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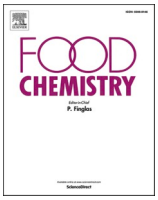

Target screening method for the quantitative determination of 118 pyrrolizidine alkaloids in food supplements, herbal infusions, honey and teas by liquid chromatography coupled to quadrupole orbitrap mass spectrometry

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ABSTRACT

An analytical procedure for the screening of 118 pyrrolizidine alkaloids (PAs) was successfully validated and applied to their quantitative determination in food supplements, herbal infusions, honey, and teas. It provides the reliable analyte identification by high-resolution tandem mass spectrometry (HRMS/MS), the accurate determination of 21 regulated PAs, and broad contamination profiles. 10% of 281 analyzed samples resulted contaminated at levels above the maximum levels (MLs) of European legislation. The contamination of herbal infusions of mixed plants can represent a possible health concern (23%; mean of PA sum above ML). A high number of PAs not included in the regulation was detected in honey and herbal food supplements, but their contribution was only relevant to the overall level in honey. The results indicate the need to continue collecting contamination data in food supplements and infusions of mixed herbs and to expand the PA-pool to be monitored in honey and related products.

1. Introduction

Occurrence of pyrrolizidine alkaloids (PAs) and their oxidized forms (pyrrolizidine alkaloids *N*-oxides, PANOs) in foods has recently become an emerging food safety issue. Numerous scientific reports have revealed a high incidence of PA contamination in foods and the number of alerts reported on the Rapid Alert System for Food and Feed (RASFF) portal has notably increased in recent years (Casado et al., 2022b). PAs can be introduced into the food chain from various contamination routes. Besides the direct consumption of PA-producing plants, the major dietary sources appear to be plant-derived products contaminated with PA-producing plants; many of these species are weeds growing in the fields, and their accidental co-harvesting lies at the basis of the presence of PA in the raw materials and hence in the processed products (Casado et al., 2022b; EFSA, 2016; Schrenk et al., 2020). Furthermore, the collection of nectar and pollen from PA-containing plants by bees can contaminate beehive products such as honey (Brugnerotto et al., 2021).

PAs are considered among the most widespread and dangerous phytotoxins capable of causing liver damage. In fact, 1,2-unsaturated PAs/PANOs exhibit a strong hepatotoxic, genotoxic, cytotoxic, tumorigenic, and neurotoxic activity. Their intake can lead to severe cases of hepatotoxicity (acute toxicity) or to slowly progress to chronic diseases following long-term exposure to low levels of PA/PANOs (Dusemund et al., 2018; EFSA, 2011; Schrenk et al., 2020).

Regarding the dietary exposure assessment, the European Food Safety Authority (EFSA) issued several scientific opinions recognizing the PAs as undesirable substances in foods and establishing a reference point of 237 μg kg^{-1} body weight per day to assess the carcinogenic risk of 1,2-unsaturated PAs (EFSA, 2011, 2016, 2017). EFSA scientific reports concluded that there is a possible human health concern related to chronic cumulative exposure to PA-contaminated food products. The main foods contributing to the human exposure to PAs are teas and herbal infusions, but also pollen and herbal food supplements can contribute significantly, although the lack of sufficient occurrence data (EFSA, 2016, 2017). Based on EFSA outcomes, the European Commission has recently amended the Regulation (EC) 1881/2006 regarding

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the maximum levels (MLs) of the sum of PAs and PANOs in the foodstuffs that contribute significantly to the human exposure, as described in the Commission Regulation (EU) 2020/2040 (Commission regulation (EC) No 1881/2006, 2006; Commission Regulation (EU) 2020/2040, 2020). MLs were set for 21 PAs and PANOs, belonging to the three most widespread families and with high toxic potential (Casado et al., 2022b): heliotrine-type, lycopsamine-type and senecionine-type. Regulation (EU) 2020/2040 extended the list of PAs to be monitored to the 14 co-eluting isomers of 21 PAs, if the chromatographic methods employed allow it. Moreover, the regulation recommends including in the sum other PAs which can be identified with the method of analysis used (Commission Regulation (EU) 2020/2040, 2020).

According to the legislation, highly sensitive and efficient analytical methods are needed to monitor these contaminants in food. Currently employed methods are based on target LC-MS/MS analyses and require the availability of reference standards (Rizzo et al., 2023; Casado et al., 2022b; Ma et al., 2018; Mulder et al., 2018; Picron et al., 2018). On the other hand, the pyrrolizidine alkaloids show a striking structural variety (more than 600 known compounds) (Moreira et al., 2018; Schramm et al., 2019), but few are available as reference substances for analytical demands. Monitoring programs should be extended to other PAs, which can potentially contaminate foods and that have a relatively high toxic potential (Casado et al., 2022b; Louisse et al., 2022). Thus, novel analytical approaches are required to broaden the knowledge about the distribution of these contaminants in foods and to identify additional compounds not yet included in the list of relevant PAs to be monitored.

In our recent study, the development of an analytical platform for the screening and identification of a high number of PAs and PANOs using an innovative approach based on high resolution mass spectrometry (HRMS) was discussed. It allows the rapid and automated screening and identification of 118 PAs and PANOs of a HRMS/MS spectral library at ppb levels in different food matrices (Rizzo et al., 2023). The high-throughput nature of this procedure allows to screen a high number of real samples, providing accurate results on the presence of the target PAs (HRMS/MS spectra), even without being dependent on availability of reference standards. This HRMS approach also offers the possibility of retrospective analyses and the simultaneous structural characterization of suspect and unknown PAs and PANOs.

In this study, the suitability of the proposed analytical procedure for quantitative purposes was evaluated with the aim of providing a quali-quantitative PA profiling of a large numbers of high-risk samples for potential non-compliant results. The analytical procedure was validated according to the European guidelines for 28 reference standards, including the 21 regulated PAs, in five food matrices susceptible to the contamination of PAs and relevant to consumer intake (honey, herbal infusion, food supplements, black and green teas). Afterwards, it was applied to the analysis of 281 commercial samples covering 5 food categories: honey, *Camelia sinensis* teas, herbal infusions at 8.4.1 and 8.4.2 of the Regulation (EC) 1881/2006, and herbal food supplements. The accurate quantitative determination of the 28 reference standards was achieved through a matrix-matched calibration or standard addition approach, while the levels of the remaining target analytes were estimated by linking them to a structurally related reference standard.

2. Materials and methods

2.1. Chemicals and standards

Analytical grade acetonitrile (MeCN), methanol (MeOH), magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$), sodium sulphate (Na_2SO_4), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4) and MS grade formic acid (HCOOH) were purchased from Merck Chemicals (Milan, Italy). MS-grade MeCN and water (H_2O) were provided by Romil (Cambridge, UK). Ultrapure water (18 MΩ) was prepared using a Milli-Q purification system (Millipore, Bedford, USA).

Reference standards (n = 28) (85–98% HPLC grade) of echimidine, echimidine *N*-oxide, erucifoline, erucifoline *N*-oxide, europine, europine *N*-oxide, heliotrine, heliotrine *N*-oxide, intermedine, intermedine *N*-oxide, jabobine, jacobine *N*-oxide, lasiocarpine, lasiocarpine *N*-oxide, lycopsamine, lycopsamine *N*-oxide, monocrotaline, monocrotaline *N*-oxide, retrorsine, retrorsine *N*-oxide, senecionine, senecionine *N*-oxide, seneciphylline, seneciphylline *N*-oxide, senkirkine, senecivernine, senecivernine *N*-oxide, trichodesmine were provided by Merck Chemicals. Standard stock solutions were prepared for each analyte (1 mg mL^{-1}) in MeOH and stored at −20 °C. Diluted solutions and standard mixtures were prepared in H_2O/MeOH 7:3 v/v.

2.2. Samples

A total number of 281 samples were collected between 2019 and 2021 from different supermarkets, herbalists, pharmacies, and online stores between the Italian and Belgian market. In detail, 60 herbal infusions of mixed plants (Foodstuffs at 8.4.1 of the Reg. EC 1881/2006), 25 herbal infusions of rooibos, anise, lemon balm, chamomile, thyme, peppermint, lemon verbena and mixtures (Foodstuffs at 8.4.2), 51 teas of *Camellia sinensis* and flavoured teas (Foodstuffs at 8.4.3), and 73 plant-based food supplements (Foodstuffs at 8.4.6) (Commission regulation (EC) No 1881/2006, 2006), including 44 formulated as solid forms, 21 as infusions, and 8 as syrups or liquid forms, were collected from various brands. Among the 8.4.1 samples, three were infusions of PA-producing plants (*Borago officinalis*, *Symphytum officinale*, and *Tussilago farfara*). Detailed information about herbal infusions, teas, and food supplements samples (product form, recommended daily intake, and composition) are reported in Table S1. In addition to the regulated food matrices, 72 samples of monofloral (n = 31) and multifloral (n = 41) honey were collected by supermarkets and beekeepers. Information on the botanical and geographical origin of the honey samples are reported in our previous study (Rizzo et al., 2022). Honey samples were stored at 4 °C and homogenized by manual stirring before the analysis. Regarding herbal infusions, teas, and solid forms of food supplements, 50% of units of each package were combined and milled to form a representative aggregate sample. Each aggregate sample was appropriately coded and kept in plastic containers at room temperature and protected from light until the analysis.

2.3. Sample preparation

The studied matrices underwent a two-steps sample preparation procedure whose purpose was to first extract the analytes from the matrix and bring them into aqueous solution and then clean-up the aqueous extracts to reduce the amount of matrix interferences (Rizzo et al., 2023). The extraction step of the procedure is described below. 25 g of each homogenized honey were diluted to 100 mL with distilled water and sonicated for 15 min. 1 g of solid forms of food supplements were extracted with 10 mL of acidic water (H_2SO_4, 0.05 M); the samples were then vortexed (1 min) and sonicated (15 min) before being centrifugated (13,000 rpm, 5 min) and re-extracted under the same conditions. 2 g of each homogenized herbal infusion and tea were brewed with 150 mL of boiling water and left to infuse for 5 min; the solution was then filtered through a fluted filter paper. Syrups and liquid forms of food supplements were properly diluted with water before being processed. After the pre-treatment, the aqueous extracts of each matrix were processed with a previously developed Salting-out Assisted Liquid-Liquid Extraction (SALLE) procedure, which was used as clean-up step (Rizzo et al., 2023). In detail, 10 mL of each aqueous extract were salted-out by adding $MgSO_4{\cdot}7H_2O$ (1 M) and Na_2SO_4 (1.5 M), brought to a pH of 9.6 using NaOH 5 M, vortexed (1 min), and centrifugated (13,000 rpm, 5 min). After that, 2 mL of the solution were extracted by adding 2 mL of MeCN, vortex-mixed for 1 min, and centrifugated again (13,000 rpm, 5 min) to separate the two liquid phases. The upper organic phase (MeCN) was quantitatively transferred into a clean tube and left to dry under a gentle nitrogen flow. The dried residues were redissolved in 125

μL (for honey), 250 μL (for solid forms of food supplements) and 200 μL (for herbal infusions and teas) of H_2O/MeOH 7:3 v/v.

2.4. UHPLC-HRMS/MS analysis

The analysis was carried out using an Ultimate 3000 UHPLC system (ThermoFisher Scientific, Milano, Italy) interfaced to a Q-Exactive mass spectrometer (ThermoFisher Scientific, Milano, Italy) equipped with a heated electrospray ionization source (HESI-II). The UHPLC-HRMS/MS analysis was performed using a previously developed and optimized instrumental method (Rizzo et al., 2023). Briefly, the chromatographic separations were performed using a Luna Omega Polar C_{18} (2.1 × 100 mm, 1.6 μm; Phenomenex, Bologna, Italy) column, thermostated at 40 °C, and a binary gradient of H_2O and MeCN both containing 0.1% of HCOOH at a flow rate of 400 μL min^{-1}. The MS detection was performed in positive ionization mode and using a Full MS data-dependent MS/MS (Full MS/dd-MS^2) acquisition mode. The resolution of the Full MS scans (scan range 250–500 *m/z*) was set at 70 k (FWHM) while that of the dd-MS^2 scans was set at 17.5 k (FWHM). The dd-MS^2 scan was triggered on an inclusion list of 112 target precursor ions and the TopN parameter was disabled to prevent precursor ions other than those contained in the inclusion list from being isolated. A normalized collision energy (NCE) between 40 and 60 was applied. Instrument control and spectra acquisition were carried out using Xcalibur software (Version 4.4, ThermoFisher Scientific).

2.5. Data processing and quantitative determination

Data processing was performed using TraceFinder software (Version 5.1, ThermoFisher Scientific). A high-throughput data processing method was developed using the "Target screening method" workflow of the software. A Compound database of 118 target PAs was created in the master method by uploading into the software a csv file generated from an in-house HRMS spectral library of the target PAs, created using mzVault software (ThermoFisher Scientific, version 2.3). The csv file contained all the information to detect and identify the 118 target PAs (retention time, molecular formula, precursor ion, five most abundant product ions and relative ion ratios). This information was used for the detection and quantitative determination of the target analytes. The detection parameters were set as follows: a single-detected detection type within a time range of 60 *sec*, a response threshold (peak area) of 10e4, a mass tolerance of ± 5 ppm, at least three product ions required for the identification, and a library match score higher than 70%. The latter was possible by uploading the HRMS spectral library into the "Library selection" section of the software configuration. The extracted ion chromatograms of target compounds (precursor ion, $[M + H]^+$) with a mass selection window of 5 ppm were used for the quantitation and semi-quantitation of 118 target PAs and PANOs (Table S2). The parameters for the quantitative determination of 28 reference standards were set as follows: external standard mode, linear calibration curve, and no weighting factor applied. The semi-quantitative determination of the remaining target PAs was estimated by indicating one of the 28 reference standards as linked compounds; the latter were chosen based on structural similarity, giving priority to the belonging of the same type of necine base first and the same type of esterification then (Table S2). After processing of raw data, the software flagged a target compound as found (green flag) every time a precursor ion was detected with a mass tolerance of ± 5 ppm at the set retention time (±0.2 min), together with the typical set of product ions and a library match score greater than 70%. Afterwards, the detected compounds were quantified by the software quantification algorithm, which directly interpolated the area of each peak in the matrix-matched calibration curve of the relative matrix, injected within the same batch of the same day. The concentration levels of PAs and PANOs of food supplements were estimated using solvent calibration curves or the standard addition method. Conversion factors (0.25 for honey, 7.5 for herbal infusions and teas, and 2.5 for solid forms of food supplements) were entered in the software sample processing system to convert the quantitative data from μg L^{-1} of SALLE extracts to μg kg^{-1} of each matrix. When calculating the total content of the samples, the analyte concentrations below the LOD (limit of detection) were considered as 0.0 μg kg^{-1} while the concentrations between LOD and LOQ (limit of quantification) were summed as 0.5 times the LOQ value.

2.6. Quality control

The stability of the reference standards in SALLE extracts and under the sample preparation conditions was checked by UHPLC-HRMS analysis. PAs and PANOs resulted quite stable, which allowed to process the samples up to three days prior to the injection. Sensitivity tests were performed before each batch of samples by analyzing in triplicate a solution of analytes at the concentration level of 2 μg L^{-1}, prepared in solvent. To ensure total absence of carryover, a sample of solvent (H_2O/MeOH 7:3 v/v) was injected after each calibration curve and after every ten runs. Additionally, the 10 μg L^{-1} level of the solvent calibration curve was injected every ten samples of a batch to ensure the stability of the detector response. A tolerated deviation of ± 15% from the theoretical concentration of the calculated values was required for the batch of samples to be considered qualified for the analysis.

2.7. Targeted method validation

The quantitative performance of the proposed target screening method was assessed according to the criteria established by the European analytical guidelines (Magnusson & Örnemark, 2014; Pihlstrom et al., 2018). The method validation was carried out for 28 reference standards in five food matrices by studying the limits of detection (LODs) and quantification (LOQs), matrix effect (ME), linearity, extraction efficiency (EE) and intra-day repeatability (expressed as relative standard deviation, RSD).

The validation experiments were performed on blank samples, previously identified through analysis, for honey and black and green teas. A representative sample of herbal infusion was prepared for the validation process by mixing the most representative herbs of the collected samples: chamomile (28%), fennel (56%), melissa (32%), mint (47%) and licorice (44%) (Table S1). Blank samples of these herbs were selected after processing them with the target screening method. On the contrary, it was not possible to select or prepare a representative sample of herbal food supplement to be used for validation experiments due to the high variability of their composition. Thus, ten blank samples of different composition were pooled to perform the calculation of the following validation parameters: sensitivity (LODs and LOQs), accuracy (EEs) and intra-day repeatability (RSDs).

LODs and LOQs of 28 reference standards in the investigated matrices were calculated using the calibration-based approach (Wenzl et al., 2016). In detail, blank samples of each matrix were fortified at concentration levels close to the expected LOD (range 0–1.5 μg L^{-1} of the SALLE extracts, including the zero level) and processed by the entire analytical procedure in duplicate (independent replicates). For some analyte/matrix combinations the experiments were repeated at higher concentrations.

The matrix-matched calibration curves were prepared by spiking blank SALLE extracts of honey, herbal infusion, black and green tea at 8 concentration levels covering the range 1–100 μg L^{-1} (corresponding to 0.25–25 μg kg^{-1} for honey, and 7.5–750 μg kg^{-1} for teas and infusions). Each concentration level was injected in triplicate to evaluate the linearity of each curve with the analysis of variance (ANOVA). A linear model was found appropriate over the tested concentration range ($R^2 \geq$ 0.999) for all analytes in the studied matrices. The MEs were evaluated by comparing the slope of the matrix-matched calibration curves (post-spiked samples) with that of the solvent calibration curves (H_2O/MeOH 7:3, v/v) of the reference standards in the concentration range 1–100 μg L^{-1} of the SALLE extracts. MEs were defined as ratio between the slopes

of matrix-matched and solvent-based calibration curves.

The accuracy of the method, expressed as extraction efficiency (EEs), was calculated by spiking the reference standards before (pre-spiked samples) and after (post-spiked samples) the sample preparation procedure at two concentration levels: a low level (LL) close to the LOQ of 2 μg L^{-1} of the SALLE extracts (corresponding to 0.5, 5 and 15 μg kg^{-1} for honey, food supplements and teas/infusions, respectively), and a high level (HL) of 100 μg L^{-1} of the SALLE extracts (corresponding to 25, 250 and 750 μg kg^{-1} for honey, food supplements and teas/infusions, respectively). The EE at low level of retrorsine *N*-oxide in green tea was evaluated with the spike level of 37.5 μg kg^{-1} (5 μg L^{-1} of SALLE extract). Experiments were conducted in triplicate. Intra-day repeatability was obtained by the same set of accuracy experiments, evaluating the responses of the pre-spiked samples at two concentration levels. Precision was expressed as RSD of the PA/PANO contents (three replicates).

3. Results and discussion

3.1. UHPLC-HRMS/MS analysis

Liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is the gold standard technique for the analysis of PAs and PANOs at low concentration levels in food matrices, thanks to its high sensitivity and selectivity (Casado et al., 2022b; Ma et al., 2018). According to EFSA, quantification limits of 10 μg kg^{-1} (total content of PAs and PANOs) and 0.1–5 μg kg^{-1} (individual content of PAs and PANOs) must be reached (EFSA, 2011). Unfortunately, this is not always possible due to the complex nature of some food matrices (food supplements, herbal infusions, honey, pollen, teas, etc.), which may have a great number of matrix interferences and decrease the sensitivity of the analytical method.

The quantitative determination of PAs and PANOs of the present study was performed using a UHPLC-HRMS/MS method, previously developed for the high-throughput target screening and identification of 118 pyrrolizidine alkaloids in complex food matrices (Rizzo et al., 2023). Briefly, the UHPLC conditions were optimized on 30 reference standards and extracts of 10 PA-producing plants with the aim of separating structural co-eluting isomers. The quantification and semi-quantification of the analytes was performed by extracting the accurate masses of precursor ions in full MS traces (<5 ppm) set in the range *m/z* 250–500. An inclusion list of precursor ions was activated to trigger the dd-MS^2 scans of the target analytes. In addition, the intensity ratios between the diagnostic product ions and the match with an HRMS spectral library of the target compounds were used to confirm their identity. The instrumental parameters were optimized to allow the HCD fragmentation of precursor ions with low ion abundances, as this is the case of PAs in complex matrices.

The adopted UHPLC-HRMS/MS method allowed to rapidly and reliably detect and identify the target analytes at ppb levels in different food matrices with high risk of contamination. The high-throughput nature of the procedure allowed to screen a large number of real samples, providing accurate results on the presence of the target analytes, even without being dependent on the purchase of further reference standards.

3.2. Sample preparation procedure

Considering the co-occurrence of PAs and related PANOs, a simultaneous extraction of both is required. Solid-liquid extraction (SLE) and liquid–liquid extraction (LLE) techniques, usually combined with a purification step by solid-phase extraction (SPE) are commonly used to extract PAs and PANOs from complex matrices like herbal products. QuEChERS methods have also been used as suitable sample preparation approach (Casado et al., 2022b, 2022a; Ma et al., 2018).

In this study, a previously proposed sample preparation procedure, based on Salting-out Assisted Liquid-Liquid Extraction (SALLE) of aqueous extracts (Rizzo et al., 2023) was applied for the determination of PAs and PANOs at low concentration levels in some of the food matrices with the highest risk of contamination, which are plant-based food supplements, herbal infusions, honey, black and green teas. In detail, for food supplements and honey (solid matrices) a preliminary extraction of the analyte from the matrix was carried out using an ultrasound assisted solid–liquid extraction with acidic aqueous solution (H_2SO_4, 0.05 M). Herbal infusions and teas were extracted by infusion with boiling water to obtain a more realistic PA exposure of population (Mulder et al., 2018; Picron et al., 2018). Syrups and liquid forms of food supplements were simply diluted with water. Afterwards, SALLE was applied to the aqueous extracts of the studied matrices as clean-up step of the procedure. The reconstitution volume of the SALLE extracts was chosen according to the required sensitivity and regulated MLs (Commission regulation (EC) No 1881/2006, 2006; Commission Regulation (EU) 2020/2040, 2020), by considering the matrix effects. For this purpose, SALLE extracts of the five investigated matrices were dissolved in different volumes (from 125 to 500 μL) and spiked at 10 μg L^{-1} of each PA and PANO.

Unlike QuEChERS, the sample preparation procedure of the present study involved an acid aqueous solution as solvent for the PA extraction from matrix rather than the mixture acetonitrile/water. Thus, the SALLE was applied to cleaner extracts reducing the risk of co-extraction of matrix interferents. Moreover, this procedure does not include the addition of clean-up sorbents as part of a sample purification step, making it faster and less expensive.

3.3. Method validation

The aim of this study was to validate an analytical procedure, previously developed for the screening and identification of PAs and PANOs, in order to perform the quantitative determination of a wide range of pyrrolizidine alkaloids and collect occurrence data from a large number of samples.

The analytical performance was assessed for 28 commercially available standards (including the 21 regulated PAs and PANOs) in five food matrices: food supplements, herbal infusions, honey, black and green teas. The analytical procedure was validated in terms of sensitivity (limits of detection, LOD, and quantification, LOQ), matrix effect (ME), linearity, extraction efficiency (EE) and intra-day repeatability (RDS). The method specificity was evaluated in our previous study and the proposed procedure provided excellent performance to discriminate the analytes from any other matrix interferences (Rizzo et al., 2023). The validation was performed using blank samples (honey, black and green teas) or simulated blank samples which represent the composition variability of food supplements (pool of ten samples with different composition) and herbal infusions (mix of five herbs).

3.3.1. Sensitivity

LODs (the lowest analytes concentrations that can be detected at a specified confidence level) and LOQs (the lowest analytes concentrations that can be quantified with a reasonable level of accuracy) were determined using the calibration-based approach (Wenzl et al., 2016). Calculated values of LOQs for selected PAs in each studied matrix are shown in the Table 1; the achieved LOQs were very low, ranging from 0.1 to 2.1 μg $kg^{-}1$ in solid matrices (honey and food supplements) and from 1 to 12 μg kg^{-1}in infusions and teas. Retrorsine *N*-oxide only showed a higher LOQ value in green tea. Overall, LOQs were within the recommended quantification range set by EFSA (EFSA, 2011), and LOQ values showed to be lower than those established by the European regulation for the 21 relevant analytes (Commission regulation (EC) No 1881/2006, 2006), which demonstrate the suitability of the method for the determination of PAs and PANOs at trace levels in all the studied matrices. The sensitivity of the proposed method was lower or comparable than quantification limits established by other analytical

Table 1
Limits of quantification (LOQs), Extraction efficiencies (EEs) and Precision (RDS) of 28 PAs and PANOs in studied food matrices.

Compound [a]	Honey			Black tea			Green tea			Herbal infusion			Food supplement		
	EE Low [b] (RSD)	EE High [c] (RSD)	LOQ (μg kg^{-1})	EE Low [b] (RSD)	EE High [c] (RSD)	LOQ (μg kg^{-1})	EE Low [b] (RSD)	EE High [c] (RSD)	LOQ (μg kg^{-1})	EE Low [b] (RSD)	EE High [c] (RSD)	LOQ (μg kg^{-1})	EE Low [b] (RSD)	EE High [c] (RSD)	LOQ (μg kg^{-1})
Echimidine	72.6 (5.4)	85.6 (2.7)	0.2	87.3 (8.1)	95.1 (2.3)	5.4	82.1 (11.6)	98.7 (7.1)	3.3	75.4 (16.8)	84.8 (8.4)	2.4	82.9 (4.0)	103.0 (1.9)	1.1
Echimidine NO	89.2 (3.8)	95.7 (2.3)	0.1	102.6 (4.9)	101.2 (3.0)	3.6	90.3 (6.0)	100.0 (7.9)	11.7	104.1 (7.0)	113.5 (5.8)	4.1	97.4 (5.2)	111.2 (0.1)	0.9
Erucifoline	88.7 (13.0)	99.6 (7.3)	0.2	80.1 (13.7)	99.2 (2.1)	5.6	83.7 (7.4)	97.1 (10.1)	2.3	80.9 (9.8)	92.1 (7.3)	3.1	99.8 (6.3)	102.1 (5.5)	2.1
Erucifoline NO	92.7 (6.7)	94.3 (1.3)	0.2	89.0 (10.2)	95.3 (11.5)	2.8	88.1 (1.4)	101.7 (5.5)	2.3	92.2 (9.7)	95.9 (7.1)	3.3	75.6 (4.4)	92.6 (3.5)	1.2
Europine	83.8 (7.9)	103.9 (4.8)	0.2	94.2 (9.6)	97.0 (1.0)	6.0	84.0 (5.0)	97.6 (1.2)	4.6	90.1 (3.1)	95.9 (7.4)	1.9	89.0 (8.4)	96.7 (8.6)	1.3
Europine NO	63.3 (14.1)	70.0 (7.1)	0.1	72.2 (6.9)	77.9 (0.3)	2.7	74.0 (6.8)	76.4 (13.6)	3.2	85.0 (12.7)	70.1 (11.3)	5.5	70.5 (4.0)	71.3 (6.0)	0.8
Heliotrine	85.2 (8.3)	93.2 (4.3)	0.2	91.3 (8.9)	99.0 (4.1)	9.2	78.3 (4.5)	92.5 (4.6)	2.4	95.9 (9.3)	101.4 (1.7)	3.2	90.4 (2.9)	97.8 (0.1)	0.7
Heliotrine NO	80.5 (0.6)	98.4 (1.0)	0.2	87.2 (7.2)	90.3 (3.8)	2.0	78.5 (6.5)	81.6 (11.4)	5.7	85.8 (8.3)	88.4 (4.2)	5.1	81.9 (2.4)	91.9 (1.1)	1.3
Intermedine	89.0 (5.1)	83.4 (0.6)	0.2	90.1 (2.2)	94.8 (2.2)	7.1	89.8 (0.1)	100.7 (9.1)	2.1	88.5 (0.4)	88.2 (1.4)	3.7	88.4 (10.0)	95.5 (1.6)	1.3
Intermedine NO	75.0 (0.6)	78.6 (2.7)	0.2	65.0 (12.6)	77.1 (2.9)	2.8	78.3 (1.7)	81.7 (6.7)	3.4	75.7 (4.5)	82.6 (0.9)	3.3	70.4 (6.3)	72.5 (2.3)	0.7
Jacobine	103.7 (11.5)	93.4 (9.5)	0.2	93.3 (4.8)	96.8 (4.8)	6.4	78.2 (9.3)	90.4 (10.7)	3.3	79.3 (4.5)	97.2 (5.0)	11.1	96.7 (12.4)	102.4 (2.7)	1.1
Jacobine NO	81.6 (4.6)	91.7 (10.4)	0.2	75.0 (10.1)	81.2 (3.3)	2.6	83.3 (0.4)	90.9 (6.5)	2.1	89.5 (4.0)	103.2 (0.8)	3.3	81.8 (6.3)	92.2 (0.6)	0.7
Lasiocarpine	88.4 (4.4)	90.3 (4.1)	0.1	90.7 (6.7)	100.2 (1.2)	9.0	111.0 (2.9)	106.6 (6.6)	2.7	86.2 (7.8)	101.3 (1.4)	3.9	92.2 (5.3)	101.5 (3.8)	0.8
Lasiocarpine NO	90.1 (0.2)	91.7 (5.0)	0.2	89.4 (8.9)	96.3 (3.3)	1.0	83.4 (7.0)	90.8 (1.4)	3.5	86.4 (11.5)	92.5 (1.7)	6.0	95.2 (2.3)	101.9 (3.4)	1.5
Lycopsamine	83.4 (2.6)	95.1 (0.2)	0.2	88.4 (4.0)	98.0 (1.0)	5.8	89.4 (2.5)	90.2 (2.1)	5.3	80.9 (5.1)	88.5 (0.2)	2.9	88.6 (5.8)	93.3 (2.6)	1.4
Lycopsamine NO	63.3 (14.7)	71.4 (5.9)	0.2	63.9 (10.0)	70.0 (3.6)	1.7	65.2 (10.4)	71.0 (10.3)	3.0	63.0 (8.1)	66.0 (3.2)	4.3	65.1 (8.4)	70.2 (14.4)	0.5
Monocrotaline	89.5 (5.3)	95.7 (6.4)	0.4	90.9 (2.9)	98.7 (3.1)	5.9	88.6 (3.9)	94.8 (5.1)	2.7	86.3 (6.1)	94.8 (2.7)	3.8	91.1 (8.0)	101.4 (2.3)	1.9
Monocrotaline NO	72.7 (7.8)	74.9 (13.0)	0.4	71.2 (8.3)	80.9 (3.2)	7.4	68.0 (8.0)	81.4 (8.4)	1.6	69.4 (10.4)	77.2 (3.8)	3.8	70.9 (9.4)	80.8 (6.1)	0.7
Retrorsine	98.4 (6.0)	100.0 (0.7)	0.1	94.1 (6.3)	102.1 (1.0)	4.4	99.7 (15.1)	104.2 (16.4)	3.1	66.4 (14.6)	78.6 (9.3)	4.3	90.9 (1.4)	101.9 (1.5)	1.2
Retrorsine NO	78.1 (6.4)	84.3 (1.3)	0.3	96.8 (3.1)	103.2 (4.1)	9.6	82.6 (8.2)[d]	91.4 (10.0)	23.4	101.0 (5.2)	116.9 (2.6)	4.7	93.5 (4.5)	96.1 (7.0)	1.9
Senecionine	71.4 (9.2)	77.1 (3.8)	0.2	84.3 (1.8)	87.4 (4.3)	9.4	94.1 (1.9)	100.4 (6.7)	3.0	100.1 (5.5)	105.6 (3.0)	3.7	98.7 (4.7)	104.8 (1.4)	1.6
Senecionine NO	93.1 (9.8)	102.0 (4.6)	0.2	93.5 (4.9)	100.4 (3.1)	6.0	80.8 (7.1)	91.1 (11.3)	2.2	98.3 (3.3)	94.6 (2.3)	5.1	87.3 (5.6)	98.1 (3.6)	1.2
Seneciphylline	80.8 (3.2)	89.4 (3.4)	0.2	91.2 (4.2)	99.4 (0.1)	9.6	80.4 (11.7)	89.3 (14.7)	3.6	82.6 (1.0)	95.2 (2.6)	2.1	97.7 (8.0)	107.9 (4.8)	1.3
Seneciphylline NO	89.6 (10.1)	95.9 (5.6)	0.2	81.1 (7.3)	90.0 (4.0)	2.3	88.8 (2.9)	95.9 (8.3)	2.6	84.8 (14.0)	95.2 (4.9)	4.1	81.1 (3.7)	92.7 (0.4)	1.2
Senecivernine	78.1 (1.8)	85.8 (4.7)	0.2	103.5 (8.6)	95.6 (0.5)	9.4	83.2 (4.0)	80.5 (9.0)	3.3	91.1 (9.0)	97.4 (2.8)	3.2	98.6 (9.9)	104.7 (4.3)	0.9
Senecivernine NO	93.5 (8.1)	100.6 (2.2)	0.2	90.5 (7.4)	100.9 (2.1)	2.6	93.7 (0.3)	101.5 (4.6)	1.9	86.8 (10.4)	93.0 (8.2)	3.9	80.8 (2.6)	98.8 (2.2)	1.0
Senkirkine	89.8 (9.8)	104.7 (7.4)	0.2	86.4 (6.0)	97.0 (0.4)	3.6	85.3 (7.9)	102.8 (2.0)	1.5	89.2 (6.6)	94.3 (0.4)	4.3	92.5 (9.6)	101.4 (5.6)	0.9
Trichodesmine	87.6 (11.4)	99.0 (6.4)	0.4	88.3 (5.8)	96.8 (1.7)	6.1	92.0 (8.2)	95.6 (7.1)	3.6	85.9 (7.7)	93.7 (2.4)	3.8	96.0 (5.1)	97.8 (0.8)	1.6

[a] NO: *N*-oxide.
[b] spike level, 2 μg L^{-1} of SALLE extract.
[c] spike level, 100 μg L^{-1} of SALLE extract.
[d] spike level, 5 μg L^{-1} of SALLE extract.

procedures based on SPE and QueChers (Mulder et al., 2015; Kaltner et al., 2019; Casado et al., 2022b, 2022a).

3.3.2. *Matrix effects and linearity*

The ME phenomenon (suppression or an enhancement of the instrumental response due to the co-elution of matrix interferences) can compromise the sensitivity of the analytical method and the accuracy of the data; therefore, in the validation process an evaluation of the ME must be performed to establish the most suitable quantification method. MEs of 28 PAs in herbal infusions, honey, black and green teas were variable (61–149%) depending on the matrix type and the type of analyte; however, the ME resulted negligible (80–120%) for 93% of the target analytes in herbal infusions, 96% and 93% of them in black and green teas respectively, and 89% of them in honey (Fig. S1). These results confirmed the efficiency of the sample preparation procedure in removing or reducing matrix interferences from all the tested matrices,

despite their complex nature. Despite the good ME values achieved, the PAs/PANOs content of contaminated samples of herbal infusions, honey and teas were quantified on matrix-matched calibration curves to obtain more accurate data. On the contrary, the PAs/PANOs content of food supplements was estimated using solvent-based calibration curves due to their high variable composition.

Linearity of the solvent and matrix-matched calibration curves of herbal infusions, honey, black and green teas, was proven within the range 1–100 μg L^{-1} of SALLE extracts, with excellent correlation coefficients for a linear model ($R^2 > 0.998$ for all 28 reference standards).

3.3.3. Accuracy and precision

The accuracy (expressed as EE) and precision (expressed as intra-day repeatability, RSD) were assessed at two contamination levels: low (2 μg L^{-1}) and high (100 μg L^{-1}). Results are shown in Table 1. EEs in the range 63–117% were obtained for the 28 reference PAs in all the studied matrices, indicating the efficiency of the proposed procedure in providing an exhaustive extraction. The lowest values of EE were observed for lycopsamine *N*-oxide in all investigated matrices. Also, the precision data were very satisfactory: RSD values below 17% were achieved for the five investigated matrices.

The proposed sample preparation procedure showed a good and repeatable extraction efficiency comparable to the previous analytical procedures employed for PA analysis (Mulder et al., 2015; Kaltner et al., 2019; Casado et al., 2022b, 2022a). However, it is simpler, faster and cheaper than clean-up techniques based on SPE or QueChers.

3.4. Analysis of commercial samples

The food matrices of this study were chosen based on the data available on their contamination incidence and consumer intake, according to EFSA reports, literature studies, and the Regulation (EC) 1881/2006 (Commission regulation (EC) No 1881/2006, 2006). Therefore, plant-based food supplements, herbal infusions, honey, black and green teas were selected, and a high number of commercial samples were collected and categorized according to the foodstuff of European regulation (Table S1). The sampling of food supplements and herbal infusions was guided by the composition of the products, mainly selecting those which contained the plants most susceptible to contamination.

The collected commercial samples (n = 281) were subjected to the target screening of 118 PAs and PANOs, which allowed to provide an accurate quantitative determination of the 28 reference standards, while the levels of the remaining target analytes were estimated by linking them to a structurally related reference standard. Quantitative data (Table S3) were expressed as concentration of each PA and PANO and total PA content, to evaluate the PA contamination profiles of the investigated matrices and to verify the compliance with the MLs.

The contamination rate of the investigated matrices as function of the total PA content is summarized in Fig. 1, where the total PA content of the contaminated samples is divided in four sections (from LOQ to0.5 times the ML; from 0.5 times the ML to ML; from ML to 2 times the ML; and more than 2 times the ML) based on regulated MLs of herbal infusions (8.4.1 and 8.4.2), teas (8.4.3), and food supplements (8.4.3) or the recommended level (RL) of the maximum daily intake of honey (Brugnerotto et al., 2021; EFSA, 2017). In general, 56% of the analyzed samples contained measurable amounts of at least one of the 118 target PAs/PANOs, with contamination levels ranging from 0.1 to 218381 μg kg^{-1} (Table S3), and 9.6% of the samples exceed the MLs (or RL) (Fig. 1).

The contamination profile of the studied matrices was assessed by grouping the target analytes into three main classes: the 21 regulated PAs/PANOs, their 14 co-eluting isomers, and additional PAs (not included in the list of 35 PAs considered by the European Regulation), to evaluate their contribution to the overall contamination level of the samples. The 21 regulated PAs and PANOs covered almost all the total PA content in herbal infusions (89%) and food supplements (87%), while the 14 co-eluting isomers significantly contributed to the total PA content of honey (36%) and tea (48%). The contribution of additional PAs and PANOs was only relevant for honey (16%) and food supplements (8%) (Fig. S2). A detailed discussion of the contamination data of the studied matrices is addressed in the next paragraphs.

3.4.1. Honey

The European regulation does not set a ML for honey, for which recommended levels (RLs) for adults (71.1 μg kg^{-1}) and children (23.7 μg kg^{-1}) were considered in this study. The RLs were calculated using the margin of exposure (MOE), which is ≥ 10,000 for genotoxic and carcinogenic substances such as PAs, and the Benchmark Dose Lower Confidence Limit 10% ($BMDL_{10}$) of 237 μg kg^{-1} body weight per day established by EFSA. The values were compared to the average human daily intake of honey, which is 20 g per day (Brugnerotto et al., 2021; EFSA, 2017).

Honey resulted the food matrix with the highest prevalence of contamination; at least one PA or PANO above the LOQ was found in 78% of the samples with concentrations ranging from 0.2 to 129.2 μg kg^{-1} (Table S3). The mean and median values of the positive samples, 15.5 and 2.9 μg kg^{-1} respectively, were well below the RL for adults. Even though 90% of the samples contained negligible levels of PAs (<0.5 RL), 7% of them (five samples of multifloral honey from extra-European countries) exceeded the RL for adults (Fig. 1), and 3% of them (two samples) exceeded the RL for children (Table S3). These data confirm that the daily consumption of honey represents a health risk for consumers, especially for children.

Regarding the contamination profile of honey, the most abundant PAs of contaminated samples were lycopsamine-type compounds (59% of the total content of all samples), mainly present as tertiary amines. Echimidine (73%, range 0.2–16.2 μg kg^{-1}), intermedine (43%, 0.2–57.0 μg kg^{-1}), lycopsamine (25%, 0.2–3.6 μg kg^{-1}), echimidine *N*-oxide (23%, 0.1–1.3 μg kg^{-1}), and intermedine *N*-oxide (14%, 0.3–0.7 μg kg^{-1}) were the most frequently encountered, among the regulated PAs (Fig. 2). These data suggest that the botanical species responsible for the PA contamination of honey belong to *Boraginaceae* and *Asteraceae*

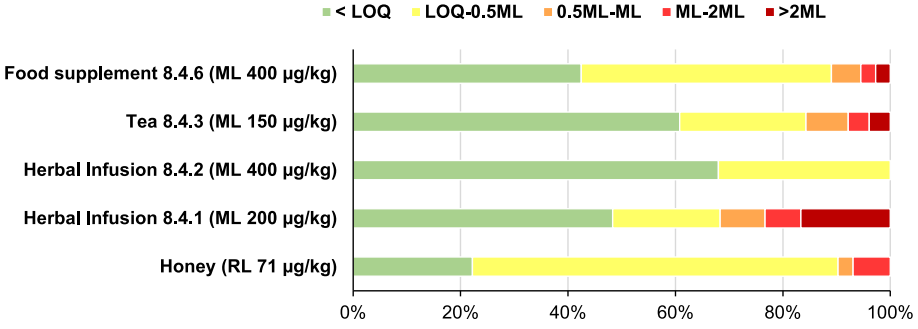

Fig. 1. Contamination rate of the studied food categories as function of the total PA content.

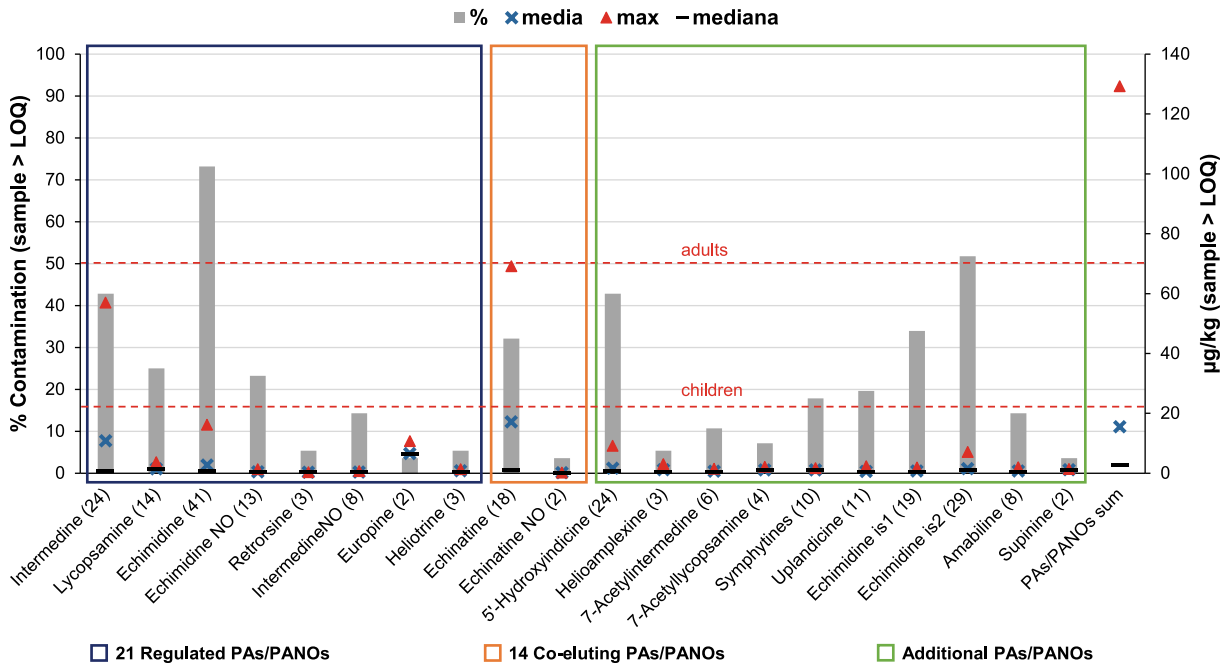

Fig. 2. Contamination profile of honey: prevalence and level of each PA and PANO in positive samples. Only PAs present in more than 2% of the positive samples are shown.

families (Brugnerotto et al., 2021). Among the 14 co-eluting isomers, echinatine was detected in 32% of the positive samples (0.2–69.1 µg kg^{-1}) with a contribution of 36% to the total PA content of all samples. In fact, some samples exceeded the RL because of the contribution of echinatine (45.8–69.1 µg kg^{-1}) to the total content of PAs (Table S3). This proves the importance to chromatographically separate co-eluting isomers of PAs from the regulated PAs to provide more accurate data on the botanical origin of the contaminated honey samples. Concerning the PAs not included in the list of regulated PAs, 11 additional compounds were detected, 7 of which in more than 10% of the positive samples: two isomers of echimidine (52% and 34%), 5-hydroxyindicine (43%), uplandicine (20%), symphytines (sum of isomers, 18%), amabiline (14%) and 7-acetylintermedine (11%) (Fig. 2). Among them, the isomers of echimidine, symphytines, as well as uplandicine, 7-acetylintermedine, 7-acetyllycopsamine and amabiline are typical PAs of the main plants responsible for the contamination of honey (*Echium* spp., *Symphytum* spp., *Senecio* spp., *Eupatorium* spp. and *Borago* spp.) (Casado et al., 2022b; Mädge et al., 2020; Rizzo et al., 2023). On the contrary, 5-hydroxyindicine and helioamplexine have been recently reported in Australian honey as result of a contamination with *Heliotropium amplexicaule* (Carpinelli De Jesus et al., 2019). These data suggest that the contamination profile of honey can be extremely variable as it depends on the botanical origin of the geographical area. Thus, there it is necessary to expand the pool of PAs to be monitored in honey and related products to provide a more accurate PA contamination profile and to adequately cover the mean total PA contents.

3.4.2. Teas and herbal infusions

Teas and herbal infusions are divided in three different categories, according to the European regulation: herbal infusions of mixed plants (8.4.1, ML = 200 µg kg^{-1}), herbal infusions of rooibos, anise, lemon balm, chamomile, thyme, peppermint, verbena, and their mixtures (8.4.2, ML = 400 µg kg^{-1}), and *C. sinensis* teas (8.4.3, ML = 150 µg kg^{-1}).

According to EFSA reports and literature studies, infusions of the category 8.4.2 and teas are the food matrices with the highest contamination rate (EFSA, 2017; Mulder et al., 2018; Picron et al., 2018). However, in this study they were resulted to be the least contaminated matrices (Fig. 1); this is likely due to the application of good agricultural and harvesting practices, adopted after the reporting of worrying levels of contamination of these food matrices. Herbal infusions 8.4.2 showed the lowest contamination rate (32% samples > LOQ) with much lower levels than the ML (6.5–97.7 µg kg^{-1}, mean and median of 44 µg kg^{-1}). A similar contamination rate was also observed in tea samples (39% samples > LOQ, range 6.9–415.7 µg kg^{-1}). However, four samples of black teas (8%) exceeded the ML (150 µg kg^{-1}) (Fig. 1, Table S3). Consistently with previous data, senecionine-type *N*-oxides were predominant in teas samples (64% of the total content found in all samples). Retrorsine *N*-oxide (30%, 33.2–258.9 µg kg^{-1}), lycopsamine *N*-oxide (40%, 6.9–20.9 µg kg^{-1}) and senecionine *N*-oxide (10%, 2.6–76.4 µg kg^{-1}) were the regulated PANOs most frequently detected. Among the 14 co-eluting isomers, echinatine *N*-oxide (60%, 8.5–76.3 µg kg^{-1}), echinatine (45%, 8.3–33.8 µg kg^{-1}) and integerrimine *N*-oxide (40%, 8.0–245.5 µg kg^{-1}) were frequently found in the contaminated samples (Fig. S3) and with a contribution to the overall contamination levels of teas (8, 14 and 22%, respectively) comparable to those of 21 regulated PAs (Fig. S2).

Differently, herbal infusions of mixed plants (8.4.1) resulted much more contaminated (52% of the samples > LOQ), with higher total levels up to 218381 µg kg^{-1} (mean = 14025 µg kg^{-1} and median = 127 µg kg^{-1}) (Table S3). In fact, 23% of these samples exceeded the ML established for this category (200 µg kg^{-1}) (Fig. 1). Among the 60 analyzed samples, three of them were labelled as containing PA-producing plants (*Borago officinalis*, *Symphytum officinale*, *Tussilago farfara*); resulted contaminated with very high levels (865–218381 µg kg^{-1}), well above the ML. Except for the infusion of *Tussilago*, where senkirkine was the main PA, the contamination of the infusions of *Borago officinalis* and *Symphytum officinale* was mainly due to lycopsamine-type monoesters (76%) (Table S3). The results of the samples containing PA-producing plants are not included in the following discussion to avoid overestimation of the contamination data of category 8.4.1. However, even excluding these samples, the contamination levels of this category resulted relevant (maximum level of 953.6 µg kg^{-1}, mean of 225 µg kg^{-1}, median of 110 µg kg^{-1}) with a mean value exceeding the ML. These data indicates a possible risk for human health associated to the consumption of infusions of mixed plants, even if they do not contain PA-producing plants.

The contamination profile of herbal infusions of the category 8.4.1 (without PA plant) showed a prevalence of heliotrine-type and senecionine-type compounds, which were responsible for 59% and 24% of the overall contamination, respectively (Fig. 3, Table S3). These results clearly demonstrate that this matrix is mainly subjected to the contamination of *Heliotropium* and *Senecio* spp. Among the 21 regulated PAs and PANOs, the most detected ones were senecionine *N*-oxide (32%) lasiocarpine *N*-oxide (32%), europine *N*-oxide (29%), heliotrine *N*-oxide (29%), seneciphylline *N*-oxide (21%) and heliotrine (21%). The main contributors to the total contents, with mean concentrations higher than 100 μg kg^{-1}, were retrorsine *N*-oxide (136–421 μg kg^{-1}), europine (30–398 μg kg^{-1}), europine *N*-oxide (39–337 μg kg^{-1}), and lasiocarpine *N*-oxide (31–217 μg kg^{-1}) (Fig. 3). Co-eluting isomers and additional PAs and PANOs contributed the least amount to the contamination level of this matrix (7% and 3%, respectively) (Fig. S2). Echinatine *N*-oxide (18%), rinderine *N*-oxide (14%) and integerrimine *N*-oxide (14%) were the main detected isomers. Among 1,2-unsatured additional PAs, heleurine *N*-oxide was found in 18% of the contaminated samples (Fig. 3); it is a supinidine-type of PAs, characteristic of *Heliotropium* spp. (Carpinelli De Jesus et al., 2019; Louisse et al., 2022) and mainly detected in the samples highly contaminated by heliotrine-type compounds of these species (Table S3).

3.4.3. Herbal food supplements

Collected samples of herbal food supplements showed a huge diversity in their composition, with added plant extracts, various herbs, spices, flowers, or roots (Table S1). Therefore, the levels of PAs and PANOs of these samples were calculated using solvent-based calibration curves as it was not possible to select or prepare a representative matrix to mimic the real samples as closely as possible. Contaminated samples with PA levels close to or above the ML (400 μg kg^{-1}) were accurately quantified using the standard addition approach.

In total, 42 out of 73 samples (58%) contained at least one target analyte above the LOQ and 6% of the samples exceeded the ML (Fig. 1); however, two of the exceeding samples contained more than 1000 μg kg^{-1} of total PAs (infusion I82 and food supplement DS44). The ingredients of these two samples (Table S1) were mainly raw plant materials of herbal products well-known to be contaminated with significant levels of pyrrolizidine alkaloids (leaves of rosemary and peppermint, and fruits of anise, cumin and fennel). Heliotrine-type PAs/PANOs covered 75% of the overall contamination of food supplement samples. According to these results, *Heliotropium spp* seemed to be the most contaminating specie of plant crops used for the manufacturing of herbal food supplements. The most frequently occurring regulated PAs in positive samples were europine (38%), heliotrine (33%), lasiocarpine (26%) and heliotrine *N*-oxide (21%). On the contrary, the quantitatively predominant analytes (mean greater than 100 μg kg^{-1}) were seneciphylline *N*-oxide (5 and 231 μg kg^{-1}), europine *N*-oxide (4–924 μg kg^{-1}), heliotrine *N*-oxide (3–1374 μg kg^{-1}) and lasiocarpine *N*-oxide (3–652 μg kg^{-1}) (Fig. 4). The highest levels of these analytes were found in food supplements formulated as infusion, and therefore containing dried plant materials. The content of co-eluting isomers was not significant for food supplements (5% of the overall contamination), and the most detected compounds were always echinatine (45%), echinatine *N*-oxide (19%) and integerrimine *N*-oxide (12%). On the other hand, numerous additional PAs (23 out of 49 detected analytes) were detected but only heleurine *N*-oxide (10%), thesinine and its glycosides (17%) frequently occurred in contaminated samples (Fig. 4).

It is noteworthy the diverse PA/PANO distribution observed for the different types of food supplements: *N*-oxide forms were predominant in the positive infusion samples (86% of the overall contamination), while tertiary amines significantly contributed to the contamination of plant extracts-based food supplements (59% of the overall contamination). This is likely due to higher water solubility of PANOs than to PAs, which leads to the loss of PANOs during the extraction processes employed to produce plant extracts used in the formulations of dietary supplement.

4. Conclusions

In this study, a previously developed analytical platform was validated for quantitative purposes and applied to the analysis of high number of real samples (n = 281) of different food matrices with high risk of contamination, which were screened against the presence of 118 PAs and PANOs. Overall, the proposed method showed for 28 reference standards good analytical performance that fulfilled the requirements of analytical methods for trace level contaminants analysis in food

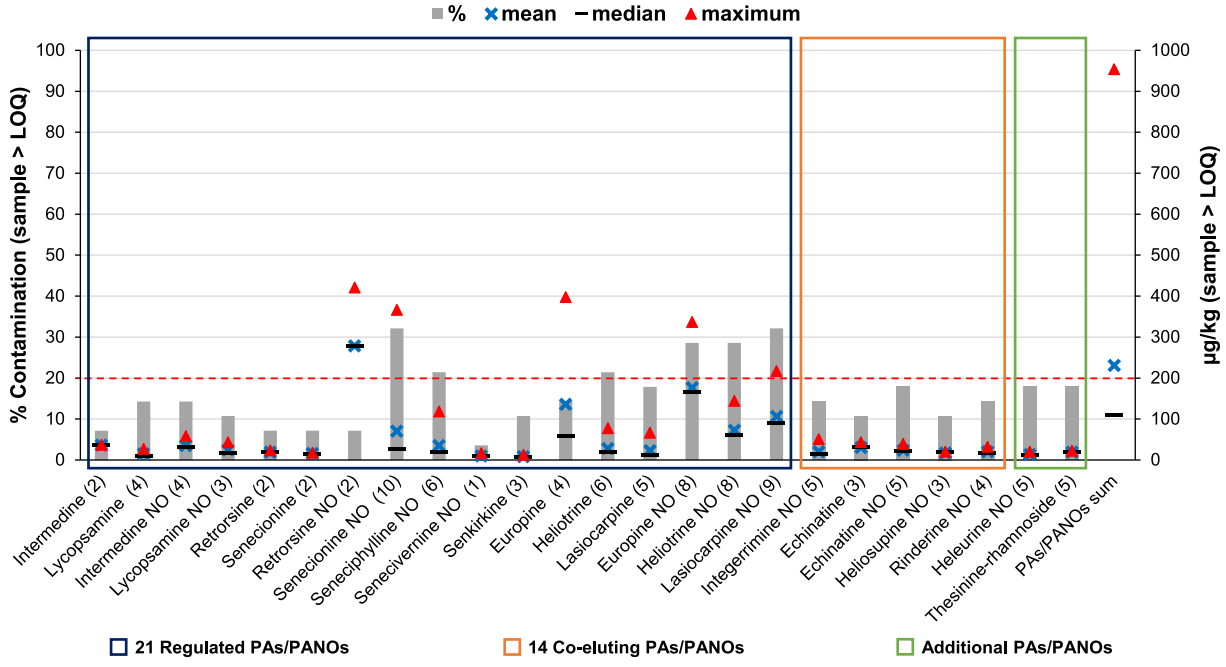

Fig. 3. Contamination profile of herbal infusion of mixed plants (8.4.1): prevalence and level of each PA and PANO in positive samples. Only PAs present in more than 5% of the positive samples are shown. Samples containing PA-producing plants were excluded.

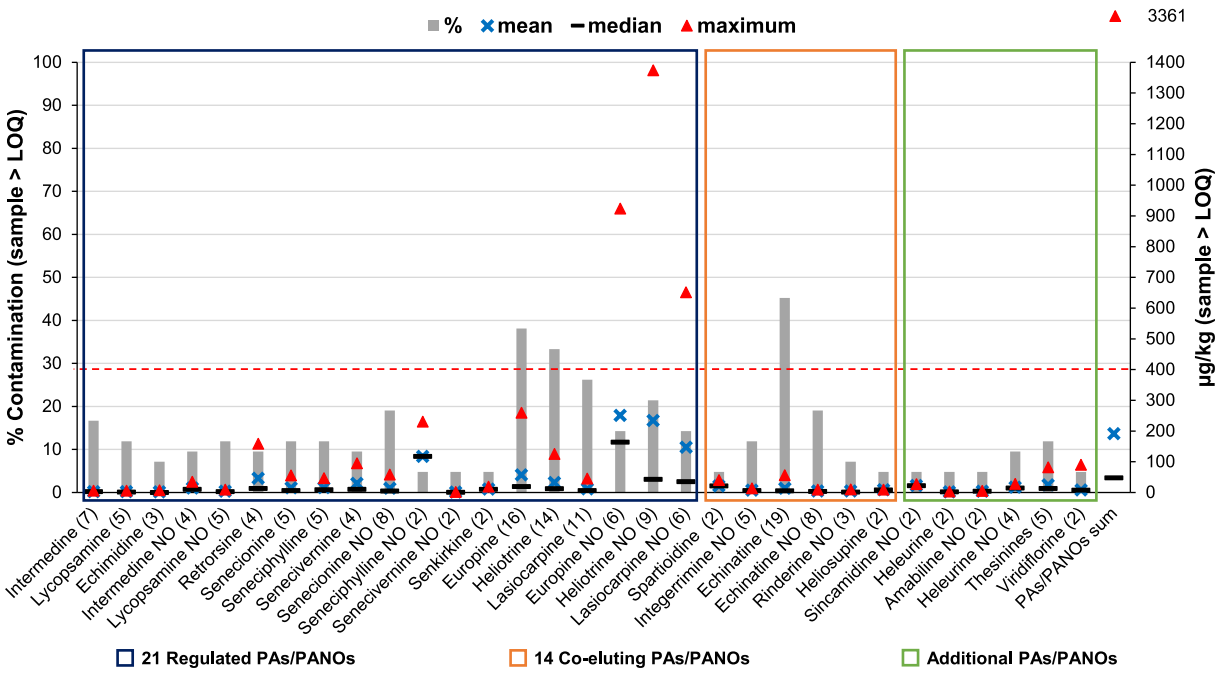

Fig. 4. Contamination profile of herbal food supplements: prevalence and level of each PA and PANO in positive samples. Only PAs present in more than 5% of the positive samples are shown.

samples. Therefore, it can be successfully used for the determination of PA and PANO levels in food supplements, herbal infusions, honey and teas.

The accurate quantification of 28 compounds, including the 21 relevant PAs and PANOs selected by European regulation, was achieved using matrix-matched calibration and standard addition approaches. Regarding the remaining target analytes, for which no reference standards were available, a semi-quantitation was performed by associating each of them to a structurally related reference standard. Even if the present study could provide accurate quantitative data for 28 analytes only, it is certainly worth using to estimate the total PA content and direct high-PA-content samples to further quality control investigations.

Given the lack of occurrence data for the pyrrolizidine alkaloids that are not part of the list of 35 PAs to be monitored in food, the aim of the present study was to collect and share data on the distribution of a wider number of compounds in the most susceptible foods to the contamination of PAs. A high prevalence of contamination (more than 50% of the samples) was determined in honey, herbal infusions of mixed plants (category 8.4.1), and food supplements. Moreover, samples exceeding the MLs were found for all the investigated food categories, except for the infusions of the category 8.4.2. Particularly, the contamination of herbal infusions of mixed plants (8.4.1) was worrying because 23% of the samples exceed the set ML, as well as the mean value of the total PA content. This means that there is a possible health risk for consumers associated with the consumption of these products, especially those with habits of drinking herbal infusions. Regarding the contamination profiles, the co-eluting isomers significantly contributed to the overall contamination levels of honey (36%) and tea (48%) and a high number of additional PAs (not included in the regulation) was detected in honey (11 out of 24) and herbal food supplements (23 out of 49), even if their contribution to the overall contamination level was only relevant for honey.

These occurrence data indicate the need to continue collecting data on PA contamination relating to plant-based food supplements and infusions of mixed herbs, which cover a large slice of the health market and may represent a source of health risks for consumers who take them regularly. Furthermore, it is necessary to expand the pool of PAs to be monitored in honey and related products to provide a more accurate contamination profile of them and to adequately cover the mean total PA contents.

The results of this study highlight that it is extremely important to collect data on of PAs and PANOs, both the regulated ones and additional compounds, in order to provide the regulatory agencies with a broader picture regarding the distribution of these toxins in foods and enable them to strengthen the current regulatory framework. This requires further improvement of the analytical methods to identify relevant PAs, which are not yet included in monitoring programmes. It is also recommended to isolate and/or synthesise these PAs to accurately assess their levels in contaminated foods and their genotoxicity.

CRediT authorship contribution statement

Serena Rizzo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Rita Celano:** Conceptualization, Investigation, Formal analysis, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Anna Lisa Piccinelli:** Conceptualization, Data curation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Mariateresa Russo:** Conceptualization, Funding acquisition, Writing – review & editing. **Luca Rastrelli:** Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136306.

References

Brugnerotto, P., Seraglio, S. K. T., Schulz, M., Gonzaga, L. V., Fett, R., & Costa, A. C. O. (2021). Pyrrolizidine alkaloids and beehive products: A review. *Food Chemistry, 342*, Article 128384. https://doi.org/10.1016/j.foodchem.2020.128384

Carpinelli De Jesus, M., Hungerford, N. L., Carter, S. J., Anuj, S. R., Blanchfield, J. T., De Voss, J. J., & Fletcher, M. T. (2019). Pyrrolizidine Alkaloids of Blue Heliotrope (Heliotropium amplexicaule) and Their Presence in Australian Honey. *Journal of Agricultural and Food Chemistry, 67*(28), 7995–8006. https://doi.org/10.1021/acs.jafc.9b02136

Casado, N., Morante-Zarcero, S., & Sierra, I. (2022a). Application of the QuEChERS Strategy as a Useful Sample Preparation Tool for the Multiresidue Determination of Pyrrolizidine Alkaloids in Food and Feed Samples: A Critical Overview. *Applied Sciences, 12*, 4325. https://doi.org/10.3390/app12094325

Casado, N., Morante-Zarcero, S., & Sierra, I. (2022b). The concerning food safety issue of pyrrolizidine alkaloids: An overview. *Trends in Food Science & Technology, 120*, 123–139. https://doi.org/10.1016/j.tifs.2022.01.007

Commission regulation (EC) No 1881/2006. (2006). Commission regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L 364, 5–24.

Commission Regulation (EU) 2020/2040. (2020). Commission Regulation (EU) 2020/2040 of 11 December 2020 amending Regulation (EC) No 1881/2006 as regards maximum levels of pyrrolizidine alkaloids in certain foodstuffs (Text with EEA relevance). Official Journal of the European Union, L 420/1-5.

Dusemund, B., Nowak, N., Sommerfeld, C., Lindtner, O., Schäfer, B., & Lampen, A. (2018). Risk assessment of pyrrolizidine alkaloids in food of plant and animal origin. *Food and Chemical Toxicology, 115*, 63–72. https://doi.org/10.1016/j.fct.2018.03.005

Efsa. (2011). Scientific Opinion on Pyrrolizidine alkaloids in food and feed. *EFSA Journal, 9*(11), 1–134. https://doi.org/10.2903/j.efsa.2011.2406

Efsa. (2016). Dietary exposure assessment to pyrrolizidine alkaloids in the European population. *EFSA Journal, 14*(8), 50. https://doi.org/10.2903/j.efsa.2016.4572, 4572.

Efsa. (2017). Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. *EFSA Journal, 15(7), 4908*, 34 pp. https://doi.org/10.2903/j.efsa.2017.4908

Kaltner, F., Stiglbauer, B., Rychlik, M., Gareis, M., & 622 & Gottschalk, C. (2019). Development of a sensitive analytical method for determining 44 pyrrolizidine alkaloids in teas and herbal teas via LC-ESI-MS/MS. *Analytical and Bioanalytical Chemistry, 411*(27), 7233–7249. https://doi.org/10.1007/s00216-019-02117-1

Louisse, J., Mulder, P. P. J., Gerssen, A., Stoopen, G., Rijkers, D., van de Schans, M. G. M., & Peijnenburg, A. A. C. M. (2022). Bioassay-directed analysis-based identification of relevant pyrrolizidine alkaloids. *Archives of Toxicology, 96*(8), 2299–2317. https://doi.org/10.1007/s00204-022-03308-z

Ma, C., Liu, Y., Zhu, L., Ji, H., Song, X., Guo, H., & Yi, T. (2018). Determination and regulation of hepatotoxic pyrrolizidine alkaloids in food: A critical review of recent research. *Food and Chemical Toxicology, 119*, 50–60. https://doi.org/10.1016/j.fct.2018.05.037

Mädge, I., Gehling, M., Schöne, C., Winterhalter, P., & These, A. (2020). Pyrrolizidine alkaloid profiling of four Boraginaceae species from Northern Germany and implications for the analytical scope proposed for monitoring of maximum levels. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 37*(8), 1339–1358. https://doi.org/10.1080/19440049.2020.1757166

Magnusson, B., & Örnemark, U. (2014). Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics. (2nd ed. 2014). ISBN 978-91-87461-59-0. Available from http://www.eurachem.org.

Moreira, R., Pereira, D. M., Valentão, P., & Andrade, P. B. (2018). Pyrrolizidine alkaloids: Chemistry, pharmacology, toxicology and food safety. *International Journal of Molecular Sciences, 19*(6), 1668. https://doi.org/10.3390/ijms19061668

Mulder P. P. J., López Sánchez, P., These, A., Preiss-Weigert, A, & Castellari, M. (2015). Occurrence of Pyrrolizidine Alkaloids in food. EFSA supporting publication 2015: EN-859, 114 pp. https://doi.org/10.2903/sp.efsa.2015.EN-859.

Mulder, P. P. J., López, P., Castelari, M., Bodi, D., Ronczka, S., Preiss-Weigert, A., & These, A. (2018). Occurrence of pyrrolizidine alkaloids in animal- and plant-derived food: Results of a survey across Europe. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 35*(1), 118–133. https://doi.org/10.1080/19440049.2017.1382726

Picron, J. F., Herman, M., Van Hoeck, E., & Goscinny, S. (2018). Analytical strategies for the determination of pyrrolizidine alkaloids in plant based food and examination of the transfer rate during the infusion process. *Food Chemistry, 266*, 514–523. https://doi.org/10.1016/j.foodchem.2018.06.055

Pihlstrom, T., Fernández-Alba, A. R., Gamón, M., Amate, C. F., Poulsen, M. E., Lippold, R., & Anastassiades, M. (2018). Analytical quality control and method validation procedures for pestice residues analysis in food and feed. *Sante/11813/2017, 42*.

Rizzo, S., Celano, R., Campone, L., Rastrelli, L., & Piccinelli, A. L. (2022). Salting-out Assisted Liquid-Liquid Extraction for the rapid and simple simultaneous analysis of pyrrolizidine alkaloids and related N-oxides in honey and pollen. *Journal of Food Composition and Analysis, 108*, Article 104457. https://doi.org/10.1016/j.jfca.2022.104457

Rizzo, S., Celano, R., Piccinelli, A. L., Serio, S., Russo, M., & Rastrelli, L. (2023). An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination. *Food Chemistry, 406*, Article 135058. https://doi.org/10.1016/j.foodchem.2022.135058

Schramm, S., Köhler, N., & Rozhon, W. (2019). Pyrrolizidine alkaloids: Biosynthesis, biological activities and occurrence in crop plants. *Molecules, 24*(3), 498. https://doi.org/10.3390/molecules24030498

Schrenk, D., Gao, L., Lin, G., Mahony, C., Mulder, P. P. J., Peijnenburg, A., … These, A. (2020). Pyrrolizidine alkaloids in food and phytomedicine: Occurrence, exposure, toxicity, mechanisms, and risk assessment - A review. *Food and Chemical Toxicology, 136*, Article 111107. https://doi.org/10.1016/j.fct.2019.111107

Wenzl, T., Haedrich, J., Schaechtele, A., Robouch, P., & Stroka, J. (2016). Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food. EUR 28099 EN. *European Union Reference Laboratory. Publications Office of the European Union, Luxembourg, ISBN 978-92-79-61768-3.* https://doi.org/10.2787/8931.