Green extraction approach based on µSPEed® followed by HPLC-MS/MS for the determination of atropine and scopolamine in tea and herbal tea infusions

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Highlights

- Solid-phase micro-extraction procedure for atropine and scopolamine determination.
- Good analytical performance of the developed methodology.
- Determination of atropine and scopolamine in 17 commercial tea and herbal tea infusions
- Concentrations above the legislated value for almost all liquid herbal infusions.

A B S T R A C T

A high throught methododology based on a green extraction technique, µSPEed®, followed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) has been proposed for the analysis of atropine and scopolamine in tea and herbal tea infusions. For this, a digiVOL® Digital Syringe was used with different sorbents and working conditions to obtain a fast and efficient µSPEed® extraction. The best performance was achieved with a PS/DVB sorbent phase, sample loading of 5 x 500 µL and elution with 2 x 100 µL aliquots of methanol. The strategy based on µSPEed® followed by HPLC-MS/MS was validated, attaining quantitation limits lower than 0.15 ng mL^{-1} and recoveries between 94 -106% for both analytes and applied to seventeen tea and herbal tea infusions. Fourteen infusions showed contamination with one or both analytes above the maximum content legislated (sum of atropine and scopolamine < 0.2 ng mL^{-1}).

Keywords: µSPEed®; atropine; scopolamine; tea and herbal teas; infusions; HPLC-MS/MS.

1. Introduction

Tropane alkaloids are a group of toxic substances with hallucinogenic effects after consumption. Their presence in foods of plant origin is caused by contamination with plant species producing tropane alkaloids, as the ones belonging to the Brassicaceae, Solanaceae and Erythroxylaceae families (Arcella et al., 2018; European Commission, 2013; González-Gómez et al., 2022). Due to the potential risk for human health that the intake of foods contaminated with tropane alkaloids may entail, it is of utmost importance to control their occurrence in foods and, for this reason, in recent years, different analytical methods have been reported. However, food matrices contain a multitude of compounds that make very difficult their analysis (Kanu, 2021; Ridgway et al., 2012). Sample treatment plays an important role in methods for food analysis since it helps to clean up the interfering matrix components and to achieve lower detection limits. An inadequate sample treatment can lead to problems in the analytical instrument or produce interferences in the analysis of the target analytes (Martins et al., 2021; Seidi et al., 2019). Among the extraction/purification techniques used for liquid samples or extracts from solid samples already extracted, solid-phase extraction (SPE) is the most widely applied (Płotka-Wasylka et al., 2017). This is because SPE usually involves analyte enrichment to meet the sensitivity needs of the analytical method. In addition, many types of sorbents are commercially available for SPE and, if suitable material is selected, appropriate interactions between the sorbent and the target analytes can be formed to separate them from the interfering compounds. This can significantly improve the capacity and selectivity of the analytical methodology. Currently, trends in sample preparation are moving towards Green Analytical Chemistry (GAC) requirements (López-Lorente et al., 2022) and, as a consequence, considerable progress has been made in the miniaturization of conventional sorptive extraction techniques (Agrawal et al., 2021). Accordingly,

microextraction by packed sorbents (MEPS), pipette-tip solid-phase extraction (PT-SPE), solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE) or micro-solid-phase extraction (μ-SPE) are techniques that have emerged over the years improving some drawbacks of the traditional SPE technique (Casado et al., 2020; Płotka-Wasylka et al., 2017). The popularity of these formats for food sample preparation is increasing because they need lower sample, solvent and sorbent amounts and perform quicker analysis (Casado et al., 2019). In this way, more environmental friendly and cost-effective sample treatment procedures can be developed. MEPS is an interesting miniaturization of the SPE technique characterized by having a small amount of sorbent (usually 1 - 4 mg, 50 μm diameter) packed in the syringe barrel, as a plug, or between the needle and the barrel as a cartridge (Pereira et al., 2022). As packing is integrated directly into the syringe, MEPS can handle volumes between 10 and 1000 μL, without compromising the extraction efficiency. Another configuration that incorporates some improvements over MEPS is μSPEed®. This format was introduced in the market by EPREP company (Victoria, Australia) and allows more efficient extractions than traditional MEPS (Pereira et al., 2019, 2022). Specifically, μSPEed® cartridges contain sorbents with even smaller particles than MEPS (≤ 3 μm) providing a higher surface area and more efficient separations. They also contain a pressure-driven one-way check valve that supports high pressures up to 1200 psi. This allows a low dead-volume connection and a single way flow path through the sorbent bed in every step of the extraction protocol. μSPEed® cartridges with different sorbents are commercialized, such as unmodified and functionalized silica and polymeric materials, that meet the different retention interactions required with the diverse target analytes (Pereira et al., 2022). Methodologies for polyphenol analysis in different food matrices using μSPEed® have been developed showing that this technique provides faster and cheaper methods than conventional SPE

(Casado et al., 2019; Porto-Figueira et al., 2015). However, as far we may know, methodologies using µSPEed® in other fields of analysis such as food safety were not reported in the literature. The interest in applying this type of technique in the determination of contaminants and toxins is because usually, these toxic compounds appear in minimal concentrations, making their detection difficult. Applying microextractive techniques such as µSPEed® would allow the development of faster, greener and cheaper protocols than traditional methods, with similar recovery rates and higher sensitivity. Moreover, the development of methodologies including µSPEed® extraction for the analysis of contaminants and toxins would allow reaching the low limits established by European Union (EU) legislation for their presence in complex matrices.

Recently, the Commission Regulation (EU) 2021/1408 (European Union, 2021) has established maximum contents of tropane alkaloids (atropine and scopolamine) in processed cereal-based foods and baby foods for infants and young children, in processed and unprocessed cereals (millet, sorghum, maize and buckwheat) and herbal infusions (dried products and liquids). The lowest maximum level allowed, established in 0.2 ng mL^{-1} as the sum of atropine and scopolamine, was set for liquid herbal infusions. An important source of human exposure to tropane alkaloids are infusions prepared with commercial dried tea and herbal teas contaminated with weeds producing tropane alkaloids (Arcella et al., 2018; European Commission, 2013). These beverages are gaining popularity due to their high content in natural bioactive compounds and there are a wide variety of such products in the market (Etheridge & Derbyshire, 2020; Kamiloglu et al., 2016). Consequently, the risk for the consumption of infusions containing high levels of atropine and scopolamine is growing very significantly. Generally, tea and herbal tea samples are analyzed as dried products by methodologies that include solid-liquid extraction (SLE) (Cirlini et al., 2019) or SLE followed by a SPE purification step

with polymeric cationic mixed-mode sorbents (Marín-Sáez et al., 2017; Marín-Sáez et al., 2019; Mulder et al., 2016; Romera-Torres et al., 2018). Specifically, the commercial Strata-X-C® cartridge evaluated by the group of Garrido Frenich, contains a polymeric sorbent chemically modified with polar and anionic functional groups. Besides the strong cation exchange interactions, this sorbent offers other retention mechanisms such as hydrophobic, hydrogen bonding and π-π that allow good recoveries for the basic target analytes. QuEChERS methodologies have been also recently used for the analysis of tropane alkaloids in dry samples of tea and herbs for infusions (León et al., 2022). However, besides the type of dry herbal product, the extraction technique and conditions employed affect significantly the recovery of tropane alkaloids in the analysed extracts. Moreover, preparation of tea and herbal tea infusions requires a heating step with boiling water for a few minutes that affects the transfer rate of tropane alkaloids, has been evidenced for brews (around 50% depending on the source of contamination) (Marín-Sáez et al., 2019; Mulder et al., 2016). Therefore, it is necessary to develop quick and sensitive analytical methods to determine the presence of both alkaloids in the beverages and to ensure compliance with Commission Regulation (EU) 2021/1408 regarding alkaloids presence in herbal infusions. Therefore, the main aim of this work was to develop and validate for the first time a powerful analytical methodology based on the use of µSPEed® extraction followed by HPLC-MS/MS for the quick and sensitive analysis of atropine and scopolamine in infusions of tea and herbal teas.

2. Materials and methods

2.1 Chemical reagents and materials

Atropine sulphate (≥ 99 %) and scopolamine hydrobromide (≥ 98 %) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH),

ammonia solution (32%) and formic acid (FA) LC–MS grade were purchased from Fischer Scientific (Loughborough, UK). Hydrochloric acid (HCl, 37%, 36.46 g mol $^{-1}$, 1.19 g mL^{-1}) were purchased from Panreac (Barcelona, Spain). Ultra-pure deionized water (18.2 MΩ cm quality) was obtained using a Millipore Milli-Q-System (Billerica, MA, USA) and was used for the preparation of mobile phases and aqueous solutions. Nylon syringe filters (0.45 µm) for the filtration of tea infusions and nylon filter membranes (0.45 µm) for mobile phase filtration were acquired from Scharlau (Barcelona, Spain).

The digiVOL® Digital Syringe and the commercial µSPEed cartridges tested were acquired to EPREP (Mulgrave, Victoria, Australia). Nine types of cartridges of different chemical nature, six silica-based (C4, 3µm/300 Å; C8, 3µm/120 Å; C18, 3µm/120 Å; C18/P-hydrophillic ODS, 3µm/120 Å; PFAS, 3µm/120 Å and silica, 3µm/120 Å) and three polymeric-based (PS/DVB, 3µm/300 Å; PS/DVB-RP, phenyl, 3µm/300 Å; PS/DVB SCX, 3µm/non-porous), were tested during the optimization of the method.

2.2 Standards solutions

Stock standard solutions of atropine (1000 µg mL^{-1}) and scopolamine (1000 µg mL^{-1}) were prepared by diluting the appropriate amount in MeOH. The resulting solutions were stored in the dark at -20 °C. The working solutions containing atropine and scopolamine were prepared by diluting the individual stock solutions (1000 µg mL^{-1}) in MeOH. The solvent calibration curve was prepared in MeOH in a range of 1.25-313 ng mL^{-1}.

2.3 Samples

Seventeen tea and herbal tea commercial samples were purchased from local markets in Funchal (Portugal) and Madrid (Spain). Specifically, the samples studied were three samples of lemon balm (Lembal), one sample of lemon verbena (Lemver), four samples of linden (Lin), two samples of camomile (Cam), four samples of peppermint (Pep) and three samples of green tea (Greetea). The samples were stored in the dark at room temperature and in a dry place until analysis.

2.4 Tea and herbal tea infusion preparation

Infusions of tea and herbal teas were prepared according to International Standard ISO 3103 protocol (ISO, 2019). For it, 2.00 ± 0.01 g of sample was weighted in a stainless-steel tea filter strainer, which was placed in a porcelain pot-cup and 100 mL of boiling ultra-pure deionized water was added. The infusion was covered with a lid for 5 min. After, the stainless-steel tea filter strainer was removed, and the sample was cooled to room temperature. Later, the infusion was filtered through a nylon syringe filter (0.45 µm) before being purified by µSPEed®. Three consecutive infusions were prepared by taking three different portions of 2 g from each sample.

2.5 µSPEed® extraction procedure of tropane alkaloids

The µSPEed® procedure was carried out using a 500 µL syringe at a constant flow rate of 800 µL min^{-1}. Fig. 1 shows the optimized operating conditions for the sample treatment. First, the cartridge (previously activated with 2 × 500 µL of MeOH) was conditioned with two different 250 µL aliquots of water (2 × 250 µL). Later, five different 500 µL aliquots of the filtered infusion were loaded into the cartridge (500 µL, 5 cycles in extract-discard mode). No washing step was carried out. Then, analytes were eluted

into a vial with two different 100 µL aliquots of MeOH (2 × 100 µL). The extract collected in the vial was directly injected into HPLC-MS/MS. Between each extraction, to ensure the reuse of the cartridge and avoid the memory effect, the cartridge was rinsed with two different 500 µL aliquots of MeOH (2 × 500 µL). Between days and after storage, the cartridges were activated with two aliquots of MeOH (2 × 500 µL). Each cartridge was reused more and 100 times.

2.6 HPLC-MS/MS analysis

The analysis of atropine and scopolamine was carried out on Varian 1200/1200L LC-MS/MS (Varian Ibérica, Spain). The equipment used contained two solvents delivery modules (ProStar 210/215), an autosampler equipped with a 100 µL loop (ProStar 410), a column heater section and a triple quadrupole mass spectrometer detector (1200L TQ) with electrospray ionization (ESI) ion source. Data acquisition was made in a system MS Workstation (version 6.3). Separation was achieved at 30ºC using a reverse C18 Kromaphase 100 column (150 mm × 2.0 mm, 3.5 µm particle size) coupled to C18 Kromaphase guard column (10 mm × 4.0 mm, 5 µm particle size). The column and guard column were acquired from Scharlab (Barcelona, Spain). A gradient program was used combining solvent A (ACN) and solvent B (ultra-pure deionized water) both phases with 0.1% formic acid as follows: from 10% to 70% A in 10 min, from 70% to 10% A in 1 min and 10% A for 4 min. The total run was 15 min. The flow rate was 0.25 mL min^{-1} and the injection volume was 10 µL (partial injection). MS data acquisition was carried out using ESI in positive ion mode. The parameters established in the MS detector were set at 350 ºC and 22 psi for drying gas (N_2), 58 psi for nebulizer gas pressure (N_2), 5000 V for capillary voltage, 600 V for shield, 1.90 mTorr for collision gas (argon) and 1480 V for the detector voltage. The ionization source parameters were optimized by direct

infusion of an individual standard solution of atropine and scopolamine in MeOH (10 µg mL^{-1}) at a flow rate of 20 µL min $^{-1}$. Multiple reaction monitoring (MRM) mode was used for atropine and scopolamine detection (mass peak width Q1 2.5; mass peak width Q3 2.5; scan width 0.70). The precursor ion for atropine was 290.1 m/z (70 V cone voltage) and the product ions were 124.1 m/z, 93.0 m/z and 90.9 m/z with a collision energy of 20.5 V, 29 V and 34 V, respectively and a dwell time of 0.25 s. The precursor ion for scopolamine was 304.1 m/z (70 V cone voltage) and the product ions were 156.0 m/z, 138.1 m/z and 121.0 m/z with a collision energy of 9.5 V, 12 V and 16 V, respectively and a dwell time of 0.25 s. The quantification was carried out with the product ion 124.1 m/z for atropine and 138.1 m/z for scopolamine.

2.7 Method validation

The methodology based on µSPEed® followed by HPLC-MS/MS was validated in terms of linearity, matrix effect (ME), method detection (MDL) and quantification (MQL) limits, selectivity, accuracy, and precision, and follow the recommendations of Guidance SANTE/12682/2019 (European Union, 2019) for pesticides, AOAC International Guidelines for Dietary Supplements and Botanicals (AOAC International, 2013) and IUPAC harmonized guidelines for single-laboratory validation (Thompson et al., 2002). These guidelines were selected because there is no specific validation recommendations for these types of analytes. A lemon balm sample (Lembal-1) was used for full validation of the method (linearity, ME, MDL, MQL, selectivity, accuracy and precision). For lemon verbena, linden, chamomile, peppermint and green tea, some analytical parameters were assessed using a representative sample of each infusion, such as linearity, ME, MDL and MQL.

To evaluate the linearity, matrix-matched calibration curves were prepared for each infusion sample type and analyte. For this, the tea or herbal infusion were prepared according to section 2.4 and the optimized µSPEed® procedure was carried out. Then, an appropriate amount of standard solution was added to the extracts to obtain the desired concentration level in the calibration curve. Simultaneously, a blank sample was treated with the same protocol to correct the signal in case the sample was contaminated with atropine or scopolamine. Eight points of the matrix-matched calibration curve were prepared between 1.25-313 ng mL^{-1}. A total of six matrix-matched calibration curves were prepared for each kind of infusion. Linearity was determined through the correlation coefficient (R^2) of each calibration curve obtained by plotting the peak area of atropine or scopolamine versus the concentration of each point of matrix-matched calibration injected in triplicate in the HPLC-MS/MS. The ME was verified in each sample by comparing the slope of the matrix-matched calibration obtained with the slope of the solvent calibration curve following the next equation: ((slope matrix-matched/slope solvent-based) − 1) × 100) (Moussa et al., 2020), both expressed in the same units and concentrations. Positive values obtained after the application of this formula mean an increase in the signal and negative values mean suppression of the signal. The ME is considered important if it exceeds +/- 20% (European Union, 2019). On the other hand, MDL and MQL were calculated as three and ten times the standard deviation for the lowest concentration in the fortified sample (0.1 ng mL^{-1}). Selectivity was tested as the absence of signal when comparing a spiked and unspiked sample without contamination. Also, the contaminated samples were compared with the doped samples to ensure that the retention time (t_R) was ± 0.1 min and ion transition ratios in unit mass resolution were verified to check that the deviation was not more than 30% (relative abundance). The accuracy and precision were evaluated by spiking 100 mL of the infusion with an

appropriate amount of atropine and scopolamine standard solution to achieve four different concentration levels: 0.1, 0.2, 5 and 25 ng mL^{-1} . The levels were selected as the lowest (0.1 ng mL^{-1}) near to the MQL, 0.2 ng mL^{-1} and according to the maximum content in liquid herbal infusions (sum of atropine and scopolamine) established in the Regulation 2021/1408 (European Union, 2021), 5 ng mL^{-1} as an intermediate level and 25 ng mL^{-1} as a high level, that correspond to the highest point of the calibration curve. The accuracy was evaluated in terms of recovery and, for this, six lemon balm infusions (n = 6) were prepared and spiked at the corresponding concentration of each level (four levels) before the µSPEed® procedure (pre-extraction spike). An additional sample (simulated sample, post-extraction spike) was purified and doped at the end of the procedure to estimate the recovery (%). The doped and the simulated samples were injected in triplicate. Precision expressed as relative standard deviation (RSD%) was evaluated as intra- and inter-day. Intra-day precision showed the repeatability of the method by evaluating six sample replicates in one day (n = 6) at the four levels evaluated. Inter-day precision evaluated the intermediate-precision of three sample replicates between three different days (n = 9). Each day three samples were evaluated at the four validated levels.

3. Results and discussion

3.1 µSPEed® optimization

The optimization of the µSPEed® extraction procedure involves different steps that include the selection of the best sorbent, flow rate, sample loading and elution cycles, loading and elution solvents.

3.1.1 Preliminary studies

Firstly, the preliminary studies assayed the extraction efficiency and reproducibility of only three type of sorbents, by setting conditioning and loading conditions, and different elution solvents. These cartridges, PS/DVB-SCX, C18 and PS/DVB-RP, were selected according to the literature. For the purification of basic tropane alkaloids, mixed-mode cation exchange polymeric cartridges have been used (González-Gómez et al., 2022). For this reason, the non-porous polymeric PS/DVB-SCX commercially available for µSPEed® was selected for the preliminary assays. On the other hand, silica-based C18 it is a highly used type of cartridge and, according to Papadoyannis et al. (1993), it shows better results compared to other sorbents. Additionally, a third cartridge (PS/DVB-RP) with reversed-phase (phenyl) porous polymeric nature sorbent was included in the preliminary study. In PS/DVB-RP cartridges, the electron density of the aromatic ring provides high selectivity for aromatics compounds compared to the other non-polar phases caused by the π-π interactions. In addition, polymeric sorbents exhibit higher loading capacities than the silica-based C18 so the inclusion of this sorbent in the preliminary assays with allow us to have a snapshot of the different chemistries, specifities and loading capacities of the µSPEed® sorbent commercially available.

Due to the solubility of tropane alkaloids in acidic aqueous solutions (Dräger, 2002) and the aqueous media of the sample infusions to be analysed in this work, water, FA 1% (pH 2.3) and HCl 0.1 M (pH 1.3) were selected as sorbent activation solvents. The cartridges were conditioned firstly with 250 µL of water, followed by other 250 µL of water, FA 1% or HCl 0.1 M. After, 500 µL of a standard solution (0.5 µg mL^{-1}) prepared in each selected solvent was loaded into the cartridge (ten loading/withdraw cycles of the same solution). This high concentration was selected to assess the capacity of the sorbent. The flow rate was set at 500 µL min^{-1}. Next, the analytes were eluted with

250 µL of MeOH followed by 250 µL of water. The results showed the best recoveries, close to 100%, for the loading solvents water and FA 1% with the PS/DVB-RP cartridge (Fig. 2A). In this sorbent, π - π interactions between the aromatic groups of the polymer and the target analytes allow higher retention. On the other hand, as shown in Fig. 2A, the non-porous sorbent PS/DVB-SCX provided the worse extraction efficiency for both target analytes, which can be justified by the lower superficial area of interaction between the sorbent particles and the alkaloids. Further, three flow rates, 250, 500 and 800 µL min^{-1}, were also assayed but no differences were found in terms of recovery percentages. For this reason, for the rest of the optimization, 800 µL min^{-1} was set to reduce the time for each extraction.

On the other hand, the elution step was optimized by using FA 1% as loading solvent and different elution solvents: MeOH, ACN, and MeOH-water and MeOH-ammonia (10%) sequential elutions. In these elutions mixtures, first 100 µL of MeOH were passed through the cartridge and then 100 µL of water or ammonia solution (10%). Fig. 2B shows the results obtained in these experiments, being MeOH the best elution solvent. Moreover, a 2 × 100 µL elution was enough to achieve good recovery percentages for both analytes in the case of PS/DVB-RP sorbent.

Recently, the new Commission Regulation (EU) 2021/1408 (European Union, 2021) has established a maximum content of 0.2 ng mL^{-1}, as the sum of atropine and scopolamine, in herbal infusions (liquids). For this reason, this concentration was fixed for the following optimization test. Following this preliminary assay with PS/DVB-SCX, PS/DVB-RP and C18 cartridges, six additional sorbent were included in the study. The PS/DVB (cross-linked polystyrene divinylbenzene) was evaluated because it is a non-functionalized polymeric sorbent and, in consequence, cheaper and greener. PS-DVB has a non-polar retention mechanism and interactions with tropane alkaloids are through π-π

interactions between the aromatic rings of their polymeric structure and the aromatic rings of the target anaytes. On the other hand, besides the conventional C18, other silica-based sorbents with different ligands, including bare silica, C4, C8, PFAS and C18/P-ODS, a modified C18 recently designed to process large volume of aqueous samples. Water and FA 1% were used as sample loading solvents and 2 × 100 µL of MeOH was used for the elution step. As it can be seen in Fig. 2C and 2D, good results were obtained for PS/DVB-RP (recovery between 100-114 %), PS/DVB (between 83-113%) and C18/P-hydrophilic ODS (between 80-113%) for both analytes, so these sorbents were selected for the optimization studies with infusion samples. On the other hand, since recoveries were not improved by using acidified water as sample loading solvent, infusions were extracted without the addition of FA.

3.1.2 Evaluation of the best sorbents for µSPEed® extraction of infusion samples

The best performning cartridges (PS/DVB-RP, PS/DVB, C18/P-ODS) were conditioned with 2 × 250 µL of water at 800 µL min^{-1} flow rate. Later, ten different 5 mL peppermint, lemon balm or linden infusion aliquots (spiked at 0.2 ng mL^{-1}) were loaded into the cartridge (10 loading cycles of 500 µL in extract-discard mode). In these assays, the loading cycles were increased to ten to increase the preconcentration. The analytes were eluted with two aliquots of 100 µL of MeOH (2 × 100 µL). To determine the recovery percentage, infusion samples spiked at the end of the extraction process (simulated sample) were compared with spiked samples at the beginning. All assays were performed in duplicate. The results of these experiments are shown in Table 1. In general, PS-DVB-RP provided acceptable recoveries for the three samples, between 70-105% for atropine and 70-93% for scopolamine. PS-DVB showed similar recoveries for atropine, 70-114%, and slightly lower for scopolamine, between 60-97%. These two cartridges

have similar characteristics, both are cross-linked polystyrene divinylbenzene cartridges, but PS-DVB-RP contains additional phenyl groups in its structure and it is ideal for the extraction from aqueous samples of polar analytes with aromatic rings that are not adequately retained on PS-DVB sorbents. On the contrary, although C18/P-hydrophilic ODS is a silica-based cartridge designed for aqueous samples, it was the cartridge that provided the worse results for both analytes in peppermint and scopolamine in lemon balm (range: 11-59 %). On the other hand, experiments were carried out to reduce to half (5×500 µL) the number of sample loading cycles to check if a washing effect could be occurring due to the large sample volume used (10×500 µL). In these experiments, six infusions, peppermint, lemon balm, linden, lemon verbena, camomile and green tea, were analysed and the best recoveries were obtained with the PS/DVB cartridge (between 91-105% for atropine and 95-115% for scopolamine). PS/DVB-RP and C18/P-hydrophilic ODS cartridges showed low recoveries in some samples, especially for scopolamine (Table 1). The different beaviour ot PS/DVB-RP cartriges in the infusion samples can be explained because the phenyl group on its structure favours the π-π interactions with other matrix compounds, such as phenolic compounds, that reduce the retention of the tropane alkaloids in the sorbent. Thus, PS-DVB was selected as the best cartridge, and to verify its capacity to retain higher amounts of the target analytes, 5 different aliquots of 5 ng mL^{-1} (500 µL, 5 cycles in extract-discard mode) were loaded. The results obtained at this concentration were similar to those obtained at the low concentration (0.2 ng mL^{-1}) with recoveries in the range 92-105% for atropine and 91-107% for scopolamine. This is a demonstration of the high loading capacity of the sorbent.

As far as we know, only one method has been previously developed and validated for the analysis of atropine and scopolamine in (herbal) tea infusions (Mulder et al., 2016). In this protocol, 37.5 mL of the tea infusion were loaded in an OASIS MCX cartridge for

SPE clean-up (150 mg of sorbent, Waters USA). MeOH was used to conditionate the cartridge (6 mL) and 1% formic acid in water was used to equilibrate the cartridge (6 mL). The washing step was carried out with 6 mL of MeOH/water/FA, 75/25/1 *v*/*v*/*v* and the cartridge was dried under vacuum for 5-10 min. Last, analytes were eluted with 6 mL of methanol containing 0.5% ammonia. This procedure involves large amounts of sample, organic solvents and time than those proposed in the present paper, which are overcomed by the the miniaturized and high-throughput methodology here proposed. Moreover, according to the ten principles of green sample preparation (López-Lorente et al., 2022), reusable and renewable materials are used in the proposed µSPEed® extraction protocol (because each cartridge can be used multiple times), the sample, chemical and materials amounts are minimized and the sample throughput is maximized. In addition, compared with protocols where dry (herbal) tea samples are analyzed, no acids are employed in the extraction step that is the main source of chemical waste generation in sample preparation. And finally, the method here proposed can be easily scaled to automatic and highthroughput systems using the ePrep® Sample Preparation Workstation (EPREP, Australia).

3.2 Validation of proposed method based on µSPEed® followed by HPLC-MS/MS

Table 2 shows the linearity, matrix-matched calibration curve for each sample, MDL, MQL and ME for atropine and scopolamine. Matrix-matched calibration curves showed good linear regression with R^2 between 0.998-1.000 in the six samples studied for both analytes. Regarding the limits, the proposed method showed low MQL between 0.09-0.12 ng mL^{-1}-for atropine and between 0.06-0.15 ng mL^{-1} for scopolamine and MDL 0.03-0.04 ng mL^{-1} for atropine and 0.02-0.05 ng mL^{-1} for scopolamine. All limits are lower than the maximum content set for liquid herbal infusions (0.2 ng mL^{-1}), published in the

Regulation (EU) 2021/1408 (European Union, 2021) that is the official regulation for the maximum levels of tropane alkaloids in foods. The ME was also evaluated in the six samples studied. As it can be seen in Table 2, no significant ME was found in the lemon balm and camomile samples since ME does not exceed +/- 20% according to the SANTE/12682/2019 document (European Union, 2019) and a slight positive ME was observed for atropine in the lemon verbena and for both analytes in peppermint and green tea infusion samples. On the contrary, in the linden sample, a large positive ME greater than 20% was found. The selectivity of the method was demonstrated with contaminated, uncontaminated and spiked samples. Firstly, it was verified that the t_R of both analytes in the sample extracts corresponded to that of the matrix-matched calibration standards, with an SD lower than ± 0.1 min (Fig. S1, see supplementary material). On the other hand, the absence of interfering peaks in uncontaminated samples was evaluated. Fig. S1 shows the absence of interfering peaks at t_R of atropine and scopolamine for an uncontaminated sample of lemon balm compared to a sample contaminated with atropine and a sample spiked with atropine and scopolamine at 0.2 ng mL^{-1}. Additionally, ion transition ratios in unit mass resolution MS/MS were verified in contaminated samples and did not deviate more than 30% (relative abundance) from the value obtained in the corresponding spiked samples.

Finally, the method based on µSPEed® followed by HPLC-MS/MS was validated for precision and accuracy in a representative sample of lemon balm at four concentration levels (0.1, 0.2, 5 and 25 ng mL^{-1}). The results shown in Table 3 show good accuracy in the four validated levels with recovery percentages between 94-103% for atropine and between 94-106% for scopolamine. On the other hand, precision also showed satisfactory results with RSD (%) ≤ 8% for atropine and scopolamine in intra-day precision and RSD (%) ≤ 10 % for atropine and ≤ 8% for scopolamine in inter-day precision.

3.3 Application of the validated methodology in infusions of tea and herbal teas

The methodology developed and validated was applied to seventeen infusions of lemon balm, lemon verbena, linden, camomile, peppermint and green tea. These samples were selected because they are popular to prepare hot infusions in Spain and Portugal. In addition, the exposure to tropane alkaloids from contaminated tea and herbal tea samples has also been documented and more information about the appearance of these toxic compounds in this type of samples has been suggested by the European Food Safety Authority (European Commission, 2013; Mulder et al., 2016). Each infusion was prepared in triplicate and the extract obtained after the µSPEed® protocol was injected three times in the HPLC-MS/MS. The peaks obtained in the chromatograms of the contaminated samples were integrated and matrix-matched calibration was applied.

Table 4 collects the results of the samples analyzed with the proposed methodology. Fourteen of the seventeen samples of infusion studied showed contamination with one of the analytes, or both, above the maximum content legislated in liquid herbal infusions (0.2 ng mL^{-1} as the sum of atropine and scopolamine). Atropine was detected in sixteen infusions with concentrations between 0.08-3.88 ng mL^{-1} and scopolamine was detected in five samples with concentrations between 0.48-1.13 ng mL^{-1}. The most contaminated samples were the green tea and peppermint infusions that contained both tropane alkaloids. In particular, Greetea-3 showed atropine concentration above 3 ng mL^{-1}, an amount almost twenty times greater than what is allowed by the current legislation. According to the scientific report published by Mulder et al. (2016) and the work of Marín-Sáez et al. (2019) who studied the extraction efficiency of tropane alkaloids from dry tea and herbal teas to the infusions, atropine and scopolamine have a transfer efficiency of around 40-50%. These studies confirm that to assess the real consumer

exposure, the content of atropine and scopolamine in infusions of tea or herbal teas, rather than their herbs samples should be analysed.

Table S1 (see Supplementary Material) shows the content of atropine and scopolamine estimated in the samples analysed and expressed in ng g^{-1} of dry weight (herbal), taking into account a transfer efficiency of 50% for both analytes. If we compare these concentrations with the concentrations found in other works, similar values have been found. For example, Shimshoni et al. (2015) found concentrations of atropine and scopolamine between 20-208 ng g^{-1} in eight peppermint samples. In turn, Romera-Torres et al. (2018) analysed eleven herbal teas sold in Spain, and found six samples positive to the presence of tropane alkaloids. Moreover, the concentration obtained for atropine in a sample of *Mentha pulegium* was 9 ng g^{-1}. In our work (Table S1), similar amounts have been found in peppermint samples, around 58-295 ng g^{-1} (sum of atropine and scopolamine). Other types of dry herbal teas, such as camomile and lemon balm, have been also previously analyzed. Cirlini et al. (2019), for instance, reported 25 ng g^{-1} of atropine and 50 ng g^{-1} of scopolamine in a sample of *Melissa officinalis* (lemon balm) and 2.1 ng g^{-1} of scopolamine in a sample o*f Chamaemelum nobile* (camomile). Slightly higher concentrations have been found in this work, around 52 ng g^{-1} of atropine for lemon balm and between 8-18 ng g^{-1} of atropine for camomile (Table S1). Mulder et al. (2016) analyzed different varieties of dry herbal teas (120 samples sold in the European market), including peppermint and camomile, and found concentrations of atropine of up to 129 ng g^{-1} and scopolamine up to 34.1 ng g^{-1}. Recently, León et al. (2022) did not find concentrations above 1.8 ng g^{-1} (cut-off level of the screening method) in a sample of camomile, three samples of green tea and a sample of linden, among other teas and herbs for infusions analysed, whereas atropine was found in 50% of the teas (8 samples) and herbal teas (16 samples) analysed by Reinhard & Zoller (2021).

4. Conclusions

This work proposes a new methodology for the analysis of atropine and scopolamine based on the application of a micro-extractive technique, µSPEed®, before analysis by HPLC-MS/MS. The developed method was successfully validated and applied for the quantification of both toxic alkaloids in infusions of commercial samples of tea and herbal teas. Contamination with atropine was found in sixteen infusions, and with scopolamine in five infusions. The highest concentrations were found in peppermint and green tea samples. The developed methodology demonstrates its potential in the quick, green and sensitive analysis of natural toxins, to control the presence of both alkaloids in beverages and to ensure compliance with Commission Regulation (EU) 2021/1408 regarding tropane alkaloids in liquid herbal infusions. Given the toxicological relevance of these compounds and to assess realistic consumer exposure, the content of atropine and scopolamine in infusions of tea or herbal teas, rather than their herbs samples should be analysed.

Declaration of Competing Interest

The authors have declared no conflict of interest.

Author Contributions: Conceptualization: L. Gonzalez-Gomez, S. Morante-Zarcero, J.S. Câmara, I. Sierra; methodology: L. Gonzalez-Gomez, J. Pereira, J.S. Câmara; validation: L. Gonzalez-Gomez, J. Pereira; writing—original draft preparation: L. Gonzalez-Gomez; writing—review and editing: L. Gonzalez-Gomez, J. Pereira, S. Morante-Zarcero, J.S. Câmara, I. Sierra. . All authors have read and agreed to the published version of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version

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Figure captions:

Fig. 1. Schematic diagram of the optimized µSPEed® procedure using the digiVOL® Digital Syringe (EPREP, Australia).

Fig. 2. Optimization of (A) sample loading solvent and (B) elution solvent and selection of the best sorbent after sample loading with (C) water and (D) formic acid (FA) 1%. See details of the full experimental conditions in section 3.1.1.

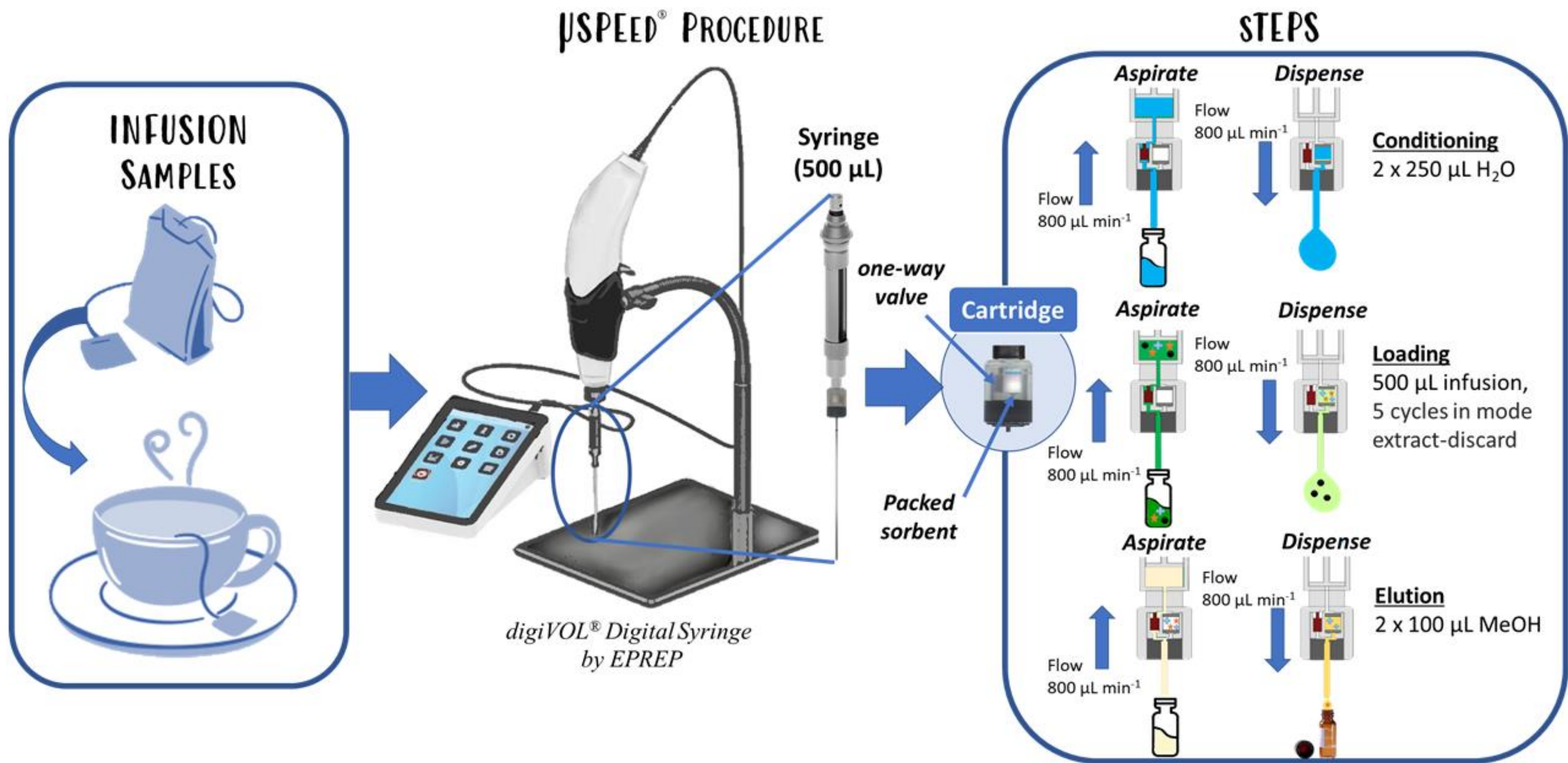

Fig. 1.

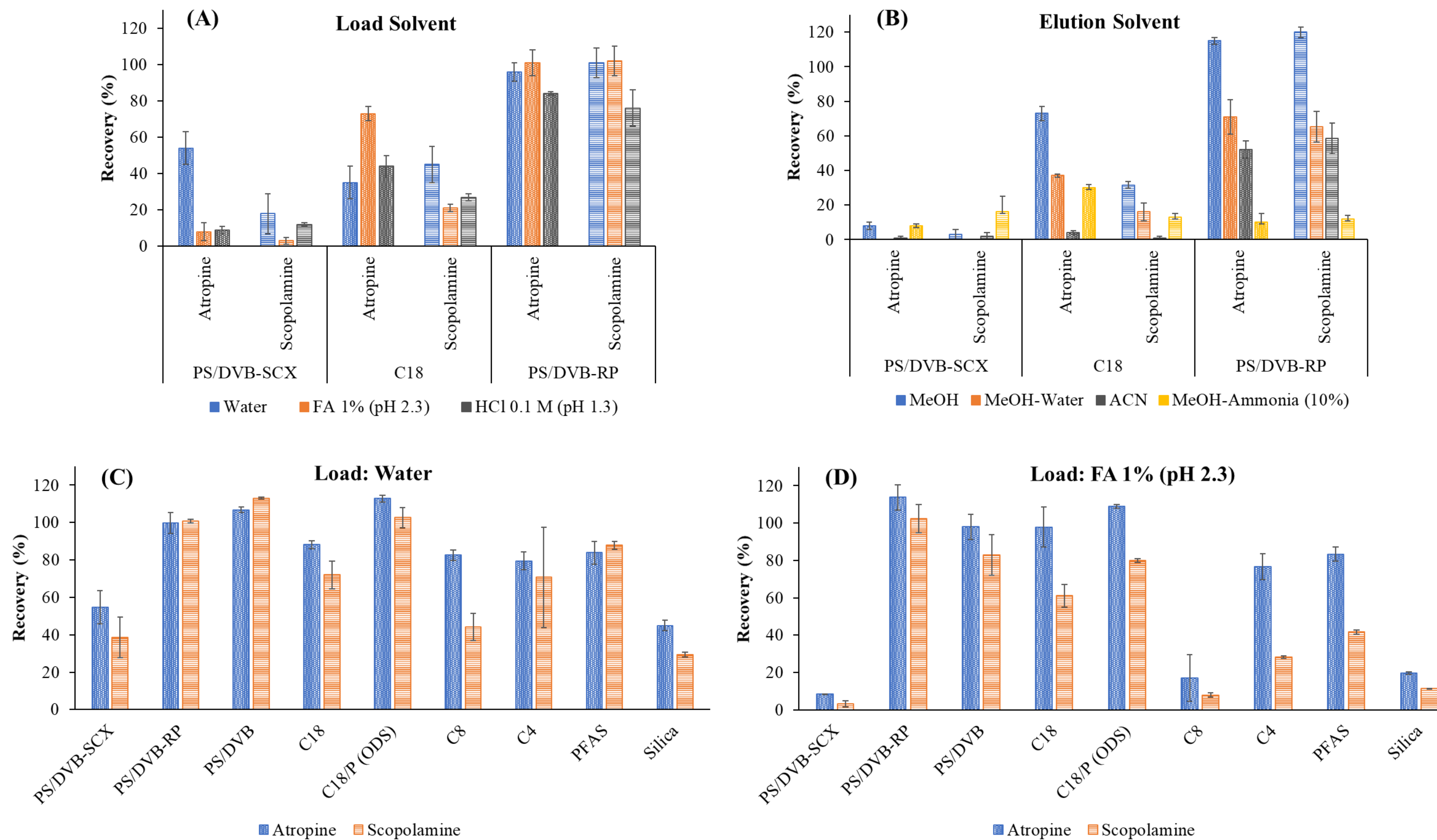

Fig. 2.

Table 1.

Recovery percentages for atropine and scopolamine using PS/DVB-RP, PS/DVB and C18/P-ODS cartridges with different samples of infusions, number of loading cycles and spiking levels in µSPEed®*.

Cycles (spiking level)	Samples[a]	PS/DVB-RP		C18/P-ODS		PS/DVB	
		At[b] (% ± SD)	Sc[c] (% ± SD)	At[a] (% ± SD)	Sc[b] (% ± SD)	At[a] (% ± SD)	Sc[b] (% ± SD)
10 (0.2 ng mL^{-1})	Pep	92 ± 1	70 ± 1	57 ± 5	11 ± 3	108 ± 6	60 ± 5
	Lembal	105 ± 4	93 ± 1	106 ± 3	59 ± 2	114 ± 7	97 ± 8
	Lin	70 ± 1	71 ± 1	87 ± 2	102 ± 5	70 ± 6	94 ± 3
5 (0.2 ng mL^{-1})	Pep	101 ± 3	75 ± 1	101 ± 5	31 ± 1	98 ± 2	99 ± 1
	Lembal	103 ± 9	93 ± 6	116 ± 10	108 ± 1	105 ± 12	95 ± 5
	Lin	74 ± 5	83 ± 5	84 ± 2	94 ± 5	91 ± 6	100 ± 6
	Lemver	69 ± 1	38 ± 7	94 ± 6	62 ± 1	94 ± 2	115 ± 2
	Cam	115 ± 3	77 ± 7	100 ± 3	125 ± 9	96 ± 4	109 ± 2
	Greetea	91 ± 1	71 ± 1	120 ± 10	118 ± 12	101 ± 4	106 ± 10
5 (5 ng mL^{-1})	Pep	-	-	-	-	92 ± 6	91 ± 8
	Lembal	-	-	-	-	98 ± 6	99 ± 7
	Lin	-	-	-	-	105 ± 4	107 ± 5
	Lemver	-	-	-	-	97 ± 1	92 ± 3
	Cam	-	-	-	-	96 ± 4	95 ± 4
	Greetea	-	-	-	-	90 ± 5	99 ± 4

* µSPEed® conditions: Conditioning – 2 x 250 µL water; Loading - 500 µL, 10 or 5 cycles in mode extract-discard; Elution – 2 x 100 µL MeOH. Flow rate: 800 µL min^{-1}.

[a] Pep: Peppermint; Lembal: Lemon balm; Lin: Linden; Lemver: Lemon verbena; Cam: Camomile; Greetea: Green tea.

[b]At: Atropine

[c]Sc: Scopolamine.

Table 2

Linearity, limits of the method and matrix effect in different infusions of tea and herbal tea with methodology based on µSPEed® followed by HPLC-MS/MS*.

Sample[a]	Linearity ($ng\ mL^{-1}$)	Atropine					Scopolamine				
		Matrix-matched calibration	R^2	MDL[b] ($ng\ mL^{-1}$)	MQL[c] ($ng\ mL^{-1}$)	ME[d] (%)	Matrix-matched calibration	R^2	MDL[b] ($ng\ mL^{-1}$)	MQL[c] ($ng\ mL^{-1}$)	ME[d] (%)
Lembal	1.25-313	$1.8\cdot10^{6}\times -6.6\cdot10^{6}$	0.999	0.04	0.12	8	$6.9\cdot10^{5}\times - 1.8\cdot10^{6}$	0.999	0.04	0.12	-8
Lemver	1.25-313	$2.1\cdot10^{6}\times -2.5\cdot10^{6}$	0.999	0.03	0.09	26	$8.8\cdot10^{5}\times -8.3\cdot10^{5}$	0.999	0.04	0.14	18
Lin	1.25-313	$2.8\ 10^{6}\times -1.3\cdot10^{7}$	0.998	0.03	0.10	70	$1.3\cdot10^{6}\times -5.9\cdot10^{6}$	0.998	0.02	0.07	72
Cam	1.25-313	$1.9\cdot10^{6}\times +1.1\cdot10^{6}$	0.999	0.03	0.09	14	$8.4\cdot10^{5}\times +8.6\cdot10^{5}$	1.000	0.03	0.10	13
Pep	1.25-313	$2.3\cdot10^{6}\times -6.9\cdot10^{6}$	0.999	0.04	0.12	38	$9.4\cdot10^{6}\times -3.2\cdot10^{6}$	0.999	0.02	0.06	26
Greetea	1.25-313	$2.2\cdot10^{6}\times -1.7\cdot10^{6}$	0.998	0.04	0.11	32	$1.0\cdot10^{6}\times -5.0\cdot10^{6}$	0.998	0.05	0.15	34

*See section 2.5 and 2.6 for experimental conditions.

[a] Lembal: Lemon balm; Lemver: Lemon verbena; Lin: Linden; Cam: Camomile; Pep: Peppermint; Greetea: Green tea.

[b]MDL: Method detection limit.

[c]MQL: Method quantification limit.

[d]ME: Matrix effect. Calculated by comparing the slopes of the matrix-matched calibration curve with the slopes of the solvent calibration curve (atropine: $y = 1.7\cdot10^{6}x + 4.8\cdot10^{6}$ and scopolamine: $y = 7.5\cdot10^{5}x + 1.3\cdot10^{6}$), both in the same units ($ng\ mL^{-1}$) following the equation: [((slope matrix-matched/slope solvent-based) − 1) × 100].

Table 3

Accuracy and precision of the proposed methodology based on µSPEed® followed by HPLC-MS/MS* for the analysis of atropine and scopolamine in a representative sample of lemon balm.

Analyte	Spiked level (ng mL^{-1})	Accuracy (recovery % ± SD)[a]	Intra-day precision (% RSD)[b]	Inter-day precision (% RSD)[c]
Atropine	0.1	94 ± 3	3	10
	0.2	96 ± 8	7	8
	5	101 ± 7	8	8
	25	103 ± 3	3	10
Scopolamine	0.1	94 ± 7	8	8
	0.2	99 ± 4	5	5
	5	99 ± 5	8	9
	25	106 ± 4	4	4

*See section 2.5 and 2.6 for experimental conditions.

[a] Accuracy (n = 6).

[b] Intra-day precision (n = 6, in one day).

[c] Inter-day precision (n=9, in three different days).

Table 4

Atropine and scopolamine content in infusions prepared with tea and herbal tea commercial samples analysed with the validated methodology.

Code	Sample type	Scientific Name	Acquired	Atropine ($ng\ mL^{-1}$)	Scopolamine ($ng\ mL^{-1}$)
Lembal-1	Lemon balm	*Melisssa officinalis, L*	Portugal	0.53 ± 0.02	ND
Lembal-2	Lemon balm	*Melisssa officinalis, L*	Portugal	0.52 ± 0.09	ND
Lembal-3	Lemon balm	*Melisssa officinalis, L*	Portugal	ND	ND
Lemver-1	Lemon verbena	*Aloysia citriodora*	Portugal	0.32 ± 0.04	ND
Lin-1	Linden	*Tilia argentum (70%), Tilia officcinalis (30%)*	Portugal	0.96 ± 0.07	ND
Lin-2	Linden	*Tilia L.*	Portugal	0.96 ± 0.04	ND
Lin-3	Linden	*Tilia L.*	Portugal	1.81 ± 0.19	ND
Lin-4	Linden	*Tilia L.*	Portugal	0.92 ±0 .08	ND
Cam-1	Camomile	*Chamaemelum nobile*	Portugal	0.18 ± 0.12	ND
Cam-2	Camomile	*Chamaemelum nobile*	Spain	0.08 ± 0.03	ND
Pep-1	Peppermint	*Mentha piperita (90%), Mentha spicata (10%)*	Portugal	0.75 ± 0.06	ND
Pep-2	Peppermint	*Mentha piperita, Mentha spicata, Mentha pulegium (1%)*	Spain	0.58 ± 0.09	ND
Pep-3	Peppermint	*Mentha piperita (95%) Mentha pulegium (5%)*	Spain	1.86 ± 0.84	1.09 ±0.30
Pep-4	Peppermint	*Mentha piperita*	Spain	1.14 ± 0.02	0.61 ±0.04
Greetea-1	Green Tea	*Camellia sinensis*	Portugal	2.78 ± 0.31	0.88 ± 0.08
Greetea-2	Green Tea	*Camellia sinensis*	Spain	2.88 ± 0.39	0.48 ± 0.08
Greetea-3	Green Tea	*Camellia sinensis*	Spain	3.88 ± 0.31	1.13 ± 0.10

*ND: Not detected.

Supplementary material

Green extraction approach based on µSPEed® followed by HPLC-MS/MS for the determination of atropine and scopolamine in tea and herbal tea infusions

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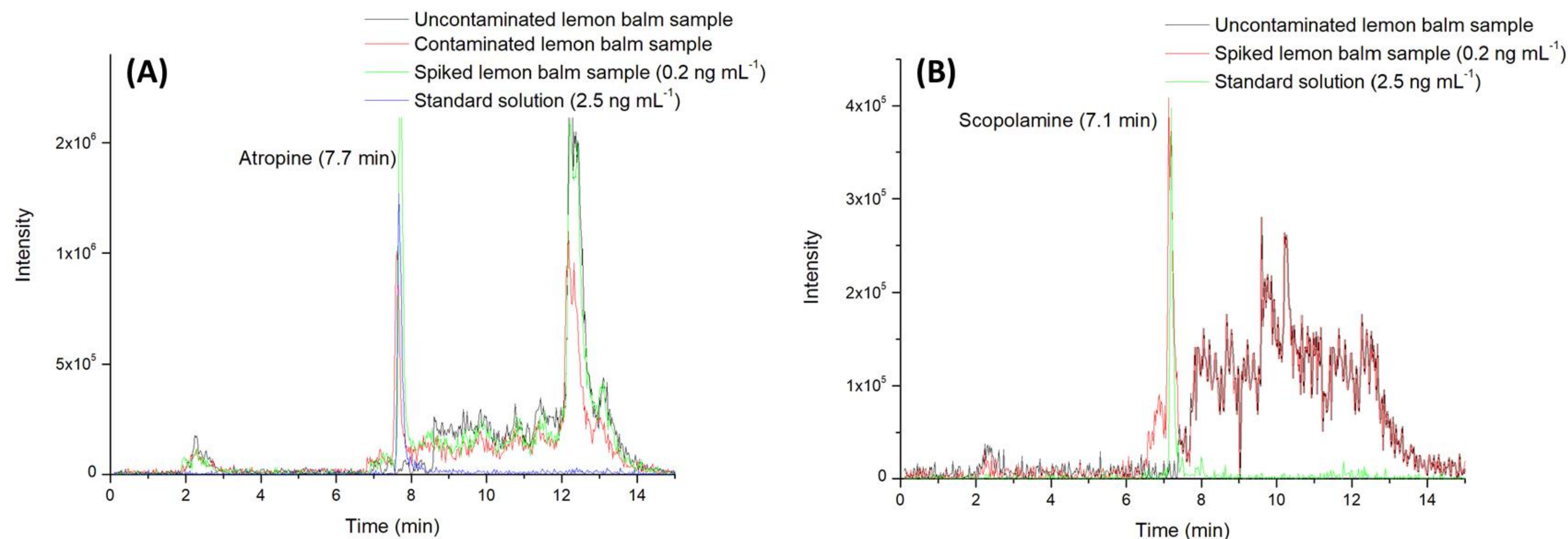

Fig. S1. Chromatograms of (A) atropine (m/z 290.1 > 124.1) and (B) scopolamine (m/z 304.1 > 138.1) in uncontaminated lemon balm sample (Lembal-3), contaminated sample (Lembal-2), spiked lemon balm sample at 0.2 ng mL^{-1} and standard solution in MeOH at 2.5 ng mL^{-1}.

Table S1

Atropine and scopolamine content expressed in ng g^{-1} of tea and herbal tea samples*.

Code	Atropine (ng g^{-1})	Scopolamine (ng g^{-1})	Total content (ng g^{-1})
Lembal-1	53	N.D	53
Lembal-2	52	ND	52
Lembal-3	ND	ND	ND
Lemver-1	32	ND	32
Lin-1	96	ND	96
Lin-2	96	ND	96
Lin-3	181	ND	181
Lin-4	92	ND	92
Cam-1	18	ND	18
Cam-2	8	ND	8
Pep-1	75	ND	75
Pep-2	58	ND	58
Pep-3	186	109	295
Pep-4	114	61	175
Greetea-1	278	88	366
Greetea-2	192	48	240
Greetea-3	388	113	501

* Atropine and scopolamine amounts in ng g^{-1} were estimated from the results obtained in infusions prepared with 2 g of dry sample and 100 mL of water (see Table 4), taking into account a transfer efficiency of 50% for both analytes.