Supplementary material - **Figure S.1**) that, even though PCA enables a discrimination for the filtrated data, the one given by ICA is superior both by its quality (better sample separation) and its ability to align chemical phenomenon on a single component. So, the detection of suspect samples and ions is eased thanks to ICA by simply sorting ions based on their weight on the discriminating component.

3.1.1 BLIND DISCRIMINATION OF SAMPLES BY ICA

Optimal number of ICs was determined as 4 for the development data set in both positive and negative modes with the random ICA method. For each ionization mode, score plots were drawn considering the different ICs prone to discriminate sample groups (an illustrative plot is given in **Figure 2**). Interestingly, all sample groups could be discriminated whatever the ionization mode, and each time IC1 was determined as the most probable meaningful component regarding group information. It is clear that group separation along IC1 is related to the level of contamination. It should be emphasized that tea samples contaminated at the low level (10 $\mu g.kg^{-1}$) could be distinguished from control tea samples, even with a high chemical diversity of contaminants. This is the first time that contaminated food samples are discriminated from control samples at this level: this opens new perspectives for food safety control, since 10 $\mu g.kg^{-1}$ is the maximum level authorized for several regulated chemicals, especially pesticides.

The next step is the annotation of discriminating ions, in order to assess if the discrimination observed is really due to the contaminants.

3.1.2 ANNOTATION AND INTERPRETATION OF ICA OUTPUT

Most of the annotation process was automated thanks to database search and data mining scripts. For each ionization mode, the filtered data matrix went through two automated steps: (i) isotopic peaks were first grouped together, and (ii) observed ions were searched through a broad-range toxicants database for testing their potential matching with different adducts. Then, for each ionization mode, ions were sorted along the discriminating component(s) and extracted with their information (*m/z*, retention time, presence of isotopes, and potential match in the database for different adducts). At the end, the results can be quickly curated by the user, who can then rapidly spot suspect samples and ions. These information can be completed, when needed, by a manual exploration of raw data files.

That way, for positive mode, the 69 first discriminating ions were putatively annotated or characterized and grouped into 20 “features” (see **Table 1a**). Over those 20 “features”, 14 were attributed to our “tracers”. Three others (#13, 18 and 19) were not expected to be present in the samples (since they were not found after a targeted analysis of the control samples, nor reported in the analysis certificate of standards used). Raw chemical formulas could be proposed, that show very strong similarities with some of our “tracers”, so they

were putatively annotated as impurities from the initial standard solution (such result has been confirmed by an *a posteriori* classical targeted analysis of the standard solution). This clearly underlines the potential of our developed method to detect unexpected compounds at trace levels since those impurities were not expected before the analysis. Three other features remained unknown.

Putative annotation and characterization of the 69 first ions in negative mode was also achieved, and these ions were grouped into 14 "features" (see **Table 1b**). For these features, raw molecular formulas hypotheses were made based on information given by the automated annotation step, enabling a putative characterization to be achieved. Thus, 12 of our "tracers" could be recovered. Two "features" (corresponding to eight ions) remained unresolved after annotation attempt (using both automated scripts and manual exploration of raw data), but they were characterized as being halogenated compounds thanks to the isotopic peaks found during the automated data mining step.

3.2 METHOD PERFORMANCE

Performance was assessed based on blind detection rates of "tracer" contaminants. In positive mode, 44% of our "tracers" were successfully putatively annotated, and 38% in negative mode. When considering both modes, the overall detection rate is 66% (since some molecules were detected in both modes, e.g. diuron and ochratoxin A). By comparing with detection performance of a dedicated targeted multi-residue method on the same samples (Cladière et al., 2018), it appears that the "tracers" not successfully annotated using the untargeted approach were also the most difficult to analyze with a targeted approach (i.e. showing high ion suppression and therefore low signal/noise ratio, high relative standard deviation of the signals and poor recoveries). A manual exploration of the raw chromatograms reveals that these molecules give very noisy peaks, which are not even extracted during the pretreatment step with XCMS. In fact, to date, it is likely that there is no algorithm that can achieve exhaustive peak extraction from raw data in untargeted LC-MS study (Coble & Fraga, 2014). In our case, XCMS managed to extract 75% of our "tracers" (i.e. 24 over 32) from the raw data files, which is still a good score even though the noisiest peaks are missed. We tested the only existing optimization algorithm for XCMS parameters (IPO) (Libiseller et al., 2015), but with no improvement. Unsurprisingly, the response factor of a compound has been found to be the main factor affecting its detectability.

Thus, the data treatment methodology applied after XCMS treatment (including filtration of the data matrix, preprocessing and multivariate analysis coupled with data mining) successfully annotated 21 tracers over the 24 extracted by XCMS (i.e. 88%). This is very satisfactory regarding the wide diversity of molecules studied, both in terms of chemical structure and response factor in LC-MS, as well as regarding the trace levels studied. Last but not least, the detection and putative annotation of unexpected impurities coming from the spiking mix highlights the ability of our untargeted approach to detect potentially unknown or unexpected trace contaminants in food products and to propose the user annotation hypotheses. It should be spotted that a molecule generating a high number of ions (adducts, isotopes, fragments, etc.) will be more easily annotated than a molecule generating only few signals.

For each annotated compound, a limit of detection (LOD) was estimated based on the calculated fold change (calculated between the group with the highest level -100 $\mu g.kg^{-1}$- and the control group). Briefly, a rule of three was made to figure out what concentration would lead to a fold change of 3, which is the most commonly used signal/noise ratio for LOD determination. For compounds annotated as impurities, assumption was made that they come from standards of similar families (i.e. atrazine for simazine, diflubenzuron or diuron for fenuron, and acid herbicides for 2,4-D isopropyl ester) and a LOD was then estimated for each by taking the standard purity into account. As shown in **Table 1a and 1b**, estimated LODs are relevant against EU regulation since they are in the range 10 $\mu g.kg^{-1}$ or below for almost every annotated compound. Again, our untargeted approach proves to have quite similar performance in terms of sensitivity as compared to our dedicated targeted multi-residue method [18], having the additional asset to detect unexpected molecules.

3.3 APPLICATION ON VALIDATION DATA SET

The developed methodology (including filtration parameters, pretreatment steps and multivariate method) was blindly applied to the validation data set, obtained based on the analysis of two different types of green tea, either non-spiked (controls) or spiked with a mix composed of only three contaminants in order to offer a much more challenging discrimination between blanks and contaminated samples.

Data from the two types of tea were treated simultaneously. Data matrix was filtrated using same parameters as for development set, and then the optimal number of ICs was determined for this data set. It was calculated as 6 both for positive and negative modes. Unsurprisingly, since the data set is more heterogeneous, the filtration led to a smaller reduction of ion number than for the development data set. Still, the number of ions

dropped from 23,391 and 17,269 (respectively for positive and negative mode) to 9,789 and 9,409 thanks to the filtration. For each ionization mode, two discriminating ICs were clearly observed (**Figure 3**). IC1 separates samples based on their brand, and IC6 separates control and contaminated samples. It should be emphasized that in ICA, ICs are not ordered by descending contribution like in PCA, meaning in other words that IC1 does not necessarily explain more variability than IC6.

Annotation of the data matrix was done as described for the development data set. IC6 was determined as bearing the separation due to contaminants thank to information provided in the automated annotation step. The three "tracers" were successfully annotated within the first 10 ions of IC6 with this methodology in at least one ionization mode. This results is of prime interest in our case since ICA, as employed here, shows its main assets which is to separate independent phenomenon. Indeed, we can see that in our case, the "natural" variability of the product is well separated from the variability brought by the spiking (these two phenomenon are likely to be mixed in less powerful methods). Moreover, the number of ions generated in a complex data set such as the validation one is very high despite of filtration strategy applied (in our case ~9,000 per ionization mode). Therefore, the use of multivariate methods enables the reduction of the dimensionality of the data and the achievement of suspect samples and ions detection. Thanks to ICA, the annotation of only 10 ions per ionization mode was sufficient to underscore a contamination of tea.

This highlight the ability of ICA to resolve complex signal mixtures and simplify annotation of relevant ions, even in cases where the information is bore by few, low intensity ions. Our proposed approach thus has a strong potential in detecting food contaminants at low levels in complex and rather heterogeneous data sets. Its applicability to other food matrices should be feasible if reference samples are available.

4. CONCLUSION

This work shows some important methodological features for untargeted approach development for food chemical contaminants detection. It gives evidence that the blind untargeted detection of contaminants in complex food matrices is feasible thanks to high resolution methods coupled to powerful data analysis strategies. A widely spread, well-known, freely available and easy to use tool (i.e. XCMS run on W4M platform) was used for peaks extraction from raw data. Then, an efficient automated strategy was set up for data filtration, using well-known easy to use tools (t-tests and fold change). Samples were separated using a

multivariate method (ICA), and discriminating ions putatively annotated by the help of automated data mining methods.

Thanks to this strategy, 66% of the "tracers" considered were successfully putatively annotated. This detection rate rises to 88% if brought back to tracers actually in the data matrix (after the peak extraction step). This shows the power of our developed data treatment strategy to detect potential food contaminants in a data matrix. In addition to the known "tracers", some unexpected molecules were detected and putatively annotated in the samples, which clearly highlights the potential of this approach. Method LODs were roughly estimated for each putatively annotated compound (both expected and unexpected), with values below or near 10 $\mu g.kg^{-1}$ for most of them, which compares favorably with a targeted multi-residue method. The approach, developed on a rather simple case, has been validated on a more complex and realistic situation, where the contamination is brought by a low number of molecules, and in which different brands of the same food product are considered simultaneously. This study opens new perspectives in the development of truly untargeted approaches based on tools and strategies from metabolomics (particularly HRMS and chemometrics) for food chemical safety assessment. Such approaches may constitute, in a near future, a major complement to targeted methods in a view of rapidly screening possibly contaminated food products. A next step towards a routine use of these approaches would be to implement them on even more complex cases like the following of a production batch. Interestingly, since our developed methodology is rather generic, it could be applied with only few development on any other UHPLC-HRMS data set, or even to other applications such as origin or authenticity issues to complement existing approaches. As a conclusion, no doubt that these results will encourage new developments on this analytical issue, both on the methodology and the tools, especially on the improvement of existing peak extraction methods or the handling of the product variability.

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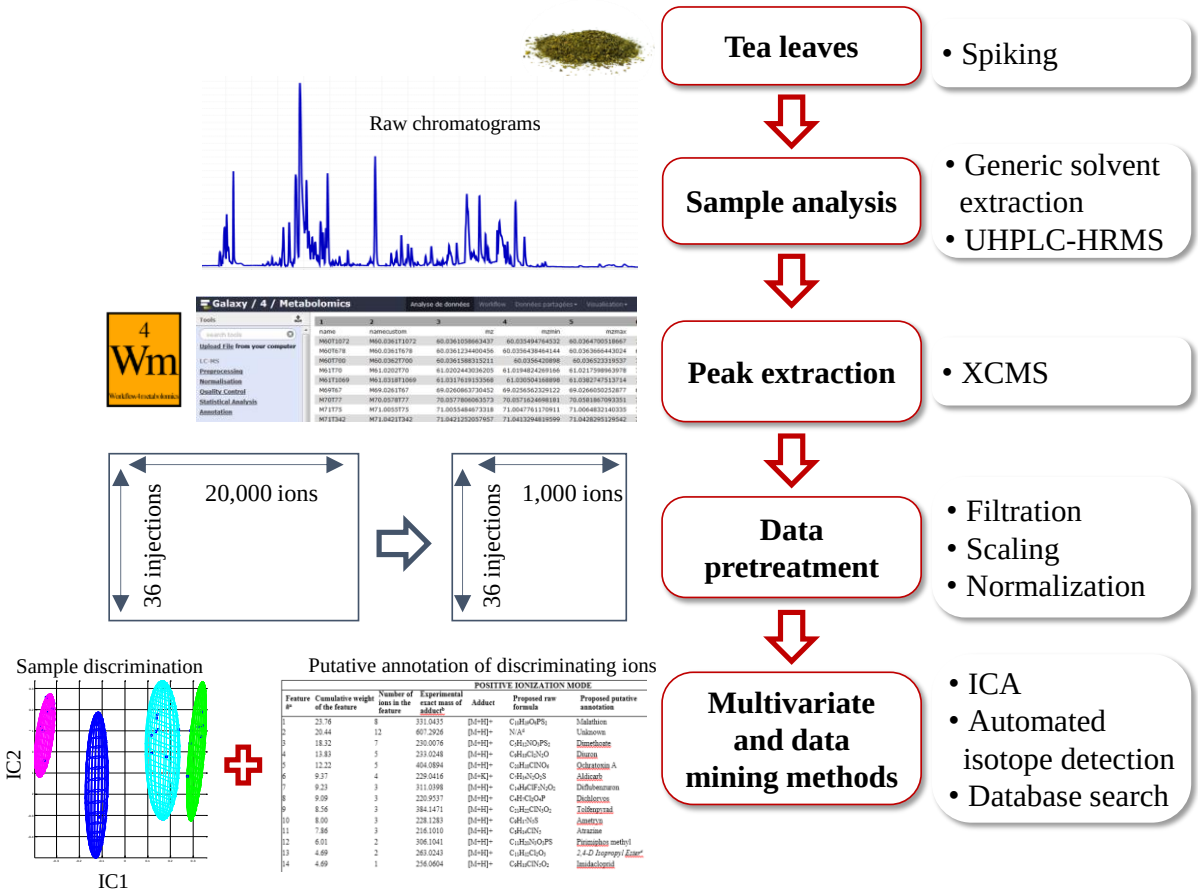
Table 1a: Discriminating features for positive ionization mode and respective putative annotation.

POSITIVE IONIZATION MODE									
Feature #[a]	**Cumulative weight of the feature**	**Number of ions in the feature**	**Experimental exact mass of adduct[b]**	**Adduct**	**Proposed raw formula**	**Proposed putative annotation**	**Mono-isotopic mass [c]**	**Delta (ppm)**	**Estimated LOD (µg.kg^{-1})**
1	23.76	8	331.0435	[M+H]+	$C_{10}H_{19}O_6PS_2$	Malathion	330.0361	0.48	1.1
2	20.44	12	607.2926	[M+H]+	N/A[d]	Unknown	N/A	N/A	N/A
3	18.32	7	230.0076	[M+H]+	$C_5H_{12}NO_3PS_2$	Dimethoate	228.9996	3.04	0.9
4	13.83	5	233.0248	[M+H]+	$C_9H_{10}Cl_2N_2O$	Diuron	232.0170	2.32	1.0
5	12.22	5	404.0894	[M+H]+	$C_{20}H_{18}ClNO_6$	Ochratoxin A	403.0823	-0.34	4.9
6	9.37	4	229.0416	[M+K]+	$C_7H_{14}N_2O_2S$	Aldicarb	190.0776	4.41	15.1
7	9.23	3	311.0398	[M+H]+	$C_{14}H_9ClF_2N_2O_2$	Diflubenzuron	310.0321	1.45	1.9
8	9.09	3	220.9537	[M+H]+	$C_4H_7Cl_2O_4P$	Dichlorvos	219.9459	2.47	3.6
9	8.56	3	384.1471	[M+H]+	$C_{21}H_{22}ClN_3O_2$	Tolfenpyrad	383.1401	-0.67	1.3
10	8.00	3	228.1283	[M+H]+	$C_9H_{17}N_5S$	Ametryn	227.1205	2.59	1.5
11	7.86	3	216.1010	[M+H]+	$C_8H_{14}ClN_5$	Atrazine	215.0938	-0.14	2.9
12	6.01	2	306.1041	[M+H]+	$C_{11}H_{20}N_3O_3PS$	Pirimiphos methyl	305.0963	1.56	0.4
13	4.69	2	263.0243	[M+H]+	$C_{11}H_{12}Cl_2O_3$	*2,4-D Isopropyl Ester[e]*	*262.0163*	*2.73*	*0.5*
14	4.69	1	256.0604	[M+H]+	$C_9H_{10}ClN_5O_2$	Imidacloprid	255.0523	3.19	16.3
15	4.36	2	621.2713	N/A	N/A	Unknown	N/A	N/A	N/A
16	4.29	2	623.2868	N/A	N/A	Unknown	N/A	N/A	N/A
17	2.73	1	251.0380	[M+H]+	$C_{12}H_{10}O_4S$	Bisphenol S	250.0300	3.08	12.1
18	2.24	1	202.0855	[M+H]+	$C_7H_{12}ClN_5$	*Simazine[e]*	*201.0781*	*0.68*	*0.7*
19	2.03	1	182.1282	[M+NH4]+	$C_9H_{12}N_2O$	*Fenuron[e]*	*164.0950*	*-3.57*	*0.9*
20	1.69	1	335.1254	[M+Na]+	$C_{19}H_{20}O_4$	Bisphenol F diglycidyl Ether	312.1362	0.12	57.0

[a] *Features sorted by descending cumulative weight of ions in the discriminating IC*
[b] *Mass measured for the ion having the highest weight in the discriminating IC*
[c] *Electron mass used: 5.485.10^{-4} Da*
[d] *Not Applicable*
[e] *Found in the spiking mix, may be considered as impurities*

Table 1b: Discriminating features for negative ionization mode and respective putative annotation.

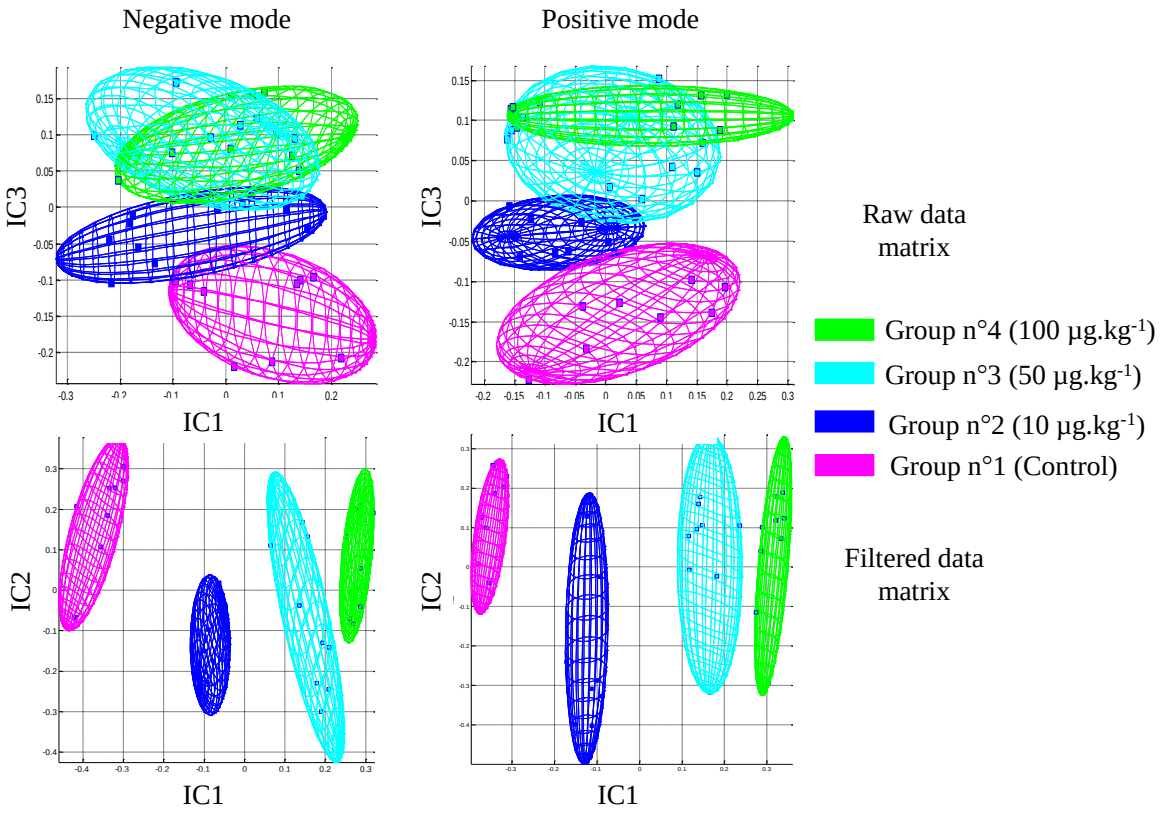
NEGATIVE IONIZATION MODE									
Feature #[a]	**Cumulative weight of the feature**	**Number of ions in the feature**	**Experimental exact mass of adduct[b]**	**Adduct**	**Proposed raw formula**	**Proposed putative annotation**	**Mono-isotopic mass [c]**	**Delta (ppm)**	**Estimated LOD ($\mu g.kg^{-1}$)**
1	27.50	10	266.9385	[M-H]-	$C_9H_7Cl_3O_3$	Fenoprop	267.9461	-1.02	2.0
2	22.07	8	252.9227	[M-H]-	$C_8H_5Cl_3O_3$	2,4,5-T	253.9304	-1.90	3.0
3	21.62	8	309.0249	[M-H]-	$C_{14}H_9ClF_2N_2O_2$	Diflubenzuron	310.0321	0.40	2.6
4	16.59	6	231.0091	[M-H]-	$C_9H_{10}Cl_2N_2O$	Diuron	232.0170	-2.97	0.9
5	14.32	8	232.9771	[M-H]-	$C_9H_8Cl_2O_3$	Dichlorprop	233.9850	-2.75	5.9
6	13.48	5	213.0313	[M-H]-	$C_{10}H_{11}ClO_3$	MCPP	214.0397	-4.98	2.5
7	11.68	5	199.0152	[M-H]-	$C_9H_9ClO_3$	MCPA	200.024	-7.72	5.2
8	8.05	6	204.9217	N/A	N/A	Unknown chlorinated	N/A	N/A	N/A
9	7.75	3	249.0223	[M-H]-	$C_{12}H_{10}O_4S$	Bisphenol S	250.0300	-1.53	6.0
10	5.37	2	239.0668	[M-H]-	$C_{10}H_{12}N_2O_5$	Dinoseb	240.0746	-2.08	10.4
11	4.61	2	402.0749	[M-H]-	$C_{20}H_{18}ClNO_6$	Ochratoxin A	403.0823	-0.13	10.3
12	3.88	2	254.0444	[M-H]-	$C_9H_{10}ClN_5O_2$	Imidacloprid	255.0523	-2.29	25.9
13	2.62	2	218.9611	[M-H]-	$C_8H_6Cl_2O_3$	2,4-D	219.9694	-4.74	15.5
14	2.36	2	771.1431	N/A	N/A	Unknown chlorinated	N/A	N/A	N/A



572

573 **Figure 1:** Workflow developed for untargeted contaminants detection at trace levels in tea.

574

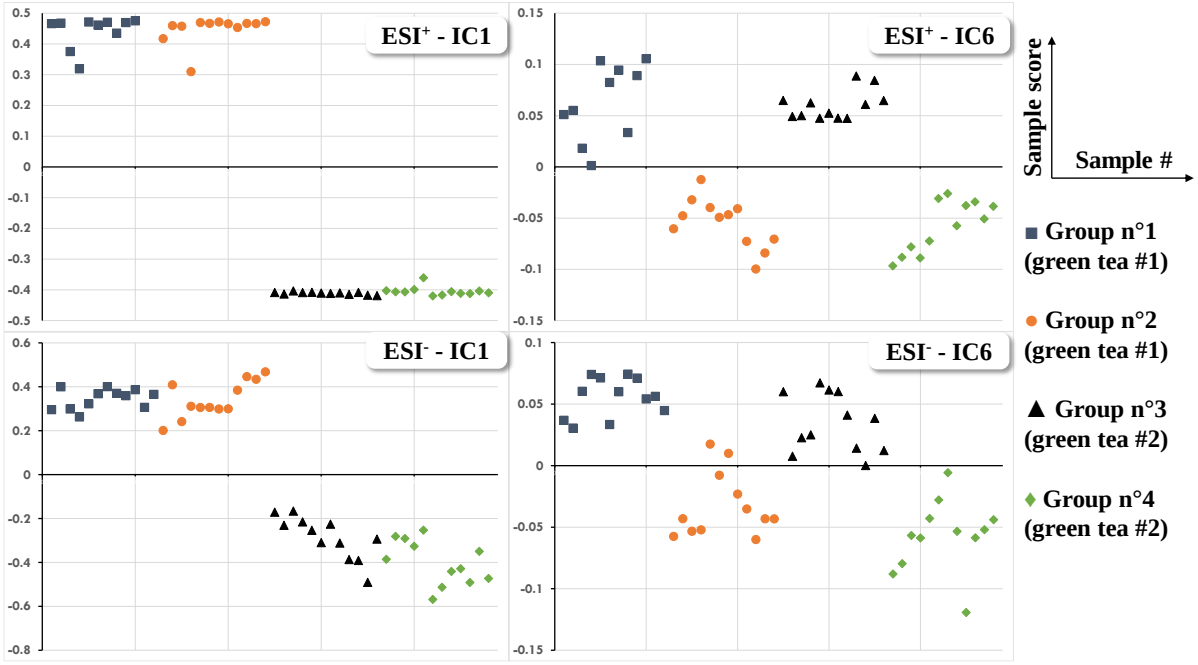


575

576 **Figure 2:** ICA score plots for filtered and unfiltered data matrix for both ionization modes on the

577 development data set.

578



579

580 **Figure 3:** Score plots of discriminating ICs (both ionization modes) for the validation data set.

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583 **SUPPLEMENTARY DATA**

584 **Table S.1**: Information on "tracer" contaminants used in this study.

N°	Name	Class	Chemical family	log K_{ow}	Ionization mode	Adduct - ESI+/ESI-
1	(2,4-dichlorophenoxy)acetic acid 2,4-D	Pesticide (herbicide)	Auxinic herbicide	-0.82	+/-	$[M+H]^+/[M-H]^-$
2	(4-chloro-2-methylphenoxy)acetic acid MCPA	Pesticide (herbicide)	Auxinic herbicide	-0.81	-	ND/$[M-H]^-$
3	2-(4-chloro-2-methylphenoxy)propanoic acid MCPP	Pesticide (herbicide)	Auxinic herbicide	-0.19	-	ND/$[M-H]^-$
4	2,4,5-trichlorophenoxyacetic acid 2,4,5-T	Pesticide (herbicide)	Auxinic herbicide	2.88	-	ND*/$[M-H]^-$
5	4-(2,4-dichlorophenoxy)butanoic acid 2,4-DB	Pesticide (herbicide)	Auxinic herbicide	1.35	+/-	$[M+H]^+$/Frag**
6	Acetamiprid	Pesticide (insecticide)	Neonicotinoid	0.8	+	$[M+H]^+$/ND
7	Acrylamide	Process-induced	Amide	-0.67	+	$[M+H]^+$/ND
8	Aldicarb	Pesticide (acaricide)	Carbamate	1.15	+	$[M+Na]^+$/ND
9	Ametryn	Pesticide (herbicide)	Triazine	2.63	+	$[M+H]^+$/ND
10	Atrazine	Pesticide (herbicide)	Triazine	2.7	+	$[M+H]^+$/ND
11	Bisphenol A	Migrant from packaging	Bisphenol	3.3	-	ND/$[M-H]^-$
12	Bisphenol A diglycidyl ether BADGE	Migrant from packaging	Diglycidyl ether	3.84	+	$[M+Na]^+$/ND
13	Bisphenol F	Migrant from packaging	Bisphenol	1.65	-	ND/$[M-H]^-$
14	Bisphenol F diglycidyl ether BFDGE	Migrant from packaging	Diglycidyl ether	Not available	+	$[M+Na]^+$/ND
15	Bisphenol S	Migrant from packaging	Bisphenol	2.91	+/-	$[M+H]^+/[M-H]^-$
16	Deoxynivalenol	Mycotoxin	Trichothecene	0.29	+/-	$[M+H]^+/[M-H]^-$
17	Dichloprop	Pesticide (herbicide)	Auxinic herbicide	2.29	-	ND/$[M-H]^-$
18	Dichlorvos	Pesticide (acaricide)	Organochlorinated	1.9	+	$[M+H]^+$/ND
19	Diflubenzuron	Pesticide (insecticide)	Benzoylurea	3.89	+/-	$[M+H]^+/[M-H]^-$
20	Dimethoate	Pesticide (acaricide)	Organophosphate	0.7	+	$[M+H]^+$/ND
21	Dinoseb	Pesticide (herbicide)	Dinitrophenol	2.29	-	ND/$[M-H]^-$

22	Diuron	Pesticide (herbicide)	Phenylurea	2.87	+/-	$[M+H]^+/[M-H]^-$
23	Fenoprop 2,4,5-TP	Pesticide (herbicide)	Auxinic herbicide	2.84	-	ND/Frag**
24	Fumonisin B1	Mycotoxin	Fumonisin	-0.5	+	$[M+H]^+$/ND
25	Fumonisin B2	Mycotoxin	Fumonisin	1.2	+	$[M+H]^+$/ND
26	Hydroxymethylfurfural	Process-induced	Furan	-0.09	+	$[M+H]^+$
27	Imidacloprid	Pesticide (insecticide)	Neonicotinoid	0.57	+/-	$[M+H]^+/[M-H]^-$
28	Malathion	Pesticide (insecticide)	Organophosphate	2.75	+	$[M+H]^+$/ND
29	Ochratoxin A	Mycotoxin	Ochratoxin	4.74	+/-	$[M+H]^+/[M-H]^-$
30	Pirimiphos-methyl	Pesticide (insecticide)	Organophosphate	3.9	+	$[M+H]^+$/ND
31	Propargite	Pesticide (acaricide)	Organosulfite	5.7	+	Frag**/ND
32	Tolfenpyrad	Pesticide (insecticide)	Pyrazole	5.61	+/-	$[M+H]^+/[M-H]^-$

**ND: Not Detected*

** *Frag=Fragment*

588 **Table S.2**: Full parameters and their corresponding values for peak extraction using XCMS.

Step	Parameter	Value
xcmsSet	scanrange	180-2400
	nSlaves	1
	method	centWave
	ppm	15
	peakwidth	5-60
	mzdiff	-0.001
	snthresh	10
	integrate	1
	noise	0
	prefilter	0
group - A	method	density
	minfrac	0.5
	bw	2
	mzwid	0.015
	sleep	0.001
retcor	method	peakgroups
	smooth	loess
	extra	1
	missing	1
	span	0.2
	family	gaussian
	plottype	mdevden
group - B	method	density
	minfrac	0.5
	bw	2
	mzwid	0.015
	sleep	0.001
	max	50
fillPeaks	method	chrom
	convertRTMinute	FALSE
	numDigitsMZ	4
	numDigitsRT	2
	intval	into
annotatediff	nSlaves	4
	sigma	6
	perfwhm	0.6
	ppm	15
	mzabs	0.015
	maxcharge	1
	maxiso	4
	minfrac	0.5
	quick	TRUE
	convertRTMinute	FALSE
	numDigitsMZ	4
	numDigitsRT	0
	intval	into

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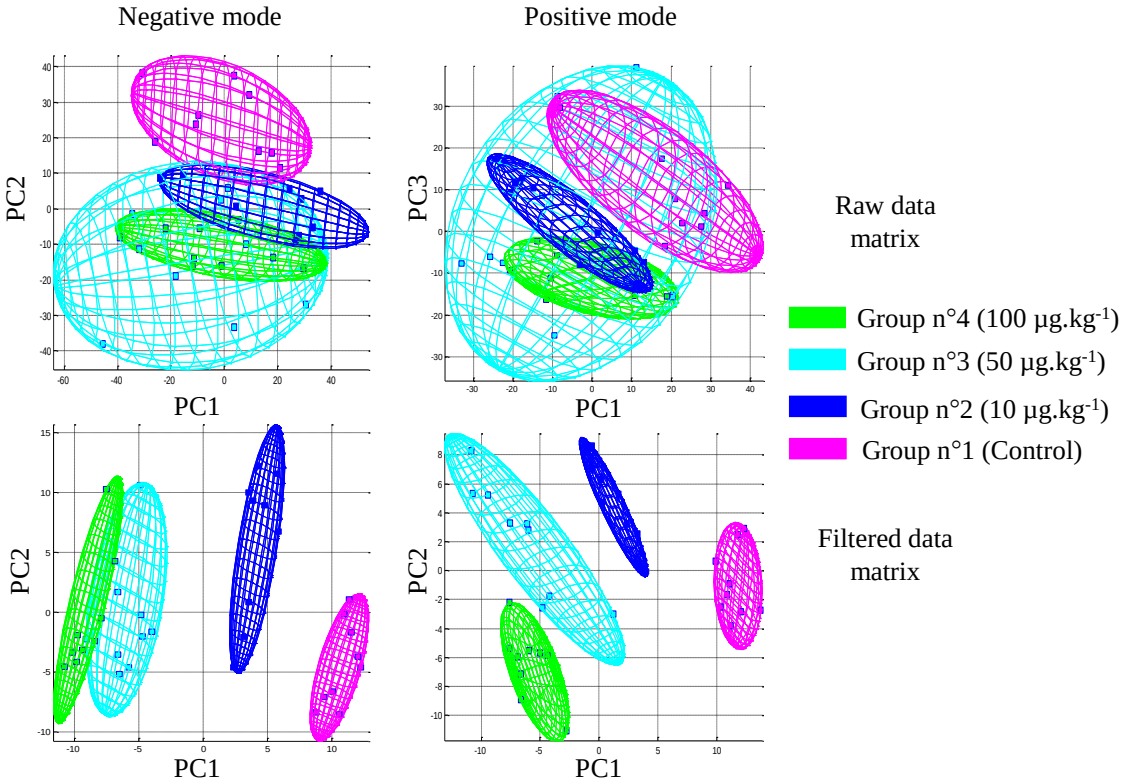

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592 **Figure S.1**: PCA score plots for filtered and unfiltered data matrix for both ionization modes on the
593 development data.

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