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RESEARCH

Structural Modifications of Certain Pyrrolizidine Alkaloids During Sample Extraction and Its Impact on Analytical Results

Florian Kaltner[1,2] · **Lisa Monika Klein**[2,3]

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Abstract

Pyrrolizidine alkaloids (PAs), a group of plant toxins often contaminating food or feed, are typically extracted from samples using liquid extraction. The crude extracts are then often purified using solid-phase extraction (SPE) cartridges before being analysed by LC–MS/MS. During the development of analytical methods based on strong cation exchange SPE, certain structurally related PAs showed unexpectedly low or significantly increased recoveries, suggesting transformation reactions may be at play. To investigate this hypothesis, sample preparations were conducted using PA-free milk as food matrix, water or organic solvents, into which PA reference standards were spiked before or after critical steps of the protocol. The results revealed a significant decrease in acetylated PA *N*-oxides to their corresponding deacetylated compounds, as well as the formation of epoxydic PAs from PA compounds containing chlorine and hydroxyl groups in the α position. Evaporation of the alkaline SPE eluates, combined with the use of the protic solvent methanol in cases of deacetylation, was responsible for these phenomena. An alkaline ester hydrolysis mechanism was hypothesised for the deacetylation, while an internal S_N2 reaction, similar to the chlorohydrin reaction was suggested for the formation of epoxy PA compounds. Consequently, using different sample preparation methods may inadvertently bias the determined PA patterns.

Keywords Plant toxins · Transformation · Strong cation exchange · Deacetylation · Epoxidation · Solid phase extraction

Introduction

Pyrrolizidine alkaloids (PAs) are a group of toxic plant secondary metabolites occurring in tribes and genera of the Boraginaceae, Asteraceae and Leguminosae families (Smith & Culvenor 1981). Currently, more than 660 compounds have been identified, occurring in approximately 6000 plant species worldwide as a defence mechanism against herbivorous insects (Boppré, 2011; Mattocks 1986). In plants, PAs primarily exist in their more hydrophilic corresponding *N*-oxide form. It is widely accepted that unintentional co-harvesting of PA-containing plants in crop fields is the primary cause of contamination of plant-based food and feed. Teas, herbal teas, spices and culinary herbs are among the most affected product groups (Bodi et al. 2014; European Food Safety Authority 2017; Kaltner et al. 2020). The ingestion of contaminated feed has been shown to transfer PAs to animal-based food such as milk, meat or eggs (Mulder et al. 2016, 2020; Taenzer et al. 2025). When ingested, only 1,2-unsaturated PAs and their corresponding *N*-oxides exhibit toxic potential to humans and mammals. Passing the intestine, these compounds are metabolised to pyrrole esters, capable of forming covalent adducts with DNA or cellular proteins (Fu et al. 2004). Consequently, both short-term and long-term intake of PAs can lead to fatal intoxications and are associated with pulmonary hypertension, hepatic sinusoidal obstruction syndrome, liver cirrhosis or liver cancer in humans (Edgar et al. 2015, 2020; Yang et al. 2017).

For the purpose of preventive consumer protection, food authorities have established regulatory limits for PAs in frequently contaminated foodstuff. In the European Union, these limits range from 1.0 µg/kg in tea and herbal infusions

✉ Florian Kaltner
florian.kaltner@lmu.de

1 Institute of Food Chemistry and Food Biotechnology, Justus Liebig University of Giessen, Heinrich-Buff-Ring 17, 35415 Giessen, Germany

2 Chair of Food Safety and Analytics, Faculty of Veterinary Medicine, LMU Munich, Schoenleutnerstr. 8, 85764 Oberschleissheim, Germany

3 Chair of Analytical Food Chemistry, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Maximus-Von-Imhof-Forum 2, 85354 Freising, Germany

for infants and young children to 1000 µg/kg in dried borage, lovage, marjoram and oregano. The regulatory limits were first implemented in 2020 and apply to a sum of 35 PAs (European Commission 2023). Besides regulatory limits, PAs have been analysed in several matrices for research purposes for decades. As PAs are considered harmful even when ingested in small amounts over a long period of time, sensitive analytical methods are necessary to detect PA trace levels down to the ppb range. To avoid signal-decreasing interfering matrix compounds as well as possible, liquid chromatography coupled to sensitive mass spectrometry (LC–MS) has been established as the instrumental "gold standard" in PA analytics (Casado et al. 2022; Crews et al. 2010). Prior to detecting PAs, they have to be properly extracted from the respective sample material. Commonly, acidic extraction solvents are used, followed by further purification and concentration steps such as the use of solid phase extraction (SPE) cartridges. Herein, SPE cartridges based on reversed phase or strong cation exchange (SCX) material are commonly used (Keuth et al. 2022; Kwon et al. 2021; Picron et al. 2018).

In earlier method development studies, discrepancies in recovery rates of certain, back then newly incorporated PA reference standards were noticed. Specifically, a near-total loss of 7-*O*-acteylintermedine *N*-oxide and 7-*O*-acteyllycopsamine *N*-oxide was observed, along with increased recoveries for the corresponding deacetylated PA *N*-oxides (Kaltner et al. 2019). In a subsequent study, poor recoveries were obtained for three PAs with an incorporated chlorine atom, while three epoxydic PAs resulted in increased recovery rates (Klein et al. 2022). In both cases, it was hypothesised that unknown transformation reactions during the sample preparation procedure using SCX SPE cartridges were responsible for these findings. While these types of reactions of PAs during sample preparation have not been described in the literature before, they can have a significant impact on the reported analytical results for a given sample. Consequently, in the current study, we investigated the individual steps of the sample preparation applied in the mentioned studies by using PA reference standards spiked prior to and after potentially critical steps of the method. As the observed effects were assumed to be caused by the method itself and to avoid matrix interference, both milk (as a model food matrix) and water or extraction solvents without food matrix were used for spiking during the experiment.

Materials and Methods

The experiments and measurements were independently performed in two separate laboratories (Oberschleissheim, Lab1; Giessen, Lab2). Lab1 performed experiments using PA-free dairy milk while in Lab2 the reference standards were spiked to water or pure solvents without any sample matrix.

Chemicals and Reagents

For all experiments, acetonitrile and methanol (both LC–MS grade) as well as formic acid (99–100%), ammonia solution (25%) and n-hexane (all of analytical grade) were achieved from Th. Geyer (Renningen, Germany). Ammonium carbonate (HPLC grade) was acquired from Fisher Scientific (Schwerte, Germany), and ammonium hydrogen carbonate (LC–MS grade) was obtained from Merck (Darmstadt, Germany). Ultra-pure water was obtained by water purification, either using an UltraClear™ system from Evoqua Water Technologies (Barsbuettel, Germany, Lab1) or a Milli-Q™ system provided by Merck (Darmstadt, Germany, Lab2). Eighteen reference standards, namely 7-*O*-acetylintermedine (AcIm), 7-*O*-acetylintermedine *N*-oxide (AcImN), 7-*O*-acetyllycopsamine (AcLy), 7-*O*-acetyllycopsamine *N*-oxide (AcLyN), heliotrine (Ht), heliotrine *N*-oxide (HtN), intermedine (Im), intermedine *N*-oxide (ImN), jacobine (Jb), jacobine *N*-oxide (JbN), jaconine (Jn), lycopsamine (Ly), lycopsamine *N*-oxide (LyN), merenskine (Mk), merenskine *N*-oxide (MkN), merepoxine (Mx), merepoxine *N*-oxide (MxN) and senkirkine (Sk) were purchased from Phytolab (Vestenbergsgreuth, Germany) or from Cfm Oskar Tropitzsch (Marktredwitz, Germany). Stock solutions of each compound were prepared with either methanol or acetonitrile/water (50/50,v/v) (1 mg/mL) and shared between Lab1 and Lab2 to conduct the experiments with identical reference standards. Spike solutions of the standards (10 µg/mL) were obtained by subsequent dilution of stock solutions in methanol. All reference standard solutions were stored at – 20 °C.

General Sample Preparation Procedure

The following general sample preparation was performed: 3.0 mL of milk (Lab1) or 2.0 g of water (Lab2) were weighed into a 50 mL polypropylene sample tube, and 30 mL formic acid (2%, aq.) and 15 mL n-hexane were added. After shaking (30 min) and centrifugation (2600 g), 15 mL of the aqueous phase was loaded onto a Bond Elut Plexa PCX 6 mL 200 mg SPE cartridge (Agilent, Waldbronn, Germany), preconditioned with 5 mL of methanol and 5 mL of formic acid (2%, aq.). After washing with 10 mL of water and 10 mL of methanol, the analytes were eluted with 6 mL of ammoniated methanol (5%). Eluates were evaporated to dryness under nitrogen at 50 °C. Therefore, Lab1 used a Turbovap II water bath apparatus (Biotage, Uppsala, Sweden) while Lab2 evaporated with a FSC400D Sample Concentrator with an aluminium heat block (Cole Parmer, St. Neots,

UK). Residues were reconstituted in 500 µL (Lab1) and 1000 µL (Lab2) methanol/water (10/90, v/v) and filtered through a 0.2-µm PVDF syringe filter (Macherey–Nagel, Düren, Germany) into a glass vial.

Instrumentation and Measurements

As LC–MS instrumentation, Lab1 used a Shimadzu HPLC system (Duisburg, Germany) hyphenated to an API 4000 triple quadrupole MS from Sciex (Darmstadt, Germany). The system of Lab2 consisted of a Dionex Ultimate 3000 RS by Thermo Scientific (Dreieich, Germany) coupled to an API 3200 triple quadrupole MS from Sciex (Darmstadt, Germany). Both Lab1 and Lab2 used chromatographic settings already published by Klein et al. (2022). In brief, separation was performed on Kinetex™ EVO C18 columns 100 × 2.1 mm with particle size 2.6 µm (Lab1) or 1.7 µm (Lab2), respectively. The columns were protected by SecurityGuard™ ULTRA EVO C18 2.1 mm pre-columns (all from Phenomenex, Aschaffenburg, Germany). At a flow of 0.3 mL/min and an oven temperature maintained to 30 °C, 10 µL were injected into the system. Eluent solvents were ammonium carbonate (Lab1) or ammonium hydrogen carbonate solution (Lab2), (10 mmol/L at pH 9.0, A) and acetonitrile (B). The used LC gradient, exact ion source parameters and selected m/z transitions are extensively described elsewhere (Klein et al. 2022). Analyte levels were calculated for each sample using calibration solutions (Lab1: 0.1, 1.0, 2.5, 5.0, 10 ng/mL; Lab2: 1.0, 2.5, 5.0, 10, 25, 50 ng/mL) of a mix of PAs in methanol/water (10/90, v/v).

Software

Mass spectrometry data acquisition, data processing and peak integration were performed using Analyst 1.6 and Multiquant 3.0 (both from Sciex, Darmstadt, Germany). Chemical structures were drawn using ChemDraw Prime V 20.1 (PerkinElmer, Waltham, MA, USA). Atomic charges and the respective figure were calculated and drawn using the browser-based tool "Atomic Charge Calculator II" (Raček et al. 2020) in automatic mode. Relative charge colouring was calculated according to split-charge equilibration with parametrized initial charges (SQE+qp) method and CCD parameters as published by Schindler et al. (2021).

Spiking Experiments

For the experiments, a total of 11 setups were prepared, comprising four using milk and seven using solely water or solvents as spiked mediums. Table 1 provides a comprehensive overview of all tested sets.

In Lab1, to examine the deacetylation process in matrix, PA-free milk was spiked with AcImN and AcLyN to a theoretical concentration of 5 ng/mL per compound in the final sample extract. HtN and Sk were already known not to undergo any changes due to sample extraction conditions and thus were added as control compounds in the same amount. The following experiments with milk were prepared in triplicates: (M1) spiking of milk aliquots before the sample preparation procedure, (M2) spiking the SPE eluate of blank milk prior to evaporation, (M3) spiking 6 mL of pure methanol prior to evaporation and subsequently reconstituting the residue with syringe-filtered (0.2 µm, PVDF) blank milk matrix extract, and, as a control (MC), by adding 50 µL

Table 1 Analytes and timepoints used for the spiked milk (M) and solvent (S) experiments

Used matrix/solvent	Name	Spiked analytes	Final *c* in vial	Timepoint of spiking
Milk	M1	AcImN, AcLyN, HtN, Sk	5 ng/mL	Prior to sample preparation
	M2			Prior to evaporation
	M3			Spiked to pure MeOH prior to evaporation, reconstituted in milk extract
	MC			Added to evaporated residue (=control)
Water	S1	AcImN, AcLyN, HtN, Jn, MkN, Sk	50 ng/mL	Prior to sample preparation
Water	S2			Prior to SPE purification
Water	S3			Prior to evaporation
MeOH + NH_3[a]	S4			Spiked to solvent prior to evaporation
ACN	S5			Spiked to solvent prior to evaporation
ACN + NH_3[a]	S6			Spiked to solvent prior to evaporation
MeOH + NH_3[a]	SC			Added to evaporated residue (=control)

AcImN, 7-*O*-acetylintermedin *N*-oxide; *AcLyN*, 7-*O*-acetyllycopsamine *N*-oxide; *ACN*, acetonitrile; *HtN*, heliotrine *N*-oxide; *Jn*, jaconine; *MeOH*, methanol; *MkN*, merenskine *N*-oxide; *Sk*, senkirkine; *SPE*, solid phase extraction

[a]5% NH_3 in the final mixture

of a standard mix in methanol and 450 µL water to an evaporated blank milk residue, followed by vortex mixing and syringe filtering into a glass vial. To calculate changes in analyte levels due to the sample preparation procedure, the mean signal responses of the four spiked analytes in experiments M1, M2 and M3 were compared to their respective mean peak area ($n = 3$) in the control experiment MC.

In Lab2, matrix-free solvents were spiked instead. Spiking was conducted in each experiment in a way to achieve a theoretical concentration of 100 ng/mL per compound in the measurement vial. First, to check for deacetylation and epoxidation of respective PAs, AcImN and AcLyN, HtN and Sk, as well as the chlorine-consisting compounds MkN and Jn, were spiked to 2.0 g of water and extracted as described above (S1). Next, water was extracted, and the six analytes were spiked after the liquid extraction step and prior to SPE purification (S2) or after SPE elution and prior to evaporation (S3), respectively. Furthermore, in three experiments, the compounds were directly spiked to different solvents, subsequently evaporated, reconstituted and filtered (0.2 µm, PVDF). Herein, the six PAs were added to ammoniated methanol (5%, S4), pure acetonitrile (S5) or ammoniated acetonitrile (5%, S6). In a control setup, 2.0 g of water was treated according to the described extraction procedure, with the evaporated residue reconstituted in 50 µL of a PA mix in methanol and 450 µL water (SC).

To express changes in the non-spiked, but arising analytes ImN, LyN, Jb and MxN, their mean signal responses ($n = 3$, Lab1) or their mean concentrations ($n = 3$, Lab2) were reported relative to levels of the control analyte HtN in the respective experiment.

Results

Deacetylation

In milk samples, the deacetylation of AcImN and AcLyN during the extraction and clean-up procedure was investigated by spiking these two compounds as well as HtN and Sk at different steps of the sample preparation (MC, M1–M3), as described above. Compared to the reference setup (MC), the mean concentrations ($n = 3$) of the control PAs ranged from 100 ± 4% (M3) to 105 ± 7% (M2) for HtN and from 93 ± 7% (M3) to 105 ± 8% (M1) for Sk, respectively, indicating the overall stability of the used control compounds (Fig. 1A). In contrast, this was not the case for the acetylated *N*-oxides. When spiked to the milk prior to any sample preparation (M1), their mean concentrations were reduced to 2.5 ± 0.7% (AcImN) or 2.3 ± 0.9% (AcLyN). Also, when spiked to SPE eluates prior to nitrogen evaporation (M2), remaining concentrations of 13 ± 2% (AcImN) or 12 ± 2% (AcLyN) were detected, respectively. Interestingly, in experiment M3, where AcImN and AcLyN were spiked into pure methanol, evaporated and reconstituted in extract of PA-free milk, the levels of both compounds were barely reduced and 95 ± 7% (AcImN) or 93 ± 7% (AcLyN) of the initial concentration was still detectable. A corresponding increase of ImN and LyN was observed due to changes in the ratio of their mean signal response, compared to the respective mean concentration of the stable reference analyte HtN. In the control setup MC, still 1.6 ± 0.1% of ImN and 2.4 ± 0.2% of LyN were detected. Also, in experiment M3, their ratio to the concentration of HtN remained low with 6.1 ± 1.2% (ImN) and 8.2 ± 0.8% (LyN), whereas in setups M1 and M2, their ratios greatly increased to 108 ± 4% and 60 ± 5% (ImN) and to 89 ± 2% and 75 ± 6% (LyN), respectively. An increase in the amount of the corresponding tertiary amine PAs, namely, AcIm, AcLy, Im and Ly, was not detected in any of the milk setups.

The observation of decreased amounts of AcImN and AcLyN due to the sample preparation procedure was consistent with findings from solvent setups conducted at Lab2 (Fig. 1B). When spiked prior to sample preparation (S1), both analytes were detected only in small amounts, at 2.3 ± 0.4% for AcImN and 2.3 ± 0.2% for AcLyN, compared to their levels in the control setup (SC). Similar results were obtained in experiment S2, where the analytes were spiked prior to the SPE purification step (2.5 ± 0.9% for AcImN, 2.5 ± 1.1% for AcLyN). When added to the SPE eluate prior to evaporation (S3), the detected levels were 11 ± 1% for AcImN and 10 ± 2% for AcLyN, consistent with the results observed in the corresponding experiment in milk (M2). In setups S4, S5 and S6, the analytes were spiked into ammoniated methanol, ammoniated acetonitrile or pure acetonitrile and subsequently evaporated. In ammoniated methanol (S4), the levels of AcImN and AcLyN were also decreased to 4.2 ± 1.0% (AcImN) or 4.4 ± 1.1% (AcLyN). In contrast, spiking the acetylated *N*-oxides to acetonitrile (S5) or ammoniated acetonitrile (S6) resulted in hardly any loss of the compounds (AcImN: 93 ± 2% or 92 ± 6%; AcLyN: 96 ± 3% or 95 ± 4%). Consistent with the findings of the milk setups, no corresponding tertiary amine PAs of the analytes (AcIm, AcLy, Im, Ly) were detected above the method's limit of detection (LOD) in any of the solvent experiments. In sum, a strong decrease of mean levels of acetyl PA *N*-oxides, corresponded with significantly increased levels of non-acetylated PA *N*-oxides, was observed for the spiked analytes both in milk and solvent samples. This deacetylation (Fig. 2A) was solely detected in setups with AcImN and AcLyN being present in ammoniated methanol and evaporated to dryness (M1, M2, S1 – S4). In summary, the current study showed a decrease of AcImN and AcLyN ranging from 77 to 93% concurrent with an increase of the corresponding analytes ImN and LyN.

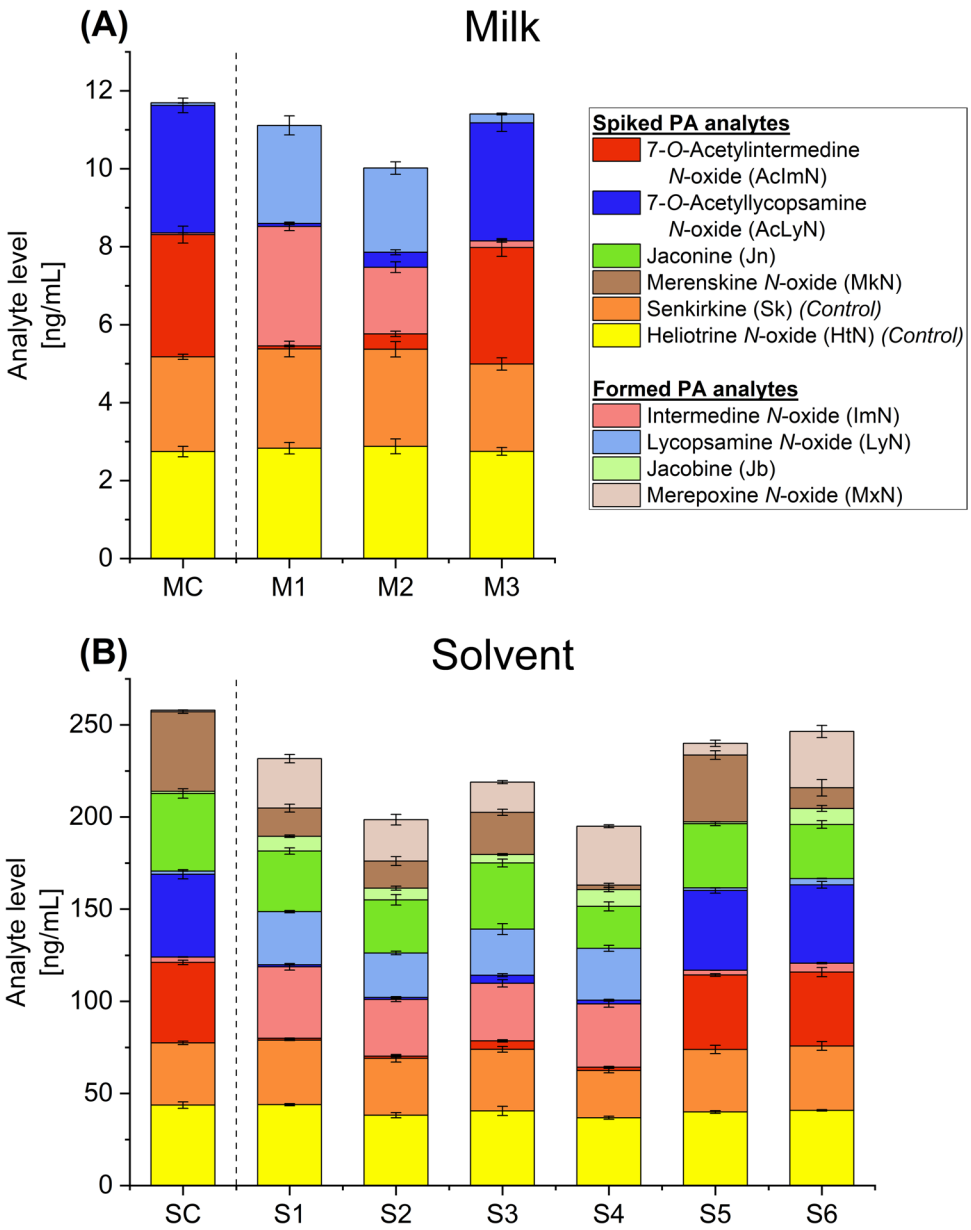

Fig. 1 Distribution of spiked and formed pyrrolizidine alkaloids (PAs) in stability experiments using milk (M) (**A**) or solvent (S) (**B**). Luscious and pastel-shades of one colour represent the corresponding spiked and formed PAs. Senkirkine and heliotrine *N*-oxide were used as controls known to be unaffected by potential deacetylation or epoxide formation. MC and SC, milk or solvent experiment control, spiked to evaporated residue; M1 and S1, spiked prior to sample preparation; S2, spiked prior to solid phase extraction (SPE); M2 and S3, spiked to alkaline SPE eluate prior to evaporation; M3, spiked to methanol prior to evaporation; S4, spiked to ammoniated methanol prior to evaporation; S5, spiked to acetonitrile prior to evaporation; S6, spiked to ammoniated acetonitrile prior to evaporation. Please see Table 1 for a concise overview of the performed experiments

Epoxidation

In the current study, the potential epoxide formation in certain PAs due to the applied sample extraction procedure was investigated. Therefore, the respective analytes Jn and MkN were spiked to the solvent setups conducted at Lab2. In the solvent control setup (SC), corresponding epoxide PAs Jb and MxN were detected, but only in traces smaller than the limit of quantitation (LOQ). In contrast, when Jn and MkN underwent at least the evaporation step of the regular sample extraction procedure (S1—S3), Jb and MxN were formed to a certain extent (Jb: 18 ± 2%, 17 ± 3% or 11 ± 1%; MxN: 61 ± 5%, 59 ± 8% or 40 ± 2%; in relation to the concentration of HtN). In setups where the analytes were directly spiked into ammoniated methanol (S4), acetonitrile (S5) or ammoniated acetonitrile (S6) and then were subsequently evaporated, the findings differed between S4 to S6. In S4, the Jn mean level was reduced to 54 ± 6% and its corresponding epoxide Jb was detected (25 ± 3%, relative to HtN). Also, MkN was almost completely lost with mean proportions of 5.6 ± 2.4%, while its corresponding epoxide PA MxN was formed to the highest extent (87 ± 2%, relative to HtN) compared to all other solvent experiments. On the other hand, in experiment S5, Jb was hardly formed (2.6 ± 0.2%), and also MxN was detected in comparably low concentrations of 16 ± 4%, relative to the mean level of HtN. In setup S6, however, the presence of ammonia in the acetonitrile solution led to reduced concentrations of Jn (70 ± 5%) and MkN

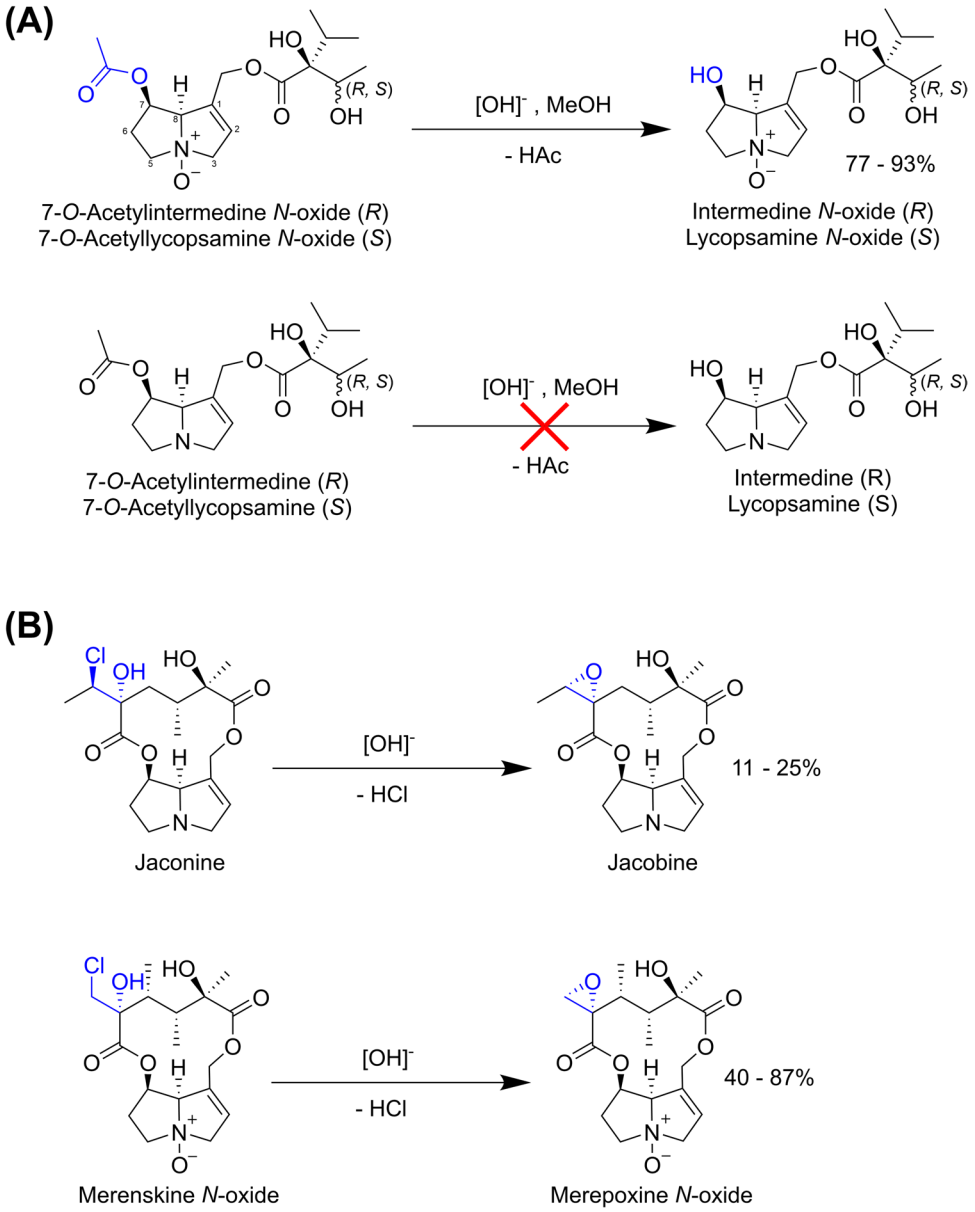

Fig. 2 Deacetylation (**A**) and epoxidation (**B**) reactions from spiked pyrrolizidine alkaloids (PAs) to their corresponding compounds detected in the milk and solvent spiking setups performed in the current study. 7-*O*-Acetylintermedine *N*-oxide and 7-*O*-acetyllycopsamine *N*-oxide were deacetylated to intermedine *N*-oxide and lycopsamine *N*-oxide when evaporated in a protic solvent (methanol) at alkaline conditions. Deacetylation does not take place for acetylated tertiary amine PAs, which was already known (Kaltner et al. 2019). Jaconine and merenskine *N*-oxide, PAs incorporating chlorine and a hydroxy group in α-position, were transformed to varying extents to their corresponding epoxy PAs jacobine and merepoxine *N*-oxide. Epoxidation was observed when the analytes were evaporated in an ammoniated solvent, regardless of whether solvents are protic (methanol) or not (acetonitrile). Given yield percentages of deacetylation were taken from solvent experiments S1 – S4 and those of epoxidation were taken from S1 – S4 and S6 (see Table 1)

(26 ± 10%) while Jb and MxN were formed (21 ± 4% and 75 ± 8%, relative to HtN). Thus, the latter results were similar to those of setups S1 – S4. The corresponding tertiary amine or *N*-oxide PAs (JnN, Mk, JbN, Mx) of the investigated analytes were not detected above the LOD in any of the solvent setups. The detected decrease of PAs with chlorine and a hydroxyl group in α position, herein Jn and MkN, correlated with a high increase of their respective epoxide compounds Jb and MxN. The formation of corresponding epoxide PAs (Fig. 2B) was observed in all solvent experiments where ammoniated solvents, regardless of whether they were protic (methanol) or aprotic (acetonitrile), were used (S1—S4; S6). Herein, the extent of this reaction was different between the structures, ranging between 11 and 25% for Jn to Jb and 40 and 87% for MkN to MxN (Fig. 2B). Consequently, MxN was generally formed to a much higher extent than Jb. It is expected that Mk and JnN, the structurally corresponding PAs to MkN and Jn, the compounds tested herein, react accordingly.

Discussion

In former method development studies, deacetylation or epoxidation of certain PAs due to sample extraction conditions had not been noticed or investigated. As comparable analytical methods did not commonly include the analytes AcImN, AcLyN, Jn, Mk or MkN, the phenomena described in this study were likely missed. Herein, it was shown that AcImN and AcLyN deacetylate to their corresponding

compounds ImN and LyN when handled in methanol under alkaline conditions (Fig. 2A). Regarding a potential mechanism of the observed deacetylation, no analogues were found in the literature. First, a nucleophilic substitution (S_N) at the C7 atom was considered. If an S_N1 mechanism was assumed, the acetylic group would have to be released spontaneously, which is unlikely, forming a carbocation intermediate stabilised by protic solvents such as methanol. A subsequent nucleophilic attack by hydroxide anions present in an alkaline environment would result in racemic mixtures of ImN and rinderine *N*-oxide (RrN), or LyN and echinatine *N*-oxide (EnN), respectively. If an S_N2 mechanism occurred, a free hydroxyl group would react via a nucleophilic backside attack, resulting in an inversion of the stereochemistry at C7, leading to RrN or EnN as the respective sole reaction product (Fig. 3A). The analytical method used in the current study was able to detect and to chromatographically separate the latter two analytes from ImN and LyN (Fig. 3B), but neither RrN nor EnN was detected in any of the sample extracts where deacetylation occurred (Fig. 3C). This demonstrated that neither a S_N1 nor a S_N2 mechanism at C7 took place. Consequently, we hypothesised a nucleophilic ester hydrolysis, initiated by a methanolate ion formed from methanol under alkaline conditions. The methanolate attacks the carbonyl C atom of the acetyl side chain, forming a negatively charged intermediate. Relocation of a lone electron pair back to the oxygen-carbon bond releases methyl acetate as a leaving group, leading to the deacetylated corresponding PA *N*-oxides while maintaining the existing stereochemistry at the C7 atom (Fig. 4A). Considering our experimental results, we currently consider the latter mechanism as the most probable one. To prove our hypothesis, reference standards labelled with stable isotopes at positions crucial for the proposed mechanism, such as the oxygen bound to the C7 atom, could be used in comparably designed future studies but are not commercially available so far.

It was demonstrated that AcImN and AcLyN deacetylate in methanol under alkaline conditions to ImN and LyN. In contrast, AcIm and AcLy did not undergo a deacetylation to Im and Ly, which was already known from earlier studies (Kaltner et al. 2019). Consequently, potential differences in the atomic charges of the C7 or the acetyl carbon atom were assumed to occur between the acetyl PA and its corresponding acetyl PA *N*-oxide. The atomic charges of AcLy and AcLyN were calculated and compared, revealing negligible charge differences of the two C7 atoms (Fig. S1). As expected, the atomic charges of the acetyl carbon atoms of AcLy or AcLyN were much more positive than of the C7 atoms, strongly supporting the proposed reaction mechanism of a nucleophilic methanolate attack at this highly electrophilic site of the molecules (Fig. 4). As the partial atomic charges did not explain the reaction taking place solely for acetylated PA *N*-oxides, it was hypothesised that the presence of an *N*-oxide may stabilise the intermediate charged molecule in a way that the deacetylation can solely occur in these molecules.

However, although this deacetylation occurs solely under specific extraction conditions (protic solvents and alkaline pH commonly present in SCX SPE-based analytical methods), it should be particularly taken into account if different extraction methods were used. While AcImN and AcLyN are not included in the PA analyte set of Commission Regulation (EU) 2023/915 (so-called PA35, 21 PAs + 14 potentially co-eluting isomers), their deacetylated forms ImN and LyN are indeed part of this analyte set. Applying a sample extraction method based on alkaline pH and protic solvents may, therefore, affect reported results and may lead to unintended variations regarding the PA profiles in samples or even to varying PA (sum) amounts in samples. For instance, if AcImN or AcLyN are considerably present in a sample, their conversion to ImN and LyN due to a SCX SPE sample preparation method using alkaline conditions might result in differing sum amounts than those obtained with another extraction technique. Based on the data from a previous study on PAs in spices and herbs (Kaltner et al. 2020), theoretical overestimations of the PA35 sum level have been calculated (Supplementary Table S1). In eight samples out of 305, AcIm and/or AcLy were detected, but not AcImN or AcLyN. A hypothetical transformation rate of 100% for AcImN and AcLyN into ImN and LyN and assumed original levels of AcImN and AcLyN, representing one- to up to fivefold the detected sum levels of AcIm and AcLy, were considered. Theoretical overestimations ranged from 0.0% (in samples with high sum levels) to a maximum of 7.7% (in samples with low sum levels). Although the results of the few samples indicated slight theoretical overestimations, the PA patterns analysed in samples may still have varied due to the chosen extraction conditions and should be taken into account in future studies.

Potential deacetylation was also estimated to have occurred in a study from Zan et al. (2023). Here, three PA-containing plants used in traditional Chinese medicine were investigated for their PA levels and PA patterns, and the authors demonstrated *Arnebia guttata* to contain AcLy and AcLyN. However, an unusual PA *N*-oxide/tertiary amine PA ratio distinctly below 1 was reported for AcLyN/AcLy in the plant samples, while for other PA compounds, this ratio was, as expected, ≥ 1, giving a hint to potential deacetylation of AcLyN. Although, using alkaline methanol to elute the PAs from the SPE and subsequently evaporating the eluates and, thus, applying conditions comparable to them in the current study, a recovery of 89% was reported for AcLyN. However, these results were contrary to findings of the current study; the reason for this remains unclear.

In this study, the epoxide formation of chlorine-containing PAs under alkaline conditions was systematically

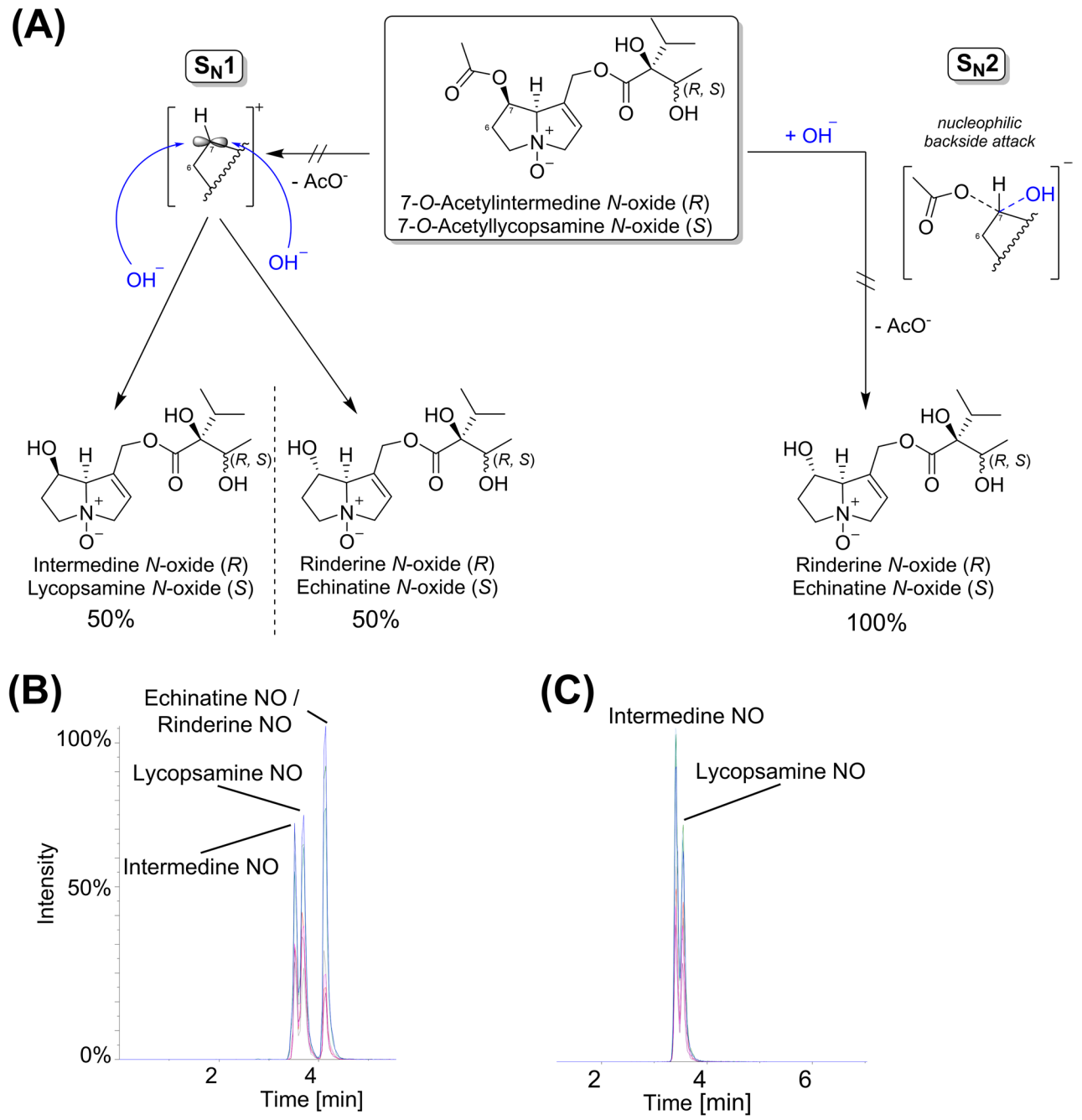

Fig. 3 Theoretical reaction products of 7-*O*-acetylintermedine *N*-oxide or 7-*O*-acetyllycopsamine *N*-oxide when a unimolecular (S_N1) or bimolecular nucleophilic substitution (S_N2) reaction mechanism of a hydroxide anion at the C7 atom is considered (**A**). If the reaction follows an S_N1 mechanism, an intermediate carbocation is formed, followed by a nucleophilic attack by the hydroxide anion. As this attack can occur on both sides of the planar carbocation, a racemic mixture results. If an S_N2 mechanism is more likely, the hydroxide anion attacks the electrophilic centre on the backside, causing an inversion of the stereochemistry at the C7 atom. In both cases, 50 to 100% of rinderine *N*-oxide or echinatine *N*-oxide would be expected. With the analytical method used in the current study, chromatographic separation is possible, as demonstrated by measuring a standard solution mix of 100 ng/mL (**B**). In samples with decreased acetylated *N*-oxide levels, neither echinatine *N*-oxide nor rinderine *N*-oxide was detected (**C**). Consequently, the reaction of the acetylated PA *N*-oxides to intermedine *N*-oxide or lycopsamine *N*-oxide did not follow an S_N1 or S_N2 reaction mechanism at the C7 atom

investigated, demonstrating the transformation of Jn to Jb and MkN to MxN to a certain degree (Fig. 2B). This transformation was attributed to conditions during sample extraction similar to those found in the literature for the so-called chlorohydrin reaction (Carrà et al. 1979). In the study by Klein et al. (2022) on the development of an analytical

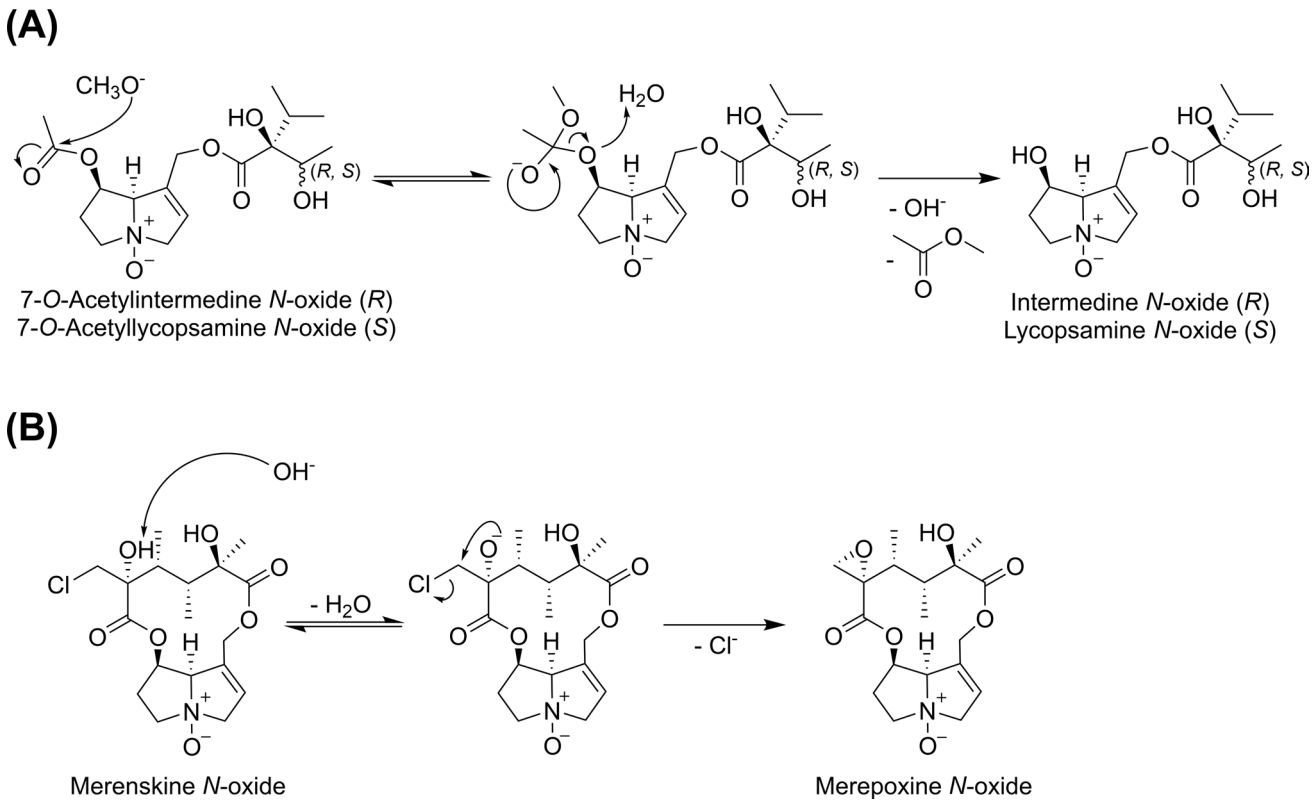

Fig. 4 Hypothesised reaction mechanisms of the deacetylation (**A**) and the epoxide formation (**B**) of certain pyrrolizidine alkaloids (PAs). In the top part of the figure, 7-*O*-acetylintermedine *N*-oxide and 7-*O*-acetyllycopsamine *N*-oxide are deacetylated to their corresponding PAs intermedine *N*-oxide and lycopsamine *N*-oxide via an addition of an methanolate anion, formed out of methanol under alkaline conditions. Methyl acetate is released from an anionic intermediate structure, where the methanolate is bound to the carbonyl carbon atom of the acetyl side chain. The bottom part of the figure shows exemplarily the chlorohydrin reaction-like epoxide formation of merenskine *N*-oxide to merepoxine *N*-oxide. The reaction of jaconine to jacobine is analogous

method to detect PAs in milk, chlorohydrin-reaction-like conditions were applied during the SPE step of sample extraction analogous to the protocol used in the current study. The authors observed decreased recoveries of Jn by up to 65%, while its corresponding epoxide compound Jb showed increased recoveries of up to 127%. Similarly, the chlorine-containing PAs Mk and MkN showed decreased recoveries of 69% (Mk) and 13% (MkN), while their corresponding epoxides, Mx and MxN, had recoveries of up to 121% (Mx) and 175% (MxN), respectively. These observations support the theory of a chlorohydrin-like reaction mechanism transforming Jn to Jb, Mk to Mx and MkN to MxN. It is likely that this epoxide formation occurred already in other earlier studies without recognising this effect (Kaltner et al. 2019).

This phenomenon also occurred during another research study on the transfer of pyrrolizidine alkaloids from dry tea to tea infusions. Herein, brewing of artificially PA-/TA-contaminated tea with buffer solutions at pH 9 (i.e. alkaline conditions like during the chlorohydrin reaction) led to significant decreases in Jn, Mk and MkN, with losses of up to 99% (Kaltner et al. 2025). Conversely, only MxN, the corresponding epoxide of the latter compound, was found in increased amounts exceeding 100%, while Jb and Mx were present at comparable levels when brewing was performed with pure water. These results suggest that the latter tertiary amine PAs are generally less stable at cooking temperatures. Nevertheless, the observed loss of PAs with chlorine and hydroxyl groups in the α position at alkaline pH aligns with the findings of the current study. Although neither the PA compounds prone to epoxide formation nor their epoxide reaction products are included in the PA35 set of analytes, unintended changes in the chemical structures of PA analytes due to certain conditions during sample extraction may affect scientific findings and, thus, should not be neglected in future studies.

Conclusions

In the current study, potential deacetylation of AcImN and AcLyN to ImN and LyN as well as epoxidation of Jn to Jb and MkN to MxN during sample preparation were

systematically investigated in spiked milk or solvent. Both effects, deacetylation and epoxide formation, were tracked back to the evaporation step of the ammoniacal methanol eluate under a stream of nitrogen at 50 °C. In case of epoxidation, these conditions are comparable to those applied in the so-called chlorohydrin reaction, and a similar reaction mechanism may be assumed. For deacetylation, a potential reaction mechanism similar to the alkaline ester hydrolysis was supposed. However, it was not fully understood why deacetylation solely affected the *N*-oxides and not the corresponding tertiary amine PAs, which should be further investigated in future studies. In consequence, our findings demonstrated that reported results of PA amounts in a sample could differ, depending on the used extraction techniques of PAs from food matrices. Consequently, the described effects should be considered when applying the PA35 set for regulated PAs, since transformation of herein not covered analytes (AcImN; AcLyN) to incorporated ones (ImN, LyN) was demonstrated.

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Data Availability The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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