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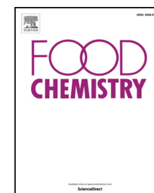

Analytical strategies for the determination of pyrrolizidine alkaloids in plant based food and examination of the transfer rate during the infusion process

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ABSTRACT

Two sample preparation methods were developed (graphitised carbon and C18 solid phase extraction clean-up) and validated in house using liquid chromatography and tandem mass spectrometry (MS/MS) for the determination of 30 pyrrolizidine alkaloids (PAs) in salads, herbs, tea, herbals teas as well as tea infusion and ice-tea beverages. Total PAs concentration of samples purchased on the Belgian market varied greatly with matrix type ranging from < LOD to 187151 ng/g for dry samples, while for infusions and ice-tea beverages the highest PAs concentration was 2106 ng/mL. Surprisingly high PAs concentrations were detected in herbs and spices mixes. The infusion study indicated a transfer rate between 16 and 28% (except for monocrotaline) which highlights the overestimation of PAs concentrations in infusions when derived from a dilution factor (transfer rate of 100%) to the measured concentrations of the dry tea sample.

1. Introduction

Pyrrolizidine alkaloids (PAs) are natural toxins, exclusively biosynthesized by a wide variety of plant species (> 6000). They are plant secondary metabolites against herbivores, are believed to be one of the most widely spread natural toxins and can affect wildlife, livestock and humans via food consumption. Outbreaks in farm animals can cause economic losses to farmers and PAs can become a significant public human health problem due to the intake of contaminated food of botanical or animal origin. Diverse cases of human poisoning in Afghanistan (kakar et al., 2010), India (Tandon, Tandon, Tandon, & Nayak, 1977), South Africa (Willmot & Robertson, 1920) and the former USSR (Bourkser, 1947) are documented and are related to animal grazing on toxic plants or fed with contaminated feed/forage. Human poisoning is mainly caused by the consumption of contaminated traditional herbal remedy, contaminated milk (cow grazing PA plants) or honey (transfer from bees). Poisoning caused by PA toxins is characterized by acute and chronic liver damage, sometimes by pulmonary hypertension, cardiac hypertrophy or kidneys degenerative injuries and can lead to death (Fu, Xia, Lin, & Chou, 2004; Wiedenfeld & Edgar, 2011; EMA, 2014). Therefore, the development of efficient analytical methods is required to detect the presence of PAs, to quantify the total level of toxins or to accurately measure the quantity of individual compounds even at very low levels (ppt/ppb range). This task is particularly challenging for PAs because of their variety, their widespread nature and their different forms. Indeed, more than 600 different PA structures are known so far and 50% of them are recognized as being toxics. Toxic PAs are 1,2-unsaturated and have in common the presence of at least one ester bond: these compounds can be activated by hepatic cytochrome P450 to reactive pyrrole metabolites (Edgar, Colegate, Boppré, & Molyneux, 2011). Moreover, they can exist in 2 different forms: the tertiary base amines and the corresponding N-oxides (PANOs), which behave differently in many analytical systems. PANOs are too polar and thermally unstable compounds, and need to be analysed by means of reduction to the tertiary amines if GC is intended to be used, which is a major drawback. Considering the large structural variety of PAs, especially the co-occurrence of PAs and corresponding N-oxides (PANOs), the extraction method has to ensure the efficient simultaneous extraction of both types.

A critical point in quantitative assessment of PA-contamination is the limited availability of analytical standards of PAs and PANOs. Indeed, EFSA (2011) recommended the monitoring of 30 PAs/PANOs in food items, these compounds are commercially available in Europe, but in small quantities and are very expensive. Many different plants or plant organs and food have been analysed for PAs in the past and most of the common analytical techniques (LC or GC hyphenated to QqQ, IT or ToF MS) were applied in the detection of these compounds (Mattocks, 1986; Crews & Krska, 2009; Crews, Berthiller, & Krska, 2010; Crews, 2013; Bodi et al, 2014; Letsyo, Jerz, Winterhalter, & Beuerle, 2017). Regarding food, there is a need to generate reliable concentration data to be able to evaluate PAs intake for the populations in a realistic way. The large variety and the complexity of the

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potentially contaminated food matrices represent analytical challenges. Previous exposure assessments reported by EFSA (2011, 2015, 2016, 2017) have been hampered by data gaps and no occurrence levels have been reported for the Belgian market. To appropriately address the level of risk to Belgian consumers, occurrence data will be generated following a structured sampling plan and using accurate methods for the determination of toxic free pyrrolizidine alkaloids and pyrrolizidine alkaloids N-oxides. As these compounds are exclusively produced by plants, the present study will focus on plant-derived food products: herbal teas and less targeted food matrices such as salad mixes, spices and ready-to-drink ice teas. In addition, we will propose a new approach to evaluate the quantity of pyrrolizidine contaminants in brewed (herbal) teas, taking into account their transfer rate during the infusion process. Indeed, whereas transfer rates of pesticides, environmental pollutants, mycotoxins, heavy metals, microorganisms, radionuclides or plant growth regulators are relatively abundant in the literature (Abd El-Aty et al., 2014), pyrrolizidine alkaloids were never targeted in such a study.

2. Material and methods

2.1. Solvents, reagents and standard solutions

Methanol and acetonitrile were HPLC grade and purchased from Biosolve BV (Valkenswaard, the Netherlands). Water was purified using a Millipore Milli-Q system (Millipore Corp., Bedford, MA, USA). Ammonia solution (28–30%) and formic acid with a purity of 98–100% were ordered from Merck (Kenilworth, New Jersey, USA). Supelclean™ ENVI™-Carb SPE tubes 0.5 g/6 mL (Supelco, Bellefonte, Pennsylvania, USA) were obtained from Sigma-Aldrich and Isolute® 500 mg/6 mL C18 cartridges were purchased from Biotage (Uppsala, Sweden).

Standards of pyrrolizidine alkaloids (PAs) and related N-oxides (PANOs) were ordered from PhytoLab GmbH and Co. KG (Germany): 16 PAs i.e. echimidine, erucifoline, europine, heliotrine, indicine, intermedine, jacobine, lasiocarpine, lycopsamine, monocrotaline, retrorsine, senecionine, seneciphylline, senecivernine, senkirkine, trichodesmine and 14 PANOs i.e. echimidine N-oxide, erucifoline N-oxide, europine N-oxide, heliotrine N-oxide, indicine N-oxide, intermedine N-oxide, jacobine N-oxide, lasiocarpine N-oxide, lycopsamine N-oxide, monocrotaline N-oxide, retrorsine N-oxide, senecionine N-oxide, seneciphylline N-oxide, senecivernine N-oxide (Compound structures available in Supplementary Data, Fig. S-1). Purity was between 89% and 100%. Individual solutions of each compound at the concentration of 1 mg/mL were prepared in methanol. In case of viscous or hygroscopic standard, the entire content of the commercial vial was dissolved in methanol. Therefrom, a stock solution containing a mixture of the 30 compounds at 10 µg/mL and successive dilutions of the later in order to obtain solutions at concentrations of 1000, 250, 100, 50, 10, 5 and 1 ng/mL were prepared in methanol. All solutions were stored at −20 °C.

2.2. Sample preparation and samples

Fresh plant products (salad samples) were freeze dried for 7 days followed by homogenization and milling. Dry plant products (teas, herbal teas and spice samples) were homogenized and milled to a fine powder with specific focus on representativeness of the purchased sample (e.g. in case of dry tea: 10 tea bags were emptied, combined and milled together). Two procedures for the sample preparation were developed, depending on the type of matrix:

2.2.1. Method 1: Dry plant material

Two grams (± 0.050 g) of sample were weighted in a 50 mL Falcon tube. Then, 15 mL of 0.1% formic acid in methanol were added (20 mL for salads), the tube was vortex-mixed for 10 min, placed in an ultrasonic bath for 15 min, shaken for 15 min and centrifuged for 15 min at 4000 rpm and 5 °C. The supernatant was transferred to a 25 mL volumetric flask and the residual solid was re-extracted with 8 mL of 0.1% formic acid in methanol (10 mL for salads), the tube was vortex-mixed for 10 min, shaken for 10 min and centrifuged for 15 min at 4000 rpm and 5 °C. Both supernatants were combined before adjusting the volume with 0.1% formic acid in methanol. Supelclean™ Envi™-Carb 0.5g/6 mL cartridges were conditioned with 8 mL methanol followed by 8 mL 0.1% formic acid in methanol. The plant crude extract (10 mL) was then filtered through the cartridge. The purified extract was collected in a 15 mL Falcon tube and the solvent was evaporated to dryness under nitrogen flow with bath temperature at 45 °C. The sample was reconstituted in 1 mL of water/methanol mixture (80:20), vortex-mixed for 10 min, placed in an ultrasonic bath for 10 min and centrifuged for 15 min at 4000 rpm and 5 °C. Finally, the supernatant was transferred to an injection vial ready for analysis.

2.2.2. Method 2: Infusions extracts (including ice-tea beverages)

Infusions of (herbal) teas were performed according to ISO 3103 (1980) standardized method for tea brewing. Two grams (± 0.050 g) of milled and homogenized (herbal) tea were weighted and transferred in a tea bag. Purified water (Milli-Q) was brought to a boil. The sample was placed in a 250 mL Erlenmeyer flask and 100 mL of boiling water was poured on it, allowing brewing for 6 min. The tea bag was then removed from the infusion and placed on a funnel above the Erlenmeyer flask for straining until return of the infusion to room temperature. The infusion was basified to pH 9–10 with 3 drops of ammonia 28–30%. Fifty milliliters of homogenized solution were transferred to a 50 mL Falcon tube and centrifuged for 15 min at 4000 rpm and 5 °C. Isolute® C18 500 mg/6 mL SPE cartridges were conditioned with 8 mL methanol followed by 8 mL water (pH 9–10). The infusion solution (40 mL) was then loaded on the cartridge, which was subsequently washed with 5 mL water (pH 9–10) and dried under vacuum. The analytes were eluted with 10 mL methanol, the purified extract was collected in a 15 mL Falcon tube and the solvent was evaporated to dryness under nitrogen flow with bath temperature at 45 °C. The sample was reconstituted in 1 mL of water/methanol mixture (80:20), vortex-mixed for 10 min, placed in an ultrasonic bath for 10 min at room temperature and centrifuged for 15 min at 4000 rpm and 5 °C. Finally, the supernatant was transferred to an injection vial ready for analysis. Ice-tea beverages were processed in the same way, starting from the basification step before SPE purification.

Samples were purchased in Brussels at 6 major supermarkets, organic shops and specialized tea houses. The food categories were selected based on the possible and expected occurrence of PAs in foodstuff. Then, a sampling list was built with the selected food items covering private labels and manufacturers brands. Moreover, five plants products for infusions were acquired online. The sampling involved 235 items comprised of pre-packaged salad mixes (N = 17), spices (N = 17), black teas (N = 43), green teas (N = 43), herbal teas (N = 79), rooibos teas (N = 8) and ice-teas (N = 28). The samples were then categorized and codified with sampling details (e.g. date of purchase, location, price and list of ingredients). It is noteworthy that some samples were labelled as containing PA producing plant species.

2.3. Chromatographic and mass spectrometric settings

The UPLC-MS/MS system consisted of an Acquity UPLC® system (sample and quaternary solvent manager, column oven) hyphenated to a Xevo™ TQ-S triple quadrupole mass spectrometer both from Waters (Millford, MA, USA), equipped with an Acquity UPLC® BEH C18 column (dimensions: 100 × 2.1 mm and particle size: 1.7 µm), thermostatted at 45 °C. The mobile phases were (A) water with 0.1% ammonia and (B) acetonitrile. All the gradient changes were performed in a linear manner. The elution was done with a gradient program of 12 min at 0.4 mL/min flow rate starting at 5% of phase B, kept for 1 min, then rising to 15% till 2 min before a new isocratic separation for

1 min, increasing to 20% (from 3 to 5 min), 25% (from 5 to 6 min), 50% (from 6 to 9 min) and 95% (from 9 to 10 min). This rate was kept for 0.5 min before returning to the initial conditions in 0.5 min and held for 1 min for re-equilibration. The injection volumes were 10 µL for plant extracts and 5 µL for infusion extracts.

The samples were ionized with an electrospray probe in positive mode operating at a cone voltage of 30 V. The capillary voltage was set at 1 kV. Cone gas flow and desolvatation gas flow were respectively 150 L/H and 800 L/H. The source and desolvation temperatures were set at 150 °C and 500 °C, respectively. Argon was used as collision gas with a flow of 0.17 mL/min and the collision energies were optimised in order to obtain two fragments for each PA/PANO. The spectrometer was programmed in MRM mode for the monitoring of 2 transitions for each compound. The dwell time for both transitions was set in order to acquire at least 12 points.

The selected quantification and confirmation fragments with their respective collision energies are listed in Supplementary Data, Table S-1.

2.4. Validation of the method

The developed methods were in-house validated following the procedure described by Van Loco and Beernaert (2003). The following parameters were evaluated and the validation criteria laid down by Directive 2002/657/CE and SANTE/11945/2015were used to evaluate the validation parameters: linearity, matrix effect, recoveries, repeatability, (intra-)reproducibility, limit of quantification (LOQ), selectivity and accuracy. The matrix used for validation was a blank "Sencha" green tea. The validation experiments were performed on 3 days at 2 concentration levels in triplicate. The spiked levels were chosen in accordance with the expected PAs/PANOs concentrations in the targeted matrix and the presence and nature of potential interferences. The repeatability (r) and within-lab reproducibility (rw) were evaluated by calculating the coefficients of variation (CV) obtained for these two parameters at each spike level. Apparent recoveries were calculated at each spiked levels and measurement uncertainties (MU) was assessed through the CVr and CVrw.

2.5. Transfer study

The dry tea materials were brewed following a standardized method described in ISO 3103 (1980): 2 g of dry material was brewed for 6 min in 100 mL boiling water. An original pre and post brewing spiking experiment was developed: on one hand, a dry "sencha" green tea sample was spiked before brewing, giving a final concentration that was representative of the real PA transfer; on the other hand, a blank dry "sencha" tea sample was brewed and the infusion solution was spiked with the same PAs/PANOs amount than above in order to represent a virtual transfer of 100% (Supplementary Data, Fig. S-2). The ratio of both results indicates the transfer rate of the targeted compounds.

The transfer rates have been calculated for each compound, at 2 concentration levels (i.e. 0.02 and 2 ng/mL) in triplicate on each day of the validation.

2.6. Quality control

Multiple parameters (sensitivity, recovery and detector response) were evaluated during the analysis of each batch. For each batch, the second concentration level of the calibration curve was spiked in solvent and taken as a control for the sensitivity of the UPLC-MS/MS system. The *S/N*-ratio of the signal related to the compound that exhibits the lowest MS response (i.e. monocrotaline N-oxide) had to be at least 10 before the samples were injected. To ensure total absence of carry-over, a solvent sample was injected after the calibration curve. Furthermore, a point in the middle of the calibration curve (1 ng/g or 0.5 ng/mL) was repeatedly injected after every 10 samples to ensure a stable response of the detector. The tolerated deviation from the theoretical concentration was set at ± 20%. The concentration of each PA/PANO was calculated from a spiked matrix-matched calibration curve with a mix of the 30 targeted compounds using the QuanLynx software (Waters, Manchester, UK). The results were automatically corrected for recoveries. The identity of the detected PAs/PANOs was confirmed with their retention time and the ratio of 2 daughter ions corresponding with 2 MS/MS transitions. The analysis of the reference standards was used for comparison.

3. Results and discussion

3.1. Optimization of the sample preparation

The sample preparations were optimized to obtain the best method sensitivity taking into account the combination of the very low targeted LOQs (0.1 ng/g for dry plant products, EFSA 2011) and the complexity of the food matrices. The following parameters were optimized: the sample aliquot, the volume of extract used for the clean-up with SPE, the volume of solvent used to reconstitute the purified extract before injection and the volume of purified extract injected on the LC column. The addition of acid to the extraction solvent lead to better extraction yields of the PAs/PANOs. In the course of the development of the extraction protocol for dry extracts, an aqueous acidic extraction was tried but this media led to irreproducible analytical results, most probably because of swelling and variable water absorption by the dry plant products. The use of methanol with formic acid gave reproducible results. This organic solvent enabled the extraction of more interfering compounds than water, but the SPE Envi™-Carb subsequent purification permitted the trapping of most of them (planar compounds, pigments and polyphenols in majority). Two successive extractions were necessary to obtain good recoveries and the volumes were controlled using a volumetric flask to ensure analytical accuracy. The combination of vortex mixing, ultrasonication and overhead shaking facilitates and quickens the extraction process (Chemat et al., 2017). Finally, the reconstitution of the extract with the injection solvent (H_2O/MeOH 80:20) led to precipitation of additional impurities that were eliminated by centrifugation. To the best of our knowledge, this is the first time that infusions – not only dry plant extracts - are considered in the framework of PAs/PANOs detection. Yet, this approach is meaningful because this is the way purchasers really consume (herbal) teas and in consequence the way they are really exposed to PAs/PANOs contamination. Infusions of (herbal) teas were performed according to ISO 3103 (1980) standardized method for tea brewing. Afterwards, the infusions were purified by SPE. Diverse SPE stationary phases were tested and the more reproducible results were obtained with an Isolute® C18 cartridge. The crucial point to obtain good recoveries during the SPE purification is the basification to pH 9–10 of the crude infusion extract. The experiments showed that the loading of the SPE cartridges with the infusion as such led to loss of analytes at this step. The retention of organic analytes from polar solutions (e.g. water) onto reversed phase C18 SPE materials is due primarily to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. These nonpolar-nonpolar attractive forces are commonly called van der Waals forces, or dispersion forces. If the compound of interest is acidic or basic (case of PAs), it is recommended to use a pH at which the compound is not charged. The retention of charged species onto the reversed phase C18 SPE stationary phase would be disturbed. At physiological blood pH (± 7.4), the nitrogen within the pyrrolizidine moiety exists in a charged form (Pomeroy & Raper, 1971). The pH of tea infusion is ± 6 which implies that the nitrogen atom is charged in this scenario too. In consequence, basification of the infusion extract was mandatory to yield a complete retention of the analytes onto the C18 stationary phase. Therefore, pH 9–10 showed empirically to be the most suitable value. The reconstitution of the extract with the injection solvent (H_2O/MeOH 80:20) led to

precipitation of additional impurities, that were eliminated with an additional centrifugation step. The optimized sample preparation procedures allowed to easily analyze hundreds of samples with complex matrices with very low LOQs without suffering from sensitivity loss.

3.2. Optimization of the UHPLC- MS/MS method

An UPLC BEH reversed phase C18 column was chosen for its universal character and the wide usable pH range. The presence of ammonia in the aqueous phase improved the separation and chromatographic peak shapes compared to the acidic media that are more regularly used for the analysis of PAs/PANOs (Gray, Porter, O'Neill, Harris, & Rottinghaus, 2004). One of the major bottlenecks in quantitative assessment of individual PAs/PANOs is the co-occurrence of isomers. It is noteworthy that the optimized LC gradient and the choice of a suitable injection (H_2O/MeOH 80:20) solvent led to chromatographic separation of the 3 PANOs isomers intermedine N-oxide, lycopsamine N-oxide and indicine N-oxide with respective retention times of 2.74, 2.84 and 2.93 min (a typical chromatogram of a standard solution is depicted in Supporting Information, Fig. S-3). However, the 3 corresponding PAs could not be resolved. As a consequence, results are expressed as a sum of these 3 isomers. Due to the use of a basic mobile phase, the PANO systematically eluted before the corresponding PA. Regarding the MS/MS parameters, the fragmentation pattern of each compound was first monitored at collision energies ranging from 5 to 30 eV by step of 5 eV by injecting individual standard solutions. From these acquisitions, the two most intense transitions exhibiting less matrix interference were chosen as quantifying transition and confirmation transition. At least one of the two toxic related fragments (m/z = 120 and 138) was found for the majority of PAs/PANOs. An overview of the selected ions is given in the Supplementary Data, Table S-1.

3.3. Validation

3.3.1. Linearity and matrix effect

Linearity was assessed at 7 concentrations levels for plant extracts (i.e. 0, 0.1, 0.5, 1, 5, 10 and 20 ng/g) and 8 concentration levels for infusions (i.e. 0, 0.01, 0.05, 0.1, 0.5, 1, 2.5 and 5 ng/mL). Each calibration level was injected in triplicate, the response of each compound was plotted against the concentration level and submitted to a Mandel's Fitting test to evaluate linearity. The results of the test indicated a linear model fit for the response in both methods. However, obtained correlation coefficients values were better ($R^2 > 0.98$ for all 30 standard compounds) and less standard deviation was experimentally observed using a quadratic model, the latest has thus been chosen. Matrix effect was assessed by a *t*-test at 95% confidence level between a calibration curve made in pure injection solvent and one in matrix. The different concentration levels for both calibration curves were end-spiked to ensure that the measurements were only due to matrix effect, without any recovery influence. The results clearly showed that there was a significant difference between the slopes of the curves, meaning that a matrix effect was present for both methods. As a result, spiked matrix matched calibrations curves were used for the quantification.

3.3.2. Selectivity and limits of quantification (LOQs)

The combination of the SPE purification with the monitoring of 2 MS/MS transitions resulted in a high level of selectivity for both methods. Consequently, no peaks were detected at the retention times of the PAs/PANOs of interest when a blank matrix was analysed. The realistic limits of quantification (LOQs) for the 30 compounds taking into account potential loss in the course of the sample preparation were assessed by spiking blank samples before the extraction/purification process. For practical reasons, LOQs were defined and set at the first calibration point ($\neq 0$) for which the signal to noise ratio was at least 10. The LOQs are extremely low and were determined to be 0.1 ng/g for most of the compounds in plant extracts (0.5 ng/g or 1 ng/g for the other compounds), and 0.01 ng/mL in infusion extracts.

3.3.3. Precision, measurement uncertainty and apparent recoveries

Repeatability and reproducibility were evaluated according to the methodology described by Van Loco and Beernaert (2003) and results are reported in Supplementary Data (Table S-2 for dry matrices and Table S-3 for infusions). Most of the CVs were below the maximum allowed values from the Horwitz equation (CVr < 14.7%, CVrw < 22%). For dry plant analysis, blank "Sencha" green tea samples were spiked with a mix of the 30 targeted compounds the day before sample preparation to obtain a low (0.5 ng/g,) and high (20 ng/g) final concentration and analysed in triplicate on three different non-consecutive days. The blank matrix (2g) was spiked with 100 µL of 10 ng/mL solution of 30 PAs/PANOs and with 40 µL of 1 µg/mL solution of 30 PAs/PANOs, respectively for the low and the high validation level. Erucifoline N-oxide, europine, europine N-oxide, jacobine, retrorsine, retrorsine N-oxide, seneciphylline N-oxide, senecivernine N-oxide and trichodesmine exhibit slightly higher CVr values, while europine, jacobine, retrorsine, retrorsine N-oxide and trichodesmine present slightly higher CVrw values. In consequence, MUs are higher for those compounds, principally at low levels. Particularly, the quantification values for retrorsine at low level should be considered as estimates. Apparent recoveries were within the range 70–120% for 28 compounds at both validation levels, which was within the performance criteria proposed by Directive 2002/657/CE, only retrorsine (123%) and trichodesmine (125%) fall out of the tolerated range for recoveries at low level. The deviations from the preferred ranges for CVr, CVrw and recovery values are attributed to a higher matrix effect at low concentrations for those compounds.

For infusions, blank "Sencha" green tea samples were brewed according to the protocol described above. The infusions were then spiked with a mix of the 30 targeted compounds to obtain a low (0.02 ng/mL) and high (2 ng/mL) concentration and analysed in triplicate on three non-consecutive days, with at least one day in between. The blank infusion (100 mL) was spiked with 200 µL of 10 ng/mL solution of 30 PAs/PANOs and with 200 µL of 1 µg/mL solution of 30 PAs/PANOs, respectively for the low and the high validation level. Intermedine N-oxide, lycopsamine N-oxide, monocrotaline and monocrotaline N-oxide exhibit slightly higher CVr values; lycopsamine N-oxide, monocrotaline and monocrotaline N-oxide present slightly higher CVrw values. In consequence, MUs are higher for those compounds, principally at low levels. Apparent recoveries were within the range 70–120% for all compounds at both validation levels, which was within the performance criteria proposed by Directive 2002/657/CE. The deviation from the preferred ranges for CVr, CVrw and recovery values is attributed to a higher matrix effect at low concentrations for those compounds.

3.3.4. Accuracy

No reference material was available at the time of investigation, the accuracy of the method for the quantification of PAs/PANOs in plant extracts was thus evaluated by means of participation in a Proficiency Test organized by ProofACS (post validation) in which 11 laboratories reported results. The proposed PT matrix was rooibos tea, which is known to be a highly challenging matrix, containing spiked and incurred PAs and PANOs. Table S-4 (Supporting Information) summarises the results obtained for the PT material. All compounds were correctly identified (7 spiked: heliotrine N-oxide, intermedine, lycopsamine N-oxide, retrorsine, seneciphylline, seneciphylline N-oxide, senkirkine, and 4 incurred: retrorsine N-oxide, senecionine, senecionine N-oxide and senecivernine N-oxide) and none false positive was reported. Remarkably, the comparability criterion was passed for all PAs/PANOs (all z-scores were below the critical allowed value of |2|) and the trueness criterion (results in the range 70–120% of the spiked level) was passed for all compounds except one (retrorsine, 64%).

3.4. Sample analysis

The concentration data relative to the 30 compounds (16 PAs and 14 PANOs) will be discussed according to their 4 main families. This classification is based on structure similarities and botanical origins (Supporting Information, Fig. S-1):

Lycopsamine-type: echimidine, echimidine N-oxide, indicine, indicine N-oxide, intermedine, intermedine N-oxide, lycopsamine, lycopsamine N-oxide.

Monocrotaline-type: monocrotaline, monocrotaline N-oxide, trichodesmine.

Heliotrine-type: europine, europine N-oxide, heliotrine, heliotrine N-oxide, lasiocarpine, lasiocarpine N-oxide.

Senecionine-type: erucifoline, erucifoline N-oxide, jacobine, jacobine N-oxide, retrorsine, retrorsine N-oxide, senecionine, senecionine N-oxide, seneciphylline, seneciphylline N-oxide, senecivernine, senecivernine N-oxide, senkirkine.

If no specification is made, the concentrations are expressed as a sum of the 30 PAs/PANOs. The calibration curve ranges were deliberately selected in order to favour the quantification at the low part of the calibration. In consequence, the maximum quantifiable individual PAs/PANOs concentration was relatively low (20 ng/g in dry extracts, 5 ng/mL in infusion extracts). In case of high PA/PANO concentration detected in a sample (> validated linearity range), the purified extract was diluted by the appropriate factor with the corresponding matrix blank extract used in the calibration curve and re-injected for a new analysis. Most of those 30 PAs/PANOs available as analytical standards were quite frequently encountered in the different food matrices, only erucifoline (+NO), indicine (+NO), jacobine (+NO), monocrotaline (+NO) and trichodesmine were clearly anecdotal in these matrices.

3.4.1. Mixed salads

None of edible salads are known to produce PAs. However, salads were included in the sampling plan because of the close shape similarities between rucola and ragwort leaves, which are PA producing plants. The study focused on pre-packaged salads (17 samples) that are more prone to be contaminated with other plant species. The entire packs were individually freeze dried before homogenization by milling in order to be able to reach the targeted LOQ values. Indeed, water content represented 92–95% of the samples weights. The PAs/PANOs concentration values in the dry products were converted to the corresponding concentration for the fresh products. The results are given in Supplementary Data (Table S-5). At least 1 targeted compound with a concentration above the LOQ (dry product) was found in 53% of the samples, but 70% of the samples contained less than 0.1 ng/g of PAs/PANOs (fresh product, Fig. 1section (a)). The average PAs/PANOs concentration in pre-packaged salad mixes was 1.13 ng/g. This can be evaluated as relatively high given the broad consumption of these food items, which was quite unexpected. Three mixed salad samples were contaminated with 2.59, 5.20 and 10.47 ng/g exclusively of senecionine-type PAs/PANOs. The major contaminants were retrorsine, retrorsine N-oxide and seneciphylline N-oxide (Fig. 2). Moreover, none of these samples were labelled as containing rucola (only mixes of escarole, curly endive, radicchio and lamb's lettuce). In these cases, the contamination was clearly due to co-harvesting.

3.4.2. Herbs and spices

Interestingly, all the 17 analysed spices samples were contaminated as can be seen in Fig. 1section (b). Quantitative results are given in Supplementary Data (Table S-6). However, none of the label description of the products reported the presence of known PAs/PANOs producing plants in their compositions. Thirty percent of the samples contained more than 50 ng/g of pyrrolizidine alkaloids. The average PAs/PANOs concentration in spices was 197 ng/g. Even up to 1770 and 1065 ng/g were found in Mediterranean herbs mixes respectively for spaghetti and pizza, with a majority of europine and europine N-oxide. Heliotrine-type PAs/PANOs were responsible of 85% among the overall contamination of spice mixes, which is a clear evidence of *Heliotropium* spp. co-harvesting or adulteration. However, an herbs mix for salads contained 239 ng/g exclusively of the senecionine-type. Lycopsamine-type compounds were also often encountered but in smaller concentrations (Fig. 3). These different PAs/PANOs patterns indicate that spices can be contaminated with a large diversity of unexpected/unintended botanical species. Unfortunately, there are currently very few reports on the subject in the literature, but the presence of *Borago* spp. in culinary herbs was reported recently (Mädge et al., 2015; Cramer, Schiebel, Ernst & Beuerle, 2013; Kapp, 2017). Moreover, herbs and spices adulteration is poorly controlled and publications recently revealed that oregano was largely adulterated (Black, Haughey, Chevallier, Galvin-King, & Elliott, 2016). This could also explain the PAs/PANOs contamination of herbs and spice mixes. Indeed, "oregano" was present in most of the contaminated samples. To the best of our knowledge, only very few studies have focused on the detection of PAs and PANOs in salads and herbs/spices. In light of the results, these matrices should be further investigated.

3.4.3. Ice-teas

Anecdotally, 7.58 ng/mL of senecionine-type compounds (mainly retrorsine N-oxide) were found in a ready-to-drink ice-tea. This sample contained the highest percentage of tea extract (95%) amongst the 28 samples analysed. All 27 others were mostly free of contaminants. Quantitative results are given in Supplementary Data (Table S-7).

3.4.4. Teas and (Herbal) teas in dry state

(Herbal) teas are quite challenging to analyse because of their huge diversities in composition, with added fruits, flavouring, various herbs, flowers or roots. In addition, black teas are oxidized. Consequently, the calibration curve was adapted to the different sample types in order to mimic the real samples as closely as possible. Three different matrix blanks were used for dry extracts as well as infusions: Sencha green tea for samples labelled as containing a majority of green tea, Keemun imperial black tea for samples labelled as containing a majority of black tea and an in-house prepared mix of chamomile, fennel and peppermint (1:1:1) dedicated to herbal teas, as these species are the most often encountered in the herbal teas purchased from the Belgian market. In addition, it appeared that teas and herbal teas containing hibiscus systematically led to false positive detects for jacobine and retrorsine (both $m/z = 352.3$ and same fragmentation patterns), with unmatched ion ratio values compared to the reference values. The fragmentation pattern of a sample containing hibiscus was studied, leading to the identification of 2 specific MS/MS transitions of the interfering compound (352.3 > 316 and 352.3 > 298). In consequence, these 2 transitions were monitored as "guard transitions": if they were detected, quantification of jacobine and retrorsine was not feasible (more visual details of extracted chromatograms are given in Supporting Information, Fig. S-4a and b. In total, 159 samples including black teas, green teas, rooibos teas and herbal teas were analysed; 87% of the samples were contaminated with at least 1 PA/PANO above the LOQ (EFSA reported 91% contaminated samples) . The term "herbal tea" refers to mixes of herbs without tea plant (*Camellia sinensis*). It should be noted that some herbal teas bought online and in organic shops were labelled as containing PA producing plants; the results for these particular mixes will be discussed separately.

The maximum level detected in (herbal) teas analysed as dry material (4246 ng/g) as well as the most contaminated categories (rooibos, black teas and mixed herbs) were in good agreement with the data published by Bodi et al. (2014) and EFSA (4805 ng/g; rooibos and mixed herbs). PANOs (73%) were largely more represented than PAs (27%) in all (herbal) teas; this behaviour is observed for the great majority of plant based products. The products bought in specialized tea-houses were much less contaminated than (herbal) teas from the classical retail sector. The distribution of total PAs concentrations in

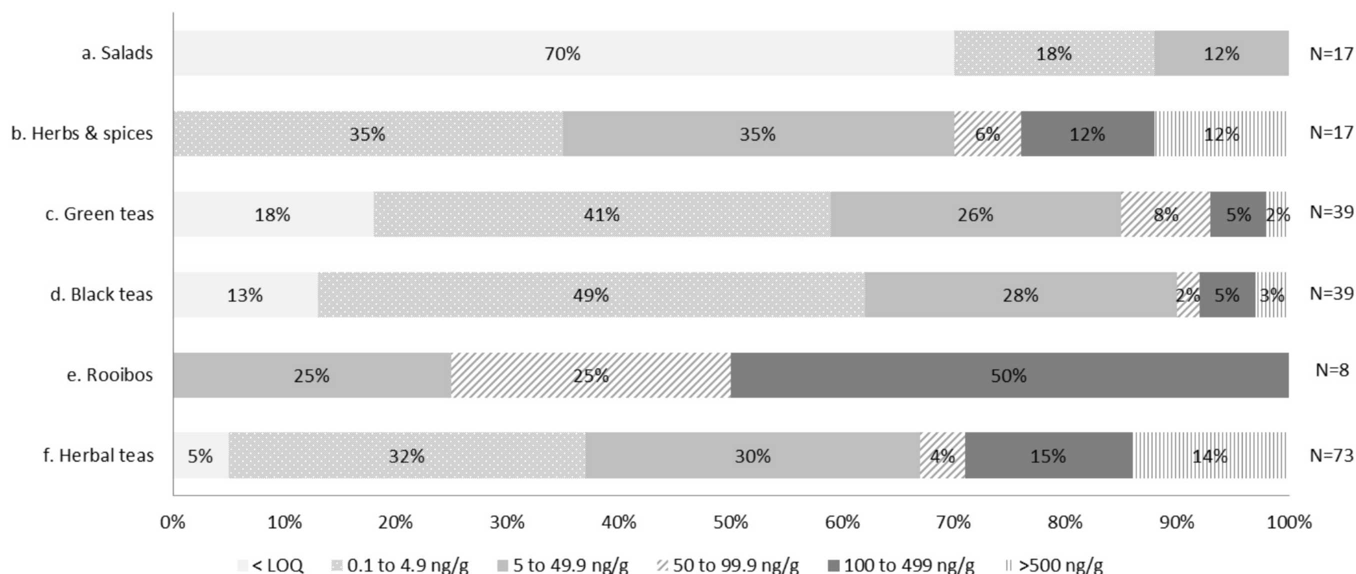

Fig. 1. Distribution of concentrations (sum of 30 PAs/PANOs) in the different food matrices targeted in this study.

each sample depending on sample types is shown in Supporting Information, Fig. S-5. Rooibos teas were included in the category "herbal teas (no PA plant)". From these results, it appears that tea based products behaves differently than herbal teas, the results will thus be discussed separately. Given the wide concentration ranges in the different tea types, it is here more relevant to treat the results in mean concentrations instead of maximum concentrations.

3.4.4.1. Green and black teas. The distributions of PAs concentration ranges in green (N = 39) and black teas (N = 39) were comparable: respectively 15% and 10% of the samples contained less than 50 ng/g of PAs/PANOs (Fig. 1section (c) and section (d)). A very high level was detected in a green tea sample (T-124, 4246 ng/g) whereas the maximum level in a black tea sample was 887 ng/g. The mean PAs/PANOs concentration in green teas was in consequence higher (137 ng/g) than in black teas (43 ng/g). However, median values were similar for both tea types: respectively 2.35 and 2.28 ng/g. The nature of contaminants in black and green teas was quite similar, with respectively 91 and 88% of the contamination due to senecionine-type PAs/PANOs. All compounds from this family were often detected (Fig. 4, histograms), but the main contributor (with highest mean concentrations) were retrorsine and its N-oxide (Fig. 4, lines).

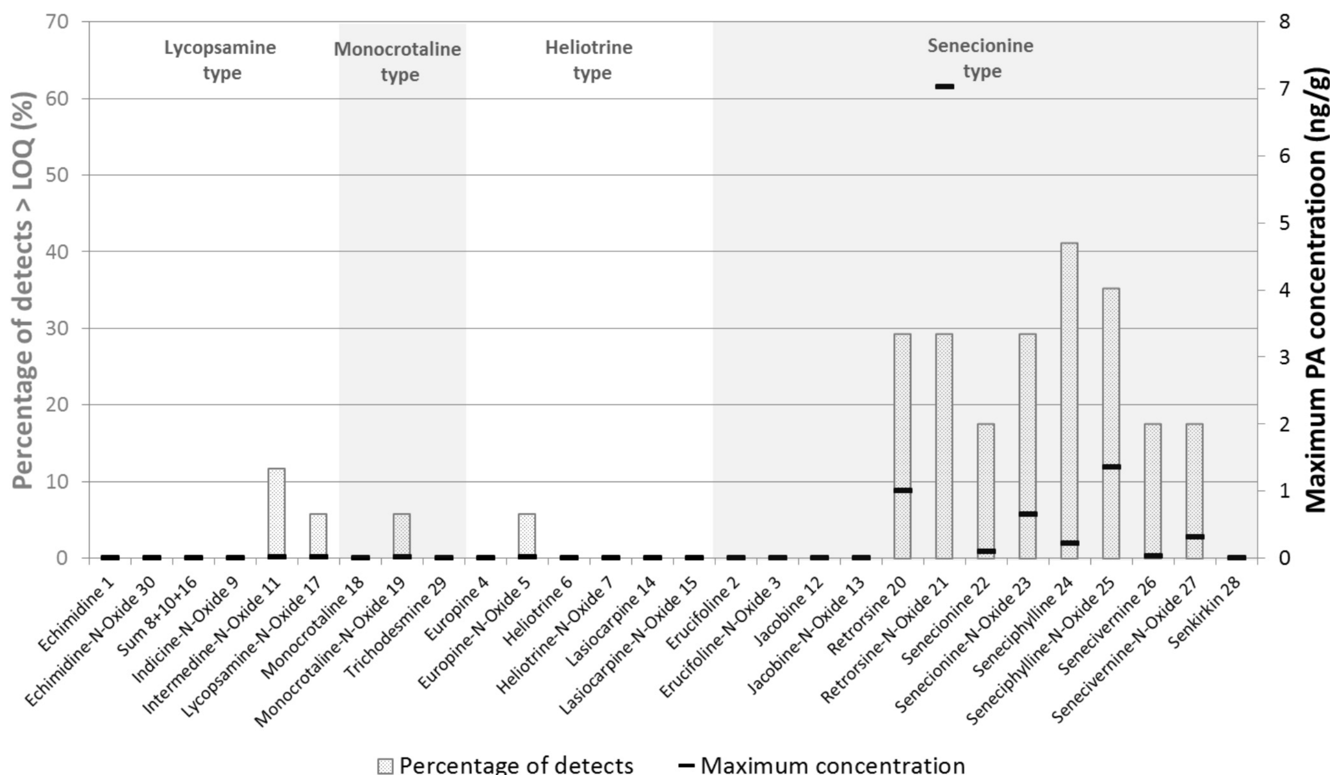

Fig. 2. Percentage of detects above the LOQ value and maximum concentration measured for each individual PA/PANO grouped by family type (grey and white background) in pre-packaged salad samples (N = 17).

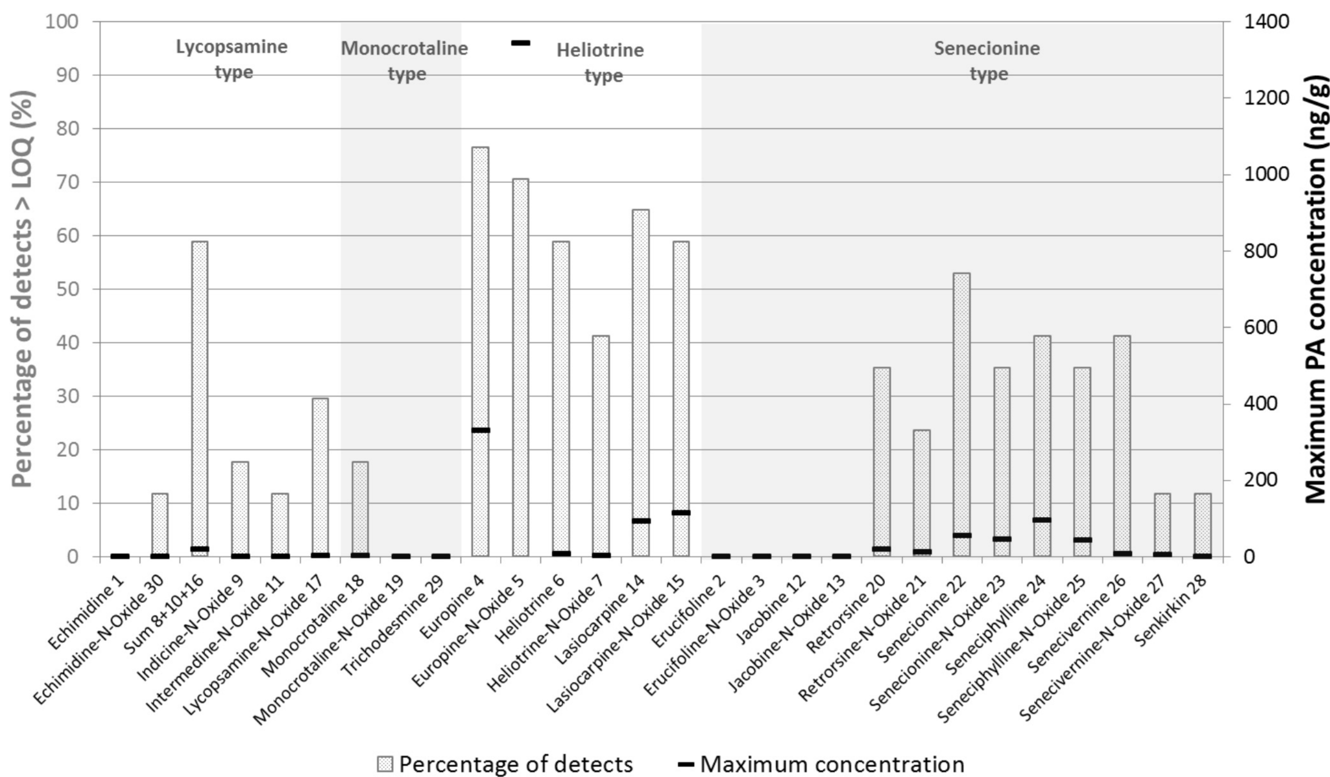

Fig. 3. Percentage of detects above the LOQ value and maximum concentration measured for each individual PA/PANO grouped by family type (grey and white background) in herbs & spices samples (N = 17).

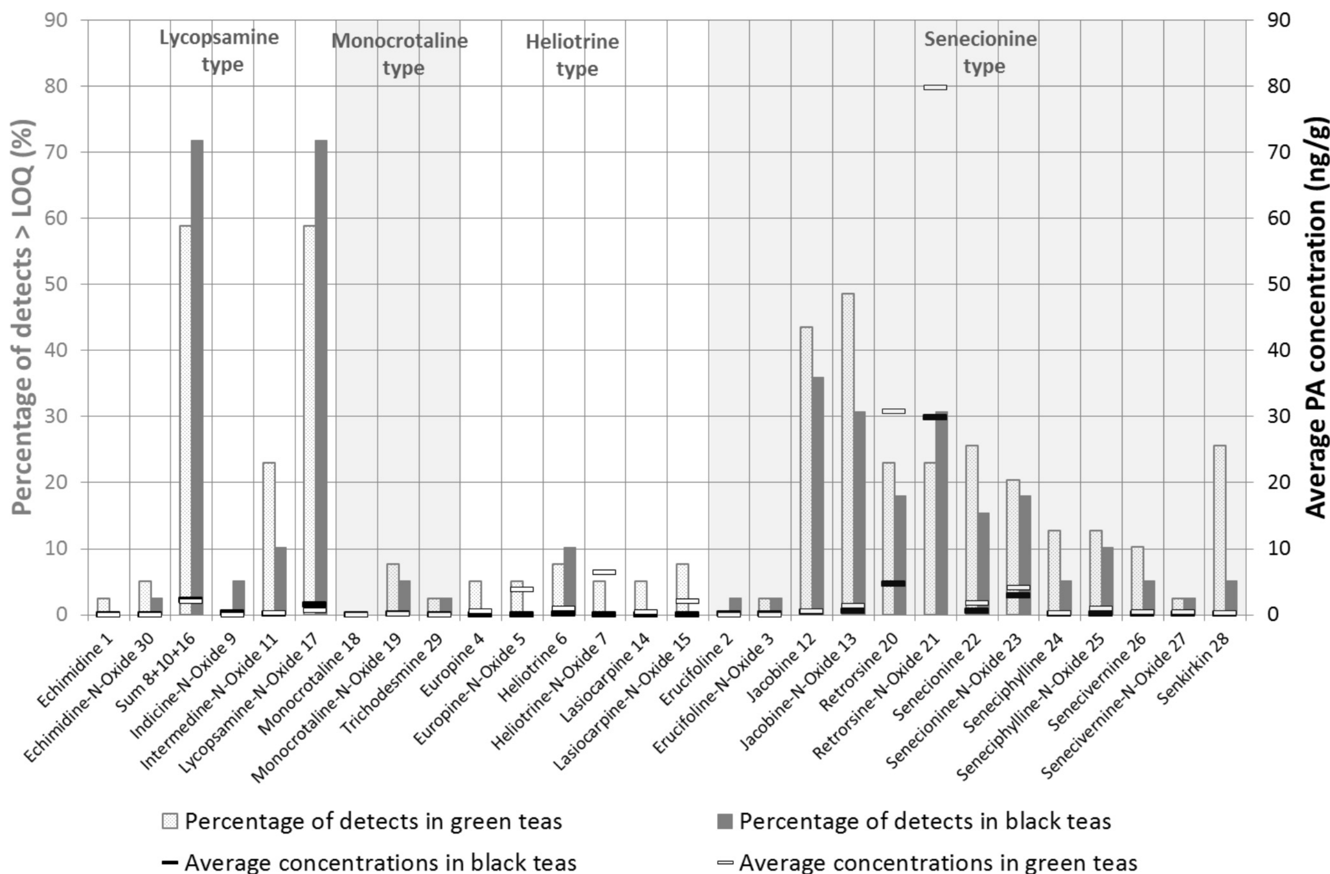

Fig. 4. Percentage of detects above the LOQ value and average concentration measured for each individual PA/PANO grouped by family type (grey and white background) in green tea samples (N = 39) and black tea samples (N = 39).

Lycopsamine/intermedine/indicine and lycopsamine N-oxide were the most frequently encountered compounds, but mainly at low concentrations. Quantitative results are given in Supplementary Data (Table S-8 for green teas and Table S-9 for black teas).

3.4.4.2. Herbal teas and rooibos teas. All the rooibos tea samples (*Aspalathus linearis*) were contaminated (Fig. 1section (e)), with levels up to 260 ng/g. The mean PAs/PANOs concentration in rooibos teas was 113 ng/g and the median value was 90 ng/g. PAs/PANOs contamination of rooibos teas has already been reported and was attributed to the co-harvesting of *Senecio angustifolius*. Indeed, studies showed that *Senecio angustifolius* occurs as a common weed throughout the rooibos production area in South Africa and that it contains high levels of the same PAs (and in the same ratios) as those found in contaminated rooibos tea (Van Wyk, Stander, & Long, 2017).

Herbal teas were also massively contaminated (95% of the samples, Fig. 1section (f)) with concentrations up to 187151 ng/g in an herbal tea containing borage (PA producing plant) used as a cold remedy (CA-074). Looking now at herbal teas without PA producing plants, the maximum level was 1936 ng/g (T-011). With and without PA plant, the mean PAs/PANOs concentrations in herbal teas were 41000 ng/g and 139 ng/g, respectively. The median values were 537 ng/g and 21 ng/g. The quantitative results for both types of herbal teas showed clearly massive contamination, but with significant difference in levels detected with and without PA producing plant in the sample (concentration data are given in Supplementary Data, Table S-10). In Fig. 1section (f), the highest concentrations were mainly attributed to herbal teas containing PA producing plants, whereas lower concentration came from herbal teas without known PA plant.

It appears clearly from Fig. 5 (histograms) that most of the targeted PAs/PANOs were often encountered in herbal teas (PA plants, no PA plants and rooibos), except monocrotaline-type compounds. This result was consistent with the very broad range of herbs that can enter in the composition of these commodities, which increase the possibilities of contaminating botanical species due to co-harvesting.

Rooibos teas were dominated by senecionine-type PAs/PANOs (85% of the total contamination), the profile of contamination is totally consistent with the one described by Van Wyk et al. (2017) for the co-harvesting of *Senecio angustifolius*. The contamination pattern of herbal teas (without PA plant) was quite more diversified with 65% senecionine-type and 28% lycospamine-type. However, the mean concentrations of senecionine-type compounds were somewhat higher, particularly senecionine N-oxide (Fig. 5, lines).

Amongst the 11 herbal tea samples labelled as containing PA producing plant, 99% of the contamination came from lycopsamine-type PAs/PANOs (exclusively lycopsamine and isomers, intermedine N-oxide and lycopsamine N-oxide, Fig. 6). The contamination pattern is thus totally different in presence of PA producing plants.

Herbal teas containing PA producing plants were found online and in organic shops. They were composed of *Eupatorium perfoliatum* (CA-037, CA-098, *Asteraceae*), *Borago officinalis* (CA-074, CA-099, *Boraginaceae*), *Pulmonaria officinalis* (CA-085, *Boraginaceae*) or *Lithospermum purpurocaeruleum L.* (CA-091, *Boraginaceae*). *Asteraceae* and *Boraginaceae* are well known to produce mainly lycopsamine type PAs. Samples based on *Echinaceae*, *Arctium* (both *Asteraceae*) or *Desmodium* (*Fabaceae*) contained only traces of PAs/PANOs.

3.5. Transfer rates of PA from dry sample into infusion using ISO 3103 harmonized brewing process

Since consumers do not "eat" dry (herbal) teas as such, the transfer rate of PAs and PANOs to the infusion in the course of the brewing

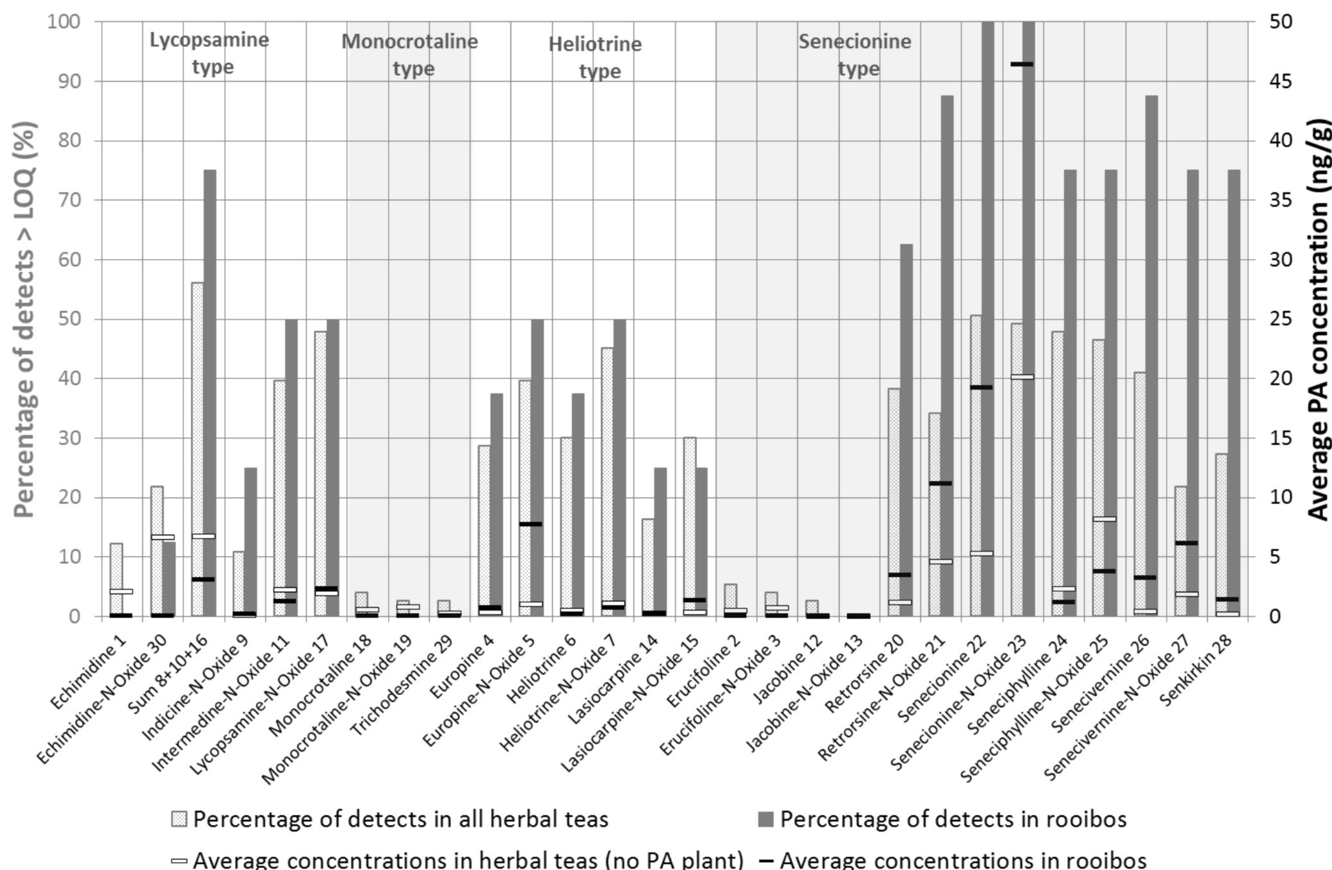

Fig. 5. Percentage of detects above the LOQ value and average concentration measured for each individual PA/PANO grouped by family type (grey and white background) in herbal tea samples without PA plant (N = 62) and rooibos samples (N = 8).

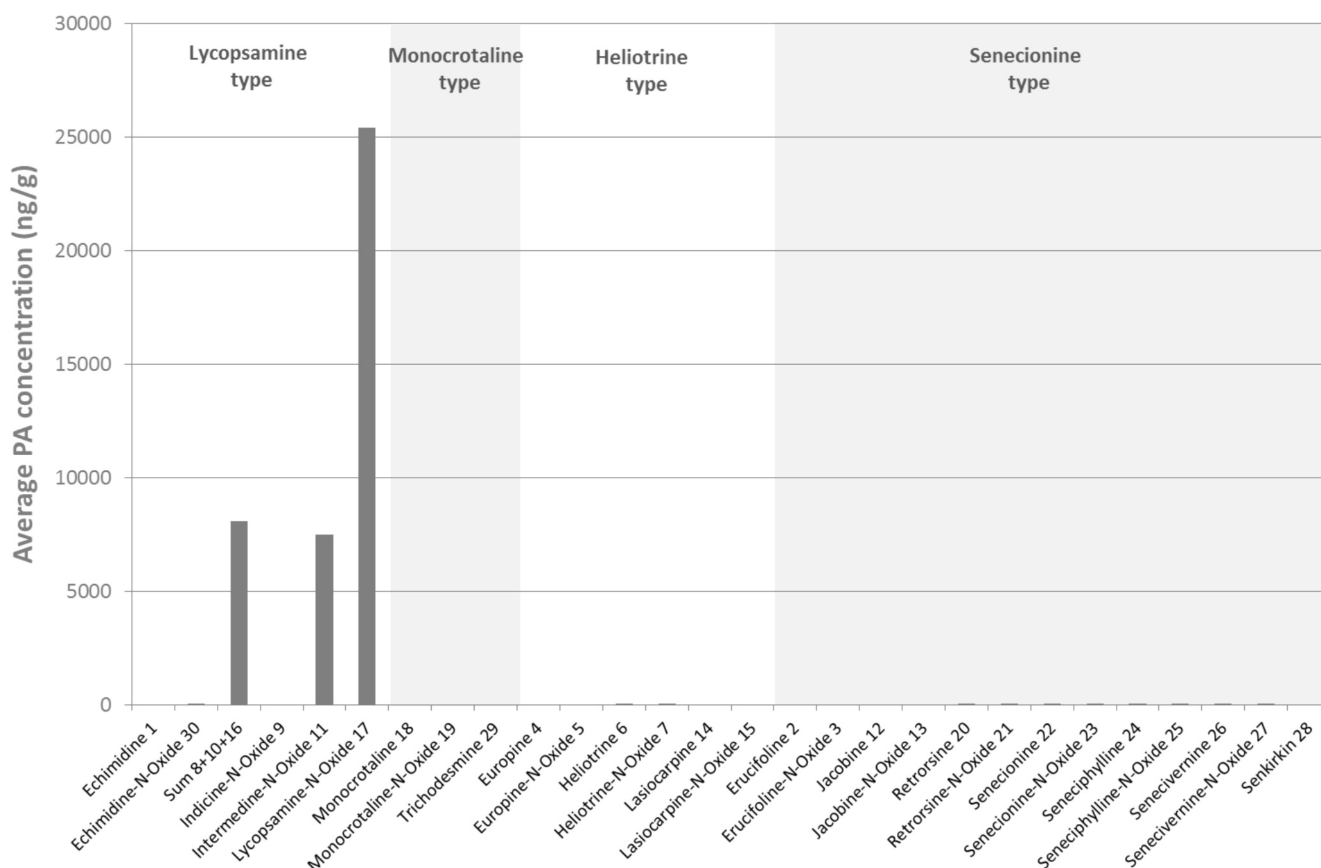

Fig. 6. Average concentration measured for each individual PA/PANO grouped by family type (grey and white background) in herbal tea samples labelled as containing PA producing plants (N = 11).

process should also be evaluated. To the best of our knowledge, only one publication reported this kind of approach very recently (Mulder et al., 2017). Moreover, EFSA usually applies a simple dilution factor to determine the PAs/PANOs concentration in the infusion from quantification experiments in the dry material, which could induce an overestimation of the actual exposure. In this study, the transfer rates were calculated as described in Section 2.5. Depending on the compounds, the results show that only 16–28% of the PAs/PANOs contamination was effectively transferred from the dry material to the infusion during the brewing process (except for monocrotaline, 45%). The calculated transfer rates were repeatable with RSD between 0.6 and 8.1%, only monocrotaline gave higher variations (Supplementary Data, Table S-11). A possible reason to explain the partial transfer of pyrrolizidine alkaloids could be the absence of acid in the brewing process.

The transfer rate model was also confirmed using a rooibos tea sample from a proficiency test containing 11 spiked and incurred PAs/PANOs. Both the dry tea and the brewed tea were analysed. Next, the expected concentration of PAs/PANOs in tea was calculated using the concentration of PAs/PANOs in the dry tea in combination with the actual transfer rates. These calculated results were then compared to the actual concentrations in the brewed tea. Results have shown that the calculated concentrations of PAs/PANOs in the infusion are slightly underestimated (ranging from 0 to −22% underestimation). However, it should be noted that the approach that is used by EFSA, meaning the application of dilution factor to the results obtained in the dry product and thus considering a 100% transfer of the PAs/PANOs lead to an enormous overestimation of the actual concentration in the brewed tea (ranging from 240 to 440% overestimation). The performance of the transfer rate model was thus confirmed (see Supplementary Data, Table S-12 for all values).

The study reported by Mulder and al. focused on a comparison of 28 PA/PANO concentrations between tea infusion in near-boiling water and extraction with aqueous sulphuric acid at room temperature. No transfer rates were mentioned but the mean PA/PANO concentrations were graphically very close for the 2 approaches. In consequence, the transfer rates of PAs/PANOs reported in the present study seem to be significantly lower. As discussed in Section 3.1., it should also be noticed that attempting to use aqueous acidic media led to incomplete PAs/PANOs extraction from the dry plant material and irreproducible analytical results.

Although the harmonized brewing method ISO 3103 (1980) was used in both studies to perform tea infusions, the parameters were interpreted differently (this study; Mulder et al. (2017)): volume of water (100 mL; 150 mL) and brewing time (6 min; 5 min). Moreover, some unspecified parameters in the ISO document could have a massive impact on the transfer rates (this study; Mulder et al. (2017)): type of water (purified water; unspecified), brewing (static; steeped), after brewing (tea bag allowed to strain, no filtration of infusion; tea bag removed, filtration of infusion).

As can been seen above, even if a harmonized method is used, discrepancies in interpretations and unspecified parameters impact greatly the analytical results. The different steps of the infusion experiments should be clearly described in papers using this approach.

3.5.1. *Contamination levels in infused (herbal) teas*

Obviously, the contamination patterns (nature of PAs/PANOs contaminants) described in the dry product approach remained valid for the infusions. However, the actual concentration levels in the infusions are different.

All the (herbal) tea samples were prepared as “dry materials” and the most contaminated samples detected in this dry product approach were brewed in order to obtain experimental PAs/PANOs concentration

values in the related infusions. PAs/PANOs levels in the other (herbal) teas were determined theoretically. The experimental and theoretical quantitative results for all targeted compounds in (herbal) infusion samples are reported in Supplementary Data, Table S-13 (green tea infusions), Table S-14 (black tea infusions) and Table S-15 (herbal tea and rooibos infusions). The distribution of PAs/PANOs concentrations in all brewed samples is given in Fig. S-6. Maximum PAs/PANOs concentrations were 5.89 ng/mL, 40.10 ng/mL and 30.04 ng/mL respectively for black tea, green tea and herbal tea infusions (without PA plant), whereas infusions containing PA producing plant exhibited contamination levels up to 2106 ng/mL. Mean levels in the infusions were 0.39 ng/mL, 1.29 ng/mL, 1.56 ng/mL and 383.02 ng/mL, respectively for black tea, green tea and herbal tea without and with PA plant. Note that PAs/PANOs levels in instant tea samples were anecdotal.

As described above, this new approach to quantify PAs/PANOs in (herbal) infusions contrasts significantly with the conventional strategy used by EFSA. As a result, major discrepancies with previously reported concentration values appear: the contamination levels are lower but much more realistic: the mean contamination in green tea infusions and herbal infusions (without PA plant) is ± 4 fold lower than previously reported values by EFSA (respectively 5.65 ng/mL and 5.82 ng/mL), even 20 times lower in black tea infusions (7.62 ng/mL). In presence of PA producing plants, the mean PAs/PANOs concentration falls for a factor 17 when applying the transfer rate model (6438 ng/mL).

4. Conclusion

Two original LC-MS/MS based analytical methods for the determination of 30 PAs and corresponding N-oxides were developed and validated in the present study, respectively for the analysis of dry plant based food items and plant infusions. It has been highlighted that the leaching of these contaminants from dry tea materials to infusions in the course of the brewing process was incomplete: only 16–28% of PAs/PANOs are effectively transferred to the tea liquor as consumed. This result strongly contrasts with the conventional strategy assuming a total transfer and enables a more realistic evaluation of the human exposure to pyrrolizidine alkaloids. Interestingly, the analytical results have shown that less targeted food matrices like herbs mixes and pre-packaged salads are prone to PAs contamination, sometimes with very high levels (ppm range), and should be further investigated. Notably, heliotrine-type compounds dominate massively the contamination profile in herbs mixes. EFSA, in accordance with the CONTAM panel, recently proposed to reduce from 30 to 17 the number of targeted compounds for PAs/PANOs monitoring in food, excluding europine, heliotrine and their N-oxides (heliotrine-type). In light of our results, the discussion about lowering the number of PAs/PANOs of interest should be prolonged.

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Conflicts of interest

The authors do not declare any conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.06.055.

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