Application Note

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µSPEed extraction and HPLC-MS/MS analysis of atropine and scopolamine in herbal infusions

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SUMMARY

The innovative µSPEed technique using digiVOL Digital Syringe (Figure 1) has been applied for the accurate and precise extraction and pre-concentration of atropine and scopolamine from different herbal infusions (rosemary, valerian and echinacea) using a PS-DVB polymeric-based cartridge. The infusion sample has been loaded 5 × 500 µL and, atropine and scopolamine have been eluted with a small amount of methanol (2 × 100 µL), pre-concentrating the analytes 12.5-fold before analysis by HPLC-MS/MS.

Figure 1. DigiVOL Digital Syringe and accessories (cartridges and syringes)

INTRODUCTION

Tropane alkaloids (TAs) are a family of natural toxins with antimuscarinic effects produced as secondary metabolites by different families of plants such as Brassicaceae or Solanaceae, among others.[1] This family is made up of more than 200 types of TAs. All of them are characterized by having a common chemical structure known as the tropane skeleton, being atropine (or (±)-hyoscyamine) and hyoscine (or (-)-scopolamine) the most representative compounds of this group (Figure 2)

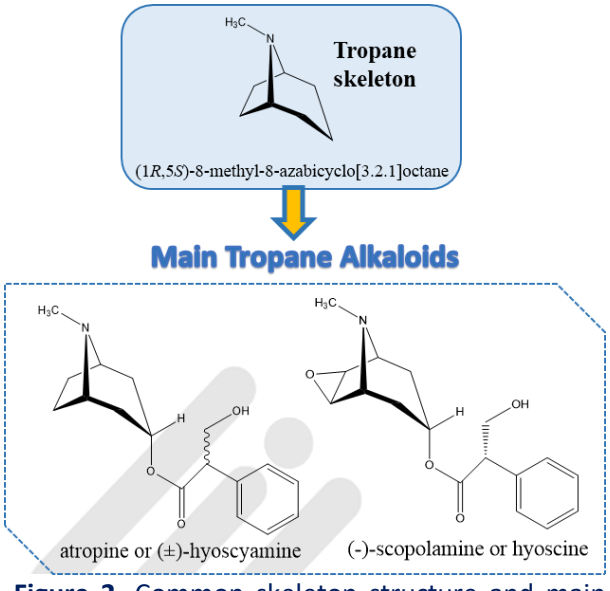

Figure 2. Common skeleton structure and main tropane alkaloids

The presence of TAs in food due to the contamination of crops with TA-producing plants is causing serious health consequences. This is because TAs are anticholinergic compounds which can generate symptoms such as tachycardia, muscle spasms, mydriasis, delirium and even death if high concentrations are consumed. The Solanaceae family is the most widespread source of accidental TAs consumption due to the highest content of these natural toxins.[2] *Datura, Brugmansia, Atropa, Hyoscyamus and Mandragora* are some of the most

problematic genera of this family. For this reason, the European Food Safety Authority (EFSA) has recommended the analysis of TAs in different matrices and has established an acute reference dose (ARfD) of 0.016 µg/kg body weight expressed as the sum of (-)-hyoscyamine and (-)-scopolamine.[1] In 2016, a maximum limit was set at 1 µg/kg of atropine and 1 µg/kg of scopolamine for processed cereal-based foods and baby foods for infants and young children[3]. And after several scientific opinions, research articles and notifications on the European Rapid Alert System for the Food and Feed (RASFF) portal demonstrating the presence of these compounds in different foods, in 2021 the European Union (EU) set maximum limits expressed as the sum of atropine and scopolamine for unprocessed or processed millet, sorghum, maize, maize for popping and buckwheat (5–15 µg/kg) and herbal infusions (25–50 µg/kg in dried products and 0.2 µg/L in liquid products).[4] To achieve these low limits, it is recommended to use high-performance liquid chromatography (HPLC) techniques coupled to mass (MS) or mass/mass (MS/MS) detectors.[5] However, sample preparation is a very important step to avoid interference in the analysis and reach the limits required by legislation. To date, solid-liquid extraction has been the most widely used technique, followed by solid-phase extraction or the QuEChERS procedure.[2] These conventional techniques require high time and reagent consumption and have high residue generation.

The innovative µSPEed technique improves on the drawbacks of traditional techniques, helping to achieve the low limits required by legislation thanks to its pre-concentration capability. Therefore, this application note describes an analytical methodology using the µSPEed approach for the extraction of atropine and scopolamine from different herbal infusions. A polymer-based cartridge PS-DVB (cross-linked polystyrene divinylbenzene) was used to load 5 × 500 µL of sample and the target analytes were eluted with 2 × 100 µL of methanol.[6]

PROCEDURE

Sample preparation

Herbal infusions of rosemary (*Rosmarinus officinalis*), valerian (*Valeriana officinalis*) and echinacea (*Echinacea purpurea L.*) were prepared according to International Standard ISO 3103 protocol [7]. For this purpose, 2 g of dried sample was weighed in a paper tea bag and infused with boiling water (100 ˚C). The infusion was covered with a lid for 5 min (Figure 3). After, the paper tea bag was removed, and the sample was cooled to room temperature. Later, the infusion was filtered through a nylon syringe filter (0.45 µm) before µSPEed process.

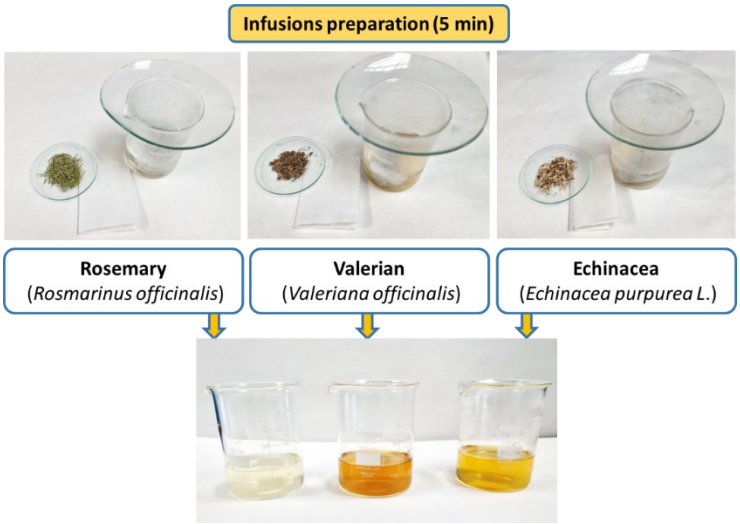

Figure 3. Preparation of herbal infusions samples

µSPEed Extraction Workflow

All extractions were performed on PS/DVB (3 µm/300 Å) µSPEed cartridge by EPREP. First, cartridge was activated with two aspiration-dispense cycles of 500 µL of methanol followed by conditioning with two aspiration-dispense cycles of 250 µL of water. Then, infusion sample was loaded onto µSPEed cartridge (five aspiration-dispense cycles of 500 µL) and eluted with two aspiration-dispense cycles of 100 µL, pre-concentrating the analytes 12.5-fold. All steps were carried out at 800 µL/min in extract-discard mode (each volume aspirated in all cycles was then discarded, after the dispense step, in a waste vial). Figure 4 shows the µSPEed extraction workflow followed and the aspiration-elution working mode.

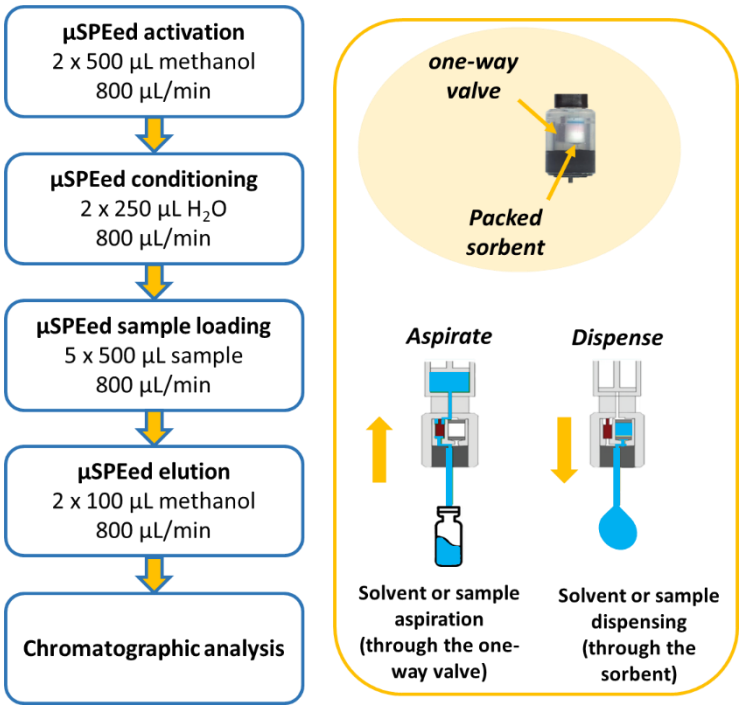

Figure 4. µSPEed extraction workflow and the aspiration-elution working mode.

Chromatographic analysis

The separation (Figure 5) and determination of atropine and scopolamine was carried out with an HPLC system coupled to triple quadrupole mass spectrometer detector with electrospray ionization (ESI) ion source (1200/1200L LC-MS/MS, Varian, Ibérica, Madrid, Spain).

Conditions:

- Column: C18 Kromaphase 100 column (150 mm × 2.0 mm, 3.5 µm particle size) with a C18 Kromaphase guard column (10 mm × 4.0 mm I.D., 5 µm particle size) (Scharlab, Barcelona, Spain)
- Flow rate: 0.250 ml/min
- Injection: 10 µL (partial injection)
- Column temperature: 30 °C

Mobile phase:

- Mobile phase A: milli-Q water containing 0.1% formic acid
- Mobile phase B: acetonitrile containing 0.1% formic acid

Gradient:

- Initial: 90% A / 10% B
- Gradient: change to 30% A / 70% B in 10 min
- Return to the initial conditions in 1 min, held 4 min.
- 1 min equilibration between injections
- Total run-time: 16 min

MS/MS detection:

- ESI in positive ion mode
- Shield: 600 V
- Capillary voltage: 5000 V
- Nebulizer gas: N_2, 58 psi
- Dry gas: N_2, 22 psi
- Dry temperature: 350 °C
- Collision gas: Argon, 1.90 mTorr
- Detector voltage: 1535 V
- Scan mode: Multiple reaction monitoring (MRM)
- Mass peak width Q1 2.5; mass peak width Q3 2.5; scan width in MRM 0.70 seconds
- Cone voltage: 70 V
- Transitions: 290.1 → 90.9 (CE = 34 V), 290 → 93.0 (CE = 29 V) and 290 → 124.1 (CE = 20.5 V) for atropine; 304 → 121.0 (CE = 16 V), 304.1 → 138.1 (CE = 12 V) and 304 → 156.0 (CE = 9.5 V) for scopolamine.

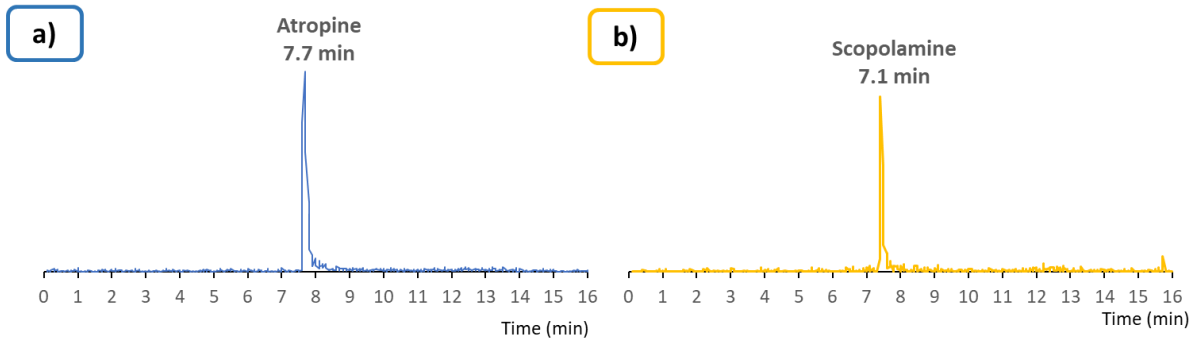

Figure 5. Chromatographic separation of TAs (2.5 µg/L): a) extracted ion chromatogram for atropine (m/z 290.1 > 124.1) and b) extracted ion chromatogram for scopolamine (m/z 304.1 > 138.1)

RESULTS

Analytical Parameters

Good performances of the method were demonstrated in terms of precision and accuracy using the proposed methodology with the µSPEed technique. The results obtained were evaluated according to the criteria established for precision and accuracy in the SANTE/11312/2021 document. [8]

Accuracy was evaluated in terms of recovery percentage (%) for standard solutions at 0.2 µg/L prepared in water and for three herbal infusions (rosemary, echinacea and valerian). The herbal infusions were spiked with an appropriate amount of atropine and scopolamine standard solution to achieve 0.2 µg/L concentration level. The 0.2 µg/L spiked level was selected according to the Commission Regulation (EU) 2021/1408 maximum level concentration established for liquid herbal infusions.[4] The standard solutions and samples were subjected to the µSPEed process before the HPLC-MS/MS analysis. An additional sample of water or infusion (simulated sample, post-extraction doping) was purified con the µSPEed procedure and doped immediately after the µSPEed procedure to estimate the recovery percentage (%). After HPLC-MS/MS analysis, the areas obtained from the sample doped before the µSPEed process and the samples doped after the µSPEed process were compared. The results of six samples purified (n=6) with µSPEed and injected in triplicate are shown in **Table 1**. According to the validation guideline, the recovery percentages should be between 70-120%.[8] The results obtained with the proposed methodology showed good accuracy with

recovery percentages between 95-102% for atropine and between 85-102% for scopolamine in the standard solutions and infusions of rosemary, echinacea and valerian.

On the other hand, the precision of the method was evaluated (expressed as relative standard deviation, RSD%) in the standard solutions and in the infusions. The precision was evaluated in terms of intra-day precision (repeatability), six replicates in one day (n = 6), and inter-day precision (reproducibility), three replicates in three different days (n = 9), all tests at 0.2 µg/L (for each analyte). According to the validation guidelines, RSD values should be ≤20%. **Table 1** shows the intra- and inter-day RSD values for the standard solutions and the three matrices studied, which are all below 20%.[8] Specifically, the intra-day values are ≤8% for both atropine and scopolamine, and the inter-day values are ≤9% for atropine and ≤8% for scopolamine in all matrices studied.

CONCLUSION

The µSPEed technique has proven to be a suitable technique for the extraction and purification of TAs, atropine, and scopolamine in samples of herbal infusions such as rosemary, echinacea and valerian. The method has provided good precision with recovery percentages between 85-102% and good intra- and inter-day accuracy (≤9%) for both analytes in the different matrices applied. These analytical parameters meet the requirements that the validation guides suggest. In addition, this technique is fast and sustainable, allowing the generation of low residue content, since the sample is loaded in water and eluted with a small aliquot of methanol (2 x 100 µL). In this sense, the good performance of µSPEed was demonstrated with its application in infusion samples for the analysis of TAs, contributing to the food safety and green analytical chemistry requirements.

ACKNOWLEDGEMENTS

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Table 1. Accuracy (recoveries ± SD %) and precision (RSD%) obtained using the µSPEed procedure proposed for the determination of atropine and scopolamine in standard solutions prepared in water at 0.2 µg/L and different herbal infusions (rosemary, echinacea and valerian) doped at 0.2 µg/L concentration.

Sample	Tropane alkaloid	Accuracy (recoveries ± SD %) n=6	Intra-day precision (RSD%) n=6, 1 day	Inter-day precision (RSD%) n=9, 3 days
Standards	Atropine	99 ± 3	5	5
	Scopolamine	95 ± 3	4	5
Rosemary infusion	Atropine	102 ± 2	2	4
	Scopolamine	102 ± 6	7	8
Echinacea infusion	Atropine	100 ± 2	5	7
	Scopolamine	102 ± 2	4	6
Valerian infusion	Atropine	95 ± 7	8	9
	Scopolamine	85 ± 11	8	8

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