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Development and validation of an analytical method for the simultaneous determination of 12 ergot, 2 tropane, and 28 pyrrolizidine alkaloids in cereal-based food by LC-MS/MS

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ABSTRACT

Alkaloids are naturally occurring compounds containing basic nitrogen atoms. They are biosynthesized mainly by plants but also by some fungi species. Many alkaloids are toxic to humans and animals, and they have been classified as food contaminants. Among them, ergot, tropane, and pyrrolizidine alkaloids have maximum levels in foods, established by the Commission Regulation (EU) 2023/915. In this study, an analytical method was successfully developed and validated for the simultaneous determination of 42 ergot, tropane, and pyrrolizidine alkaloids and their N-oxides in cereal-based food. The method includes QuEChERS-based extraction followed by liquid chromatography coupled with tandem mass spectrometry. The proposed method was validated providing recoveries ranging from 71 to 119 %, intra- and inter-day precision lower than 19 %, and limits of quantification between 0.5 and 1.0 μg kg^{-1}. Finally, the analysis of reference materials coming from FAPAS proficiency tests demonstrated the suitability for purpose of the methodology (z-scores < 2). Nine cereal-based products samples were analyzed of which ergot alkaloids were detected in two of them, while one sample showed the presence of three pyrrolizidine alkaloids.

1. Introduction

Alkaloids are organic compounds found in nature, known for their distinctive ring structure containing at least one nitrogen atom. These compounds serve as a defense mechanism for plants or fungi against potential threats from predators and have also gained recognition for their valuable medicinal and pharmacological properties (Al-Subaie et al., 2022). With over 12,000 alkaloids described (Ziegler & Facchini, 2008), tropane, pyrrolizidine, and ergot alkaloids have maximum levels in food (EU, 2023).

Tropane alkaloids are compounds found in certain flowering plant families like Solanaceae, Brassicaceae, Erythroxylaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae, and Convolvulaceae. One well-known species in the Solanaceae family, *Datura stramonium*, has a particularly high concentration of tropane alkaloids (Adamse et al., 2014). This plant is widespread in warm and temperate regions worldwide and is considered a harmful weed in important agricultural crops (Griffin & Lin, 2000). While over 200 tropane alkaloids have been identified, (-)-hyosciamine and (-)-scopolamine have been extensively studied due to their natural occurrence, unlike their corresponding (+)-enantiomers. The racemic mixture of (-)-hyosciamine and (+)-hyosciamine is known as atropine. The toxic effects of tropane alkaloids stem from their ability to block muscarinic acetylcholine receptors in both the central nervous system (CNS) and the autonomic nervous system (ANS). Some of the adverse effects include hallucinations, changes in heart rate, dilated pupils, and dry mouth, among others (EFSA, 2013). According to data from the European Food Safety Authority (EFSA), tropane alkaloids in food are mainly found in cereal-based products and herbal teas (Mulder et al., 2016).

Pyrrolizidine alkaloids are secondary metabolites produced by over 6,000 plant species (Schrenk et al., 2022). Currently, approximately 600 pyrrolizidine alkaloids have been identified. They exist in two forms: the free base form and as N-oxides. The presence of a double bond at position 1,2 within the pyrrolizidine ring system is responsible for their toxicity. This toxicity manifests as developmental toxicity, hepatotoxicity, genotoxicity, and carcinogenicity (EFSA, 2011). The European

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Commission has identified 28 pyrrolizidine alkaloids as the most relevant in food samples, including echimidine, echimidine-N-oxide, heliotrine, heliotrine-N-oxide, lycopsamine, lycopsamine-N-oxide, intermedine, intermedine-N-oxide, erucifoline, erucifoline-N-oxide, senecionine, senecionine-N-oxide, seneciphylline, seneciphylline-N-oxide, monocrotaline, monocrotaline-N-oxide, jacobine, jacobine-N-oxide, senecivernine, senecivernine-N-oxide, retrorsine, retrorsine-N-oxide, europine, europine-N-oxide, lasiocarpine, lasiocarpine-N-oxide, senkirkine, and trichodesmine. In 2016, EFSA estimated the dietary exposure to pyrrolizidine alkaloids in the European population and recommended monitoring these alkaloids in honey, tea, and herbal infusions (EFSA, 2016).

Ergot alkaloids are mycotoxins produced by various fungi species belonging to the *Claviceps* genus. Among these fungi, *Claviceps purpurea* is the most prevalent in Europe and can infect over 400 plant species, particularly cereal grains such as rye, millet, wheat, and barley (EFSA, 2012). Certain ergot alkaloids, such as ergotamine and ergometrine, have recognized pharmacological effects and are used in treating migraines and managing the third stage of labor and postpartum hemorrhage, respectively (Koleva et al., 2012). However, the consumption of contaminated grains or excessive use of drugs containing these alkaloids can lead to ergotism, which can manifest as gangrenous ergotism or convulsive ergotism (Al-Omari et al., 2018). The most concerning ergot alkaloids include ergotamine, ergometrine, ergosine, ergocristine, ergocornine, ergokryptine, and their corresponding -inine epimers. In a 2017 study by EFSA on human dietary exposure to ergot alkaloids, rye and rye-containing products were found to have the highest levels of contamination, with other cereal grains like wheat, spelt, oats, and corn also being affected (EFSA et al., 2017).

The maximum levels of these three groups of alkaloids in food are stipulated by Commission Regulation (EU) 2023/915 dated 25th April 2023 (EU, 2023). Regarding ergot alkaloids, these maximum levels are applied to processed cereal-based foods for infants and young children, wheat gluten, as well as grains or milling products of barley, wheat, spelt, oats, rye, and similar grains. In the case of tropane alkaloids, the EU regulation establishes limits for unprocessed millet, sorghum, maize, buckwheat, as well as milling products, baby food and processed cereal-based food for infants and young children, herbal infusions, and ingredients used for herbal infusions. Lastly, concerning pyrrolizidine alkaloids, the maximum allowable contents are set for borage, lovage, marjoram and oregano leaves, as well as for teas, herbal infusions, cumin, food supplements containing botanical preparation, and products derived from pollen.

To ensure compliance with the prescribed maximum levels of ergot, tropane, and pyrrolizidine alkaloids in food, it is essential to develop analytical methodologies capable of accurately detecting and quantifying these diverse compounds even at low concentrations. Indeed, in the year 2023, the European Union has recorded a total of 46 food alerts related to the presence of alkaloids in various food commodities (European Commission, 2023).

In most studies that determine alkaloids in cereal-based food, the focus has been on a single alkaloid family (Baslé et al., 2020; Di Mavungu et al., 2012; Kokkonen & Jestoi, 2010). However, only a limited number of papers have investigated the presence of two or more alkaloid families in cereal-based food (Mulder et al., 2015; Veršilovskis et al., 2020; Bessaire et al., 2021; Tölgyesi et al., 2023). In these cases, two widely used techniques in the extraction step are solid–liquid extraction (SLE) (Carbonell-Rozas et al., 2021; Di Mavungu et al., 2012; Gonçalves et al., 2020; Guo et al., 2016) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), which has gained popularity recently (Baslé et al., 2020; Huybrechts et al., 2021). Following the extraction process, a cleanup procedure is often necessary to remove interferences and concentrate the analytes. The commonly used cleanup techniques include solid-phase extraction (SPE) (Marín-Sáez et al., 2017; Storm et al., 2008) and dispersive solid-phase extraction (d-SPE) (Krska et al., 2008; Huybrechts et al., 2021), which usually follows QuEChERS extraction. Finally, the extracts obtained are analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Arroyo-Manzanares et al., 2018; Guo et al., 2016), but also by liquid chromatography combined with fluorescence detection (LC-FLD) (Storm et al., 2008).

This study describes the development and validation of an analytical method that enables the simultaneous determination of two tropane alkaloids (atropine and scopolamine), twelve ergot alkaloids, and twenty eight pyrrolizidine alkaloids and their N-oxides in cereal-based foods. The experimental procedure consists of a simple QuEChERS extraction followed by LC-MS/MS determination.

2. Material and methods

2.1. Chemicals and reagents

Standards for ergometrine, ergometrinine, ergotamine, ergotaminine, ergocristine, ergocristinine, ergocornine, ergocorninine, α-ergokryptine, α-ergocryptinine, ergosine, and ergosinine were acquired from Romer Labs (Getzersdorf, Austria). To prepare individual stock standard solutions of ergot alkaloids, the instructions provided by the manufacturer were followed. The contents of each vial were dissolved in 5 mL of acetonitrile, resulting in a concentration of 100 μg mL^{-1} for each -ine epimer and 25 μg mL^{-1} for each -inine epimer. These stock solutions were then stored at −20 °C.

Analytical standards for atropine, monocrotaline, retrorsine, scopolamine, senecionine, seneciphylline, and senkirkine were provided by Merck (Darmstadt, Germany). Additionally, echimidine, echimidine N-oxide, erucifoline, erucifoline N-oxide, europine, europine N-oxide, heliotrine, heliotrine N-oxide, intermedine, intermedine N-oxide, jacobine, jacobine N-oxide, lasiocarpine, lasiocarpine N-oxide, lycopsamine, lycopsamine N-oxide, monocrotaline N-oxide, retrorsine N-oxide, senecionine N-oxide, seneciphylline N-oxide, senecivernine, senecivernine N-oxide, and trichodesmine were supplied by PhytoLab (Vestenbergsgreuth, Germany). Each tropane and pyrrolizidine alkaloid were individually prepared as a stock standard solution at a concentration of 500 μg mL^{-1} in methanol and stored at −20 °C.

A multicomponent standard solution containing ergot, tropane, and pyrrolizidine alkaloids at 1 μg mL^{-1} was prepared in methanol and stored in the freezer for further use.

Scopolamine-d_3, acquired from Sigma-Aldrich (Round Rock, TX, USA) was used as surrogate for the analysis of real samples.

QuEChERS extraction packets, EN15662 method (1 g of sodium citrate, 0.5 g of sodium hydrogencitrate sesquihydrate, 4 g of $MgSO_4$, and 1 g of NaCl), purchased from Scharlau (Barcelona, Spain), and QuEChERS d-SPE 15 mL tubes (150 mg PSA, 150 mg C18, and 900 mg $MgSO_4$), acquired from Merck (Darmstadt, Germany), were used in sample preparation. Acetonitrile HPLC grade, from PanReac (Barcelona, Spain), and formic acid reagent grade, from Scharlau (Barcelona, Spain), were also used in this step.

Methanol LC-MS, from Scharlau (Barcelona, Spain) and Mili-Q water, obtained from an Adrona B30 Bio purification system, were used as solvents in the chromatographic analysis. Formic acid eluent additive for LC-MS, from Scharlau (Barcelona, Spain), was added to mobile phase solvents.

2.2. Instrument and apparatus

A Digicen 20 centrifuge from Orto Alresa (Madrid, Spain) was used to centrifuge samples. To evaporate extracts, a rotary evaporator R-215 with a heating bath B-491, a vacuum pump V-700 and a vacuum controller V-850 from Buchi (Barcelona, Spain) were used.

Analysis of alkaloids was conducted using an Agilent 1100 Series (Santa Clara, CA, USA) equipped with a quaternary pump (G1311A), an autosampler (G1329A) and an autosampler thermostat (G1330B). For chromatographic separation, a Luna 3u C18(2) column (100 × 2.0 mm,

Table 1
MRM transitions, collision energy and retention time for the 42 analytes and surrogate.

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)		RT (min)
		Quantification (CE)	Confirmation (CE)	
Mon	326.16	120.08 (30)	94.06 (30)	3.84
Eru	350.16	120.08 (30)	138.09 (35)	5.73
Lyc	300.18	94.06 (20)	156.10 (30)	6.18
Int	300.18	94.06 (20)	156.10 (30)	6.18
Mon-NO	342.15	137.08 (15)	120.08 (25)	6.34
Jac	352.18	120.08 (20)	94.06 (15)	6.36
Eur	330.19	138.09 (25)	94.06 (15)	6.43
Eru-NO	366.15	120.08 (25)	118.06 (30)	6.78
Eur-NO	346.19	172.10 (30)	138.09 (35)	6.96
Scp	304.15	138.09 (30)	110.09 (15)	7.01
Scp-d_3	307.15	141.09 (30)	113.09 (30)	7.01
Jac-NO	368.17	120.08 (20)	296.15 (35)	7.09
Lyc-NO	316.17	172.10 (30)	94.06 (25)	7.23
Int-NO	316.17	172.10 (30)	94.06 (25)	7.23
Tri	354.19	120.08 (25)	94.06 (20)	7.80
Ret	352.18	120.08 (20)	94.06 (15)	7.89
Egm	326.22	223.17 (25)	208.15 (30)	7.96
Hel	314.20	138.09 (30)	156.1 (20)	8.16
Sep	334.16	138.09 (30)	94.06 (20)	8.19
Ret-NO	368.17	120.08 (20)	94.06 (30)	8.24
At	290.17	124.11 (20)	93.07 (25)	8.71
Hel-NO	330.19	172.10 (20)	138.09 (25)	8.76
Sep-NO	350.16	120.08 (30)	118.06 (20)	8.78
Sec	336.18	120.08 (30)	138.09 (20)	9.12
Sev	336.18	120.08 (30)	138.09 (20)	9.12
Sec-NO	352.18	120.08 (20)	94.06 (15)	9.60
Sev-NO	352.18	120.08 (20)	94.06 (15)	9.60
Egmn	326.22	208.15 (30)	223.17 (25)	9.67
Ech	398.22	120.08 (30)	138.09 (35)	10.45
Ech-NO	414.21	254.14 (30)	120.08 (15)	10.46
Sek	366.19	168.10 (20)	122.06 (15)	10.67
Las	412.23	120.08 (20)	94.06 (25)	11.87
Las-NO	428.23	254.14 (25)	94.06 (20)	12.86
Esi	548.32	223.17 (35)	277.12 (25)	13.80
Esin	548.32	223.17 (35)	277.12 (25)	13.80
Etm	582.27	223.17 (35)	208.15 (40)	14.10
Etmn	582.27	223.17 (35)	208.15 (40)	14.10
Eco	562.35	268.17 (25)	223.17 (35)	14.18
Econ	562.35	223.17 (35)	305.15 (30)	15.05
Ekr	576.35	223.17 (35)	305.15 (30)	15.06
Ecr	610.35	223.17 (35)	305.17 (30)	15.26
Ekrn	576.35	223.17 (35)	305.15 (30)	15.67
Ecrn	610.35	305.17 (30)	223.17 (35)	15.88

CE: collision energy (V).
RT: retention time (min).
Atr: atropine; Scp: scopolamine; Scp-d_3: scopolamine deuterated; Ech: echimidine; Ech-NO: echimidine N-Oxide; Eru: erucifoline; Eru-NO: erucifoline N-oxide; Eur: europine; Eur-NO: europine N-Oxide; Hel: heliotrine; Hel-NO: heliotrine N-Oxide; Jac: jacobine; Jac-NO: jacobine N-Oxide; Las: lasiocarpine; Las-NO: lasiocarpine N-Oxide; Lyc: lycopsamine; Lyc-NO: lycopsamine N-Oxide, Int: intermedine; Int-NO: intermedine N-Oxide; Mon: monocrotaline; Mon-NO: monocrotaline N-Oxide; Ret: retrorsine; Ret-NO: retrorsine N-oxide; Sec: senecionine; Sec-NO: senecionine N-Oxide; Sev: senecivernine; Sev-NO: senecivernine N-Oxide; Sep: seneciphylline; Sep-NO: seneciphylline N-Oxide; Sek: senkirkine; Tri: trichodesmine; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.

3 μm particle size), supplied by Phenomenex (Torrance, CA, USA), was employed.

The HPLC system was coupled to a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer with electrospray ionization (ESI), from Thermo Fisher Scientific (Bremen, Germany). Data processing was conducted using Xcalibur™ version 2.2.

2.3. Samples and reference materials

Nine commercial samples of cereal-based foods were acquired from various local stores of Valencia city for their analysis using the proposed method. The samples consisted of two wheat flours, two wheat breadcrumbs, two rye flours, one rye breadcrumbs, one corn flour, and two multigrain baby foods. A wheat flour sample with no previous contamination of alkaloids was used for both the preparation of a matrix-matched calibration and the preparation of spiked samples during the method validation.

Two reference materials of pyrrolizidine alkaloids in Ispaghula Husk and ergot alkaloids in baby food, provided by FAPAS-Food Chemistry Proficiency Test (test number 22,194 and 22185, respectively), were also analyzed to ensure the suitable of purpose of the methodology.

2.4. Sample preparation

2.5 g of sample was weighted in a 50 mL polypropylene Falcon tube and spiked with 5 μL of a 1 μg mL^{-1} scopolamine-d_3 solution, used as surrogate. Then, 30 mL of extraction solvent containing 75 % ACN and 25 % H_2O, along with 0.1 % formic acid were added to the tube. Additionally, the contents of a QuEChERS extraction packet, which included 1 g of sodium citrate, 0.5 g of sodium hydrogencitrate sesquihydrate, 4 g of $MgSO_4$, and 1 g of NaCl, was also added. The mixture was vigorously shaken by hand for 1 min and centrifugated at 5000 rpm for 5 min. The resulting supernatant was carefully transferred to another 50 mL Falcon tube containing 150 mg of PSA, 150 mg of C18, and 900 mg of $MgSO_4$. The tube was shaken again by hand for 1 min and centrifugated at 5000 rpm for 5 min. Subsequently, the supernatant was transferred to a glass conical-bottom tube and evaporated to dryness using rotary evaporator. The residue was reconstituted in 250 μL of MeOH. Before proceeding to chromatographic analysis, the extract was filtered through a 0.22 μm nylon syringe filter.

2.5. LC-MS/MS analysis

5 μL of extracts were injected in the HPLC system. The mobile phase consisted of an aqueous solution containing 0.1 % formic acid (A) and methanol with 0.1 % formic acid (B). The eluent gradient profile was as follows: 0 min: 2 % B, 15 min: 70 % B, 19 min: 70 % B, 20 min: 2 % B and 32 min: 2 % B. The flow rate was set at 0.3 mL min^{-1}.

The triple quadrupole mass spectrometer operated in positive electrospray ionization mode (ESI +). The instrument was configured with the following parameters: spray voltage of 3 kV, sheath gas pressure of 10 a.u., auxiliary gas pressure of 15 a.u., and capillary temperature of 280 °C. The instrument was programmed in multiple reaction monitoring (MRM) mode and the specific transitions monitored for each compound are presented in Table 1.

LC-MS/MS chromatograms illustrating a matrix standard containing 50 μg kg^{-1} of all alkaloids and the analysis of a wheat breadcrumbs sample are presented in Fig. 1.

3. Results and discussion

3.1. Study of extraction conditions

The study of extraction procedure involved the study of two extraction techniques, SLE and QuEChERS. For SLE study, a solvent mixture of MeOH:H_2O (60:40 v/v) was used. As for QuEChERS extraction, a mixture of ACN:H_2O (75:25 v/v) was employed along with 1 g of sodium citrate, 0.5 g of sodium hydrogencitrate sesquihydrate, 4 g of $MgSO_4$, and 1 g of NaCl. A d-SPE step with 150 mg of PSA, 150 mg of C18, and 900 mg of $MgSO_4$ was applied to the QuEChERS extracts. Subsequently, the extracts were evaporated and reconstituted in MeOH. The sensitivity obtained with SLE was not good enough to align with regulatory standards. In contrast, the addition of a cleanup step and the

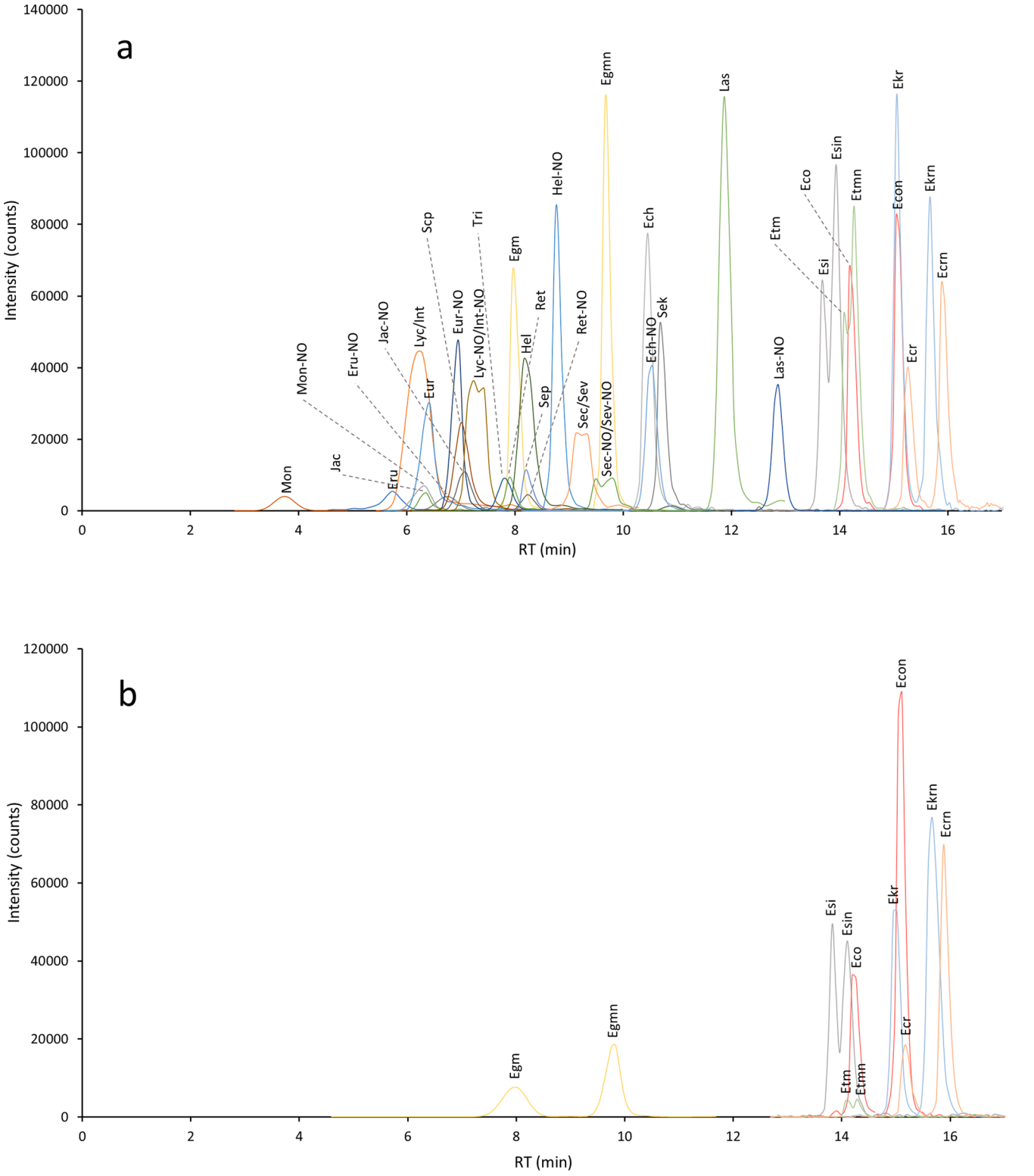

Fig. 1. LC-MS/MS chromatograms of **(a)** a matrix standard with a concentration of all alkaloids of 5 μg kg^{1} **(b)** and a wheat breadcrumbs sample. Atr: atropine; Scp: scopolamine; Ech: echimidine; Ech-NO: echimidine N-Oxide; Eru: erucifoline; Eru-NO: erucifoline N-oxide; Eur: europine; Eur-NO: europine N-Oxide; Hel: heliotrine; Hel-NO: heliotrine N-Oxide; Jac: jacobine; Jac-NO: jacobine N-Oxide; Las: lasiocarpine; Las-NO: lasiocarpine N-Oxide; Lyc: lycopsamine; Lyc-NO: lycopsamine N-Oxide, Int: intermedine; Int-NO: intermedine N-Oxide; Mon: monocrotaline; Mon-NO: monocrotaline N-Oxide; Ret: retrorsine; Ret-NO: retrorsine N-oxide; Sec: senecionine; Sec-NO: senecionine N-Oxide; Sev: senecivernine; Sev-NO: senecivernine N-Oxide; Sep: seneciphylline; Sep-NO: seneciphylline N-Oxide; Sek: senkirkine; Tri: trichodesmine; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.

Table 2
Limits of detection, limits of quantification, matrix effect, and coefficient of determination of matrix-matched calibration obtained during validation.

Analyte	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	ME (%)	$R^{2,a}$
Atr	0.05	0.5	−61	0.996
Scp	0.04	0.5	2	0.996
Ech	0.02	0.5	−57	0.997
Ech-NO	0.04	0.5	−63	0.998
Eru	0.20	1.0	−69	0.990
Eru-NO	0.07	0.5	27	0.996
Eur	0.04	0.5	58	0.997
Eur-NO	0.02	0.5	46	0.996
Hel	0.05	0.5	4	0.993
Hel-NO	0.04	0.5	−62	0.995
Jac	0.12	0.5	−40	0.996
Jac-NO	0.11	0.5	−13	0.997
Las	0.05	0.5	−63	0.996
Las-NO	0.14	0.5	−64	0.993
Lyc + Int	0.05	0.5	16	0.998
Lyc-NO + Int-NO	0.11	0.5	26	0.997
Mon	0.15	0.5	−64	0.992
Mon-NO	0.13	0.5	26	0.997
Ret	0.28	1.0	19	0.996
Ret-NO	0.25	1.0	−4	0.999
Sec + Sev	0.29	1.0	−58	0.992
Sec-NO + Sev-NO	0.13	0.5	−70	0.998
Sep	0.11	0.5	−7	0.993
Sep-NO	0.14	0.5	−66	0.998
Sek	0.04	0.5	−66	0.996
Tri	0.09	0.5	−9	0.992
Eco	0.04	0.5	−60	0.996
Econ	0.06	0.5	−70	0.998
Ecr	0.11	0.5	−61	0.993
Ecrn	0.12	0.5	−72	0.999
Egm	0.08	0.5	−14	0.997
Egmn	0.06	0.5	−72	0.997
Ekr	0.04	0.5	−61	0.999
Ekrn	0.14	0.5	−9	0.999
Esi + Esin	0.14	0.5	−68	0.997
Etm + Etmn	0.20	0.5	−69	0.997

LOD: limit of detection; LOQ: limit of quantification; ME: matrix effect.
Atr: atropine; Scp: scopolamine; Ech: echimidine; Ech-NO: echimidine N-Oxide; Eru: erucifoline; Eru-NO: erucifoline N-oxide; Eur: europine; Eur-NO: europine N-Oxide; Hel: heliotrine; Hel-NO: heliotrine N-Oxide; Jac: jacobine; Jac-NO: jacobine N-Oxide; Las: lasiocarpine; Las-NO: lasiocarpine N-Oxide; Lyc: lycopsamine; Lyc-NO: lycopsamine N-Oxide, Int: intermedine; Int-NO: intermedine N-Oxide; Mon: monocrotaline; Mon-NO: monocrotaline N-Oxide; Ret: retrorsine; Ret-NO: retrorsine N-oxide; Sec: senecionine; Sec-NO: senecionine N-Oxide; Sev: senecivernine; Sev-NO: senecivernine N-Oxide; Sep: seneciphylline; Sep-NO: seneciphylline N-Oxide; Sek: senkirkine; Tri: trichodesmine; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.
[a] Range studied: 0.5–100 μg kg^{-1}.

Table 3
Recoveries (mean values), with their standard deviations, obtained at validation.

Analyte	Recovery (%) 0.5 (μg kg^{-1})	1.0 (μg kg^{-1})	50 (μg kg^{-1})	100 (μg kg^{-1})
Atr	84 ± 7	100 ± 10	117 ± 3	111 ± 8
Scp	91 ± 3	89 ± 6	102 ± 2	102 ± 3
Ech	88 ± 9	104 ± 10	117 ± 4	109 ± 3
Ech-NO	75 ± 6	96 ± 18	77 ± 3	79 ± 6
Eru	<LOQ	89 ± 8	119 ± 3	103 ± 3
Eru-NO	110 ± 6	98 ± 13	87 ± 10	99 ± 9
Eur	83 ± 5	76 ± 3	96 ± 2	95 ± 2
Eur-NO	85 ± 9	78 ± 6	95 ± 3	98 ± 6
Hel	82 ± 11	85 ± 8	108 ± 3	105 ± 4
Hel-NO	81 ± 4	78 ± 2	90 ± 2	92 ± 5
Jac	105 ± 16	112 ± 7	119 ± 3	111 ± 13
Jac-NO	101 ± 19	79 ± 9	82 ± 8	94 ± 18
Las	98 ± 5	102 ± 6	114 ± 3	104 ± 6
Las-NO	75 ± 12	96 ± 11	73 ± 4	89 ± 8
Lyc + Int	84 ± 15	92 ± 10	111 ± 3	109 ± 4
Lyc-NO + Int-NO	72 ± 6	89 ± 11	87 ± 2	86 ± 12
Mon	91 ± 17	109 ± 5	118 ± 4	111 ± 4
Mon-NO	87 ± 11	87 ± 4	94 ± 15	97 ± 14
Ret	<LOQ	82 ± 7	81 ± 2	102 ± 5
Ret-NO	<LOQ	78 ± 3	92 ± 13	94 ± 13
Sec + Sev	<LOQ	94 ± 3	119 ± 4	109 ± 6
Sec-NO + Sev-NO	82 ± 4	82 ± 11	87 ± 6	93 ± 13
Sep	100 ± 15	93 ± 6	113 ± 5	107 ± 4
Sep-NO	110 ± 16	81 ± 3	87 ± 5	98 ± 13
Sek	85 ± 6	101 ± 4	106 ± 3	98 ± 3
Tri	106 ± 15	82 ± 3	109 ± 3	107 ± 3
Eco	110 ± 3	98 ± 3	72 ± 2	72 ± 3
Econ	99 ± 10	94 ± 3	97 ± 6	97 ± 10
Ecr	106 ± 15	99 ± 12	88 ± 2	83 ± 2
Ecrn	110 ± 3	112 ± 6	94 ± 6	96 ± 9
Egm	93 ± 4	83 ± 3	74 ± 3	79 ± 5
Egmn	89 ± 3	91 ± 5	86 ± 2	90 ± 5
Ekr	96 ± 14	95 ± 3	77 ± 2	75 ± 2
Ekrn	92 ± 17	89 ± 10	93 ± 5	93 ± 8
Esi + Esin	89 ± 9	101 ± 8	81 ± 2	92 ± 7
Etm + Etmn	101 ± 5	80 ± 3	84 ± 4	92 ± 5

Atr: atropine; Scp: scopolamine; Ech: echimidine; Ech-NO: echimidine N-Oxide; Eru: erucifoline; Eru-NO: erucifoline N-oxide; Eur: europine; Eur-NO: europine N-Oxide; Hel: heliotrine; Hel-NO: heliotrine N-Oxide; Jac: jacobine; Jac-NO: jacobine N-Oxide; Las: lasiocarpine; Las-NO: lasiocarpine N-Oxide; Lyc: lycopsamine; Lyc-NO: lycopsamine N-Oxide, Int: intermedine; Int-NO: intermedine N-Oxide; Mon: monocrotaline; Mon-NO: monocrotaline N-Oxide; Ret: retrorsine; Ret-NO: retrorsine N-oxide; Sec: senecionine; Sec-NO: senecionine N-Oxide; Sev: senecivernine; Sev-NO: senecivernine N-Oxide; Sep: seneciphylline; Sep-NO: seneciphylline N-Oxide; Sek: senkirkine; Tri: trichodesmine; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.

removal of water from the extraction solvent enabled the evaporation of the extracts and the preconcentration of the analytes. Consequently, the QuEChERS extraction yielded the best results.

3.2. Study of chromatographic conditions

Since it is necessary to cover 3 alkaloid families, a mobile phase that ensured suitable ionization of all analytes had to be selected, even though this resulted in poor resolution for some isomers. Consequently, a mobile phase composed of (A) an aqueous solution of 0.01 M ammonium formate and (B) acetonitrile was firstly employed. In the case of ergot alkaloids, excellent resolution was achieved for the "-ine" and "-inine" epimers, consistent with the findings reported in the literature (Arroyo-Manzanares et al., 2018; Holderied et al., 2019; Uhlig et al., 2021). However, ionization obtained for pyrrolizidine alkaloids jacobine, jacobine N-oxide, senecionine N-oxide and senecivernine N-oxide was notably weak.

In order to address this issue, acid conditions were explored, resulting in the adoption of a new mobile phase consisting of (A) water and (B) methanol, both containing 0.1 % formic acid. This adjustment successfully resolved the ionization challenges associated with the previously mentioned pyrrolizidine alkaloids. Additionally, a notable enhancement in sensitivity was observed for most of the analytes. However, when it comes to ergot alkaloids, coelution was found for two pairs of epimers (ergotamine/ergotaminine and ergosine/ergosinine).

Notwithstanding the challenges posed by ergot alkaloids resolution, acidic chromatographic conditions were chosen due to the successful ionization that was attained for jacobine, jacobine N-oxide, senecionine N-oxide, and senecivernine N-oxide. It is noteworthy that, in some pyrrolizidine alkaloids, quantification of isomers had to be performed as the sum of them due to their coelution (e.g., intermedine/lycopsamine, intermedine N-oxide/lycopsamine N-oxide, senecionine/senecivernine and senecionine N-oxide/senecivernine N-oxide). Regarding ergot alkaloids, it is essential to achieve the separation of the R-epimer ("-ine"),

Table 4
Occurrence of pyrrolizidine and ergot alkaloids in the reference materials of Ispaghula husk and baby food from FAPAS and evaluation of results.

Analyte	FAPAS proficiency test sample	Amount found (μg kg^{-1})	Reference value (μg kg^{-1})	z-score
Eur	Ispaghula Husk	1.61	1.37	0.8
Eur-NO	Ispaghula Husk	16.80	16.20	0.2
Hel	Ispaghula Husk	10.74	7.42	2.0
Hel-NO	Ispaghula Husk	87.82	82.60	0.3
Las	Ispaghula Husk	1.54	1.12	1.7
Las-NO	Ispaghula Husk	8.97	12.00	−1.1
Eco + Econ	Baby food	9.42	11.98	−1.4
Ecr + Ecrn	Baby food	36.77	37.33	−0.1
Egm + Egmn	Baby food	7.36	8.10	−0.6
Ekr + Ekrn	Baby food	20.11	19.36	0.3
Esi + Esin	Baby food	6.22	5.80	0.5
Etm + Etmn	Baby food	16.19	14.30	0.9

Eur: europine; Eur-NO: europine N-Oxide; Hel: heliotrine; Hel-NO: heliotrine N-Oxide; Las: lasiocarpine; Las-NO: lasiocarpine N-Oxide; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.

which exerts a toxic effect on humans, from the S-epimer ("-inine"), which demonstrates limited pharmacological activity (Komarova & Tolkachev, 2001). The coelution of ergotamine/ergotaminine and ergosine/ergosinine epimers has the potential to lead to an overestimation of the risk associated with food products containing these contaminants. Consequently, it is important to clarify that the developed method is primarily intended for ensuring food safety control and not for conducting a comprehensive risk assessment of these contaminants. Since Commission Regulation (EU) 2023/915 set maximum limits for the total concentration of all pyrrolizidine and ergot alkaloids, coelution of isomers is not a problem to comply with regulatory standards.

3.3. Method validation

The following analytical parameters were evaluated to conduct an in-house validation of the developed method: linearity, precision, accuracy, limits of detection, and limits of quantification.

The limit of detection (LOD) was estimated as 3 times the signal-to-noise ratio in chromatograms of the lowest level of calibration (0.5 μg kg^{-1}). The limit of quantification (LOQ) was established as the minimum concentration level that could be accurately (70–120 %) and precisely measured (RSD < 20 %) for each analyte. LODs achieved ranged between 0.02 μg kg^{-1} and 0.29 μg kg^{-1}, while LOQs obtained were 0.5 μg

Table 5
Intra-day and inter-day precision expressed as relative standard deviation.

Analyte	RSD (%) intra-day (n = 3)				RSD (%) inter-day (n = 5)			
	0.5 (μg kg^{-1})	1.0 (μg kg^{-1})	50 (μg kg^{-1})	100 (μg kg^{-1})	0.5 (μg kg^{-1})	1.0 (μg kg^{-1})	50 (μg kg^{-1})	100 (μg kg^{-1})
Atr	8.6	9.5	2.6	7.0	8.3	7.5	8.4	8.1
Scp	3.6	7.0	2.4	3.2	8.0	12.1	13.4	5.7
Ech	10.1	9.8	3.2	2.4	12.7	11.1	8.9	12.8
Ech-NO	7.8	19.0	4.3	8.0	15.7	7.4	12.8	9.9
Eru	<LOQ	8.5	2.8	2.7	<LOQ	14.4	11.1	9.0
Eru-NO	5.3	13.6	11.2	9.2	13.8	15.0	5.1	7.0
Eur	6.3	4.2	2.5	2.5	13.1	9.0	8.0	5.7
Eur-NO	10.2	7.6	3.5	6.5	8.9	8.8	10.7	11.9
Hel	13.6	9.7	2.7	3.8	8.3	12.5	7.6	12.3
Hel-NO	4.4	2.6	2.5	5.6	5.9	9.5	9.3	8.9
Jac	15.4	6.6	2.4	11.6	14.5	13.9	14.0	7.2
Jac-NO	18.4	11.7	9.6	18.9	12.3	13.2	7.2	6.5
Las	5.2	6.0	2.6	5.4	8.1	10.5	11.4	8.5
Las-NO	16.0	11.2	5.4	8.6	9.8	13.7	11.8	8.0
Lyc + Int	16.7	10.6	2.9	3.8	11.6	10.1	13.7	4.7
Lyc-NO + Int-NO	8.2	12.8	2.5	14.4	10.6	8.2	10.2	5.9
Mon	18.3	4.6	3.4	4.0	17.5	16.4	13.2	11.2
Mon-NO	13.2	4.3	15.5	14.3	13.9	14.4	16.4	11.0
Ret	<LOQ	8.9	2.8	4.6	<LOQ	9.0	6.7	7.5
Ret-NO	<LOQ	4.4	13.9	13.9	<LOQ	14.2	9.4	11.4
Sec + Sev	<LOQ	3.2	3.0	5.6	<LOQ	8.6	10.3	9.2
Sec-NO + Sev-NO	4.9	13.3	6.8	13.6	10.6	12.9	6.1	8.7
Sep	14.6	6.2	4.4	3.9	9.4	13.0	8.4	7.1
Sep-NO	14.6	4.1	5.8	13.3	11.6	5.4	10.9	5.3
Sek	6.5	4.4	2.4	2.8	11.1	7.6	8.4	6.0
Tri	14.6	3.5	2.4	2.4	8.5	4.4	11.1	10.3
Eco	2.8	3.4	2.6	4.0	13.2	10.6	13.8	8.0
Econ	10.5	3.3	6.4	10.2	9.8	11.6	11.5	11.4
Ecr	18.9	12.0	2.7	2.6	13.9	10.1	12.8	12.4
Ecrn	4.9	5.1	5.9	9.5	11.1	6.6	14.4	12.5
Egm	4.7	3.4	3.4	6.7	12.6	11.7	8.9	10.5
Egmn	2.9	5.6	2.8	5.2	9.6	6.7	8.1	13.5
Ekr	14.1	3.2	3.2	3.0	9.7	9.7	8.2	7.5
Ekrn	18.1	11.5	5.9	8.5	11.8	10.8	9.0	12.3
Esi + Esin	10.2	8.3	2.9	7.5	13.3	7.6	12.9	5.8
Etm + Etmn	4.8	3.9	4.6	5.5	9.6	8.0	12.7	8.0

RSD: relative standard deviation.
Atr: atropine; Scp: scopolamine; Ech: echimidine; Ech-NO: echimidine N-Oxide; Eru: erucifoline; Eru-NO: erucifoline N-oxide; Eur: europine; Eur-NO: europine N-Oxide; Hel: heliotrine; Hel-NO: heliotrine N-Oxide; Jac: jacobine; Jac-NO: jacobine N-Oxide; Las: lasiocarpine; Las-NO: lasiocarpine N-Oxide; Lyc: lycopsamine; Lyc-NO: lycopsamine N-Oxide, Int: intermedine; Int-NO: intermedine N-Oxide; Mon: monocrotaline; Mon-NO: monocrotaline N-Oxide; Ret: retrorsine; Ret-NO: retrorsine N-oxide; Sec: senecionine; Sec-NO: senecionine N-Oxide; Sev: senecivernine; Sev-NO: senecivernine N-Oxide; Sep: seneciphylline; Sep-NO: seneciphylline N-Oxide; Sek: senkirkine; Tri: trichodesmine; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.

kg^{-1} and 1.0 μg kg^{-1}, as can be seen in Table 2. According to the EU regulation (EU, 2023), the strictest limit on alkaloid content is set at 1 μg kg^{-1} for atropine and scopolamine in baby food and processed cereal-based food for infants and young children. Therefore, the LOQ values obtained align with the regulatory standards.

Accuracy was assessed by conducting recovery experiments by triplicate. A blank wheat flour sample was spiked at four concentration levels (0.5, 1.0, 50, and 100 μg kg^{-1}). The obtained results demonstrated a range of values from 71 to 119 %, as can be observed in Table 3. Recoveries values achieved indicated successful correction for potential matrix effects, ensuring a satisfactory accuracy.

Method trueness was also evaluated by comparing the levels of pyrrolizidine and ergot alkaloids in the reference materials with their respective reference values. To assess the performance, z-scores were calculated using the following expression:

$$z = \frac{x_{lab} - x_{ref}}{\sigma_{pt}}$$

where x_{lab} represents the amount detected by the proposed method, x_{ref} is the reference value and σ_{pt} is the standard deviation for proficiency assessment, which varies for each analyte. Z-score absolute values below 2 reveal satisfactory performance, while values between 2 and 3 indicate questionable performance and z-score absolute values above 3 denote unsatisfactory performance. In the present study, the obtained z-scores ranged from −1.4 to 2.0, demonstrating an adequate trueness for the developed method. Z-score values for ergot and pyrrolizidine alkaloids are shown in Table 4. In the case of ergot alkaloids, reference values were given as the sum of epimers -ine and -inine.

Precision was assessed in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). Triplicate analyses of spiked wheat flour samples were conducted to evaluate repeatability. For the evaluation of intermediate precision, a spiked wheat flour sample was analyzed over five consecutive days. As can be observed in Table 5, repeatability values ranged from 2 to 19 % and intermediate precision values ranged between 4 and 17 %.

Matrix effect (ME) was evaluated due to it was an important issue in previous studies focusing on the analysis of alkaloids in cereal-based foods (Arroyo-Manzanares et al., 2018; Holderied et al., 2019; Marín-Sáez et al., 2019; Veršilovskis et al., 2020). To assess this matrix effect, a comparison was made between the slope of an external calibration curve (prepared using methanol) and the slope of a matrix-matched calibration curve (obtained by spiking the extracts of a non-contaminated wheat flour sample). To calculate the matrix effect, the following equation was used:

$$\text{ME}\,(\%) = \left(\frac{\text{external calibration slope}}{\text{matrix} - \text{matched calibration slope}} - 1\right) \times 100$$

The obtained results are shown in Table 2. A significant modulation in signal, either suppression or enhancement, was observed for most analytes. Due to the difficulty of finding deuterated standards of all the alkaloids and their excessive costs, a matrix-matched calibration was proposed to correct the matrix effect.

Matrix-matched calibration curves were constructed using a non-contaminated wheat flour sample as the representative matrix. Various concentrations of spiked extracts, ranging from 0.5 to 100 μg kg^{-1}, were used to examinate the linearity of the 42 analytes. The obtained calibration curves demonstrated excellent linearity in the concentration range studied. Residuals standard deviation obtained were below 20 % and coefficients of determination (R^2) were above 0.99, which are presented in Table 2.

In addition to wheat flour, other matrices were also evaluated. Different matrix-matched calibration curves were constructed using samples of rye flour, wheat breadcrumbs, buckwheat flour, and multigrain baby food. Similar to the matrix effect calculation, the slopes obtained from these samples were compared with the slope derived from the wheat flour sample used during method validation.

Table 6
Matrix effect obtained for different matrices in comparison with wheat flour (%).

Analyte	Rye flour	Wheat breadcrumbs	Buckwheat flour	Multigrain baby food
Atr	4	12	28	6
Scp	5	1	3	2
Ech	−16	5	2	14
Ech-NO	−14	0	15	15
Eru	2	10	12	10
Eru-NO	3	6	30	18
Eur	12	6	3	5
Eur-NO	−14	11	−10	9
Hel	−18	0	−17	8
Hel-NO	−2	4	1	10
Jac	−14	−7	−16	−2
Jac-NO	10	3	16	7
Las	−3	4	13	12
Las-NO	−4	−2	−27	3
Lyc + Int	7	8	11	4
Lyc-NO + Int-NO	7	−1	21	−3
Mon	−3	−4	−10	3
Mon-NO	15	7	1	12
Ret	−10	6	4	8
Ret-NO	−10	0	−17	17
Sec + Sev	−19	−9	−7	−8
Sec-NO + Sev-NO	−19	5	−6	2
Sep	−15	−10	−39	3
Sep-NO	11	8	1	17
Sek	−5	7	21	8
Tri	1	3	21	11
Eco	3	0	−15	12
Econ	−12	−1	−14	−6
Ecr	−10	15	−18	45
Ecrn	−15	8	−12	−3
Egm	−14	−4	−19	4
Egmn	−3	1	30	−5
Ekr	−18	8	54	38
Ekrn	6	2	−16	5
Esi + Esin	−19	−2	−26	−14
Etm + Etmn	−9	9	−14	3

Atr: atropine; Scp: scopolamine; Ech: echimidine; Ech-NO: echimidine N-Oxide; Eru: erucifoline; Eru-NO: erucifoline N-oxide; Eur: europine; Eur-NO: europine N-Oxide; Hel: heliotrine; Hel-NO: heliotrine N-Oxide; Jac: jacobine; Jac-NO: jacobine N-Oxide; Las: lasiocarpine; Las-NO: lasiocarpine N-Oxide; Lyc: lycopsamine; Lyc-NO: lycopsamine N-Oxide, Int: intermedine; Int-NO: intermedine N-Oxide; Mon: monocrotaline; Mon-NO: monocrotaline N-Oxide; Ret: retrorsine; Ret-NO: retrorsine N-oxide; Sec: senecionine; Sec-NO: senecionine N-Oxide; Sev: senecivernine; Sev-NO: senecivernine N-Oxide; Sep: seneciphylline; Sep-NO: seneciphylline N-Oxide; Sek: senkirkine; Tri: trichodesmine; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.

Following the criteria described in the SANTE/11312/2021 document (European Commission, 2021), Table 6 shows that no significant matrix effect were observed for rye flour and wheat breadcrumbs, due to the values obtained ranged from −20 % to 20 %. Regarding multigrain baby food, matrix effect was observed only for ergocristine and α-ergokryptine. However, notable variations were identified in the case of buckwheat flour, where certain alkaloids exhibited significant signal modulation compared to wheat flour, either through ionization or enhancement. Consequently, it is recommended to employ a specific matrix-matched calibration when analyzing this particular matrix. When analyzing grain matrices not addressed in this study, it would become crucial to assess matrix effects to ensure the accuracy of the analysis.

3.4. Analysis of real samples

The developed method was finally applied to 9 samples purchased from various local markets. The samples were categorized as flours, breadcrumbs, and multigrain baby foods. Out of the samples analyzed, a

Table 7
Amount detected (μg kg^{-1}) of alkaloids in commercial samples[a].

Alkaloid	Rye flour	Wheat breadcrumbs	Multigrain baby food
Eur	n.d.	n.d.	0.6
Hel	n.d.	n.d.	1.3
Las	n.d.	n.d.	1.0
Eco	1.8	2.7	n.d.
Econ	2.8	4.7	n.d.
Ecr	1.2	6.0	n.d.
Ecrn	0.7	6.9	n.d.
Egm	1.7	1.3	n.d.
Egmn	0.6	0.9	n.d.
Ekr	3.8	8.0	n.d.
Ekrn	3.0	6.3	n.d.
Esi + Esin	2.4	3.1	n.d.
Etm + Etmn	<LOQ	<LOQ	n.d.

n.d.: non detected (<LOD).
Eur: europine; Hel: heliotrine; Las: lasiocarpine; Eco: ergocornine; Econ: ergocorninine; Ecr: ergocristine; Ecrn: ergocristinine; Egm: ergometrine; Egmn: ergometrinine; Ekr: α-ergokryptine; Ekrn: α-ergokryptinine; Esi: ergosine; Esin: ergosinine; Etm: ergotamine; Etmn: ergotaminine.
[a] In the rest of the samples analyzed, non alkaloid was detected.

rye flour sample and a wheat breadcrumbs sample were found to contain ergot alkaloids (detected amounts are summarized in Table 7). In both cases, α-ergokryptine presented the highest amount of alkaloid and the sum of ergotamine and its epimer ergotaminine was detected and identified below the LOQ. The sum of ergot alkaloids in the rye flour was 18.0 μg kg^{-1}, while in the wheat breadcrumbs was 42.9 μg kg^{-1}. However, none of the detected alkaloids exceeded the limits set by the EU regulation (EU, 2023). Additionally, a multigrain baby food sample revealed the presence of pyrrolizidine alkaloids, specifically europine, heliotrine, and lasiocarpine. Their detected amount can be observed in Table 7. It is noteworthy that pyrrolizidine alkaloids are typically found in honey, tea, and herbal infusions. Thus, this finding could prove valuable for EFSA, as it suggests the need for further data collection regarding the occurrence of pyrrolizidine alkaloids in cereal-based food products. Nevertheless, additional sample analyses are required to establish a correlation between these alkaloids and their occurrence in this specific food category. It would be crucial to ensure that the detected sample has not experienced any cross-contamination.

3.5. Comparison of the proposed method with other published methods

The main analytical features of the method were compared with similar studies involving the analysis of one or more alkaloid families in cereal-based food products. This comparison is shown in Table 8.

Although numerous analytical methods can identify a single family of alkaloids in cereal-based products, only four published methods have been found in the literature for the simultaneous detection of multiple alkaloid groups in cereal-based foods. The studies of Mulder et al. and Veršilovskis et al. introduced a method to detect 20 ergot alkaloids and 6 tropane alkaloids in grain-based products designed for infants and young children and in bread, respectively. Both methods yielded favorable results, including excellent recovery rates and low relative standard deviation values, with a limit of quantification below 1 μg kg^{-1}. In 2021, Bessaire et al. presented a method capable of identifying 99 alkaloids spanning five distinct alkaloid families in plant-based ingredients and cereal products. However, the authors noted that their method did not provide quantitative results for ergot alkaloids at concentration levels of μg kg^{-1} with a sufficient level of confidence. In 2023, Tölgyesi et al. introduced another method designed to detect 12 ergot and 2 tropane alkaloids out of a total of 285 analytes. Nevertheless, this method was primarily suitable for screening and semiquantitative analysis of ergot alkaloids, due to lack of their internal standards.

In summary, the absence of an analytical method capable of providing simultaneously quantitative results of ergot, pyrrolizidine and tropane alkaloids at low concentrations had been apparent. The method introduced in this study fulfills this need, providing analytical features comparable to the methods presented in Table 8 and, as a result, proving to be a valuable tool for ensuring food safety compliance.

4. Conclusions

This study presents the successful development and validation of an analytical method for the determination of 42 ergot, pyrrolizidine, and tropane alkaloids in cereal-based food. The experimental procedure involves a simple and fast QuEChERS extraction followed by LC-MS/MS analysis. To avoid matrix effect, a matrix-matched calibration is needed. The proposed method was successfully validated according to the EU requirements terms of linearity ($R^2 > 0.99$), precision (RSD < 19 %), and accuracy (71–119 %). The analysis of two reference materials of alkaloids and pyrrolizidine alkaloids revealed that the developed method is suitable for purpose. The achieved LOQs were found to be below the maximum levels specified by the European Commission, proving this method to be highly suitable for ensuring effective food safety control. Finally, the method was applied to 9 samples of cereal-based products in which ergot alkaloids were detected in two of them and some pyrrolizidine alkaloids were found in a different one. Anyway, none of the concentrations detected were above the maximum legal contents.

In the future, the adequacy of the method for the analysis of more foodstuffs will be studied and the analysis of opium alkaloids will be included.

Table 8
Comparison of the proposed method with other published methods.

Number of alkaloid families	Number of alkaloids	Sample preparation	Analytical technique	MLOQ (μg kg^{-1})	Recovery (%)	RSD (%)	Reference
1	2	QuEChERS	LC-MS/MS	0.5	82–114	1–23	Baslé et al., 2020
1	12	SLE/LLE	LC-MS/MS	0.15–0.96	88–119	2–26	Di Mavungu et al., 2012
1	10	SLE/SPE	LC-MS/MS	0.01–10	59–126	2–14	Kokkonen & Jestoi, 2010
1	12	QuEChERS	LC-MS/MS	0.50–3.92	85–107	3–9	Carbonell-Rozas et al., 2021
1	25	SLE/d-SPE	LC-MS/MS	0.02–0-05	77–120	1–13	Guo et al., 2016
1	12	QuEChERS	LC-MS/MS	0.1–0.5	80–111	2–34	Huybrechts et al., 2021
2	26	SLE-UF	LC-MS/MS	0.1–0.5	68–91	4–14	Mulder et al., 2015
2	26	SLE-UF	LC-MS/MS	0.3–1.2	65–94	3–17	Veršilovskis et al., 2020
2	14	QuEChERS	LC-MS/MS	0.2	67–119	3–22	Tölgyesi et al., 2023
5	99	QuEChERS	LC-HRMS	n.a.-20	31–161	2–60	Bessaire et al., 2021
3	42	QuEChERS	LC-MS/MS	0.5–1.0	71–119	2–19	Present study

MLOQ: method limit of quantification.
n.a.: not attributed.
LLE: liquid–liquid extraction.
UF: ultrafiltration.

CRediT authorship contribution statement

Alejandro García-Juan: Methodology, Validation, Investigation, Data curation, Writing – original draft. **Nuria León:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Sergio Armenta:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Olga Pardo:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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