

ePrep | Application 2022

μSPEd extraction and UHPLC-MS/MS analysis of pyrrolizidine alkaloids in herbal infusions

Pub No. 98-35030 Rev 01

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are natural toxins produced as secondary metabolites by plants belonging to different families (Asteraceae, Fabaceae, Boraginaceae, Orchidaceae and Apocynaceae) as a defense mechanism against herbivores and insects (Figure 1). In recent years, the high levels found of these toxins in food and feed have become one of the main current food safety problems, since these alkaloids can be considered potential contaminants. In this sense, the intake of PAs can produce both acute and chronic effects. Their ingestion has been mainly associated to liver damage, being particularly regarded as one of the major causes of hepatic veno-occlusive disease (HVOD), which can lead to liver cirrhosis and liver failure. However, some of them can also produce genotoxic and carcinogenic effects at long-term exposure and have been classified in Group 2B as potential carcinogens to humans by the International Agency for Research on Cancer (IARC).¹

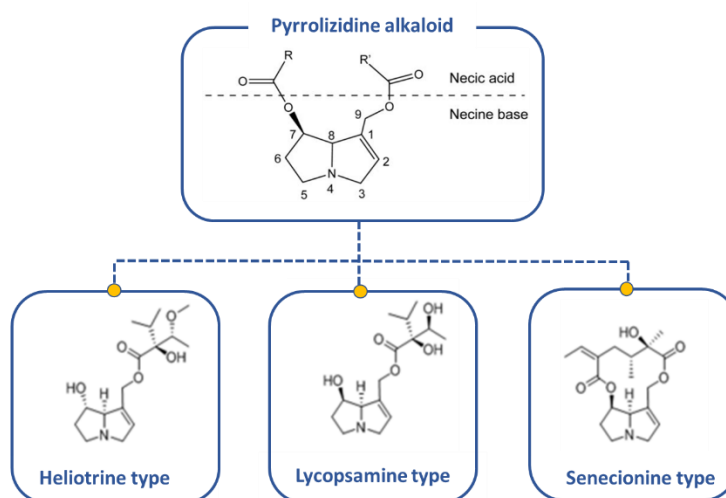
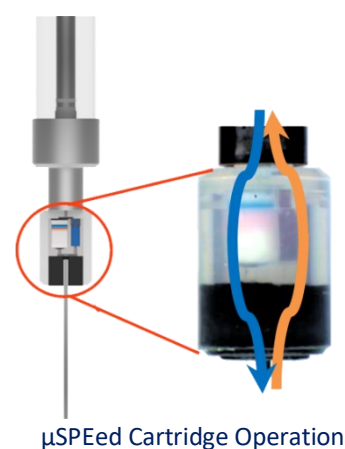


Figure 1. Common chemical structure of pyrrolizidine alkaloids and main 1,2-unsaturated pyrrolizidine alkaloids types based on their structural similarities and botanical origin.

These alkaloids can be introduced into the food chain from different vegetables and botanical sources. Some of these PA-producing plants can be directly consumed by animals (through forage) and humans (e.g., borage), while others can extensively grow in crop fields as weeds, leading to the contamination of other food products and feed. In this context, it was first assumed that the contamination of non-PA producing plants was due to the accidental inclusion of weeds or impurities from PA-producing plants during harvest or processing. However, in the last years, several works have demonstrated that besides cross-contamination during harvesting processes, other contamination paths are possible, such as natural horizontal transfer through soil, animal feed, food fraud and adulteration, etc.² In this sense, according to the food alerts notified in the last years, the main food items likely to be contaminated with high levels of these alkaloids are honey, pollen, teas, herbal teas, food supplements, spices and aromatic herbs. Particularly, a 15% of these alerts have been for teas and infusions made from plants and flowers (e.g., chamomile, spearmint, rooibos, nettle and herbal mixes), as they are products that are increasingly consumed by the population for curative and dietary purposes.² Accordingly, due to the potential risk for human health that the continuous and frequent intake of these products may entail, a regulation to monitor the occurrence of these alkaloids in some food products has recently been published in Europe, which includes maximum concentration levels for tea and herbal infusions (75-400 µg/kg for dried products and 1.0 µg/kg in liquid form).³

Given the large number of PAs to be monitored, powerful and efficient methods are required for their determination, which must include multiresidue extraction with high selectivity and sensitivity, as well as being quick and environmentally friendly procedures. To date, conventional solid-phase extraction (SPE) has been the sample preparation technique of choice for the extraction and purification of PAs from food and feed samples.^{2,4} However, compared to microextraction procedures, conventional SPE requires more time and reagents to be performed (higher volumes of sample, organic solvents, as well as greater amounts of sorbents).

Hence, this application note describes a sustainable and sensitive analytical methodology with the innovative analytical approach µSPEed (Figure 2) for the accurate extraction of PAs from different herbal infusions (linden, chamomile and green tea with mint) using C18 µSPEed cartridges in 1 min, and only 300 µL of methanol and 300 µL of sample per extraction.⁵

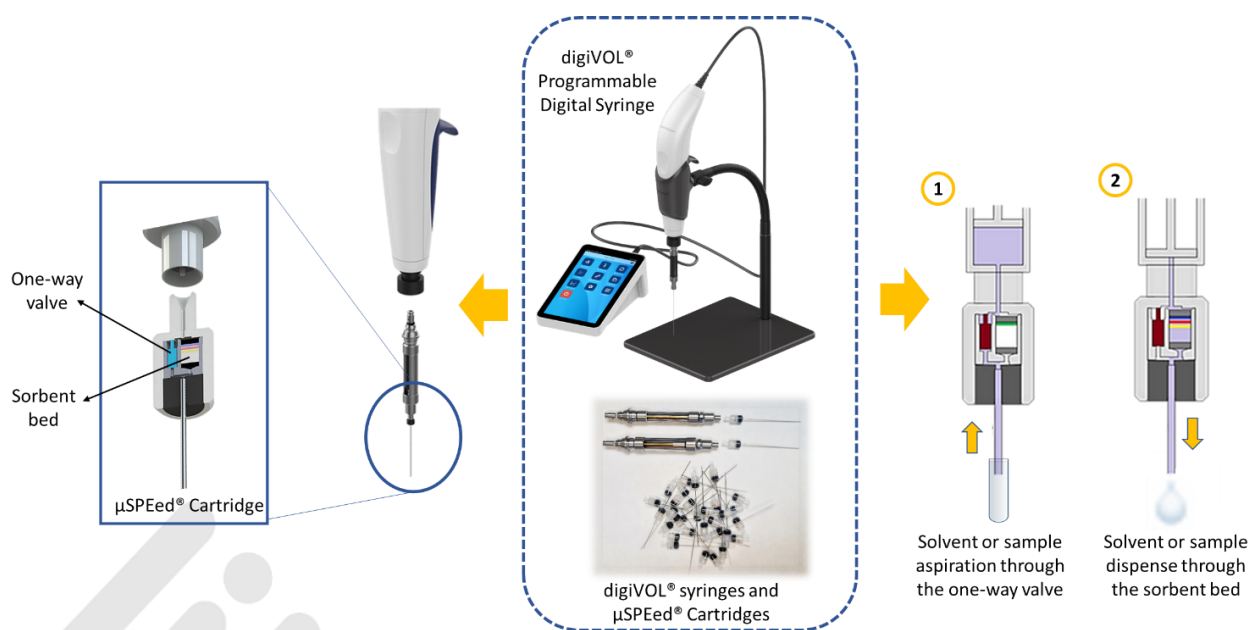
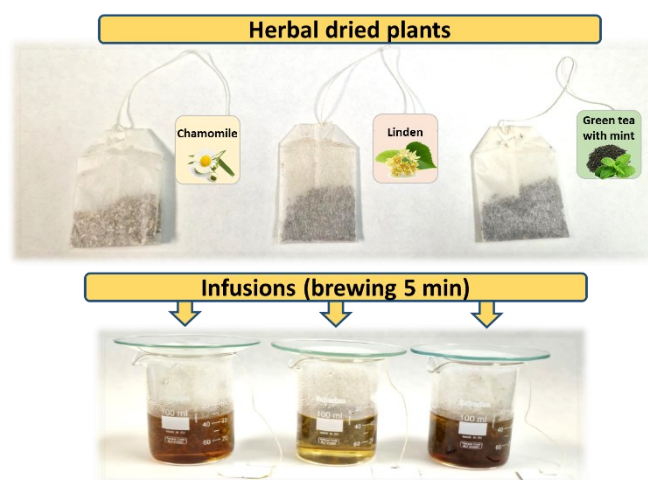


Figure 2. digiVOL® programmable digital syringe, µSPEed® cartridges and µSPEed® operation.

PROCEDURE

Sample preparation

Herbal infusions (linden, chamomile and green tea with mint) were prepared according to the manufacturers' instructions. Accordingly, 5 g of the dried plants were infused with 200 mL of boiling water (100 °C) and allowing brewing for 5 min (Figure 3). The infusions obtained were strained and kept at 4 °C until analysis. Before extraction, the infusions were filtered through a 0.45 µm PTFE filter membrane.



µSPEed Extraction Workflow

µSPEed cartridges C18RPS-3µm/120Å (EPREP) were first conditioned and equilibrated with two aspiration-dispense cycles of 100 µL of MeOH followed by two aspiration-dispense cycles of 100 µL of water. Then, infusion samples were loaded onto µSPEed cartridges (three aspiration-dispense cycles of 100 µL) and then eluted using 100 µL of methanol which were collected in a vial for the subsequent chromatographic analysis. No washing step was performed. All steps were carried out at 20 µL/s in extract-discard mode (the volume aspirated in each cycle was discarded in a waste vial after each extraction cycle). Figure 4 shows the µSPEed extraction workflow followed.

Figure 3. Preparation of herbal infusion samples

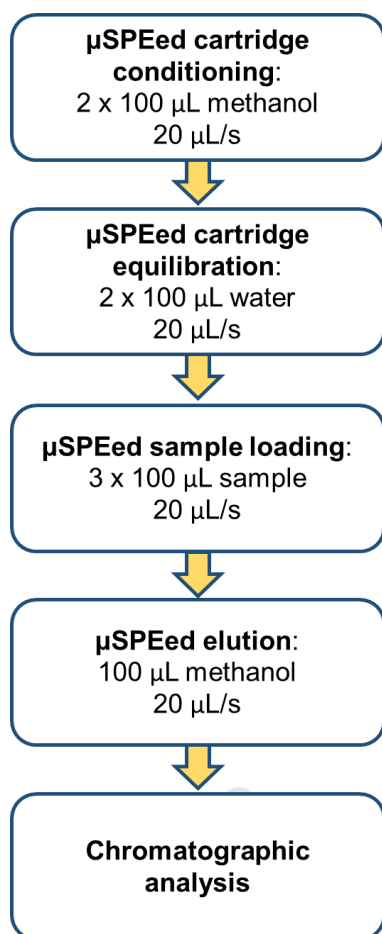


Figure 2. µSPEed extraction workflow

Chromatographic analysis

The separation and determination of 21 PAs was performed on an UHPLC system (Dionex UltiMate 3000, Thermo Scientific, Waltham, MA, USA) coupled to an ion-trap mass spectrometer detector (ESI-ITMS amaZon SL, Bruker, Billerica, MA, USA).

Conditions:

- Column: Luna Omega Polar C18 column (100 mm x 2.1 mm, 1.6 µm particle size, Phenomenex, Torrance, CA, USA)
- Flow rate: 0.250 mL/min
- Injection: 5 µL
- Column temperature: 25 °C

Mobile phase:

- Mobile phase A: methanol containing 10 mM ammonium acetate
- Mobile phase B: ultra-pure water containing 5 mM ammonium acetate and 0.2% formic acid

Gradient:

- Initial: 5% A / 95% B held for 0.5 min.
- Gradient 1: change to 50% A / 50% B over 6.5 min and held for 0.5 min.
- Gradient 2: change to 100% A / 0% B over 3.5 min and held for 1 min.
- Return to the initial conditions in 2 min and 1 min for equilibration.

MS/MS detection:

- Electrospray ionization interface (ESI) in positive ion mode
- End plate offset: -500 V
- Capillary voltage: -4500 V
- Nebulizer gas: 20 psi
- Dry gas: 10 L/min
- Dry temperature: 200 °C
- Scan mode: Multiple reaction monitoring (MRM)⁶

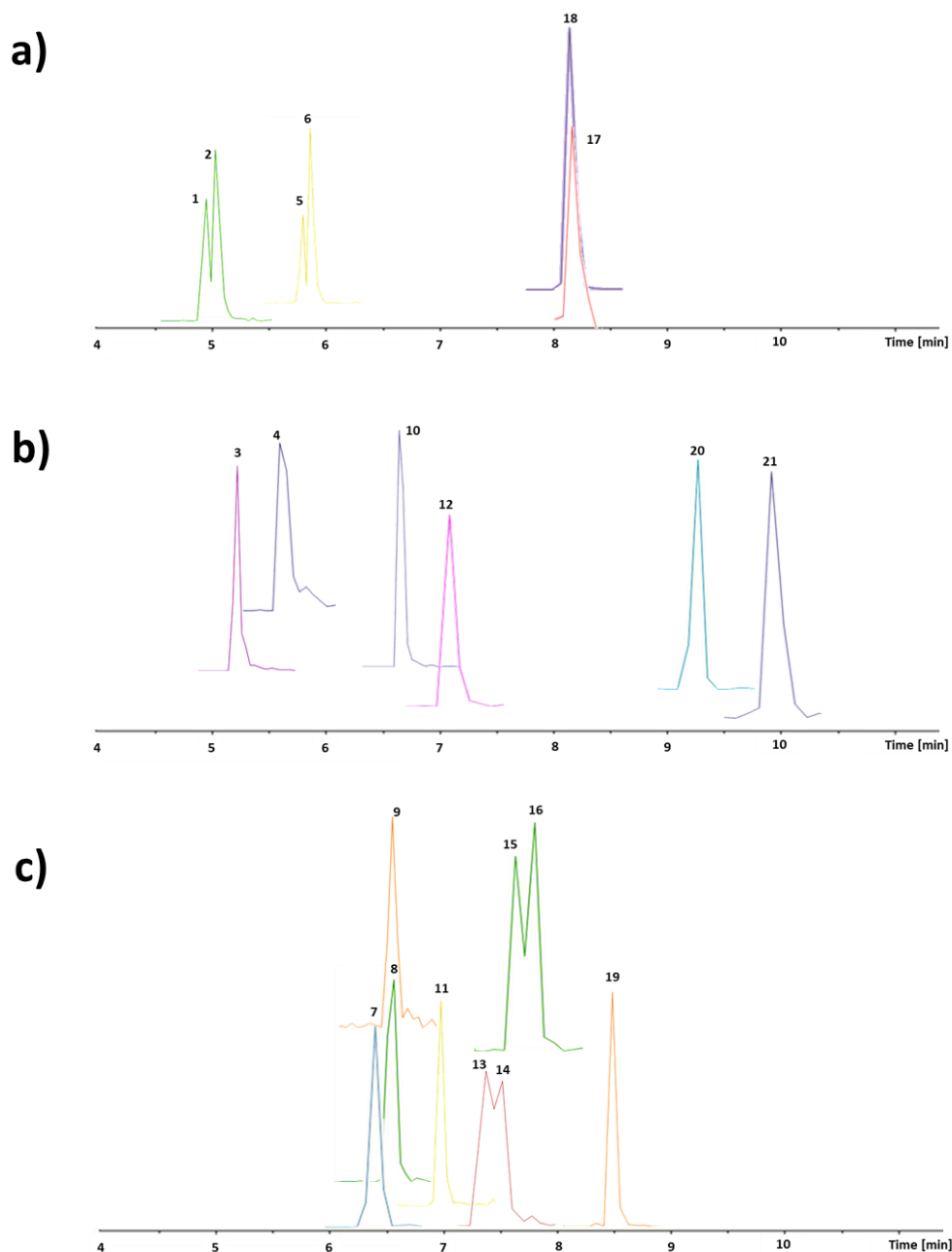


Figure 5. UHPLC separation of the pyrrolizidine alkaloids: (a) lycopsamine-type PAs, (b) heliotrine-type PAs, and (c) senecionine-type PAs. Elution order: intermedine (1), lycopsamine (2), europine (3), europine *N*-oxide (4), intermedine *N*-oxide (5), lycopsamine *N*-oxide (6), retrorsine (7), retrorsine *N*-oxide (8), seneciphylline (9), heliotrine (10), seneciphylline *N*-oxide (11), heliotrine *N*-oxide (12), senecivernine (13), senecionine (14), senecivernine *N*-oxide (15), senecionine *N*-oxide (16), echimidine (17), echimidine *N*-oxide (18), senkirkin (19), lasiocarpine (20), lasiocarpine *N*-oxide (21).

RESULTS

Analytical Parameters

Good analytical performance was achieved in terms of accuracy and precision using the extraction procedure proposed with the μ SPEed technique according to the criteria set in the European Commission SANTE/12682/2019 document and in regulation EC No 401/2006.

The accuracy was assessed in terms of recovery for each type of infusion. Recovery assays were carried out by spiking the infusions at a concentration level of 5 μ g/L (for each analyte) and, afterwards, subjecting them to the microextraction procedure. Additionally, the same procedure was carried out with a standard prepared in water at the same concentration level. The areas obtained from the chromatographic analysis of these sample extracts were then compared with the areas obtained from the analysis of simulated sample extracts (nonspiked infusion samples subjected to the microextraction procedure and spiked afterward their extraction at the same concentration level before their chromatographic analysis). The results were expressed as the mean recovery obtained from twelve samples ($n = 12$) extracted in different days. According to the validation guidelines, the recovery values should be between 70 and 120%, so as it is showed in Table 1, this was fully accomplished as the overall average recovery values were in the range 79-103, 76-101, and 76-100% in the linden, chamomile and green tea with mint matrices, respectively.

Method precision (expressed as relative standard deviation percentage, RSD%) was evaluated for each herbal infusion in terms of intra-day (repeatability) and inter-day (reproducibility) precision at a concentration level of 5 μ g/L (for each analyte). Intraday precision was assessed with the recovery values of six replicate extracts ($n = 6$) obtained on the same day from an infusion sample spiked with the analytes. Inter-day precision was determined through the recovery of replicate extracts of a sample spiked with the analytes, which were carried out throughout three different days ($n = 12$). Additionally, the same procedure was carried out with a standard prepared in water at the same concentration level. According to the validation recommendations, RSD values for the precision parameters should be $\leq 20\%$. Therefore, the method precision was also satisfactory in the three matrices, as RSD values for the target analytes were in all cases $\leq 20\%$ (Table 2). As it can be observed, for intra-day precision, the RSD values obtained were lower than 11% and for inter-day precision the results were lower than 15% (Tables 2) in the infusion samples.

CONCLUSION

μ SPEed proved to be a very suitable technique to develop quick and sustainable analytical procedures to monitor the occurrence of PAs in herbal infusion samples, such as linden, chamomile and green tea with mint. It involved minimal consumption of organic solvents and sample (300 μ L of methanol and 300 μ L of sample per extraction), providing high extraction efficiency in very short extraction time (1 min), being easier and more advantageous than other conventional extraction techniques, such as SPE. The method provided suitable overall recoveries (76-103%) and precision ($\leq 15\%$ RSDs). In this sense, the analytical parameters tested fully accomplished the validation guidelines. Therefore, the good analytical performance of the μ SPEed procedure developed was demonstrated, allowing its reliable application to the analysis of PAs in herbal infusions samples and contributing to improve the quality control and food safety of these products.

ACKNOWLEDGEMENTS

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Table 1. Mean recoveries (% \pm SD) obtained using the μ SPEed procedure proposed for the determination of PAs in a standard solution with water and different infusion samples (linden, chamomile and green tea with mint)

Pyrrolizidine alkaloid	Standards in water	Linden infusion	Chamomile infusion	Green tea with mint infusion
Intermedine	96 \pm 3	84 \pm 9	87 \pm 11	98 \pm 6
Lycopsamine	99 \pm 3	88 \pm 6	89 \pm 7	97 \pm 6
Europine	102 \pm 6	82 \pm 10	87 \pm 6	100 \pm 6
Europine N-oxide	98 \pm 2	103 \pm 8	101 \pm 6	99 \pm 6
Intermedine N-oxide	95 \pm 3	85 \pm 6	82 \pm 8	84 \pm 7
Lycopsamine N-oxide	98 \pm 3	88 \pm 9	80 \pm 9	76 \pm 7
Retrorsine	103 \pm 4	79 \pm 9	91 \pm 9	98 \pm 5
Retrorsine N-oxide	104 \pm 4	93 \pm 7	87 \pm 8	97 \pm 5
Seneciphylline	102 \pm 5	87 \pm 9	80 \pm 6	92 \pm 8
Heliotrine	91 \pm 3	92 \pm 9	82 \pm 8	95 \pm 11
Seneciphylline N-oxide	102 \pm 3	87 \pm 10	94 \pm 9	89 \pm 8
Heliotrine N-oxide	102 \pm 4	98 \pm 9	94 \pm 10	93 \pm 8
Senecivernine	95 \pm 3	84 \pm 6	88 \pm 9	84 \pm 8
Senecionine	103 \pm 4	92 \pm 8	93 \pm 8	96 \pm 6
Senecivernine N-oxide	101 \pm 3	102 \pm 5	92 \pm 5	92 \pm 7
Senecionine N-oxide	97 \pm 5	92 \pm 9	76 \pm 6	81 \pm 8
Echimidine	101 \pm 2	90 \pm 7	86 \pm 9	97 \pm 7
Echimidine N-oxide	98 \pm 4	92 \pm 6	81 \pm 7	98 \pm 7
Senkirkin	105 \pm 5	94 \pm 6	79 \pm 6	86 \pm 11
Lasiocarpine	97 \pm 2	87 \pm 13	90 \pm 12	96 \pm 10
Lasiocarpine N-oxide	103 \pm 2	93 \pm 5	84 \pm 11	91 \pm 10

Table 2. Precision (RSD %) in terms of intra-day and inter-day precision of the μ SPeEd procedure proposed for the determination of PAs in a standard solution with water and different infusion samples (linden, chamomile and green tea with mint)

Pyrrolizidine alkaloid	Intra-day precision (RSD%)				Inter-day precision (RSD%)			
	Standards in water	Linden infusion	Chamomile infusion	Green tea with mint infusion	Standards in water	Linden infusion	Chamomile infusion	Green tea with mint infusion
Intermedine	1	9	8	7	3	10	13	7
Lycopsamine	2	5	8	7	3	7	8	7
Europine	1	3	4	5	6	12	7	6
Europine N-oxide	1	7	3	6	2	8	6	7
Intermedine N-oxide	2	7	9	6	3	7	10	9
Lycopsamine N-oxide	2	10	6	8	3	11	11	10
Retrorsine	3	7	8	3	4	11	10	5
Retrorsine N-oxide	4	7	8	4	5	7	9	6
Seneciphylline	5	5	5	7	5	11	7	8
Heliotrine	2	7	8	6	4	10	10	12
Seneciphylline N-oxide	3	11	9	9	3	12	9	10
Heliotrine N-oxide	3	9	10	3	4	10	10	9
Senecivernine	3	5	6	4	3	7	11	9
Senecionine	4	5	5	5	5	9	9	6
Senecivernine N-oxide	3	4	4	6	3	5	5	7
Senecionine N-oxide	2	9	7	10	5	10	8	11
Echimidine	2	6	5	3	2	8	11	7
Echimidine N-oxide	2	5	4	5	4	7	8	7
Senkirkin	4	2	7	6	5	7	8	13
Lasiocarpine	2	8	8	5	2	15	14	10
Lasiocarpine N-oxide	1	5	8	11	2	6	13	14

REFERENCES

- [1] Dusemund, B., Nowak, N., Sommerfeld, C., Lindtner, O., Schäfer, B., & Lampen, A. (2018). Risk assessment of pyrrolizidine alkaloids in food of plant and animal origin. *Food and Chemical Toxicology*, 115, 63–72.
- [2] Casado, N., Morante-Zarcero, S., Sierra, I. (2022). The concerning food safety issue of pyrrolizidine alkaloids: An overview. *Trends in Food Science & Technology*, 120, 123–139.
- [3] Commission Regulation (Eu). 2020/2040 of 11 December 2020, amending Regulation (EC) No 1881/2006 as regards maximum levels of pyrrolizidine alkaloids in certain foodstuffs. Available online at: <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020R2040&from=ES>.
- [4] Casado, N., Morante-Zarcero, S., Sierra, I. (2022). Application of the QuEChERS strategy as a useful sample preparation tool for the multiresidue determination of pyrrolizidine alkaloids in food and feed samples: A critical overview. *Applied Sciences*, 12, 4325.
- [5] Casado, N., Fernández-Pintor, B., Morante-Zarcero, S., Sierra, I. (2022). Quick and green microextraction of pyrrolizidine alkaloids from infusions of mallow, calendula and hibiscus flowers using ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry analysis. *Journal of Agricultural and Food Chemistry*, 70, 7826-7841.
- [6] Izcara, S., Casado N., Morante-Zarcero, S., Sierra, I. (2020). A miniaturized QuEChERS method combined with ultrahigh liquid chromatography coupled to tandem mass spectrometry for the analysis of pyrrolizidine alkaloids in oregano samples. *Foods*, 9, 1319.



