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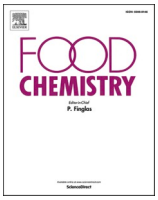

# Transfer of pyrrolizidine and tropane alkaloids from tea and herbal tea to infusions

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ABSTRACT

Pyrrolizidine alkaloids (PAs) and tropane alkaloids (TAs) are phytotoxins that occur worldwide and are important contaminants of food, especially (herbal) teas. Their transfer rates to tea infusions strongly influence exposure estimates, but conflicting results have been obtained so far, making risk assessment uncertain. Therefore, infusions prepared under controlled conditions from artificially contaminated black and herbal tea and naturally PA-containing comfrey root (*Symphytum officinale*) were analyzed by UPLC-MS/MS. Brewing time, repeated infusion, pH and temperature of the infusion were evaluated. Infusions of black tea and herbal tea produced under standardized conditions resulted in PA/TA transfer rates between 30 % and 68 %, with an average of 49 %. Surprisingly, brewing time was not found to be a significant factor. Instead, the polarity of the compounds and the pH of the tea infusion due to the tea matrix were identified as main factors. Consequently, teas should be analyzed as infusion for realistic exposure estimates.

## 1. Introduction

Pyrrolizidine alkaloids (PAs) and tropane alkaloids (TAs) are two groups of plant secondary metabolites that have attracted increasing attention from food and feed authorities in recent years. The group of PAs, including their corresponding PA *N*-oxides, comprise of more than 660 compounds that are presumed to occur in more than 6000 plant species worldwide, particularly in the families Boraginaceae, Asteraceae (Senecioneae, Eupatorieae), Fabaceae (genus *Crotalaria*) and also in Apocynaceae (Echiteae) (Boppré, 2011; Smith & Culvenor, 1981). PAs occur in various chemical structures and can be subdivided according to their grade of esterification (monoester, open-chain diester, cyclic diester) or their respective core structure (Fig. 1). Compared to PAs, the group of TAs is smaller, with up to now more than 200 known compounds (Gadzikowska & Grynkiewicz, 2002). TAs naturally occur in plants of the Solanaceae family, e.g. the genera *Datura, Atropa* and *Brugmansia* (Griffin & Lin, 2000).

Both PAs and TAs are toxicologically relevant to humans and animals. Despite their structural similarities, they induce different physiological effects. Atropine (racemic mixture of (±)-hyoscyamine) and scopolamine are the best-investigated and most abundant TAs. They can act as competitive muscarinic receptor antagonists inhibiting the neurotransmitter acetylcholine (Kohnen-Johannsen & Kayser, 2019). Intoxication symptoms typically include anticholinergic effects: patients may suffer from dry mouth, red skin, increased heart rate (tachycardia), disorientation and visual hallucinations (Adamse et al., 2014; Perharič et al., 2013). While saturated PAs (e.g. esters of platynecine) are currently seen as non-toxic, the 1,2- unsaturated PAs act as protoxic agents: After metabolic activation of the PAs by liver enzymes to highly reactive pyrrolic esters, the metabolites can form DNA or protein adducts (Edgar et al., 2020; Fu, 2017; Fu et al., 2004). The 1,2-unsaturated PA *N*-oxides undergo identical metabolic activation as these compounds are reduced to their corresponding tertiary amines in the gut (Yang et al., 2017). Acute poisoning due to PAs is relatively rare, but long-term exposure can cause hepatic sinusoidal obstruction syndrome (HSOS), pulmonary arterial hypertension (PAH) or carcinoma. The carcinogenic

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potential of PAs was recently reviewed, summarizing several PA compounds that were demonstrated to induce carcinoma in rodents (Fu, 2017). Since PA toxicity varies based on their structure, interim relative potency factors have been proposed (Merz & Schrenk, 2016).

PAs and TAs can enter the food and feed chain via unintended co-harvesting of plants containing these toxins, such as (herbal) teas, spices and culinary herbs, cereals and cereal products (Kaltner et al., 2020; Mulder et al., 2015; Mulder et al., 2016; Schrenk et al., 2021). Honey can also be contaminated if bees collect pollen and nectar from PA or TA producing plants (Edgar et al., 2002; Romera-Torres et al., 2018). Herbal teas have been regarded as a major source for the overall PA intake of consumers due to high PA concentrations detected in (herbal) teas (EFSA, 2016; Mulder et al., 2018). TAs can also pose a risk in (herbal) teas, and due to a similar way of contamination, PAs and TAs can co-occur in such products (Shimshoni et al., 2015).

In response to the toxic effects of both alkaloid groups, in the European Union (EU) regulatory limits for PAs and TAs in certain food matrices were implemented in Commission Regulation (EU) No 2023/915, 2023. In case of TAs, maximum levels are set for the sum of atropine and scopolamine in cereal products and (herbal) teas as well as for the sum of both analytes. Concerning PAs in foodstuff, regulatory limits for the sum of 35 PAs cover herbal teas, herbal tea infusions, food supplements as well as spices and culinary herbs.

In order to properly assess the potential health risks of PAs and TAs to consumers and to derive adequate regulatory limits, data on the occurrence of these toxic compounds in food as well as intake data are

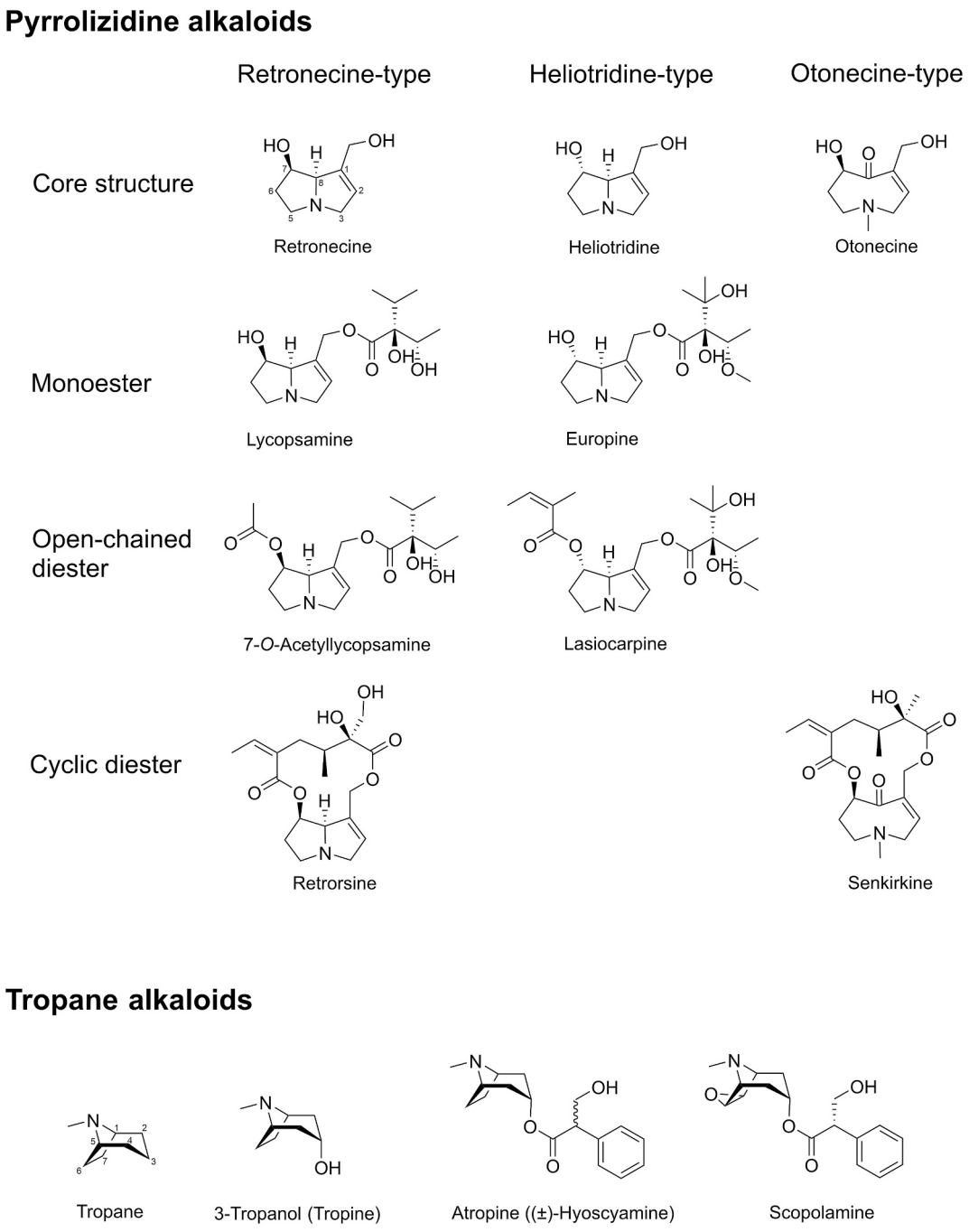


**Fig. 1.** Examples of chemical structures of 1,2-unsaturated pyrrolizidine alkaloids, grouped according to their core structure and their grade of esterification. Chemical structures of important tropane alkaloids are also given.

necessary. Particularly for (herbal) teas, the exposure estimates are still subject to uncertainties. PA and TA levels are generally analyzed in dry matter and, so far, in risk assessment transfer rates from dry products to the infusions have not been considered due to a lack of consistent data. Earlier studies reported greatly varying transfer rates of PAs and TAs from dry herbal teas to herbal tea infusions. In a study using pre- and post-brewing spiking experiments (to compare actual versus theoretical content in infusions) relatively low transfer rates, between 16 % and 45 % were reported (Picron et al., 2018). In contrast, comparing analyte levels in hot water extracts with aqueous acidic extracts resulted in estimated average transfer rates from dry teas to tea infusions of about 50 % for TAs and 85 % for PAs (Mulder et al., 2016; Mulder et al., 2018). Reinhard and Zoller (2021) studied the transfer of PAs from ten naturally PA-contaminated teas and reported transfer rates ranging from 38 % to 100 %. However, a study is still lacking that systematically investigates the potential effects of individual brewing parameters on PA and TA transfer to (herbal) tea infusions.

Consequently, the current study aimed to elucidate the impact of different brewing parameters on the transfer rates of single PA and TA compounds from dry (herbal) teas to tea infusions. Therefore, effects of brewing time, temperature, infusion pH, extent of grinding, and repeated infusions on the transfer rates were investigated. For these experiments, PA- and TA-free black tea and herbal tea as well as naturally PA-containing comfrey (*Symphytum officinale*) root, which is consumed as an herbal tea (Mei et al., 2010), were used. The results are discussed with regard to the available literature data and the consequences for exposure and risk assessment of PAs and TAs in teas and herbal teas.

## 2. Material and methods

### 2.1. Chemicals and reference standards

Acetonitrile and methanol (both LC-MS grade) obtained from Biosolve (Valkenswaard, The Netherlands), ammonia solution 25 % (analytical grade) from VWR (Amsterdam, The Netherlands) and ammonium carbonate (analytical grade) purchased from Fluka (Buchs, Switzerland). Ultra-pure water was obtained by purifying deionized water with a Milli-Q system from Merck (Darmstadt, Germany). Formic acid, sodium acetate, potassium dihydrogen phosphate, sodium carbonate and sodium hydrogen carbonate were each of analytical grade and purchased from Merck (Darmstadt, Germany).

In total, 62 PA and 2 TA reference standards were used in the study, predominantly from Phytoplan (Heidelberg, Germany) and Phytolab (Vestenbergsgreuth, Germany). In Supplementary Table 1, further information can be found on vendors and purity of standards. Three PA *N*-oxide standards were in-house synthesized according to a previously published method (Chou et al., 2003).

Stock solutions (200 μg/mL) of all 64 analytes were prepared in methanol. A mixed standard solution (1.667 μg/mL) in methanol containing all compounds was prepared from the stock solutions. From this mixed solution two dilutions (0.333 μg/mL and 0.033 μg/mL) were prepared in methanol. The solutions were used for spiking of the blank black tea and herbal tea samples as described below.

### 2.2. Sample material

For all spike experiments a black tea ("Ceylon") and an herbal tea mix were used, which are hereafter referred to as in-house samples. The herbal tea mix consisted of two parts of rosemary tea, ten parts of "tranquility tea" and five parts each of mint and hibiscus tea herbs. The black tea material was free of PAs (< 5 μg/kg), but the herbal tea mix contained small amounts of senkirkine (17 μg/kg), rinderine *N*-oxide (9 μg/kg), and echinatine *N*-oxide (8 μg/kg). Furthermore, a set of 20 retail samples (fennel tea, chamomile tea, black tea, green tea and rooibos tea, four different brands each; hereafter referred to as retail samples) was used to test the pH of (herbal) tea infusions prepared according to vendor instructions. All samples were ground to a particle size <1 mm using a Peppink 200 AN grinding mill (Veerman, Olst, The Netherlands) and stored at room temperature in a dry, dark place until analysis. Naturally PA-containing comfrey (*Symphytum officinale*) root was used to test the transfer rate effects of brewing time, brewing temperature and extent of grinding. For the latter, comfrey root was ground using an A 11 Analytical mill (IKA, Staufen, Germany).

For tea (in-house black tea and herbal tea) the following variables were studied: Brewing time, repeated infusion, pH of the brewing water, polarity of the compound. For comfrey root, the following variables were studied: brewing time, temperature of the brewing water and degree of grinding.

### 2.3. Preparation of tea infusions

The standard tea infusion preparation procedure was performed based on the standardized procedure ISO 3103 "Tea – Preparation of liquor for use in sensory tests". In brief, a tea bag (size 2, t-sac GmbH, Hanover, Germany) was placed in a 200 mL glass beaker and 2.00 ± 0.05 g of ground and homogenized (herbal) tea were weighed into the tea bag. Ultra-pure water (Milli-Q) was boiled using a water cooker and then transferred immediately into the beaker up to the 150 mL mark. Then, the tea bag was dipped by hand five times into the hot water and the tea was brewed for 5 min in total. Afterwards, the tea bag was gently squeezed against the beaker wall using a disposable plastic spoon, and removed from the beaker. The tea infusion was allowed to cool to room temperature (approx. 45 min). All tea infusions were prepared in triplicates.

To determine the transfer rates of PAs and TAs, a pre- and post-brewing spike experiment was performed at target concentrations of 0.5 ng/mL or 2.5 ng/mL in the tea infusions. Therefore, the in-house herbal tea and the black tea samples were used (see 2.2.). Dry (herbal) tea (2.00 ± 0.05 g) in a tea bag was fortified with the mixed standard solution (1.667 μg/mL) at 37.5 μg/kg (45 μL, i.e. 0.5 ng/mL in infusion) or 187.5 μg/kg (225 μL, i.e. 2.5 ng/mL in infusion), respectively. The samples were let to stand for 30 min prior to the brewing process. A second batch of the same herbal tea or black tea was used to prepare blank herbal tea and black tea infusion. After cooling to room temperature, 20 mL aliquots were fortified to 0.5 and 2.5 ng/mL, respectively, representing a theoretical transfer of 100 %. In addition, the tea bags fortified with 187.5 μg/kg were brewed a second time to investigate the transfer rates of PAs and TAs in a second infusion, which is particularly common for high-quality green or white teas. Therefore, the tea bags were moved to a new beaker after the first infusion, and treated equally to the procedure of the first infusion of the respective experiment.

To investigate the impact of the brewing time, herbal tea and black tea were fortified to two levels (37.5 and 187.5 μg/kg) and were brewed for 2.5 min, 5.0 min, 7.5 min, 10 min or 15 min. Implications of a varying pH of the boiling water were tested with black tea (fortified to 187.5 μg/kg) using 50 mM buffer solutions providing pH 3.0, 5.0, 7.0 and 9.0 at boiling point for the brewing process. To generate the buffer solutions, the following salts were dissolved in 800 mL of purified water and filled to 1.0 L: pH 3 buffer, 0.67 g sodium acetate trihydrate and 4.05 g formic acid; pH 5 buffer, 4.76 g sodium acetate and 1.35 g formic acid; pH 7 buffer, 6.80 g potassium dihydrogen phosphate and 1.19 g sodium hydroxide; pH 9, 1.07 g sodium carbonate and 3.36 g sodium hydrogen carbonate. Because the pH of the buffers is affected by temperature, the pH of the buffers was adjusted to 3.1, 5.0, 7.0 or 9.4, respectively, using hydrochloric acid or sodium hydroxide solution, to accommodate for these temperature effects during brewing. Potential effects of the water temperature on the transfer rates were examined by means of infusions from naturally PA-containing comfrey root at water temperatures of 40 °C, 60 °C, 80 °C or 100 °C. The impact of particle size was evaluated by means of infusions from non-ground (particle size ~5 to 15 mm) and ground comfrey root (particle size <1 mm).

### 2.4. Extraction/purification of tea infusions

After cooling to ambient temperature, the tea infusions were stirred to ensure homogenization. An aliquot of 20 mL was transferred to a 50 mL centrifugal tube. The pH was adjusted to 7–8 with ammonium carbonate solution (1 mol/L) in water, using pH 6.5–10.0 indicator strips from Merck (Darmstadt, Germany). The infusion was centrifuged (3500 ×*g*, 15 min, 20 °C) and loaded onto a Strata-X Polymeric reversed phase 200 mg/6 mL solid phase extraction (SPE) cartridge (Phenomenex, Utrecht, The Netherlands), pre-conditioned with 6 mL of methanol and 6 mL of water. The loaded cartridges were washed with 6 mL of formic acid (1.0 % in water) and 6 mL of water. After drying the cartridges under vacuum, the analytes were eluted into 10 mL plastic tubes using 6 mL of methanol. The eluates were dried at 50 °C under a stream of nitrogen using a Turbovap II (Biotage, Uppsala, Sweden). The residues were reconstituted in 50 µL of methanol, then 450 µL of water was added and the tubes were shaken by means of a vortex laboratory shaker. Finally, the sample extracts were transferred to a 500 µL Mini-UniPrep filter vial with a PTFE 0.45 µm membrane from Whatman (Little Chalfont, United Kingdom) and closed with a suitable filter vial compressor.

Cooled infusions of comfrey root were filtered through a folded paper filter. Aliquots (5 µL) were diluted 1/100 (*v*/v) with 495 µL of methanol/water (10/90, v/v), transferred to a 500 µL filter vial and used directly for LC-MS/MS analysis. If not analyzed immediately after extraction, sample extracts were stored at −20 °C.

### 2.5. Acidic extraction of PA-containing comfrey root after infusion

To calculate transfer rates from comfrey root to their infusions, a sufficiently accurate approximation of its PA levels was necessary. First, the samples were brewed two times via the regular infusion procedure (see section 2.3.). The sample residues were quantitatively transferred from the tea bags into a 50 mL centrifugal tube and 40 mL of aqueous formic acid (0.2 %) was added. After 30 min of overhead-shaking, the tubes were centrifuged (3500 ×*g*, 15 min, 20 °C) and the supernatants were filtered through a folded filter. Aliquots (20 µL) of the filtered extracts were diluted 1/375 (v/v) with 7480 µL of 10 % methanol. Subsequently, 500 µL of the diluted solution were transferred to filter vials. The total PA content of the comfrey root was calculated by summing the levels from the two brewing steps and the acidic extraction.

### 2.6. Instrumentation, LC-MS/MS conditions and software

Analysis was performed using an LC-MS/MS system comprised of an Acquity UPLC device coupled to a Xevo TQ-S tandem mass spectrometer (Waters, Milford, MA, USA). Two or three multiple reaction monitoring (MRM) transitions were measured per analyte in positive electrospray ionization (ESI) mode, with the one showing the best signal-to-noise-ratio (S/N) was used for quantification, while the additional transitions were used to confirm the analyte. The ion source parameters were set as follows: capillary voltage, 3.0 kV; source temperature, 150 °C; source offset, 60 V; desolvation temperature, 600 °C; cone gas flow, 150 L/h; desolvation gas flow, 800 L/h; collision-induced dissociation (CID) gas, argon, $4.3 \bullet 10^{-3}$ mbar (0.17 mL/min). See Supplementary Table 2 for an overview of analyte retention times, MRM transitions and MS settings used.

For chromatographic separation of the analytes, an Acquity UPLC BEH C18 1.7 µm 150 × 2.1 mm column (Waters, Milford, MA, USA) was used. The solvents were (A) ammonium carbonate buffer (10 mmol/L, pH 9.0 ± 0.1) and (B) acetonitrile. At a flow rate of 0.4 mL/min, the linear gradient conditions were: 0.0 min, 0 % B; 0.1 min, 5 % B; 3.0 min, 10 % B; 7.0 min, 24 % B; 9.0 min, 30 % B; 12.0 min, 70 % B; 12.1 min, 0 % B; 14.2 min, 0 % B. The injection volume was 4 µL and the column oven temperature 50 °C. A solvent delay to the waste was set from 0 to 1.5 min and 13.2–14.2 min. MassLynx and QuanLynx software (both Version 4.1, Waters, Milford, MA, USA) were used for data acquisition and processing. Statistical analysis (One-way analysis of variance, $p = 0.05$) of the processed data was performed using OriginPro software (Version 2021b, OriginLab, Northampton, MA, USA), which was also used for preparing the figures, as well as ChemBioDraw Ultra (Version 14.0, PerkinElmer, Waltham, MA, USA).

### 2.7. Quantification of PAs and TAs and quality assurance of results

Transfer rates of PAs and TAs in fortified infusions were determined by means of a pre- and post-pike experiment. The ratio of the actual concentration to the theoretical concentration was defined as transfer rate of the respective compound. Mixed standard solutions of 0.033, 0.333 and 1.667 µg/L were used to prepare matrix-matched standards (MMS) in blank black or herbal tea infusions. The fortified concentrations of the MMS in blank tea infusions prior to SPE were 0.00 (blank) and 0.05, 0.10, 0.25, 0.50, 1.00, 2.50 and 5.00 ng/mL, representing analyte equivalents from 3.75 µg/kg to 375 µg/kg of each analyte in dry plant material. The extraction of the MMS samples was conducted according to section 2.4. In case of comfrey root infusion, the quantification was performed by means of standard addition to the final sample extracts. Therefore, 15 µL of the mix standard solution (1.667 µg/mL) were added to 485 µL of the final sample extract, representing an added concentration of 50 ng/mL in the sample vial.

For quality control, acceptance criteria for MMS and the calibration curves were defined according to SANTE Guideline 11,312/2021 (Guidance SANTE 11312/2021, 2021): linearity ≥0.990, accuracy between 60 % and 130 % (MMS 0.1 ng/mL) or between 70 % and 120 % (MMS 1.0 ng/mL), maximum deviation of ion ratio of 30 %, maximum deviation of retention time of 0.1 min. A matrix matched recovery standard (MMRS) was prepared in each series to check for the performance of the SPE extraction. Therefore, a blank black or herbal tea infusion were prepared according to 2.3. and purified as described in 2.4. The dried SPE eluates were finally reconstituted in 50 µL of a mixed standard solution in methanol (400 ng/mL), followed by 450 µL water. Thus, the MMRS of 40 ng/mL corresponded to the MMS of 1.0 ng/mL in the initial tea infusion. The ratio of the analyte concentrations obtained from the MMS and MMRS samples were defined as SPE recovery rates.

### 2.8. Evaluation of transfer data available from studies on retail samples of (herbal) teas

Data on individual PA and TA transfer using retail herbal tea samples were retrieved from four published studies (Reinhardt et al., 2021, Picron et al., 2018, Mulder et al., 2018, Mulder et al., 2016). The individual data on samples are available from the supplementary data published alongside the first two studies and from unpublished in-house data for the latter two studies. For comparison and evaluation of the available data, the following criteria were applied. Only data obtained with a brewing protocol comparable to that of ISO 3103 were included, brewing protocols according to vendors descriptions were excluded. Only data of the first brewing step were used. For assessment of the content in the dried (herbal) tea, data provided from acidic extraction were used, including the combination of multiple extractions if available. Analytes were only included when quantified data of individual analytes for both the infusion and the dried material were reported. Analytes for which only a quantity was reported in dried material and not in infusion and vice versa (representing theoretical transfer rates of 0 % and 100 %, respectively) were removed from the dataset. Calculated transfer rates exceeding 100 % were included in the evaluation. (Very) high or very low transfer rates can be due to inhomogeneity of the materials used for the experiments, and this cannot be completely avoided. However, for a few samples an extreme discrepancy in results between dried material and infusion was reported and these were excluded from the dataset. The data sets, before and after data cleaning, are provided as Supplementary Table 3. Due to the data cleaning steps, the transfer rates may deviate from the originally published results.

## 3. Results and discussion

### 3.1. Methodology validation

The tea brewing procedure used to determine transfer rates of PAs and TAs was based on the standardized procedure ISO 3103. Both the tea brewing procedure and the analytical methodology were in-house validated. Infusions of herbal tea and black tea were prepared and fortified in triplicate at concentrations of 0.1, 0.5 and 2.5 ng/mL of each analyte in the tea infusion. These spike levels corresponded to 7.5, 37.5 and 187.5 μg/kg in dry tea. The artificially contaminated infusions were subsequently purified using SPE (see 2.4.) and quantified using MMS calibration curves (see 2.7.).

Acceptable recoveries were obtained, ranging from 73 % to 126 % for all 64 analytes at 0.5 and 2.5 ng/mL fortification levels (Supplementary Table 4). The repeatability at these levels, expressed as relative standard deviation ($RSD_r$) ranged between 0.4 % and 18 %, meeting the SANTE Guideline requirements for all 64 analytes in black and herbal tea. Only at spiking level 0.1 ng/mL, which was close to the limit of quantification (LOQ), some analytes showed poor results. In herbal tea, e.g. a low recovery of 37 % was obtained for jacobine, combined with a high $RSD_r$ of 39 %. At spiking level 0.1 ng/mL in black tea and herbal tea infusion, in total 15 analytes did slightly undercut at least one of the SANTE Guideline requirements for recovery and repeatability for few percent. However, the mean recoveries met prerequisites between 70 % and 120 %. The calibration curves showed good linearities ($r^2 \geq 0.990$) for all analytes.

Since the lowest calibration point of 0.05 ng/mL already fulfilled the in-house MMS quality control criteria for each analyte (see 2.7.), this level was defined as LOQ for each compound, although lower limits might have been possible for several analytes. In conclusion, the analytical procedure was shown to be suitable for the detection of PAs and TAs in (herbal) tea infusions and a reliable determination of transfer rates into infusions.

### 3.2. Effect of the brewing time

The effect of the brewing time on the individual transfer rates was tested using fortified black tea and herbal tea samples with brewing times varying from 2.5 min to 15 min. The determined minimum, mean, median and maximum transfer rates with respect to brewing time are given in Table 1 (See Supplementary Table 5 for individual transfer rates of the analytes). Over all brewing times, spike levels and analytes of the first infusions, the mean transfer rates varied from 19 % to 88 %, with RSD values ($n = 3$) ranging from 0.1 % to 35 %. The average RSD in black tea infusion at 37.5 and 187.5 μg/kg spike levels (i.e. 0.5 and 2.5 ng/mL in infusion), respectively, were 10 % and 6.5 %, while for herbal tea it was 14 % and 11 %. At the 0.5 ng/mL level in black tea, the singular transfer rates ranged from 35 % (usaramine *N*-oxide after a brewing time of 15 min) up to 75 % (lycopsamine at 10 min), with means from 49 % (15 min) to 61 % (7.5 min) over all 64 analytes. In herbal tea at the same spike level, transfer ranged from 27 % (7-*O*-acetyllycopsamine at 15 min) to 83 % (heliotrine at 10 min) and means from 44 % (15 min) to 58 % (10 min). The mean transfer rates at the 0.5 ng/mL level were slightly higher in black tea compared to those in herbal tea, and comparable results were obtained for the 2.5 ng/mL level. Overall, transfer rates did not change significantly at different levels and brewing times, indicating a balanced distribution of the transfer rates across the set of compounds.

In our study, the transfer rates of the alkaloids were determined in a manner similar to the methodology of Picron et al. (2018), who used a pre and post brewing spiking experiment at levels of 0.02 and 2.0 ng/mL. Interestingly, our findings revealed much higher mean transfer rates under standard brewing conditions (30 % to 68 %, see Table 1) compared to those published by Picron et al. (16 % to 45 %). Herein, the low transfer rates were attributed to the absence of acid in the brewing process, which was assumed to be crucial to enhance the extraction yield of PAs. Acidic extraction solvents are widely used in analytical extraction methods for PAs and TAs (Bodi et al., 2014; Dzuman et al., 2020; Klein et al., 2022). Han et al. recently investigated the effects of tea manufacturing on the PA pattern and stability in tea and reported for the brewing process (applying a two-fold infusion) transfer rates of 56 % for tertiary amine PAs and 75 % for PA *N*-oxides, which were similar to our findings (Han et al., 2022). We observed differing results for the second infusions, ranging from 7 % (jacobine at 7.5 min, herbal tea) to a maximum of 65 % (lasiocarpine *N*-oxide at 5 min, black tea), with mean transfer rates over all analytes from 14 % to 42 % (Table 1). Substantial transfer rates in second infusions were also reported by Han et al. (2022) and Reinhard and Zoller (2021).

Interestingly, in our study the brewing time did not exert significant effects on the transfer rates of PAs and TAs into the (herbal) tea infusions. With regard to the comparable transfer rates of individual analytes over the investigated brewing times (2.5 to 15 min), it is important to highlight that the black and herbal tea samples were fortified. Thus, the analytes were presumably located predominantly on the tea particle surface. However, as a second infusion of the samples still resulted in considerable amounts of extracted analytes, it is obvious that the transfer of PAs and TAs is incomplete after one infusion. This is probably due to physical effects such as adherence of the analytes to the tea matrix. Overall, our findings indicate that an extraction equilibrium between solid tea and its infusion is established very quickly, in less than

**Table 1**
Transfer rates of pyrrolizidine (PAs) and tropane alkaloids (TAs) in black tea and herbal tea, calculated as ratio of actual to theoretical content, at varying brewing times from 2.5 min to 15 min and at two spike levels[a]. A consecutive brewing was conducted for the samples of the 2.5 ng/mL level.

| Matrix | Brewing time [min], infusion | Transfer rate [%] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Minimum | | Mean | | Median | | Maximum | |
| | | Spike level[b] [ng/mL] | | | | | | | |
| | | 0.5 | 2.5 | 0.5 | 2.5 | 0.5 | 2.5 | 0.5 | 2.5 |
| Black tea | 2.5 (1st) | 39 | 33 | 51 | 51 | 50 | 52 | 67 | 68 |
| | (2nd) | | 26 | | 38 | | 39 | | 52 |
| | 5 (1st) | 37 | 19 | 51 | 36 | 50 | 35 | 67 | 54 |
| | (2nd) | | 23 | | 42 | | 42 | | 65 |
| | 7.5 (1st) | 47 | 27 | 61 | 46 | 62 | 49 | 74 | 66 |
| | (2nd) | | 15 | | 27 | | 28 | | 44 |
| | 10 (1st) | 39 | 28 | 54 | 52 | 52 | 53 | 75 | 75 |
| | (2nd) | | 13 | | 29 | | 31 | | 49 |
| | 15 (1st) | 35 | 30 | 49 | 52 | 48 | 54 | 71 | 71 |
| | (2nd) | | 15 | | 29 | | 30 | | 48 |
| Herbal tea | 2.5 (1st) | 30 | 35 | 46 | 43 | 47 | 44 | 68 | 51 |
| | (2nd) | | 16 | | 23 | | 23 | | 32 |
| | 5 (1st) | 35 | 37 | 55 | 47 | 56 | 48 | 78 | 62 |
| | (2nd) | | 17 | | 23 | | 22 | | 39 |
| | 7.5 (1st) | 38 | 39 | 57 | 51 | 58 | 52 | 75 | 63 |
| | (2nd) | | 7 | | 14 | | 14 | | 24 |
| | 10 (1st) | 42 | 41 | 58 | 55 | 58 | 55 | 83 | 69 |
| | (2nd) | | 9 | | 15 | | 19 | | 32 |
| | 15 (1st) | 27 | 44 | 44 | 57 | 43 | 58 | 70 | 74 |
| | (2nd) | | 11 | | 24 | | 27 | | 50 |

[a] Fortification of dry matter was conducted with a mixture of 64 PA and TA standards prior to the brewing procedure. Both (herbal) teas were investigated in triplicates at each spike level at each brewing time. Minimum, mean, median and maximum values were calculated from mean transfer rates of each analyzed compound of these triplicates.
[b] Theoretical level of each PA and TA in the infusion.

2.5 min.

### 3.3. Influence of the compound polarity

As to be expected, polar analytes seem to be transferred more easily to infusions of black and herbal tea than less polar compounds. In Fig. 2 the average transfer rate of individual alkaloids is plotted against their respective logarithmic octanol-water partition coefficient (logP) as a measure for polarity. Due to the lack of empirical logP values of most compounds, computed logP values from the PubChem database were used instead (Supplementary Table 2; PubChem, 2022). By plotting the compounds according to the corresponding analyte group, a linear correlation between transfer rates and logP of a compound is obtained (Fig. 2). Pearson coefficients for black tea and herbal tea were 0.61 and 0.73 for tertiary amine PAs and 0.43 and 0.82 for PA *N*-oxides, respectively. It should be noted that computed logP values have a relatively large uncertainty, which may explain why some values, such as for europine and its *N*-oxide (computed logP of −1.3 and − 1.9, respectively), are much lower than expected based on their retention behavior on the UPLC column. Concludingly, this could have affected the robustness of the correlations found between polarity and transfer rates. Notwithstanding these limitations, however, the results clearly indicate that more polar compounds show higher transfer rates into infusions of black tea and herbal tea.

For pesticide residues in tea, the water solubility expressed as logP, was identified as a main parameter describing the varying transfer rates from dry matter to infusions (Wang et al., 2019; Xiao et al., 2017). A prediction model for calculating the transfer rates of certain pesticides was derived, indicating that more polar compounds are generally better transferred to the infusion. Thus, intrinsic factors of a matrix possibly affecting the polarity (e.g. the pH in a solution or an infusion) might greatly influence the resulting transfer rates. With regard to potential matrix effects of black tea and herbal tea, this particularly is the case for analytes of the group of tertiary amine PAs, while the transfer rates of PA *N*-oxides seemed to be less influenced (Fig. 2).

Another potential explanation for our observations might be physical effects such as adherence of compounds to the tea and herbal tea substrate. Due to the low concentrations of PAs and TAs in the tea and herbal tea infusions, limited solubility of the analytes is not a factor of relevance, but differences in physical adherence to the tea matrix may influence the equilibrium. Less polar compounds might stick a bit stronger to the matrix, causing the observed differences of the transfer rates.

### 3.4. Effect of brewing water pH

Next, the effect of the brewing water pH on PA and TA transfer rates was investigated. Four buffer solutions (pH 3, 5, 7, 9) were used to prepare infusions of black tea (5 min of brewing) and the pH was measured right after removal of the tea bags and after 60 min, when the infusions had cooled to room temperature. As the pH of a solution depends on the temperature, pH values after 5 min and after 60 min were slightly different (Supplementary Table 6).

The results revealed a correlation between the transfer rates near the boiling point and the pH in infusion. With increasing pH, the average transfer rates of the alkaloid compounds steadily decreased from 62 % at pH 3 to 44 % at pH 9 (Fig. 3, see Supplementary Table 7 for specific transfer rate information). The average transfer rate for tertiary amine PAs decreased from 52 % to 35 %, and for PA *N*-oxides from 67 % to 52 %. The relative distribution of PAs and TAs correlated with their

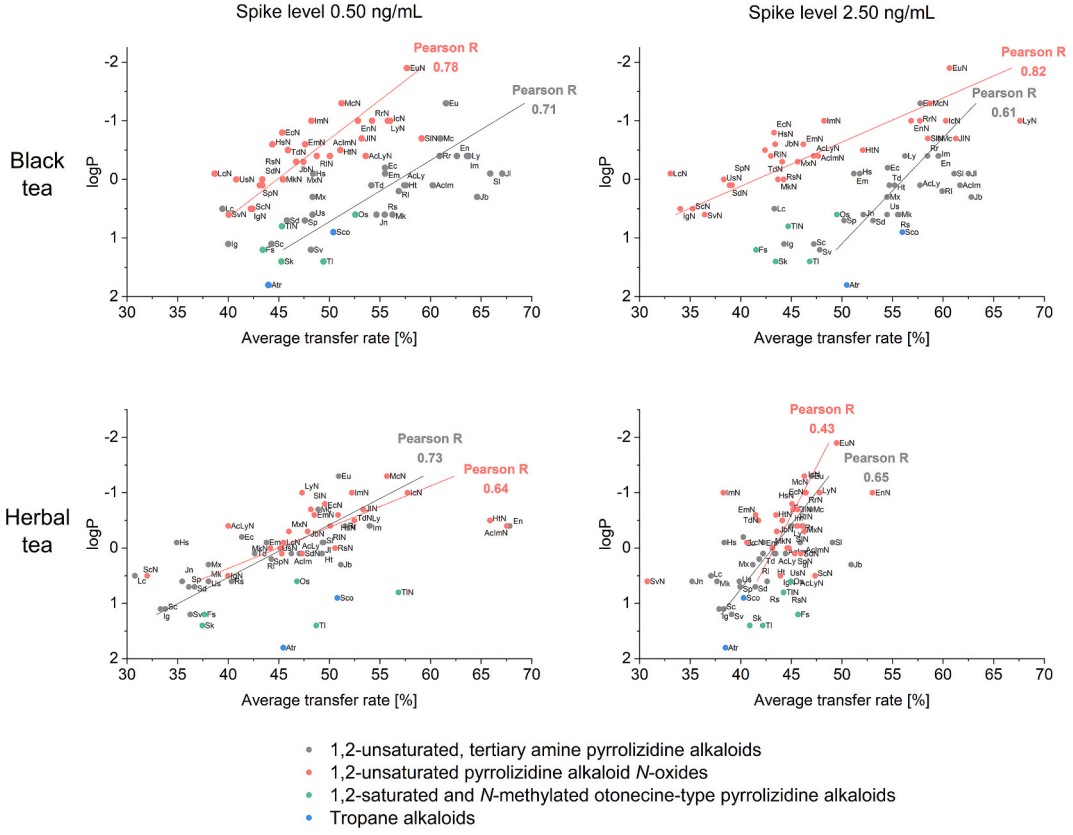


**Fig. 2.** Correlation of individual alkaloid mean transfer rates ($n = 3$) and their theoretical polarity, expressed as logP, in black tea and herbal tea at two fortification levels. The tea infusions were brewed for 2.5 min and the (computed) logP values of the compounds were taken from PubChem database. The correlation between the transfer rates and the logP values of a compound group was expressed as absolute Pearson correlation coefficient. For the abbreviations of the compounds see Supplementary Table 1.

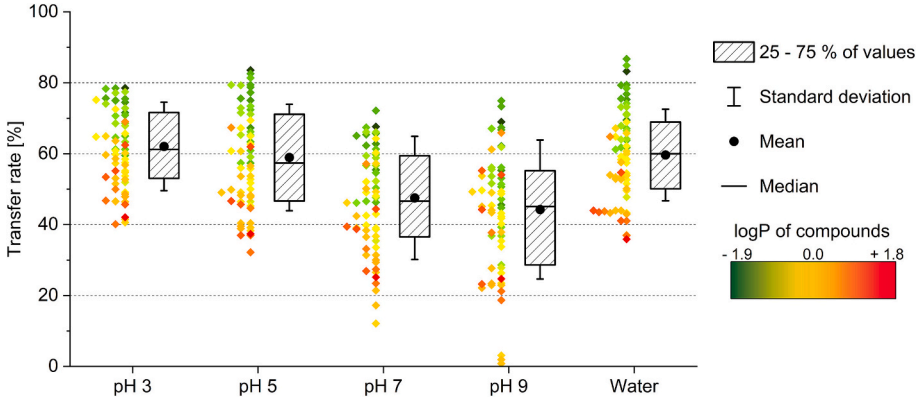


**Fig. 3.** Transfer rates of pyrrolizidine alkaloid and tropane alkaloid compounds in black tea infusions brewed with buffer solutions (pH 3.0, 5.0, 7.0, 9.0) or water. Each dot on the left to the corresponding box plot represents the mean transfer rate (n = 3) of an analyte and is colored according to their logP (low logP is equal to high polarity), ranging in total from −1.9 (europine *N*-oxide) to +1.8 (atropine). The dry black tea was fortified to a theoretical concentration of 2.5 ng/mL in the infusion and was brewed for 5 min. Tea infusions were prepared in triplicate with each pH buffer.

polarity, expressed as logP (see section 3.3) and was generally not strongly influenced by the pH. Exceptions were jaconine, merenskine and merenskine *N*-oxide. With increasing pH of the buffer solution, the transfer rates of these compounds decreased in pH 7 and pH 9 buffer, respectively, to 17 % and 1 % (jaconine), 21 % and 2 % (merenskine), and 12 % and 3 % (merenskine *N*-oxide). In contrast, under acidic conditions their transfer rates were comparable to compounds with a similar logP and thus in the expected range. Merenskine and its *N*-oxide were already reported to cause analytical problems (Kaltner et al., 2019). Jaconine, merenskine and merenskine *N*-oxide share a chemical structure feature, namely a chlorine and a hydroxy group at the α-position in their side chain (halohydrin configuration). Recently, it was shown that the formation of their corresponding epoxide compounds (jacobine, merepoxine and merepoxine *N*-oxide) is promoted under alkaline conditions via an internal $S_N2$ reaction and elimination of hydrogen chloride (Kaltner & Klein, 2025). This can explain the transfer rates observed, in particular for merepoxine *N*-oxide, but degradation of these compounds cannot be fully ruled out.

Reinhard and Zoller reported a pH-dependence of transfer rates into infusions of teas and herbal teas, and concluded that more acidic tea or herbal tea types generally showed enhanced transfer rates particularly of tertiary amine PAs (Reinhard & Zoller, 2021). To verify this observation, we measured the pH of infusions prepared with the in-house black tea and herbal tea samples as well as a set of retail samples consisting of five tea types (Table 2). Mean pH values of the hot infusions ranged from 6.2 ± 0.1 (fennel tea) to 4.9 ± 0.2 (black tea). The pH values of the black tea infusions were always slightly lower compared to the herbal tea infusions. As black tea is fermented green tea, the fermentation process may have caused the lower pH of black tea infusions.

**Table 2**
Obtained pH values of retail and in-house tea samples, expressed as mean and standard deviation (SD) of four biological replicates (retail samples) or three technical replicates (in-house samples). The pH measurements were conducted after removing the tea bag (5 min) and after cooling to room temperature (60 min). Statistical analysis was performed via one-way analysis of variance (ANOVA) at a significance level of 5 % (p = 0.05) Different superscript letters represent significant differences of the pH.

| Tea matrix | N | pH of tea infusion | |
|---|---|---|---|
| | | After 5 min | After 60 min |
| *Retail samples* | | | |
| Fennel | 4 | 6.2 ± 0.1 $^{A}$ | 6.2 ± 0.1 $^{A}$ |
| Rooibos | 4 | 5.1 ± 0.1 $^{B}$ | 5.3 ± 0.1 $^{C}$ |
| Chamomile | 4 | 5.6 ± 0.2 $^{DE}$ | 5.7 ± 0.1 $^{D}$ |
| Black tea | 4 | 4.9 ± 0.2 $^{BF}$ | 5.2 ± 0.2 $^{BCG}$ |
| Green tea | 4 | 5.3 ± 0.0 $^{C}$ | 5.5 ± 0.0 $^{E}$ |
| *In-house samples* | | | |
| Black tea | 3 | 4.9 ± 0.0 $^{F}$ | 5.2 ± 0.0 $^{G}$ |
| Herbal tea | 3 | 5.7 ± 0.0 $^{D}$ | 5.9 ± 0.0 $^{H}$ |

Taking the pH dependency of the compounds' transfer rates into account (Fig. 3), this might explain the observed differences in the transfer rates obtained for the infusions of black and herbal tea matrices (Table 1, Fig. 2). As the pH values for in-house and retail black tea samples were close to 5 (Table 2), this may explain the good fit of the distribution of individual transfer rates observed for brewing of black tea with pH 5 buffer solution and water (Fig. 3). Consequently, our findings strongly indicate a pH dependency of the transfer rates of PAs and TAs. This is also in line with the high recovery rates reported for extraction procedures that use aqueous sulfuric acid or formic acid, which are commonly used in analytical methods for PAs and TAs (Bodi et al., 2014; Dzuman et al., 2020; Klein et al., 2022). The pH of these extraction procedures is in the range of 1 to 2, thus significantly more acidic than the conditions tested during brewing.

### *3.5. Effect of brewing time, water temperature and extent of grinding on extraction of PA from comfrey root*

As discussed above, in case of fortified teas, the transfer rates were influenced by the pH of the infusion (Fig. 3), while variation of the brewing time did not show clear effects (Table 1). The latter findings were additionally examined using naturally PA-containing comfrey root. In this context, potential effects of the brewing water temperature and the extent of grinding on the PA transfer were investigated as well. To obtain an estimate of the total amount of PAs of the comfrey root material, each tea bag was brewed twice under identical conditions (depending on the respective experiment). Subsequently, the material was extracted a third time under acidic conditions. The summed PA levels of these three extractions were defined as the total amount of PAs. The transfer rates of the first and second infusions were referenced to this estimated total amount.

First, the impact of brewing time on the transfer rates of PAs was investigated by brewing for 2.5 min, 7.5 min or 15 min. Due to the botanical origin, only a limited number of PAs and no TAs were found in comfrey root infusions. The detected PAs were intermedine, lycopsamine, 7-*O*-acetylintermedine, 7-*O*-acetyllycopsamine and their corresponding *N*-oxides. The extraction of the four tertiary amine PAs in the first infusion showed no trend with transfer rates of around 27 % for the different brewing times (Fig. 4A). The corresponding PA *N*-oxides showed a similar behavior, but with higher transfer rates of around 50 %. This is in agreement with a higher polarity of the PA *N*-oxides

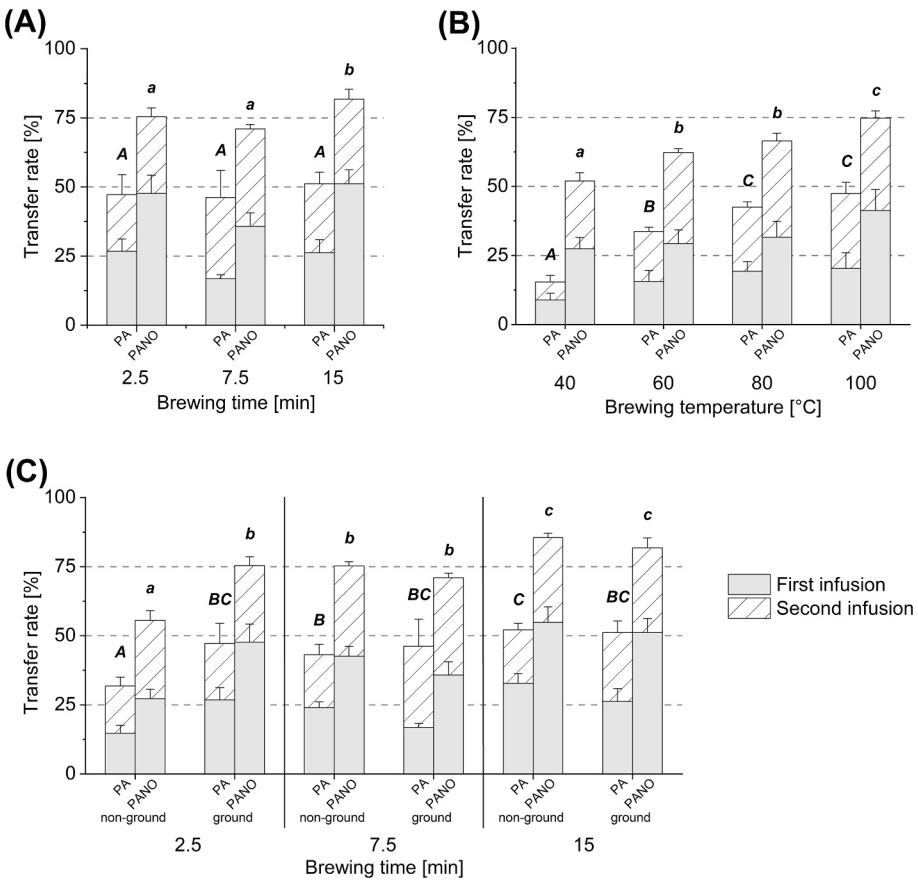


**Fig. 4.** Transfer rates of tertiary amine pyrrolizidine alkaloids (PAs) and PA *N*-oxides (PANOs) into infusions from comfrey (*Symphytum officinale*) root. Each pair of bars represents the average transfer rates of 4 tertiary amine PAs (intermedine, lycopsamine, 7-*O*-acetylintermedine, 7-*O*-acetyllycopsamine) or the 4 PA *N*-oxides (intermedine *N*-oxide, lycopsamine *N*-oxide, 7-*O*-acetylintermedine *N*-oxide, 7-*O*-acetyllycopsamine *N*-oxide) detected in the sample. Stacked bars represent the average transfer rate obtained from first and a consecutive second infusion. Transfer rates of the analytes from ground comfrey root were determined with regard to brewing time (A) and brewing temperature (B), transfer rates obtained for non-ground (particle size ~5 to 15 mm) and ground comfrey root (particle size <1 mm) were shown in (C). Statistical analysis was performed via one-way analysis of variance (ANOVA) at a significance level of 5 % ($p = 0.05$), using average summed transfer rates of analytes obtained from first and second infusion. The different letters on top of stacked bars represent significant differences of the summed transfer rates (capital letters: tertiary amine PAs; small letters: PA *N*-oxides).

compared to the corresponding tertiary amine PAs (logP PA *N*-oxides: intermedine *N*-oxide/lycopsamine *N*-oxide: −1.0; 7-*O*-acetylintermedine *N*-oxide/7-*O*-acetyllycopsamine *N*-oxide: −0.4; logP tertiary amine PAs: intermedine/lycopsamine: −0.4; 7-*O*-acetylintermedine/7-*O*-acetyllycopsamine: +0.1). On the other hand, the average transfer rates for the four tertiary amine PAs in comfrey root (27 %) were substantially lower from those obtained in fortified black tea (62 %) and herbal tea (54 %), while the results of their corresponding PA *N*-oxides were comparable between comfrey root (50 %), black tea (52 %) and herbal tea (58 %). Based on the fact that transfer rates are influenced by the pH (see 3.4.), the relatively neutral pH of the comfrey root infusions (6.2 ± 0.3) may have attributed to the lower transfer of tertiary amine PAs observed. It has to be taken into account that the natural PA levels in comfrey root were much higher than the levels in the fortified tea samples, and that therefore differences in transfer dynamics of the alkaloids could have occurred. Furthermore, since roots are a much tougher material than leaves, extraction may be more difficult, limiting the direct comparison with results obtained in fortified tea leaves.

The brewing time did not lead to obvious variations in the compounds' transfer rates into the comfrey root infusions (Fig. 4A), except for slightly increased transfer rates of the PA *N*-oxides after 15 min brewing time, just enough to be statistically significant ($p = 0.05$). So far, only one study investigated PA transfer rates from a naturally PA-containing matrix (Fernández-Pintor et al., 2023). The group studied the transfer rates of PAs from chamomile artificially contaminated with 5 % *Echium vulgare* or *Senecio vulgaris*, thus mimicking natural contamination, although for an extreme situation, to infusions prepared for 5 min or 10 min. The PA transfer rates ranged over all analytes from 13 % to 87 % and only slight differences between the two infusion times were observed.

In the current study, a subsequent second infusion was performed, resulting in still substantial transfer. In fact, the rates of the four tertiary amine PAs (20 % to 29 %) were comparable to those of the first infusion (17 % to 27 %). The four PA *N*-oxides showed somewhat lower rates during the second infusion (28 % to 35 %) compared to the first infusion (36 % to 51 %). This trend was observed for all brewing times. Thus, after the first infusion an average of 76 % of tertiary amine PAs and 55 % of PA *N*-oxides were still present in the residue, while after a second infusion still an average of 52 % of tertiary amine PAs and 24 % of PA *N*-oxides remained in the sample material. These findings indicate that consecutive infusions of PA-containing plant material, at least of comfrey root, may still contain a considerable amount of the alkaloids.

To investigate the impact of the brewing water temperature on the transfer rates of PAs, ground comfrey root was brewed for 5 min using a range of water temperatures (40, 60, 80, 100 °C). Regarding the first infusion, a higher temperature of the brewing water resulted in significantly increased transfer rates of PAs (Fig. 4B). Mean transfer rates for tertiary amine PAs steadily rose from 9 ± 3 % (40 °C) to 20 ± 6 % (100 °C) and for PA *N*-oxides from 27 ± 4 % (40 °C) to 41 ± 8 % (100 °C). Moreover, at all temperatures the transfer rates of tertiary

amine PAs from comfrey root to its infusion were lower than those of the corresponding PA *N*-oxides. The results were in line with the alkaloid caffeine that showed a temperature-dependent transfer into tea infusions (Suteerapataranon et al., 2009). In general, using heated solvents results in a higher extraction efficiency from solid sample materials. Such effects are known in accelerated solvent extraction systems, commonly using higher temperatures and pressures to increase the compounds yield, e.g. to extract catechins from green tea (Kellogg et al., 2017).

The impact of particle size was investigated by comparing transfer rates of tertiary amine PAs and PA *N*-oxides in non-ground (particle size ~5 to 15 mm) and ground (particle size <1 mm) comfrey root. Interestingly, the average transfer rates of non-ground samples for both groups differed significantly ($p = 0.05$) between the three brewing times (Fig. 4C). At a brewing time of 2.5 min, the average transfer rates of non-ground samples (tertiary amine PAs: 15 %; PA *N*-oxides: 27 %) were significantly lower compared to those of ground samples (tertiary amine PAs: 27 %; PA *N*-oxides: 48 %). However, at longer brewing times (7.5 min, 15 min), the transfer rates obtained from ground and non-ground samples were not significantly different.

Our results were partially contradictory to former studies addressing the effect of grinding of the infused material. While we did not find significant differences in the transfer rates between ground and non-ground comfrey root at 7.5 or 15 min brewing time, in a study comparing intact and comminuted leaves of naturally PA-containing herbals, the latter showed 1.1 to 4.1 times higher transfer rates after 10 min of brewing (Chen et al., 2019). In contrast, the transfer of caffeine from black tea into infusions showed increased rates from non-ground tea when longer brewing times were applied, while the transfer from ground leaves was independent from the infusion time (Suteerapataranon et al., 2009). Thus, our results were more in line with the findings of the latter study. The non-ground comfrey root material consisted of relatively large particles of up to 15 mm, probably needing more time to be wetted and extracted during infusion. In conclusion, the PA transfer from naturally PA-containing (ground) comfrey root was shown to be independent from brewing time but was affected by the brewing water temperature and the degree of grinding, at least at short brewing times. Nevertheless, it should be kept in mind that due to their natural occurrence in comfrey root only a limited number of PAs and their N-oxides were analyzed and that the conclusions on the effects of the investigated factors may be limited to these compounds.

### 3.6. Implications for exposure of consumers to PAs and TAs from tea infusions

In its exposure assessment on PAs, the European Food Safety Authority (EFSA) applied a simple conversion factor of 1/75 from dry matter to infusions (based on the standard protocol of 2 g of dried plant material and of 150 mL boiling water), assuming a transfer rate of 100 % (European Food Safety Authority (EFSA), 2016). Instead, using fortified samples we obtained mean transfer rates between 30 % and 68 % (Table 1) for a set of 64 PAs and TAs, suggesting a discrepancy between the estimated and the true PA intake in the EFSA assessment – an observation made by others as well (Picron et al., 2018).

**Table 3**
Summary of reported transfer rates (first infusion) for tertiary amine pyrrolizidine alkaloids (PAs), PA *N*-oxides (PANOs) and tropane alkaloids (TAs) in (herbal) teas, using brewing protocols that are based on ISO 3103.

| No. of analytes | Tea matrix | Conc. range in infusion [ng/mL] | Tea:water ratio (w:v) | Brewing time | Transfer rates * | Remarks | Reference |
|---|---|---|---|---|---|---|---|
| 16 PAs, 14 PANOs | Green, black, rooibos, herbal tea | 0.01–27.1 | 1:50 | 6 min | Median: PAs: 58 %; PANOs: 82 %; all: 71 % [c] | Contaminated teas, 51 samples | Picron et al., 2018 |
| 8 PAs, 8 PANOs | *Borago, Eupatorium, Lithospermum, Pulmonaria* | 0.01–1700 | 1:50 | 6 min | Median: PAs: 86 %; PANOs: 57 %; all: 70 % [e] | Inherent PA herbal teas, 6 samples | |
| 12 PAs, 12 PANOs | Black, green, rooibos, chamomile, peppermint, mixed herbal tea | 0.4–58 | 1:75 | 5 min | Median: PAs: 66 %; PANOs: 79 %; all: 72 % [c] | Contaminated teas, 38 samples | Mulder et al., 2018 |
| 16 PAs, 17 PANOs | Black, green, peppermint, mixed herbal tea, rooibos, senna tea | 0.005–4.1 | 1:50 | 6 min | Median: PAs: 65 %; PANOs: 94 %; all: 80 % [c,d] | Contaminated teas, 10 samples | Reinhart and Zoller, 2021 |
| 13 TAs | Peppermint, chamomile, mixed herbal teas | 0.13–54 | 1:75 | 4.5 min, + 0.5 min swirling of tea bag | Median: 50 % [c] | Contaminated teas, 20 samples | Mulder et al., 2016 |
| 33 PAs, 29 PANOs, 2 TAs | Black tea, mixed herbal tea | 0.5–2.5 | 1:75 | 5 min | 30–68 %, mean: 49 % | Fortified tea | This study |
| 4 PAs, 4 PANOs | Comfrey root | 60–7200 | 1:75 | 2.5 min | 16–58 %, mean 37 % | Inherent PA herbal tea, 1 sample | |
| 8 PAs, 7 PANOs | Green tea | 0.2–2 | 1:50 | 4 min | 25–75 %, mean: 44 % | Fortified tea | Han et al., 2022 |
| 16 PAs, 14 PANOs | Green, black, herbal tea | 0.01–0.4 | 1:50 | 6 min | 16–45 %, mean: 22 % | Fortified tea | Picron et al., 2018 |
| 7 PAs, 6 PANOs | Chamomile tea | 7.5–7500 | 1:40 | 5 min | 13–87 %, mean: 43 % | Artificially contam. Tea (5 % of weight) | Fernández-Pintor et al., 2023 |
| 2 TAs | Fennel tea | 0.13–0.30 | 1:50 | 5 min | 47–88 %, mean: 69 % | Contaminated teas, 4 samples | González-Gómez et al., 2023 |
| 1 PA, 1 PANO | Green tea | Not provided | 1:50 | 4 min | 27 % (PA) [a], 53 % (PANO) [b] | Contaminated teas, 4 samples | Han et al., 2022 |

* For studies with a larger number of samples ($n > 5$; five upper rows), the weighted median transfer rates for total content in the samples, based on the respective cleaned data (see Supplementary Table 3), are given. For studies with fortified samples or with a small number of contaminated samples or analytes ($n \leq 5$) the range and mean transfer rates for individual analytes are given. [a] jacobine was the only tertiary amine PA analyzed. [b] jacobine N-oxide was the only PANO analyzed. [c] Results for contaminated teas were calculated based on the individual data for PAs and TAs provided in the supplementary material of the corresponding publication. Only data were included for analytes for which a quantitative result was reported in both infusion and dried tea. The weighted median transfer rates for individual analytes were used to calculate the overall transfer rates in the infusion from dried tea. Calculated values may differ from the data originally published. [d] Data obtained with ISO protocol for preparing tea infusions. [e] Results include a number of analytes that were present at low concentrations and that were not linked to the specific plant species.

It is important to note that EFSA bases its exposure assessments preferably on occurrence data obtained for 'real' samples. Therefore, to put our results in a broader perspective, data of literature on retail tea samples was evaluated. A summary of relevant publications reporting transfer rates of PAs or TAs from tea to infusions is provided in Table 3. Further details from the three major studies on PAs in teas, based on data obtained from six or more samples (Mulder et al., 2018; Picron et al., 2018; Reinhard & Zoller, 2021) are provided in Supplementary Table 3. However, the calculation of accurate transfer rates from contaminated (herbal) tea samples is not straightforward. Not only that in these studies the transfer rates were calculated in different ways (e.g. as ratio 'analyzed concentration to theoretical concentration', or as ratio 'analyzed concentration in infusion' to 'analyzed concentration in dry material'), also sample inhomogeneity could have influenced the calculated transfer rates. To reduce a potential bias, the data reported in the three studies were carefully evaluated and a data cleaning was performed (PAs not present in both dry tea and infusion were removed; compounds measured in infusion but not in dry tea were set to a transfer rate of 100 %). This was necessary, otherwise it would have resulted in a substantial number of transfer rates being either 0 % or 100 % (4 % to 54 % of the data points, depending on the study).

However, even after data cleaning frequently very low (< 10 %) or very high (> 100 %) transfer rates were derived. This is likely due to insufficient homogeneity of the respective sample, what can result in different concentrations determined in the replicates of a tea sample, independent whether analyzed as infusion or as a diluted acidic extract. Because of the wide range of PA patterns present in the samples, the number of data points for individual analytes in the data sets was highly variable. It was also noted that a few very high individual transfer rates could have a distinct impact on the mean values of a certain analyte, especially when the number of data points (i.e. samples) for a certain PA was limited. To eliminate the extremes, in each dataset the weighted median transfer for each analyte was used for the calculation of the overall transfer rates.

In the three major studies on PAs in contaminated teas providing usable data sets based on six or more samples (Mulder et al., 2018, Picron et al., 2018 and Reinhard & Zoller, 2021), the weighted median transfer rates for tertiary amine PAs varied between 58 % and 66 % and for PA *N*-oxides between 79 % and 94 % (Table 3). Averaged over all analytes, the range was between 70 % and 80 %. In all three studies, the median transfer rates for PA *N*-oxides were higher than for the tertiary amine PAs, an observation which is in agreement with the spiking results of the current study. Nevertheless, overall, the calculated transfer rates were clearly higher than the results obtained in our and other studies with fortified samples. The reason for this discrepancy remains to some extent unresolved, but probably inhomogeneity of the retail samples investigated in those studies plays an important role.

Less data is available on the transfer rates of TAs from contaminated herbal teas. From the study of Mulder et al. (2016), for 13 TAs a weighted median transfer rate of 50 % was calculated. For atropine and scopolamine, the median transfer rates were 40 % and 37 %, respectively ($n$ = 19), while the mean transfer rates were 52 % and 47 %, respectively. This is in reasonable agreement with the study of González-Gómez et al. (2023), who reported mean transfer rates for atropine and scopolamine of 75 % and 50 % ($n$ = 4, resp. $n$ = 3). In the current study, the mean transfer rates of fortified atropine and scopolamine were 43 % and 51 % in black tea and 45 % and 50 % in herbal tea (both at 2.5 ng/mL fortification level, Supplementary Table 5) and thus in accordance with the previous studies. It is noted that the observed transfer rates are in good alignment with EU regulation 2023/915, in which transfer rates of 50 % for atropine and scopolamine into herbal infusions are implicated.

In the current study, the actual transfer rate of PAs or TAs to the infusion was demonstrated to depend on the compounds' polarity, the pH of the infusion itself and the matrix constitution. The most accurate way to account for the individual transfer rates and ways of preparing a tea infusion would be to apply maximum levels both for dry (herbal) teas and for their infusions. In contrast, the most practical way would be to apply an average transfer rate to all analytes. In case of TAs, this has already been established in Commission Regulation (EU) No. 2023/915. Herein, ratios of 1:125 for dry herbal products and 1:250 for anise seeds were set, derived from the regulatory limits for liquid vs. dry herbal infusions products (liquid: 0.20 μg/kg; dried product: 25 μg/kg // 50 μg/kg).

Furthermore, to avoid uncertainties, we suggest to prepare tea infusions in a more comparable manner by strictly following the ISO 3103 procedure prior to analyzing the infusion for their PA and TA contents. Whether this may have an impact on the future regulation of maximum levels and the routine analysis of PAs and TAs in (herbal) teas within the official food control remains subject of further discussion.

## 4. Conclusion

In the present study, the effects of several parameters on the individual transfer rates of 62 PAs and the TAs atropine and scopolamine into herbal tea infusions were systematically investigated. Overall, the transfer rates into infusions of fortified black and herbal tea under standard brewing conditions ranged from 30 % to 68 %, with an average of 49 %. While the duration of tea brewing had no distinct effect on the transfer rates, the polarity of the compounds and the pH of the infusion had an impact on the transfer rates of PAs and TAs into tea infusions. The extent of grinding showed only a minor influence on the transfer of the alkaloids, as was shown in the case of naturally PA-containing comfrey root, while the brewing time did not affect the transfer from ground material to the infusions. In case of comfrey root, the transfer rates increased with higher brewing temperatures. An evaluation of the available literature data on the transfer rates of PAs and TAs from herbal teas showed that, for contaminated commercial tea samples, the overall median transfer rates vary from 50 % for TAs to 70–80 % for PAs. In summary, the varying transfer rates of PAs and TAs due to different brewing parameters can be seen as an uncertainty affecting the risk assessment of these alkaloids in (herbal) tea infusions. Therefore, we propose that infusions should be brewed according to a standardized procedure mimicking a realistic transfer of PAs and TAs into infusions to properly assess the toxic alkaloids' risk to the health of consumers. As this may still require considerable efforts, a more practical approach would be to apply an average transfer rate factor for all regulated PAs, as has already been established for TAs. Based on the available data this average transfer factor could be in the order of 50 % (based on the transfer rates in fortified tea) to 75 % (based on the average transfer calculated from contaminated samples).

## CRediT authorship contribution statement

**Florian Kaltner:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Christoph Gottschalk:** Writing – review & editing. **Elena de Vries:** Investigation. **Patrick P.J. Mulder:** Writing – review & editing, Supervision, Resources, Methodology, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Florian Kaltner reports travel was provided by Technical University of Munich Graduate School. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The authors dedicate this article to Dr. Patrick Mulder, who sadly passed away in March 2025 after a short, serious illness. His groundbreaking work significantly advanced the understanding of plant toxins in food and feed and earned him great recognition across Europe and around the world. Patrick was not only a brilliant researcher and plant toxin expert, but also a respected colleague, a loyal friend, and an inspiring mentor. His legacy will continue to influence the field and those who had the privilege to work with him. He will be deeply missed.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.145026.

## Data availability

Data will be made available on request.

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