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Evaluation of the thermal stability and transfer rate of pyrrolizidine alkaloids during the brewing of herbal infusions contaminated with *Echium vulgare* and *Senecio vulgaris* weeds

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are currently in the spotlight of food safety due to the potential health risk that their intake may entail for consumers. Among the wide variety of food products likely to be contaminated with PAs, teas and herbal teas stand out because of their cross-contamination with weeds from PA-producing plants. Accordingly, this work simulates the cross-contamination of chamomile tea samples with weeds of *Senecio vulgaris* and *Echium vulgare* to evaluate the effect of brewing on the PA content at different temperature (100 and 75 °C) and time (5 and 10 min) conditions. The results of the thermal degradation study revealed PAs are quite stable compounds under the conditions assayed, being the initial PA concentration the variable that strongly affected the thermal stability of these compounds, rather than the brewing temperature and time. On the other hand, it was confirmed that not all PAs are effectively transferred from the dry material to the infusion during the brewing process, leading to overall transfer rates between 13 ± 1 to 87 ± 10%. Also, it was observed that the extraction efficiency may be influenced by the type of matrix, since transfer rates were lower when the PA migration came from *Echium vulgare* than from *Senecio vulgaris*. Moreover, it was confirmed that the real content of PAs in a cup of tea can be overestimated in 2–7 times if their transfer rate is not considered during the brewing process, highlighting the importance of analyzing the infusion samples instead of the dry tea samples to correctly assess the risk exposure of consumers to these contaminants.

1. Introduction

The reduction of risks for consumers and improving the functioning of food systems are basic priorities within the food safety field. For this reason, food safety is part of the focus of the Sustainable Development Goal (SDG) 2, since it is an essential factor determining the quality of life for an individual (United Nations). Consequently, lately greater food control has been demanded, highlighting the need for a rigorous control of the entire food supply chain including all stages from production to consumption. Among the different health risks related to food intake, the occurrence of contaminants still to be one of the main issues within food control. Recently, the occurrence of natural plant toxins such as pyrrolizidine alkaloids (PAs) in food has raised special concern due to their potential acute and chronic toxicity (hepatotoxicity, genotoxicity, and carcinogenicity) (Casado, Morante-Zarcero, & Sierra, 2022; Dusemund et al., 2018; Schrenk et al., 2020; Xu et al., 2019). In fact, the monitoring of PAs in food is a topic to which the scientific community has recently begun to pay attention because of the different calls emitted by the European Food Safety Authority (EFSA) to gather information on their presence in food (EFSA-European Food Safety Authority, 2017; EFSA-European Food Safety Authority, 2016; Mulder et al., 2015; EFSA-European Food Safety Authority, 2011; EFSA-European Food Safety Authority, 2007). Accordingly, PAs are currently in the spotlight of risk assessment for human health as they can be found in a wide variety of products regularly included in our daily diet (Casado et al., 2022a, 2023).

PAs are a group of alkaloids of more than 660 compounds produced by different families of plant species, such as Asteraceae (tribes Senecioneae and Eupatorieae), Boraginaceae (most genera), Fabaceae (genus *Crotalaria*), Apocynaceae (tribe Echiteae) and Orchidaceae (Reinhard & Zoller, 2021). For instance, *Jacobaea vulgaris* (ragwort, Asteraceae family), *Senecio vulgaris* (common groundsel, Asteraceae

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family), *Borago officinalis* (borage, Boraginaceae family) and *Echium vulgare* (viper's bugloss, Boraginaceae family) are some of the most common PA-producing plants (Mulder et al., 2016, 2020). Although some of these plants can be directly consumed by animals (e.g., through forage) and humans (e.g., borage as vegetables), they often find their way into the food chain as natural contaminants of food since they usually grow like weeds in crop fields and are very difficult to remove after harvest. In consequence, this leads to the accidental contamination of plant-based products (including food and feed) with PA-producing plants. This contamination pathway is the most frequent and it is known as cross-contamination (Casado, Morante-Zarcero, & Sierra, 2022; Kaltner et al., 2020; Schrenk et al., 2020). Nonetheless, other possible contamination pathways have been reported in recent years, such as the horizontal natural transfer through the soil (Izcara et al., 2020; Izcara et al., 2022; Jiao et al., 2022; Letsyo et al., 2021; Chmit et al.; Selmar et al., 2019). Therefore, PAs can contaminate a wide variety of plant-based products, and they can even be found in animal-based products (Casado et al., 2022a, 2023). Hence, it is of utmost importance to monitor the occurrence of these alkaloids and exhaustive controls must be performed to determine their levels in food and feed due to their potential toxicity for consumers.

Among the wide variety of food products likely to be contaminated with PAs, teas and herbal teas stand out (Casado et al., 2022a, 2023). In fact, these products have received in the Rapid Alert System for Food and Feed (RASFF) the largest number of alerts related to high levels of PAs (13% of the food alerts notified within the last 10 years) after herbs and spices. For instance, in 2018 and 2021 high concentrations of PAs (12541 and 5400 μg/kg, respectively) were detected in chamomile teas from Denmark (RASFF notifications No. 2018.2434 and No. 2021.7080, respectively). In consequence, many works have addressed the analysis of PAs in teas and herbal tea samples, revealing concerning concentration values of these alkaloids, even in teas made from non-PA-producing plants (Casado, Fernández-Pintor, et al., 2022; Chen et al., 2019; Han, Jiang, Wang, Wang, et al., 2022; Kaltner et al., 2019; Leon et al., 2022; Reinhard & Zoller, 2021). Accordingly, due the potential risk that the intake of PAs may entail for human health and the wide spread of these contaminants in food, the EU Commission has recently published maximum levels of PAs in some food products, including teas and herbal teas (75–400 μg/kg for dry solid tea and herbal tea samples, and 1 μg/L in the case of liquid products for infants and children). This regulation has entered into force in July 2022 (EU 2020/2040).

Nonetheless, it is worth highlighting that the regulation and many of the works published in the literature refer to the content of PAs in the dry tea sample instead of the infusion (León et al., 2022; Kwon et al., 2021; Kaltner et al., 2019; Shimshoni et al., 2015; Schulz et al., 2015; Griffin et al., 2014; Bodi et al., 2014). This is an important issue, as it seems that the analysis of the infusions is more appropriate than the analysis of the dry samples to avoid overestimation of the real intake and exposure of consumers to PAs through these beverages. This is mainly due to the fact that the transfer rate of these alkaloids during the brewing process is not complete (Casado et al., 2023). To date, there are only a few publications that have investigated this phenomenon. In addition, the current data related to the transfer rate of PAs in infusion samples show many discrepancies. For instance, Picron et al. (2018) carried out a transfer study spiking teas and herbal teas at two concentration levels with a standard solution of 30 PAs before and after the brewing process, reporting transfer rates between 16% and 28%. Accordingly, these results suggested an overestimation of the risk associated to the intake of PAs through beverage infusions. However, on the contrary, in the survey of Mulder et al. (2018) 38 naturally contaminated herbal teas were studied. The outcome was an average extraction efficiency of 85% for infusions compared to acidic extraction. However, the range was considerable, between 40 and 250%. Likewise, Reinhard and Zoller (2021) analyzed 10 naturally contaminated herbal teas and derived transfer rates depending on the method of brewing and type of tea, ranging from 38 to 100%. Han, Jiang, Wang, Lu, et al. (2022) also determined the transfer rate of PAs and their *N*-oxide forms (PANOs) during tea brewing, obtaining average transfer rate values of 57% and 75% for 8 PAs and 7 PANOs, respectively. Nonetheless, it is worth pointing out that transfer rate of PAs during brewing may be affected by the water temperature required to prepare the infusion, which in some cases may increase the extraction of the analytes but in other cases it can also lead to their thermal degradation. Accordingly, the transfer rate can be lower as a result of a poor extraction efficiency, but also because PAs and PANOs may be heat sensitive (Casado et al., 2023). However, to the best of our knowledge, this aspect has not been investigated or discussed in any of the works currently available in the literature. Moreover, these studies that determine the transfer rate of PAs during the brewing process of teas and herbal teas have been performed by spiking the samples with standard solutions (Han, Jiang, Wang, Lu, et al., 2022; Picron et al., 2018) or with naturally contaminated samples (Mulder et al., 2018; Reinhard & Zoller, 2021) but none of them have proposed a real simulation of contamination by spiking the samples with a known amount of PA-producing plants to reproduce a cross-contamination situation. This aspect is also interesting because the extraction of PAs from the plant tissues during the brewing process is not the same as when they are directly added with standard solutions, so transfer rate may probably be affected by this parameter. Likewise, the extraction efficiency of PAs may also vary depending on the type of plant.

Hence, this work aimed to evaluate the transfer rate of PAs and PANOs as well as their associated thermal degradation when they are subjected to different brewing conditions during the preparation of contaminated chamomile tea. Accordingly, the chamomile tea samples were contaminated in a controlled way with two PA-producing plants (*Senecio vulgaris* and *Echium vulgare*) that frequently grow as weeds among the crops in order to simulate a real cross-contamination process. Moreover, the samples were purified using a microextraction procedure based on μSPEed® that meets the Green Analytical Chemistry principles, followed by ultrahigh-performance liquid chromatography coupled to ion-trap tandem mass spectrometry (UHPLC-IT-MS/MS) analysis.

2. Materials and methods

2.1. Solvents, materials and standard solutions

Methanol (MeOH) LC-MS grade, acetonitrile (ACN) LC-MS grade and dimethyl sulfoxide (DMSO) were acquired from Scharlab (Barcelona, Spain). Acid formic LC-MS grade and ammonium acetate were purchased from Fluka (Bush, Switzerland). A Millipore Milli-Q system (Billerica, MA, USA) was used to obtain the Milli-Q water (resistivity 18.2 MΩ cm). Nylon syringe filters (0.45 μm, 13 mm) were purchased from Mervilab (Madrid, Spain). The μSPEed procedure was performed with an electronic digiVol digital syringe (250 μL) and μSPEed cartridges (octadecylsilane, C18 silica-based, 3 μm, 120 Å) acquired from EPREP (Mulgrave, Victoria, Australia).

Standards of PAs and PANOs (>90% of purity) were purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). The standard solutions of europine, europine *N*-oxide, heliotrine, heliotrine *N*-oxide, lycopsamine, intermedine, senecionine, seneciphylline and retrorsine (1000 mg/L) were prepared in ACN/DMSO (4/1, *v/v*). The standard solutions of echimidine, echimidine *N*-oxide, lycopsamine *N*-oxide, intermedine *N*-oxide, lasiocarpine, lasiocarpine *N*-oxide, seneciverine, senecivernine *N*-oxide, senkirkine, seneciphylline *N*-oxide, retrorsine *N*-oxide and senecionine *N*-oxide were prepared in MeOH. From these standard solutions, a solution containing the twenty-one analytes (1 mg/L) was prepared in water. All the solutions were stored at −20 °C.

2.2. Samples

Bulk bags of chamomile were obtained from a local supermarket of Madrid (Spain). In order to homogenize the samples, the bulk bags were

emptied and grounded with an A11 basic analytical mill (IKA, Staufen, Germany) to obtain a fine powder. On the other hand, samples of PA-producing plants *Echium vulgare* (viper's bugloss) and *Senecio vulgaris* (common groundsel) were collected in a wild field of Arroyomolinos (surroundings of Madrid, Spain). These samples were lyophilized for 24 h in a LyoBench −55 °C laboratory freeze-dryer (Noxair Life Sciences S. L., Barcelona, Spain) and afterwards they were grounded for their homogenization. The lyophilized grounded samples were stored in a desiccator until their use.

2.3. Analysis of PA-producing plants used for cross-contamination

Due to the high levels of PAs/PANOs that can be found in the PA-producing plants and to obtain concentration levels that could be compared with those used in the transfer study, the content of these alkaloids in the PA-producing plants was determined by preparing first mixtures of chamomile (which was previously subjected to the same sample procedure to verified that it was not contaminated with PAs) with a 5% (*p/p*) of lyophilized *Echium vulgare* or a 5% (*p/p*) of lyophilized *Senecio vulgaris*. Afterwards, 0.315 g of these samples were mixed with 50 mL of acidified water (formic acid 1% *v/v*) and the mixture was kept in maceration for 24 h at room temperature to avoid thermal degradation (Fig. 1). Subsequently, the mixture was centrifuged (6000 rpm, 5 min) and the supernatant was recovered, while the solid residue was subjected to a second acid maceration with 50 mL of acidified water (formic acid 1% *v/v*) at room temperature for 48 h to ensure a complete extraction of the target analytes. Afterwards, the mixture was centrifuged (6000 rpm, 5 min) and the resultant supernatant was collected with the previous one. Nonetheless, the two supernatants were also analyzed separately to confirm that the extraction was complete. The sample extracts were basified with ammonia solution to pH 6.0 (to obtain the same pH conditions as in the chamomile infusion samples). Finally, the sample extract was purified by μSPEed. All samples were extract and analyzed in triplicate.

The quantification of PAs in the PA-producing plants was achieved by interpolating in matrix-matched calibration curves the areas obtained in the analysis of the chamomile samples contaminated with a 5% (*p/p*) of *Echium vulgare* or *Senecio vulgaris*. These matrix-matched calibration curves were prepared with a dry blank chamomile sample (sample verified it was not naturally contaminated with PAs) macerated at room temperature in acid conditions and then subjected to the μSPEed procedure. Subsequently, after elution and prior to the chromatographic analysis, the sample extracts were spiked at different known concentration levels. Accordingly, the linear range evaluated was 1–500 μg/L (which corresponds to a range of 40–20000 μg/kg in the dry product). Before interpolating in the matrix-matched calibration curves, the areas of the chamomile contaminated samples were corrected according to the recovery of the analytes found in the PA-producing plants. For this purpose, a recovery assay was carried out at a concentration level of 50 μg/L (for each analyte) in the infusion (corresponding to 2000 μg/kg in the dry product) spiking the chamomile contaminated samples with a standard solution and subjecting them to the μSPEed procedure and the subsequent UHPLC-IT-MS/MS analysis. The areas obtained were compared with those of simulated samples (contaminated chamomile infusion extracts subjected to the μSPEed procedure and spiked afterwards at the same concentration level prior to their chromatographic analysis), as explained in S1 (see supplementary material).

2.4. Thermal degradation study

The thermal degradation assay was carried out at four concentration levels (200, 400, 1000 and 10000 μg/kg). For this purpose, 5.0 g of a dry blank chamomile sample (weighted in an analytical balance (±0.1 mg)) were spiked at the desired concentration level with a standard solution and were then capped infused in 200 mL of boiling water 5 min according to the manufacturer's instructions. Additionally, a lower temperature (75 °C) was also evaluated because sometimes some consumers do not get to boil the water when preparing tea. Likewise, sometimes consumers let the tea stand for more than 5 min, until it tempers a bit, so a higher brewing time (10 min) was also evaluated. After the infusion, the samples were filtered prior to μSPEed extraction and UHPLC-IT-MS/MS analysis (Fig. 1). All samples were infused in triplicate, and each infusion extract was purified and analyzed in triplicate.

The thermal degradation degree was determined as indicated in S2 (see supplementary material). Accordingly, the difference between the 100% and the analyte percentage finally found in the spiked sample after infusion corresponds to the thermal degradation degree of each analyte.

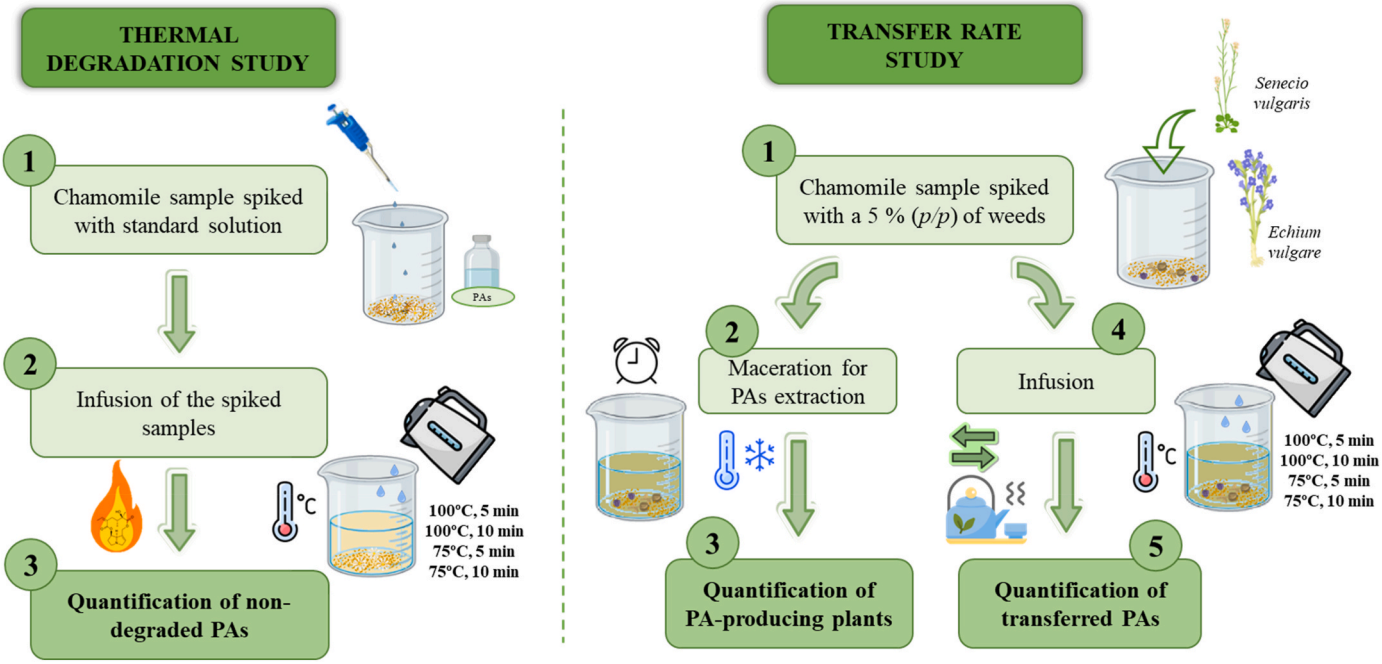


Fig. 1. Graphical scheme of thermal degradation procedure, quantification of PA-producing plants and transfer rate study.

2.5. Transfer rate study

For the transfer rate assay, dry blank chamomile samples were contaminated with a 5% (*p/p*) of lyophilized *Echium vulgare* or a 5% (*p/p*) of lyophilized *Senecio vulgaris* to simulate a real cross-contamination process. Afterwards, 5.0 g of these contaminated samples were weighted in an analytical balance (±0.1 mg) and capped infused in 200 mL of water using different temperature (100 or 75 °C) and brewing time (5 or 10 min) conditions as in section 2.5. These conditions of temperature and time were selected based on the same criteria previously explained for the thermal degradation assay. After infusion, the samples were filtered and subjected to μSPEed extraction and UHPLC-IT-MS/MS analysis (Fig. 1). All samples were infused in triplicate, and each infusion extract was purified and analyzed in triplicate.

To determine the transfer rate, the areas obtained were corrected according to the corresponding recovery value and then interpolated in the matrix calibration curves. The concentration value obtained was compared with the concentration value determined in the PA-producing plant samples. Accordingly, the difference between these concentrations in terms of percentage corresponds to the transfer rate.

2.6. Extraction and purification procedure of PAs by μSPEed

The extraction and purification of the sample extracts of the different trials was carried out using a μ-SPEed extract-discard procedure previously optimized and validated in edible flower infusion samples within the research group (Casado, Fernández-Pintor, et al., 2022), with slight modifications. Briefly, the C18 μ-SPEed cartridge was conditioned with two aspiration-dispense cycles of 100 μL of MeOH (20 μL/s) followed by two aspiration-dispense cycles of 100 μL of water (10 μL/s). Afterwards, the sample was loaded in three aspiration-dispense cycles of 100 μL (10 μL/s). To avoid the loss of the most polar compounds, the washing step was omitted. Finally, the analytes were eluted on a chromatographic vial with 100 μL of MeOH for its subsequent analysis on the UHPLC-IT-MS/MS. After each extraction the cartridge was cleaned with four aspiration-dispense cycles of 100 μL of MeOH (20 μL/s) to avoid memory effects.

2.6.1. Analytical feasibility of the method

The feasibility of the method was confirmed in chamomile samples in terms of accuracy (assessed as recovery) and precision (assessed as inter- and intra-day precision) according to the criteria establish in the European Commission SANTE/12682/2019 document and in regulation EC No 401/2006 (see details in S1, supplementary material).

2.7. UHPLC-IT-MS/MS analysis

The purified extracts were analyzed by an UHPLC system (Dionex UltiMate 3000, Thermo Scientific, Waltham, MA, USA) coupled to an ion-trap tandem mass spectrometer detector (IT-MS/MS amaZon SL, Bruker, Billerica, MA, USA). The chromatographic separation was achieved on a Luna Omega Polar C18 column (100 mm × 2.1 mm, 1.6 μm particle size, Phenomenex, Torrance, CA, USA) at 25 °C using a mobile phase gradient elution with water containing 5 mM ammonium acetate and 0.2% formic (solvent A) and MeOH containing 10 mM ammonium acetate (solvent B) as follows: 5% B (0–0.5 min), 5–50% B (0.5–7 min), 50% B (7–7.5 min), 50–100% B (7.5–11 min), 100% B (11–12 min), 100–5% B (12–14 min), and 1 min for re-equilibration to initial conditions. The total run time was 15 min. The flow rate was set constant to 0.250 mL/min and the injection volume was 5 μL. Under these conditions the separation of all the target analytes was achieved in less than 10 min. Table S1 shows the retention time for the different PAs/PANOs analyzed.

The ionization parameters for the mass spectrometry detection were set as follows: electrospray ionization interface (ESI) was used in positive ion mode, the capillary voltage was −4500 V, the end plate offset −500 V, the drying gas was at 10 L/min, the nebulizer gas was at 20 psi and the drying temperature was at 200 °C. Multiple reaction monitoring (MRM) scan mode was used for all analytes. The mass spectrum parameters for each analyte were determined by direct infusion of individual standard solutions (5 μg/mL) at a flow rate of 4 μL/min (Table S1). Accordingly, the precursor ion ($[M+H]^+$) of each analyte was identify, isolated and fragmentated, so the mass spectra (MS^2) with the corresponding product ions for each analyte were determined. In the MS^2 spectrum of each analyte, the most intense product ion was used for quantification, whereas the other ion products were used for confirmation (Table S1).

2.8. Statistical analysis

To check if there were statistically significant difference in the conditions assayed, several tests were performed. For the evaluation of the thermal degradation, a three factor two level full factorial design was applied to determine the effect of the infusion temperature (A), the brewing time (B) and the spiked concentration (C). Two concentration levels were selected for this study: a low level (200 μg/kg) and a high level (10000 μg/kg). This study pretended to evaluate all the parameters that may influence the process as a whole and it was carried out with Statgraphics Centurion software (version 19.3.03). Moreover, in order to study individual conditions of each analyte an analysis of variance (ANOVA) using Duncan's multiple range test was carried out both in the evaluation of the thermal degradation and the transfer rate. Same letters mean not statistically significant differences, while different letters mean statistically significant differences were found. The ANOVA analysis was performed using SPSS software (19.0 version).

3. Results and discussion

3.1. Analytical feasibility of the method

The feasibility of the different assays carried out has been confirmed throughout this work. Hence, the accuracy and precision of the μSPEed® procedure for the 21 target analytes were evaluated based on the criteria established in the European Commission SANTE/12682/2019 document and in regulation EC No 401/2006. Accordingly, the overall recovery values were in the range 76–101% (Table S2), so the method accuracy successfully accomplished the requirements set in the validation guidelines (recovery should be between 70 and 120%). Likewise, the RSD values achieved at the validation level were lower than 11% and 15% for intra- and inter-day precision, respectively. Therefore, the method precision also fulfilled the requirements of the validation guidelines, as RSD values for the target analytes were in all cases ≤20%. Moreover, matrix-matched curves and recovery values at different levels have been evaluated for the different assays (quantification of PAs and transfer rate). These results are included in Tables S3–S5. As it can be observed, the linearity ($R^2 > 0.99$) and accuracy (recovery ≥70%) values obtained for the different analytes found in the PA-producing plants fulfill the criteria of the validation guidelines.

3.2. Occurrence of PAs in PA-producing plants

Senecio vulgaris and *Echium vulgare* are two PA-producing plants well known to contain high concentrations of these natural toxins. *Senecio vulgaris* it is characterized by having a great predominance of senecionine-type PAs (e.g., retrorsine, senecionine, seneciphylline, senecivernine, their *N*-oxides and senkirkine). On the other hand, *Echium vulgare* has great predominance of lycopsamine-type PAs (e.g., echimidine, intermedine, lycopsamine and their *N*-oxides), particularly highlighting echimidine and echimidine *N*-oxide as their main PAs (Wang et al., 2022). These plants frequently grow as weeds in crops, grasslands and fallow farmlands with low fertilization and altered mowing frequencies (Kalac & Kartner, 2021), so their occurrence by

cross-contamination is probable in tea and herbal tea samples, such as chamomile. For this reason, the quantification of PAs in these plants was performed by simulating a cross-contamination in a chamomile dry sample with these plants. Some authors have directly analyzed PAs in dry samples. For instance, Leon, 2022 evaluated the efficiency of both solid-phase extraction (SPE) and QuEChERS methodologies to extract PAs from teas and herbal infusions. However, they concluded that the QuEChERS strategy did not allow the complete extraction of the analytes because of the high concentrations of PAs in the sample. Therefore, the direct quantification of PAs in the two PA-producing plants (i.e., using a 100% of each plant) was discarded due to the high levels of these alkaloids that can be found in both plants (up to thousands ppm). Hence, it would be very difficult to achieve a total extraction of PAs from the plant matrix, even performing successive extraction cycles. To avoid this problem, the extraction was carried out contaminating the chamomile sample with a 5% (*p/p*) of these weeds since this percentage allows the complete extraction of the analytes.

On the other hand, the extraction of PAs from the PA-producing plants needed to be carried out at room temperature and under conditions that avoided thermal degradation, so that it was possible to differentiate between heat degradation and the migration of the analytes from the plant to the liquid that can take place during the infusion process, which it is independent of the amount of analyte lost with high temperatures. Accordingly, some authors have performed the extraction of PAs from teas and herbal teas with acid solvents, such as H_2SO_4 (0.1 M) (Han, Jiang, Wang, Lu, et al., 2022), H_2SO_4 (0.05 M) (Reinhard & Zoller, 2021) or methanol acidified with formic acid (0.1%) (Picron et al., 2018). Likewise, Larcher et al., (2019) used a mixture of solvents (H_2O/MeOH/formic acid (49.5/49.5/1, *v/v/v*)) to extract PAs from *Senecio vulgaris*, whereas Mulder et al. (2020) used acidified water with 2% formic acid for the extraction of *Senecio vulgaris* and *Echium vulgare*. Therefore, based on the above works, the extraction of PAs from the chamomile samples contaminated with a 5% of the PA-producing plants was finally carried out with acidified water with 1% formic acid in two maceration cycles as previously described in Section 2.3. It was confirmed that two maceration cycles were enough to achieve the complete extraction of the target analytes, as the concentrations obtained in the second cycle were very small (concentration values lower than the limit of quantification). Thus, it was concluded that a third maceration cycle was not necessary.

Fig. 2 shows the quantification of all the PAs found in the two PA-producing plants analyzed under the extraction conditions described above. As it can be observed, 6 of the 21 target PAs were found in *Echium vulgare*, belonging all of them to the lycopsamine-type (intermedine, echimidine, lycopsamine and their corresponding *N*-oxides). High concentration levels of these compounds were quantified in this plant, ranging from 36 to 6082 mg/kg, highlighting the great presence of echimidine *N*-oxide as the most predominant PAs. To date, there are only a few works in the literature that describe the extraction of PAs from *Echium vulgare*, but the great majority focuses on the analysis of products that may be contaminated with this plant, such as honey, pollen, or derived products because of the flower pollination of this plant species by bees, which contaminate the honeycomb with these toxins (Kast et al., 2017, 2019). Nonetheless, the results obtained agree with the work of Mulder et al. (2020), in which the same PA profile was reported, although smaller amounts of echimidine *N*-oxide were detected in *Echium vulgare* (925 mg/kg).

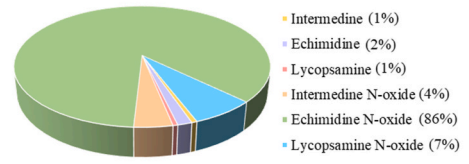
On the other hand, 7 of the 21 target PAs were found in *Senecio vulgaris,* belonging all of them to the senecionine-type (senecivernine, senecionine, senkirkine, seneciphylline, seneciphylline *N*-oxide, senecivernine *N*-oxide and senecionine *N*-oxide), as expected (Fig. 2). Although lower concentrations of PAs and PANOs were found in this plant than in *Echium vulgare,* relevant concentration levels of these alkaloids were quantified, ranging from 6 to 322 mg/kg, highlighting the great presence of seneciphylline *N*-oxide, seneciphylline, senecivernine *N*-oxide and senecionine *N*-oxide as the main predominant PAs in this plant (Fig. 2). These results are similar to those described in previous works (Flade et al., 2019; Larcher et al., 2019).

Moreover, it is worth mentioning that in both plants the concentrations of *N*-oxide forms were higher than the PA forms (Fig. 2). This trend has also been reported in previous works dealing with the contamination of PAs in infusion and tea samples (Casado, Fernández-Pintor, et al., 2022).

3.3. Thermal degradation of PAs during tea brewing

This study was carried out to evaluate the thermal stability of the target PAs at four concentration levels (200, 400, 1000 and 10000 μg/kg) spiking dry blank chamomile samples with standard solutions of the 21 PAs and PANOs, as explained in Section 2.4. These concentration levels were selected according to the concentrations of PAs quantified in the chamomile samples contaminated with a 5% (*p/p*) of the PA-

		mg/kg PAs-containing plant ± sd	% RSD
Echium vulgare 	Intermedine	40 ± 2	5
	Echimidine	109 ± 13	12
	Lycopsamine	36 ± 1	3
	Intermedine *N*-oxide	288 ± 51	18
	Echimidine *N*-oxide	6082 ± 624	10
	Lycopsamine *N*-oxide	490 ± 34	7
Senecio vulgaris 	Senecivernine	71 ± 10	15
	Senecionine	40 ± 6	15
	Senkirkine	6 ± 1	10
	Seneciphylline	174 ± 9	5
	Seneciphylline *N*-oxide	322 ± 19	6
	Senecivernine *N*-oxide	157 ± 5	3
	Senecionine *N*-oxide	162 ± 6	4



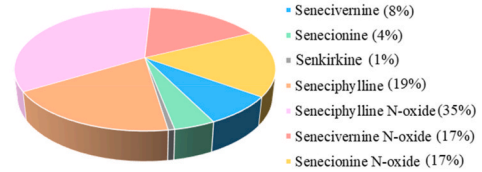


Fig. 2. Concentration of the 13 PAs of the PAs-containing plants (*Echium vulgare* and *Senecio vulgaris*) whose quantification was carried out with the extraction of a chamomile infusion spiked with a 5% (p/p) of these plants. The circle graph shows the percentage of each analyte in the plant that contains them.

producing plants. Afterwards, the spiked chamomile samples were capped infused in water under different conditions (100 °C, 5 min; 100 °C, 10 min; 75 °C, 5 min; 75 °C, 10 min). The temperature and time conditions evaluated were selected to reproduce and simulate the frequent and real preparation conditions of chamomile tea when it is drink by consumers, as previously explained in section 2.4., so that they can simulate the culinary preparation of this herbal tea by consumers to know what the thermal stability of these compounds is at conditions as close as possible to those of a real exposure scenario. Accordingly, Figs. S1–S3 and Fig. 3 show the thermal degradation of PAs at each concentration level assayed under the different brewing conditions. The degradation values were in the range 0–22%, 1–34%, 4–38% and 3–31% for the concentrations 200 μg/kg, 400 μg/kg, 1000 μg/kg (Figs. S1–S3) and 10000 μg/kg (Fig. 3), respectively. Considering the average of the four concentration levels, europine showed the highest degree of average thermal degradation (33%, at 1000 μg/kg), whereas senecionine and echimidine *N*-oxide presented the lowest degrees of average thermal degradation at the four concentrations (Table S6). As the results show, although there is some thermal instability, it seems that PAs are quite stable compounds in studied conditions because the thermal degradation observed was small and did not significantly change with the brewing time. Therefore, these stability results agree with those reported in other works subjecting PAs to mild temperatures like 68 °C and 100 °C (De Nijs et al., 2017; Jansons et al., 2022). This is an important point, because under real tea preparation conditions, the PAs found in the weed impurities of tea samples would possibly be somewhat less exposed to heat in the plant matrix than when they are added as standard solutions, so they probably would be even more heat stable.

Nonetheless, the interactions among the experimental variables (temperature (A), time (B) and concentration (C)) should be evaluated, because they can directly or indirectly affect the brewing process by mutually interacting. Hence, the statistical significance of these experimental variables A-C and their interactions were determined by ANOVA. In addition, Pareto charts were used to show the estimated standardized effects on response variables. Accordingly, this statistical study provides information on the statistically significant differences between the experimental variables studied, and individually evaluates each analyte under the brewing conditions assayed. As it can be observed, none of the three experimental variables significantly affected the thermal degradation of senecivernine, seneciphylline *N*-oxide, senkirkine, lasiocarpine *N*-oxide, echimidine *N*-oxide, europine *N*-oxide, heliotrine *N*-oxide, lycopsamine *N*-oxide, retrorsine *N*-oxide and senecionine *N*-oxide (Figs. S4 and S5). Only in the case of europine, senecionine, lycopsamine and intermedine *N*-oxide the concentration (factor C) strongly affected the thermal degradation of these compounds, observing higher degradation when the concentration increases. Likewise, the temperature (factor A) also showed a significant effect on the thermal degradation of intermedine *N*-oxide, whereas the combination of temperature (factor A) and concentration (factor C) highly affected the thermal degradation of lycopsamine (Fig. 4). For the rest of the analytes (intermedine, heliotrine, lasiocarpime, retrorsine, echimidine, seneciphylline and senecivernine *N*-oxide) the plots showed some negative influence of the concentration (factor C) on their thermal degradation, causing more analyte loss when this variable increased but without becoming statistically significant (Figs. S4 and S5). Therefore, these results suggest that the degradation kinetics may not be the same for all analytes, since in some cases the concentration have an effect, while in others does not affect. According to the literature, the thermal degradation process depends on the oxygen available and consumed by the reaction, what may change the kinetic order (Germano et al., 2021; Wang et al., 2006). This fact depends on the compound, so it could be one of the reasons why the concentration may only influence the thermal degradation of some PAs, as observed.

On the other hand, it is worth mentioning that the brewing time (factor B) did not show in any case significant effect on the thermal degradation of the target analytes. However, it is important to point out that during the brewing process, the temperature is not kept constant. When preparing tea, the dry product is usually added when the water starts to boil, but then it is left to cool, and the temperature drops fast. Accordingly, the temperature can be a crucial factor in the extraction and degradation of these compounds. Moreover, when the analytes are in the plant matrix, they also require time to be extracted. Thus, if the combination of these two factors (temperature + time) is not too drastic,

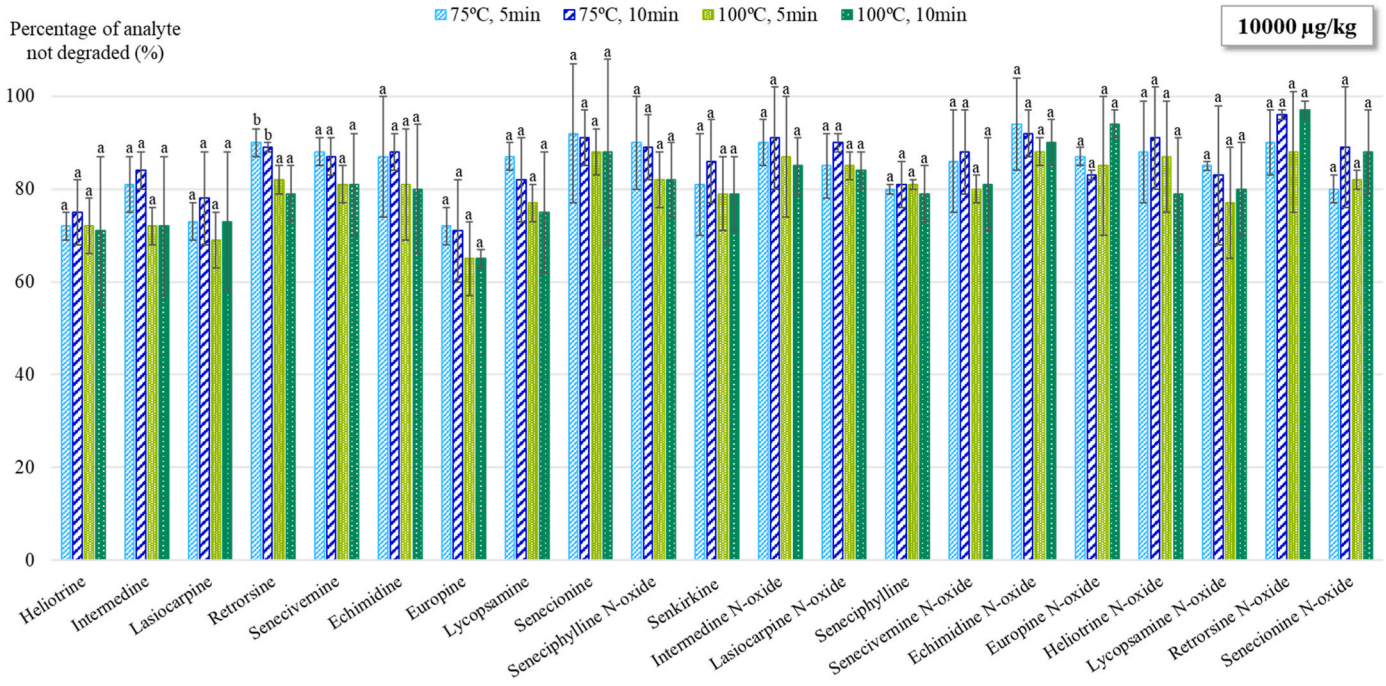


Fig. 3. Percentage of analyte not degraded (n = 3) of the 21 PAs obtained in the thermal degradation study carried out with standard solutions (10000 μg/kg). Same letters means that there are no statistically significant differences ($p > 0,05$) and different letter means that there are significant differences ($p \leq 0,05$).

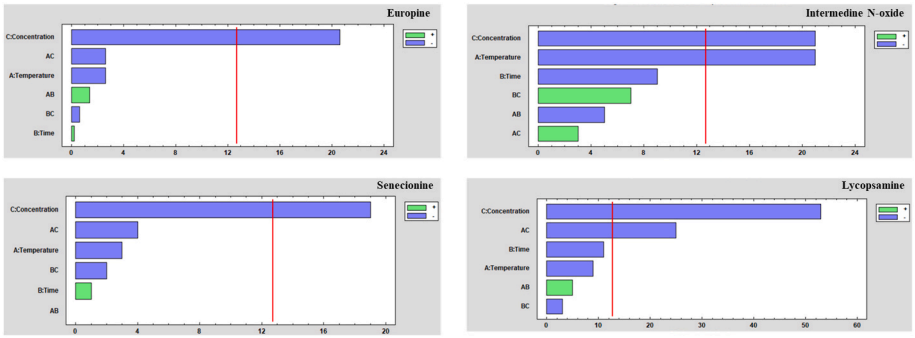


Fig. 4. Response to PAs who showed statistically significant differences from full factorial design $2^3$by the Pareto Chart of the standardized effect of each of the responses (thermal degradation), showing the three factors: (A) temperature (°C), (B) time (min), (C) concentration spiked (μg/kg).

their effect on the extraction and degradation of these analytes is not enough significant. To the best of our knowledge, this is the first time that thermal degradation of PAs during the brewing process of tea and herbal teas is determined. Moreover, to date, the heat stability of PAs is not clear since there are many inconclusive and contradictory results within the literature (Casado et al., 2023). However, from this work it can be assumed that brewing conditions usually used by consumers do not have a large impact on the thermal stability of PAs.

3.4. Transfer rate of PAs during tea brewing

The transfer rate in the brewing process is the result of the combination of two factors: the migration of the analytes from the plant tissues into the infusion and the possible thermal degradation that they can experience with the hot water. Several works have evaluated the transfer rate of PAs during the preparation of tea and herbal tea infusions (Han et al., 2022; Reinhard & Zoller, 2021; Picron et al., 2018). However, to date none of them have carried out this evaluation simulating a real cross-contamination of tea samples with a controlled amount of PA-producing plants. Accordingly, some of these works have evaluated the transfer rate using samples spiked with a known concentration of standard solutions before and after the brewing process to calculate the percentage of PAs transferred to the infusion. Nevertheless, this is an important point, because the extraction and stability of PAs from the vegetable tissues is different than when they are directly added with standard solutions, so this is important to be considered when assessing the risk exposure to these alkaloids through food. Hence, in this work the transfer rate of PAs during the brewing process was evaluated using dry blank chamomile samples contaminated with a 5% (*p/p*) of *Echium vulgare* or *Senecio vulgaris*, as previously explained in section 2.5. This contamination level of 5% was chosen based on the concentration of individual PAs found in the different PA-producing plants analyzed. Thus, since a wide range of PAs was determined in the PA-producing plants (36–6082 mg/kg and 6–322 mg/kg in *Echium vulgare* and *Senecio vulgaris*, respectively) it was necessary to choose a contamination level that ensured the correct detection and quantification of all the PAs found in the plants, so that the effect of the brewing process could be assessed in all of them. Afterwards, these chamomile contaminated samples were capped infused in water under different conditions (100 °C, 5 min; 100 °C, 10 min; 75 °C, 5 min; 75 °C, 10 min) to simulate the culinary preparation of this herbal tea by consumers, so that a real exposure scenario could be assessed as explained in section 2.4. Fig. 5 shows the transfer rate estimated for each analyte under the different conditions assayed. Transfer rates ranged from 13 to 87%. It has to be pointed out these transfer rates have been determined also considering thermal degradation. The apparent transfer rates when

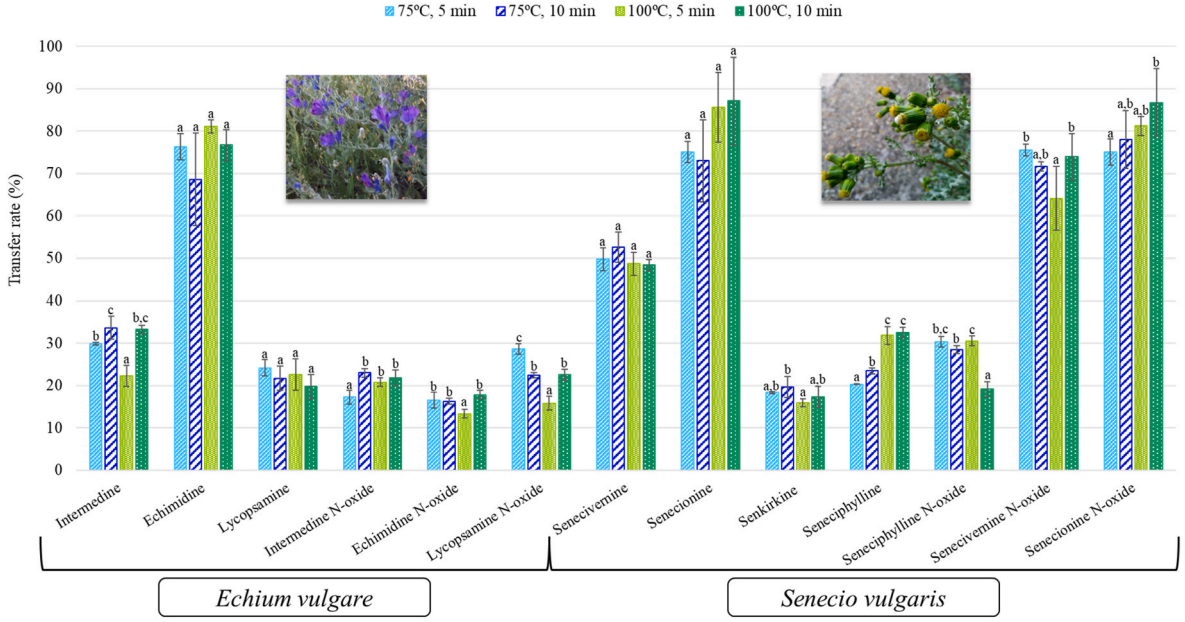


Fig. 5. Transfer rate (n = 3) of the 13 PAs obtained in the transfer study carried out with a 5% of PAs-containing plants (*Echium vulgare* and *Senecio vulgaris*) in chamomile infusion. Same letters means that there are no statistically significant differences ($p > 0,05$) and different letter means that there are significant differences ($p \leq 0,05$).

degradation is not considered cannot be estimated because it is not possible to relate the results obtained in the thermal degradation study with the ones obtained in the transfer rate study, because both things have been assessed in a different way. Thermal degradation effect has been evaluated using standard solutions, so it may not be the same as the thermal degradation that happens when PA-producing plants are used, although it might be similar. Hence, subtracting the thermal degradation determined in the first trial from the estimated transfer rate of the second study is not possible because it cannot be ensured that the effect of thermal degradation is the same in both scenarios.

The current data in the literature show many discrepancies on the transfer rate of PAs during the preparation of tea samples (Casado et al., 2023). For instance, some authors have no observed differences between the dry product and the infusion (Mathon et al., 2014; Rosemann, 2007), while many others have determined transfer rates ranging approximately between 80 and 100% (Mulder et al., 2018; Lüthy et al., 1981). Nonetheless, in many of these works the samples have been spiked with standard solutions instead of PA-producing plants. This is an important issue because the way of spiking the sample could explain the high transfer rates estimated by other authors. Accordingly, when the analytes come from plant tissues the extraction efficiency can be lower because it is more difficult to extract the analytes from the plant matrix than when they are directly added with standard solutions on the sample surface. For this reason, the transfer rates obtained in this work may be lower, but probably they are closer to the real transfer rate of PAs. Nonetheless, there are other published works which have also reported lower transfer rates of PAs (Picron et al., 2018; Reinhard & Zoller, 2021; Schulz et al., 2015). For instance, although Reinhard and Zoller (2021) concluded a global median transfer rate of 95%, they obtained very wide transfer rates intervals (38–100%), observing transfer rates below the 50% for some analytes when the teas were prepared following vendor's instructions or the ISO standard procedure (Reinhard & Zoller, 2021).

As it can be observed (Fig. 5), the transfer rates in this work were different depending on the analyte, so not all the PAs and PANOs were effectively transferred from the dry material to the infusion during the brewing process. Nonetheless, all of them followed a similar trend at all the brewing conditions tested. Thus, only four analytes (echimidine, senecionine, senecivernine *N*-oxide and senecionine *N*-oxide) showed high transfer rates (>70%). The average transfer rates of these compounds were 76 ± 5%, 80 ± 7%, 85 ± 7% and 82 ± 5%, respectively. On the other hand, senecivernine showed a medium transfer rate (50 ± 2%), while the rest of the analytes (intermedine, lycopsamine, intermedine *N*-oxide, lycopsamine *N*-oxide, echimidine *N*-oxide, senkirkine, seneciphylline and seneciphylline *N*-oxide) showed significantly lower transfer rates ranging between 13 ± 1% and 30% (Fig. 5). The low transfer rates could be attributed to a low stability of the compounds, however, as observed in the thermal degradation study, the brewing conditions are not drastic enough to produce a significant degradation of these compounds during the process. Moreover, in this case the brewing process was carried out using plant material instead of standard solutions, so probably PAs are even more stable this way because they can be protected from heat exposure by the plant matrix. For this reason, it seems that the lower transfer rates observed are due to lower extraction efficiency, leading to low migration of the analytes from the vegetable tissues into the infusion. Nonetheless, this could be confirmed by also analyzing the solid residue left after brewing. Moreover, it is worth mentioning that in general transfer rates were lower in the PAs from *Echium vulgare* than in the PAs from *Senecio vulgaris* (Fig. 5). This is also relevant because the extraction efficiency may vary depending on the type of matrix.

The EFSA currently considers a 100% transfer rate for all PAs. However, this study confirmed that the transfer rate of PAs simulating a real scenario of brewing conditions for the preparation of herbal tea samples contaminated with PAs is not a 100%, and that it is actually lower than those claimed by some authors. Accordingly, based on the results obtained in this work, the risk associated to the occurrence of these alkaloids in contaminated tea samples would be lower. This point is important to be considered to estimate the real intake level of these toxins by consumers, so that a proper risk and toxicological assessment can be achieved without overestimating the exposure. For instance, according to the results obtained in the quantification of the PAs in the PA-producing plants evaluated in this work, it can be estimated that a cup of tea (200 mL) prepared with a dry chamomile sample contaminated with a 5% (*p/p*) of *Echium vulgare* or *Senecio vulgaris* following the vendor's instructions (100 °C, 5 min) would lead to contents of 8.8 or 1.2 mg/L of PAs in the infusion, respectively, considering a transfer rate of 100%. However, considering the transfer rates calculated in this work the content of PAs would be 1.3 and 0.6 mg/L, respectively. Therefore, considering a 100% transfer rate leads to an overestimation of the risk exposure to these alkaloids, because the real concentration of PAs in the infusion can actually be 2 or 7 times lower, respectively. Hence, this highlights the importance of analyzing the infusion sample rather than the dry tea sample to avoid an overestimation of the real intake and determine in a reliable way the degree of exposure and risk of consumers to these contaminants. Nonetheless, despite the overestimation of the risk, concentrations of 1.3 or 0.6 mg/L would still present a serious health concern. Therefore, this highlights the importance of monitoring the occurrence of these alkaloids in foodstuffs.

4. Conclusions

The transfer rate of PAs during the brewing process of (herbal) teas can be influenced by different factors, such as their extraction efficiency from the plant tissues and their thermal degradation. To the best of our knowledge, this is the first time that the transfer rate of PAs has been evaluated by simulating a real cross-contamination of an herbal tea sample contaminated with weeds of PA-producing plants instead of standard solutions. As it has been observed, this is an important point to be consider because the migration of PAs from the plant tissues during the brewing process is not as effective as when they are directly added with standard solutions. Consequently, the exposure risk to these compounds is overestimated, because a 100% transfer rate is currently being considered by the EFSA. On the other hand, it has been confirmed that PAs are quite stable compounds during the brewing process, and their thermal stability is more strongly influenced by the initial contamination level of the sample than by other parameters, such as the brewing temperature and time. Currently, maximum concentration levels of PAs are regulated for dry (herbal) tea samples. However, according to the results obtained in this work it seems more appropriate to monitor the content of these alkaloids in the infusion, so that the real intake and exposure of consumers to these compounds can be correctly assessed and avoid an overestimation.

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CRediT authorship contribution statement

Begoña Fernández-Pintor: Methodology, Formal analysis, Investigation, Writing – original draft. **Natalia Casado:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing, Supervision. **Sonia Morante-Zarcero:** Conceptualization, Methodology, Investigation, Supervision. **Isabel Sierra:** Conceptualization, Investigation, Supervision, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.109926.

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