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Chiral HPLC-MS/MS determination of hyoscyamine enantiomers in baby herbal infusions after preconcentration with sulfonic HMS mesostructured silica synthesized by co-condensation

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ABSTRACT

Tropane alkaloids are considered a major food safety issue in recent years, due to their worrying appearance in many foods, such as herbal infusions, usually due to cross-contamination with weeds during harvest. (−)-Hyoscyamine is synthesized by tropane alkaloids-producing plants and its ingestion can cause all types of disorders in humans. Its enantiomerization occurs easily over time, so the inactive (+)-hyoscyamine can be found in foods in variable proportions. Therefore, the goal of the current work was to develop a chiral high performance liquid chromatography tandem mass spectrometry method for the enantiomeric determination of (−)- and (+)-hyoscyamine. Chromatographic separation was optimized, and the best chiral resolution ($R_s = 1.59$) was achieved in 6.5 min with a Chiralpak® AY-3 polysaccharide-based stationary phase column using ethanol with 0.05 % diethylamine as mobile phase. For the preconcentration of the sample, two types of mesostructured silicas with sulfonic groups were synthesized, by grafting and co-condensation methods, and characterized. The materials were evaluated as sorbents for strong-cation exchange solid-phase extraction of hyoscyamine in baby herbal infusions. Based on the findings gathered, silica denoted as HMS-SO_3^-(co) was the best sorbent due to the high number of functional groups attached to silica (2.63 mmol/g) and its textural properties. Under optimal conditions, the preconcentration factor obtained was 167, using only 50 mg of the material, and a very low racemization of the (−)-hyoscyamine to the (+) enantiomer was found (<20 %). The method was validated with recoveries of 94–99 %, good linearity ($R^2 > 0.99$), precision (RSD < 20 %), no matrix effect for both analytes and low method quantification limit (0.089 μg/L and 0.092 μg/L for (+)-hyoscyamine and (−)-hyoscyamine, respectively) to meet its applicability in monitoring real samples. Two types of herbal teas for babies (instant and teabags) were analyzed with the developed methodology. Two of the instantaneous infusions analysed were contaminated, one of them with 0.21 μg/L of (−)-hyoscyamine and 0.09 μg/L of (+)-hyoscyamine (ratio 70 (−)/30(+)) and the other with 0.075 μg/L of (−)-hyoscyamine (the (+)- enantiomer was below the limit of quantification of the method). Taking these results into account, it is important to evaluate the content of hyoscyamine enantiomers in the infusions to avoid overestimation of (−)-hyoscyamine exposure.

1. Introduction

Plants have active compounds that are used in the pharmaceutical industry, including alkaloids such as tropane alkaloids (TAs). These compounds are secondary metabolites generated by a wide variety of plants from the Brassicaceae, Convolvulaceae, Erythroxylaceae, Euphorbiaceae, Proteaceae, Rhizophoraceae and Solanaceae families. There are more than 200 TAs, however, the most well studied are (−)-scopolamine and (−)-hyoscyamine. The racemic mixture of (±)-hyoscyamine is called atropine. Although only (−)-hyoscyamine is biosynthesized in plants, its enantiomerisation occurs easily over time. Therefore, both hyoscyamine enantiomers can be found in plants in varying (−)/(+) ratios [1]. Furthermore, hyoscyamine enantiomers have different pharmacological action and toxicological effects, despite having the same physical and chemical properties [2]. The (−)-hyoscyamine is of pharmacological interest because it inhibits the presence of acetylcholine, being a more potent antimuscarinic agent than the (+)-enantiomer, which appears to be almost inactive [1]. In case of

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overdose, increased antimuscarinic side effects as tachycardia, ataxia, delirium, coma, respiratory failure, and circulatory collapse may occur [3]. Therefore, considering the potential variability of the enantiomeric ratio of hyoscyamine, the separation of both enantiomers is of some importance. However, despite some stereo-selective analytical methods have been published for this task, they were used mainly to determine the purity of (−)-hyoscyamine in pharmaceuticals, extracts of plants, or human plasma, and are not appropriate for the analysis of food [4].

Interest in the determination of TAs has increased in recent years due to their impact on food safety. This is because TAs can appear in foods due to accidental cross-contamination by mixing with parts of TAs-producing plants during harvest. In this regard, the European Food Safety Authority (EFSA) considers that certain cereals, cereal products and herbal infusions are likely to be contaminated with these alkaloids [2]. Commission Regulation (EU) 2023/915 has recently established the maximum level of TAs in certain food products. These values are expressed as the sum of scopolamine and atropine, without differentiation between the hyoscyamine enantiomers, because analytical methods commonly used are unable to separate the (−)- and (+)-hyoscyamine. However, it is recommended to develop chiral analytical methods for an acute dietary exposure assessment of the population to these compounds, and to provide data on their possible enantiomerization during food preparation [4,5].

Herbal infusions are becoming increasingly popular because they are a source of natural bioactive compounds (for example flavonoids, carotenoids or terpenoids) that can exert certain biological effects, such as antioxidant, antiviral, antimicrobial, anti-inflammatory, antimutagenic, anticancer, anti-ageing and other effects [6,7]. In the case of herbal infusions for babies, they are usually used because they help with sleep, calm digestion, soothe the throat, soothe irritation, or can contribute to the relaxation of babies. However, these products are not exempt from possible accidental contamination with TAs, as mentioned above. For this reason, they must be investigated, given the greater sensitivity and vulnerability of infants to toxic substances, as they are less capable of metabolizing and eliminating them efficiently. In herbal infusions (liquid) a maximum 0.20 ng/g level (equivalent to 0.2 ng/mL, assuming a density of 1 mg/mL for these beverages) has been established for TAs [5]. In that respect, no work has developed a method for the enantioselective determination of (−)- and (+)-hyoscyamine in baby herbal infusions. This lack of analysis highlights the need for studies that delve into the possible toxicity of these products for vulnerable groups such as children.

The determination of TAs in foods is a complicated task, due to the multiple matrix interferents that hinder their extraction and detection, given the complexity of food samples. In addition, it must be considered the low concentration of these toxins in baby herbal infusions, which makes it necessary to implement a prior extraction and preconcentration process, such as solid-phase extraction (SPE). This approach is essential to improve the sensitivity of detection methods and to ensure an accurate assessment of the presence of these toxins in products intended for children. In the case of TAs, as these alkaloids are nitrogen atom-containing basic compounds, they are well retained in strong-cation exchange (SCX) SPE and interferences can be removed using a specific washing step [8]. In this sense, different mixed mode polymeric commercial sorbents with anionic functional groups (i.e., Oasis MCX®, Strata-X-C®) have been used to extract TAs in acidic solutions [9]. However, this commercial packed cartridges for SPE contain high amounts of sorbents (usually 150 or 200 mg) [8,10]. In that respect, many authors have developed new materials with the aim of designing potential sorbents which improve extraction efficiency using a minimal amount of them to meet the Green Analytical Chemistry (GAC) requirements [11].

In recent years, the evaluation of new materials for sample preparation has attracted the attention of scientists and researchers around the world [12]. Among these materials are mesostructured silicas, widely used in food sample preparation for analysis of contaminants [13,14]. This is due to their great advantages such as ordered structure, controlled pore size or their large surface area and pore volume. In addition, they offer the possibility of functionalization with different moieties, thus expanding their potential use for the extraction of analytes with different nature and properties [15]. There are many mesostructured silicas that can be evaluated as SPE sorbents and, in that respect, the choice of the silica type and synthesis method plays a crucial role in the extraction and recovery capability in analytical processes [14]. Thus, besides the interactions of the analytes with the functionalities present on the material, it is also essential to consider the importance of other parameters (e.g., particle morphology and size, pore arrangement, textural properties), which are decisive for retaining and releasing analytes in SPE procedures [16]. On the other hand, the incorporation method of the functional groups on the mesostructured silicas can influence their selectivity and efficiency of extraction. For example, in the post-synthetic grafting (g) technique, a ligand is introduced on the surface of a previously prepared silica. However, this method does not guarantee a homogeneous distribution of the grafted groups on the surface and may reduce the porosity of the functionalised material. An alternative approach is co-condensation (co) or direct synthesis, where hydrolysis and co-condensation of silanes result in materials with uniform pores with accessible functional groups on their wall surfaces. Moreover, the functional groups of the materials prepared by (co) have better chemical stability [15].

In previously published reports, our group has successfully demonstrated the use of mesostructured silicas functionalized by (g) technique with sulfonic groups as SCX-SPE sorbents for the analysis of scopolamine and atropine in gluten-free grains and flours, insect-based foods and culinary aromatic herbs [17,18]. Under optimized conditions very good recovery percentages were achieved for the target analytes, using only 75 mg of the mesostructured material [18]. Furthermore, the results were better than those obtained for an analogous commercial sorbent, which was attributed to the good textural properties of the mesostructured silicas that provides better accessibility to the functional groups [17,18]. However, to the best of our knowledge, mesostructured silicas functionalized with sulfonic groups by (co) technique have never been evaluated for SPE of hyoscyamine enantiomers.

Therefore, this work presents the development and evaluation of a method for the chiral HPLC-MS/MS analysis of hyoscyamine enantiomers in baby herbal infusions. For this purpose, Santa Barbara Amorphous-15 (SBA-15) and Hexagonal Mesoporous Silica (HMS) mesostructured silicas were functionalized with sulfonic groups via (g) and (co) methods. The materials (denoted as SBA-15-SO_3^-(g), SBA-15-SO_3^-(co), HMS-SO_3^-(g) and HMS-SO_3^-(co)) were characterized and evaluated as SCX-SPE sorbents and, under the optimized SPE conditions the enantiomerization of the (−)-hyoscyamine into the (+)-enantiomer was evaluated. The method was validated and applied to the analysis of instant infusions from soluble plant extracts and herbal infusions from dried plant mixtures in teabag format. In this context, our work becomes significantly relevant as it focuses on the chiral analysis of hyoscyamine enantiomers in baby herbal infusions, thus making a valuable contribution to scientific knowledge and the safety of products intended for children.

2. Materials and methods

2.1. Reagents and materials

The (−)-hyoscyamine standard (≥98 %) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and the atropine standard (racemic mixture) (≥99 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock standard solution of (−)-hyoscyamine and atropine of 1000 mg/L was prepared by weighing the exact amount of solid substance and dissolved in ethanol (EtOH absolute HPLC grade, Barcelona, Spain) and stored at −20 °C in the dark. Working solutions at the desired concentrations were prepared in EtOH and stored at −20 °C

in the dark. Milli-Q water (resistance 18.2 MW cm) was obtained from a Millipore Milli-Q-System (Billerica, MA, USA). Methanol (MeOH) HPLC-MS grade, acetonitrile (ACN) HPLC-MS grade, trifluoroacetic acid (TFA) 99 % HPLC grade, formic acid 99 % Optima™ LC-MS grade, triethylamine (TEA) ≥ 99 % pure and diethylamine (DEA) ≥ 99,5% were purchased from Fisher Scientific (Madrid, Spain). To filter the samples, 1 and 10 mL syringes, nylon syringe filters (0.45 μm, 25 mm and 13 mm) were used from Scharlab (Barcelona, Spain). For SPE, 3 mL empty cartridges, polyethylene frits (0.20 μm), nylon membrane filters (0.45 μm) and ammonia 32 % (v/v) were purchased from Scharlab (Barcelona, Spain). Hexane (Hex) HPLC grade (≥95 %) and isopropanol (*i*-PrOH) LC-MS grade from Merck (Barcelona, Spain).

For materials synthesis dodecylamine 98 % (DDA, MW = 185.35 g/mol; CAS 124–22-1), tetraethylorthosilicate 98 % (TEOS, MW = 208.33 g/mol CAS 78–10-4), poly (ethylene glycol)-block-poly (propylene glycol)-block-poly (ethylene glycol) (EO20PO70EO20, Pluronic® 123) were obtained from Sigma-Aldrich (St. Louis, MO, USA). (3-Mercaptopropyl) triethoxysilane (MPTES) 94 % was from Alfa Aesar (Karlsruhe, Germany). Hydrochloric acid 37 % (v/v) and hydrogen peroxide 30 % (v/v), were obtained from Scharlab (Barcelona, Spain).

2.2. Synthesis of sulfonic acid-functionalized mesostructured silicas

2.2.1. Synthesis of functionalized mesoporous silicas via grafting

Mesostructured silicas, SBA-15 and HMS were firstly prepared according to previous works [18,19]. SBA-15 was synthetized as follow: 19.4 g of Pluronic® 123 were dissolved in 576 mL of 2 M HCl and 144 mL of Milli-Q water. The solution was stirred at 35 °C until Pluronic® 123 was completely dissolved in acid solution. Then, 41 g of TEOS were added drop by drop. Later, the mixture was stirred for 20 h. After this time, the stirring was stopped, and the temperature was raised to 80 °C. This temperature was held for 24 h to carry out an ageing process. The material was collected by filtration, washed with Milli-Q water, air-dried and calcined (ramp at 1 °C/min up to 500 °C and held for 12 h).

For preparation of HMS material, DDA (30 g) was dissolved in 227 mL of EtOH and 389 mL of Milli-Q water. The mixture was mixed at room temperature until completely dissolved. Later, 125 g of TEOS was added drop by drop balanced addition funnel. The solution was stirred for 18 h. After this time, the white suspension was filtered under vacuum and washed with Milli-Q water until no foam remained. The material obtained was dried at 80 °C overnight. For removed the rest of surfactant, the calcination program used was from room temperature to 550 °C in 12 h and then held at 500 °C for 18 h.

Both materials, SBA-15 and HMS, were subsequently functionalized via grafting, as described by González-Gómez et al. [17]. In a typical synthesis procedure, 2.5 g of silica (SBA-15 or HMS) were suspended in 250 mL of 0.1 M HCl and 1.0 g of MPTES was added. The mixture was stirred at 180 rpm for 7 h, at room temperature, and transferred to a reactor for 24 h at 100 °C. The resultant solid was filtered, washed with Milli-Q water and left at 50 °C overnight. The recovered materials (SBA-15-SH or HMS-SH) were mixed with 325 mL 2 M HCl and, next, 11.4 g of H_2O_2 (30 %) were added for oxidation of thiols to sulfonic acid. After 5

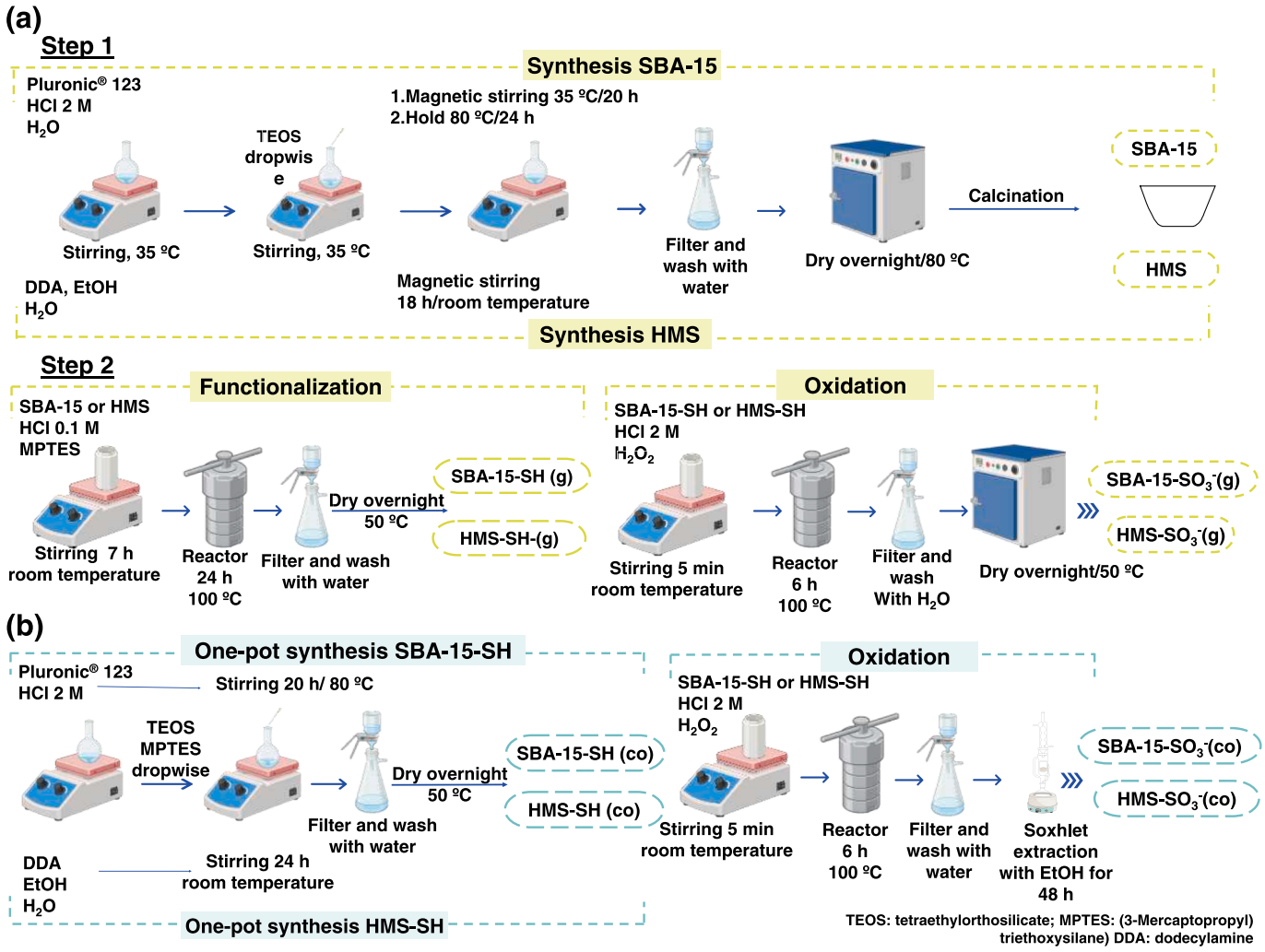

Fig. 1. Schematic diagram showing the synthesis process of the mesostructured silicas.

min under agitation at room temperature, the mixture was heated in a reactor for 6 h at 100 °C. The obtained materials were denoted as SBA-15-SO_3^-(g) or HMS-SO_3^-(g), were filtered and washed with Milli-Q water. Fig. 1a shows the schematic diagram showing the synthesis process.

2.2.2. Synthesis of functionalized mesostructured silicas via co-condensation method

Mesostructured silica materials functionalized with sulfonic groups were prepared by co-condensations following the procedure describe by Rác et. al. [20] with some modifications.

In a typical synthesis procedure for HMS-SO_3^-(co) material, 2.5 g of DDA was dissolved in 35 mL of Milli-Q water and 27.5 mL of EtOH and stirred vigorously until a homogeneous solution was obtained. Follow adding dropwise the mixture of 9.4 g of TEOS and 3.3 g of MPTES. The reaction mixture was allowed to react under stirring at 200 rpm and ambient temperature for 24 h. The surfactant was removed from HMS by solvent extraction with EtOH heated to boiling temperature (78 °C) for 2 days in Soxhlet. The oxidation of thiol group was performed as describe in section 2.2.1. The obtained material denoted as HMS-SO_3^-(co) was filtered and washed with Milli-Q water (Fig. 1b).

SBA-15-SO_3^-(co) was prepared as follow: 5 g of Pluronic® 123 was dissolved with stirring in 150 g of 2 M HCl at room temperature. The solution was heated to 40 °C and TEOS (10.5 g) was added to this solution at same time that the appropriate amount of MPTES (4.2 g) was adding. The mixture was stirred at this temperature during 20 h and aged under static conditions for 24 h at 100 °C. The resulting solid was recovered by filtration and dried at 70 °C. The surfactant was removed with EtOH heated to boiling temperature (78 °C) in Soxhlet during 48 h. Oxidation of thiol groups was performed as mentioned in section 2.2.1 (Fig. 1b).

2.3. Characterization of mesostructured silicas materials

N_2 gas adsorption–desorption isotherms were recorded using a Micrometrics ASAP 2020 analyzer. The surface specific area was calculated by Brunauer-Emmett-Teller (BET) method and the pore size distribution was obtained using Baret-Joyner-Halenda (BJH) model on the desorption branch. Elemental analysis (% S) was performed with a Flash 2000 Thermo Fisher Scientific Inc. analyzer. ^{13}C cross-polarization magic angle spinning nuclear magnetic resonance (^{13}C CP-MAS-NMR) and pulse decoupling angle ^{29}Si solid-state nuclear magnetic resonance (^{29}Si-PDA-MAS-NMR) spectra were recorded on a Bruker Avance III/HD 9.4 Teslas Fourier-Transform Infrared (FT-IR) spectra were recorded with a Spotlight 200i, Perkin Elmer (USA) spectrometer in the region 4000–400 cm^{-1}. Spectrometer operating at 400 MHz proton frequency. Conventional transmission electron microscopy (TEM) was carried out with a JEOL F200 ColdFEG microscope operating at 200 kV, with a resolution of 0.23 nm, using a copper sample holder.

2.4. Samples

Two types of products to prepare baby herbal infusions were purchased from a local pharmacy in Madrid (Spain) and from an online shop: soluble plant extracts for instant infusions (three different batches of the same product) and dried plant mixtures in teabag format (five different products). Products for instant infusions were prepared with soluble extracts of linden, lemon balm and chamomile (for details see Table S1 in supplementary material). Products in teabag format contained mainly the following dried herbs: lemon balm, chamomile, linden, green aniseed, caraway and fennel (Table S1). The samples were named with the first two letters indicating the type of product (SE for soluble extracts format and DP for dried plants in teabag format) followed by a hyphen with a number indicating the sample number, in the case of samples with different batches they were named with the letter B followed by the sample number. Samples were stored at room temperature until analysis. Each sample was analysed in triplicate and results are shown as mean ± standard deviation (SD).

2.5. Preparation of baby herbal infusions

Baby herbal infusions were prepared according to the manufacturer's instructions, as illustrated in Fig. 2. The procedure for the preparation of instant infusion samples (denoted as I-SE-B1 to I-SE-B3) from soluble extracts consisted of weighing 2.5 g of sample into a polypropylene centrifuge tube. Then it was added 50 mL of Milli-Q water at room temperature and vortexed (Rx^3 Velp Scientifica, Usmate, MB, Italy) until complete dissolution. The pH was adjusted to 2.5 using formic acid with a CRISON BASIC 20 pH meter (L'Hospitalet de Llobregat, Barcelona) and then was centrifuged for 10 min at 6000 rpm (ROTOFIX 32A Hettich, Tuttlingen, Germany). Finally, the supernatant was filtered through a 0.45 μm nylon filter prior to the purification and preconcentration step by SPE. In the case of infusions prepared from the dried plants (denoted as I-DP-1 to I-DP-5), one teabag (1.5 g) was placed in a 250 mL beaker. Then, 200 mL of boiling Milli-Q water was poured into the beaker, completely submerging the teabag, which was allowed to stand for 5 min (see Fig. 2). During this time, the teabag was continuously shaken by hand. Subsequently, the teabag was removed, and the liquid was drained into the beaker. The pH was adjusted to 2.5 and then a 50 mL aliquot of the infusion was transferred to a polypropylene tube and centrifuged for 10 min at 6000 rpm. The supernatant was then filtered prior to the SPE step.

2.6. Evaluation of mesostructured silicas as sorbent for SPE

Firstly, an optimisation process was carried out testing the mesostructured silicas previously synthesised in the laboratory as sorbents, SBA-15-SO_3^-(g), SBA-15-SO_3^-(co), HMS-SO_3^-(g) and HMS-SO_3^-(co), and was compared with the commercial sorbent Waters OASIS® MCX, which is a mixed-mode polymeric sorbent optimized to achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups. Different amounts of sorbent packed in the cartridges ranging from 50 to 150 mg and various sample load volumes, from 6 to 50 mL, were studied. The sample was loaded at 0,8 mL/min. All studies were performed in triplicate using an instant infusion sample (I-SE-B2) spiked with a standard solution of atropine and results were expressed as recovery percentage. All tests were carried out following the method proposed by Mulder et al. [8] with some modifications.

2.7. Optimized SPE conditions

SPE polypropylene cartridges were filled with 50 mg HMS-SO_3^-(co) and sealed at both ends with polyethylene frits. In addition, a nylon filter membrane (0.45 μm) was incorporated at the base of the bed to prevent sorbent loss during the process. The cartridges were conditioned with 6 mL of MeOH, equilibrated with 6 mL of 1 % formic acid in water and loaded with 50 mL of pH-adjusted tea infusion at 0.8 mL/min. This was followed by a wash using 6 mL of a mixture of MeOH/water/formic acid, 75/25/1 (v/v/v), followed by vacuum drying. For this step, a 12-port vacuum manifold (Supelco Visiprep SPE, Sigma Aldrich, St. Louis, MO, USA) connected to a vacuum pump at 7.6 psi for 5 min was used. Analytes were eluted from the cartridges with 6 mL of MeOH containing 0.5 % ammonia. Finally, the 6 mL of elution was evaporated to dryness and reconstituted with 0.3 mL of EtOH (pre-concentration factor of 167) for subsequent injection into the HPLC-MS/MS system (Fig. 2).

2.8. Evaluation of chiral chromatographic columns

Three types of CSPs based on amylose or cellulose polysaccharides with different types of ligands were evaluated for the enantioseparation of (+)- and (−)-hyoscyamine (see Figure S1 in supplementary materials). Chiralpak® AD-H [amylose tris-(3,5-dimethylphenylcarbamate) with a length of 250 mm x 4, 6 mm ID and 5 μm particle diameter]

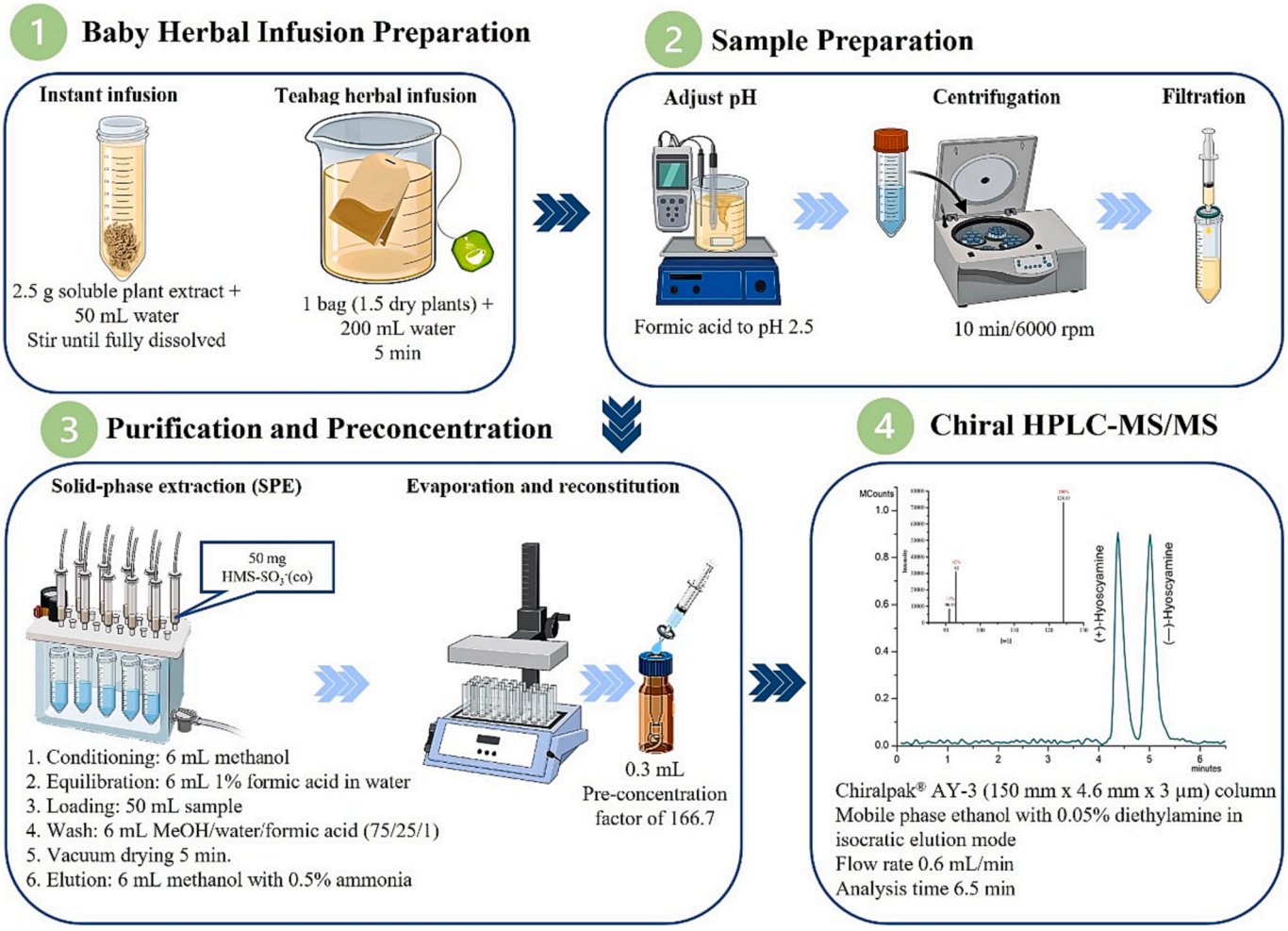

Fig. 2. Schematic representation of the optimized methodology for atropine enantiomer determination in baby herbal infusions.

(Chiral Technologies Europe, Strasbourg, France), Lux® Cellulose-1 [cellulose tris-(3,5-dimethylphenylcarbamate) with a length of 250 mm x 4.6 mm ID and 5 µm particle diameter] (Phenomenex, Madrid, Spain) and Chiralpak® AY-3 [amylose tris-(5-chloro-2-methylphenylcarbamate) with a length of 150 mm x 4. 6 mm ID and 3 µm particle diameter] (Análisis Vínicos, Tomelloso, Spain). For this purpose, an Agilent 1260 Infinity II HPLC system (Agilent Technologies, Madrid, Spain), equipped with a flexible pump (G7104C 1260 Flexible Pump), automatic multisampler (G7167A 1260 Multisampler), thermostated column compartment (G7116A 1260 MCT) and a diode array detector (G7117C 1260 DAD HS) at 230 nm was used. Agilent OpenLab CDS ChemStation Edition software (Agilent Technologies) was used for data processing. Normal phase (NP), polar organic phase (PO) and reversed phase (RP) elution conditions were tested with different mobile phase additives. Other experimental chromatographic conditions such as flow rate, column temperature, solvent and injection sample volume were evaluated too.

2.9. Optimized chiral HPLC-MS/MS analysis

A Varian 1200/1200LC HPLC-MS/MS system (Varian Ibérica, Madrid, Spain) was used, equipped with a ProStar 410 autosampler with 100 µL loop, coupled to a triple quadrupole tandem mass spectrometer detector (1200L TQ) with an electrospray ionisation (ESI) ion source. A Chiralpak® AY-3 column equipped with a Chiralpak® AY-3 pre-column (10 x 4 mm, 3 µm particle size, Análisis Vínicos, Tomelloso) was used. In this case EtOH with 0.05 % DEA was used as mobile phase in isocratic elution mode for 6.5 min. at a flow rate of 0.6 mL/min. The column temperature was 30 °C and the injection volume 10 µL (partial injection). For mass spectrometry detection of (+)-hyoscyamine and (−)-hyoscyamine was performed by ESI in positive mode (ESI +) and detection using a multiple reaction monitoring (MRM) mode (mass peak width Q1 2.5; mass peak width Q3 2.5; MRM scan width 0.70). N_2 was used as drying gas at 350 °C and 22 psi and as nebuliser gas at 58 psi and argon was used as collision gas at 1.90 mTorr. Capillary voltage of 5000 V, 600 V for the shield and 1535 V for detector voltage was used. The analytes were monitored at 70 V cone voltage and MS/MS parameters are shown in Table S2. MS Workstation Varian software version 6.8 (Varian Ibérica) was used for data processing.

2.10. Method validation

The method validation was performed considering the document SANTE/11312/2021 stablishing the analytical quality control and method validation procedures for the analysis of pesticide residues in food and feed and the International Council for Harmonisation (ICH) [21,22]. I-SE-B2 spiked sample prepared according to the manufacturer's specifications as described in section 2.5 was used for validation. Thus, linearity was assessed by matrix-matched calibration curves. For this purpose, extracts obtained after the SPE procedure were spiked with an aliquot of a standard solution of atropine at six desired concentration level of the calibration curve. A regression analysis of the area of each enantiomer versus concentration was used to verify the good linearity of the method with correlation coefficients (R^2) close to 1. Matrix effects

(ME) were established, for each enantiomer, by comparing the slope of equations obtained for the matrix-matched calibration curves and slope of the solvent calibration curves, both expressed in μg/L using the expression ME = (slope matrix-matched calibration/slope solvent calibration) × 100. When the ME values are in the range 80 to 120 %, ME can be ignored. A ratio below 100 % points to signal suppression, whereas a ratio above 100 % suggests signal enhancement. The selectivity of the method was evaluated by comparing the signals of precursor ions, product ions and retention time for each enantiomer in the standard solutions with the signals obtained for the samples. A blank sample was also analysed, which did not show any peak for the target analytes. It was considered satisfactory when the retention time of the analytes was in the range of ± 0.1 min and the unit mass MS/MS ion ratios were within ± 30 % (relative abundance) of the reference value. The sensitivity of the method was determined by the method detection and method quantification limits (MDL and MQL, respectively) of the two enantiomers expressed in μg/L in the baby herbal infusions. These values were estimated as the concentration that produce a signal-to-noise ratio (S/N) of 3 or 10, respectively, measured at the lowest concentration analysed in the matrix-matched calibration curve and considering the sample treatment procedure. Recovery studies were carried out by comparing the areas obtained in spiked infusion samples spiked with a known concentration of the analytes and subjected to the SPE procedure with the areas obtained for simulated samples spiked after the SPE process and before evaporation and prior to chromatographic analysis. The recovery assays were performed at three concentration levels: low (0.08 μg/L), medium (0.1 μg/L) and high (0.2 μg/L) of each enantiomer and results were expressed as the average recovery of six assays (n = 6). These recovery values should be in the range of 70 to 120 %. The precision of the method was evaluated in terms of repeatability and reproducibility, expressed as RSD (%). Repeatability was obtained by evaluating six spiked sample replicates injected in triplicate in one day (n = 6). Reproducibility was obtained by analysing a spiked sample in triplicate and injected in triplicate on three different days (n = 9). According to the validation guidance these RSD values should be ≤ 20 %.

2.11. Statistical analysis

Statistical analysis was carried out using SPSS 25.0 software (SPSS INC., Chicago, IL, USA). A two-tailed Student's *t*-test with a 95 % confidence interval was used, assessing only two levels of a single factor. Differences were considered significant for p-values ≤ 0.05, and different letters were used to denote them.

3. Results and discussion

3.1. Characterisation of mesostructured silicas

The N_2 adsorption–desorption isotherms for ordered mesostructured silicas prepared are shown in Fig. 3. As can be seen, both the SBA-15 type (Fig. 3a), and the HMS type (Fig. 3b) isotherms were type IV according to IUPAC categorisation with narrow hysteresis loop typical of mesoporous solids. SBA-15 type displayed a H1 hysteresis loop, indicative of uniform cylindrical pores, while HMS exhibited a H2 hysteresis loop, indicative of wormhole-like pores that cause some bottleneck effect. In SBA-15-SO_3^-(g) and HMS-SO_3^-(g) (Fig. 3a), the volume of nitrogen adsorbed increased at a relative pressure (P/P0) of approximately 0.14 and 0.15, respectively, representing capillary condensation of nitrogen within the mesopore structure. The inflection position moved slightly to lower relative pressures and the hysteresis loop decreased in materials synthetized by co-condensation method (SBA-15-SO_3^-(co) and HMS-SO_3^-(co)) as can be seen in Fig. 3b. Pore size distribution of mesostructured silicas was calculated according to Barret-Joyner-Halenda (BJH) method, obtaining narrow pore size distribution, which supplied evidence for uniform framework mesoporosity (see Fig. S2 in supplementary material). Table S3 shows the textural properties of the mesostructured silicas. As shown, S_{BET} and pore diameters of HMS-SO_3^-(co) and SBA-15-SO_3^-(co) synthetized by (co) are smaller than their analogues functionalized by (g) method, being particularly pronounced in the case of SBA-15 type materials.

The functional groups attached to the mesostructured silicas (L_o) were estimated from the sulphur content calculated by elemental analysis (Table S3). Regarding the % S, it was estimated the presence of 1.03 mmol/g and 1.11 mmol/g of S in the SBA-15-SO_3^-(g) and HMS-SO_3^-(g), respectively. For the mesostructured silicas obtained by (co) route, L_o was higher (2.18 mmol/g and 2.63 mmol/g of S for SBA-15-SO_3^-(co) and HMS-SO_3^-(co), respectively) despite maintaining the MPTES/SiO_2 molar ratio for both functionalisation routes. These results confirm that (co) gives materials with higher organic content than the (g) route, as has been indicated previously by other authors [23]. The lower L_o in (g) synthesis may be since if the ligands react preferentially in the pore openings during the initial stages of the grafting process, the subsequent diffusion of the ligands towards the pore centre may be hindered and the pore can be blocked. However, these drawbacks are overcome in (co) procedures by hydrolysis and co-condensation of a TEOS with an MPTES in the presence of a structure-directing agent (Pluronic® 123 or DDA), resulting in a hybrid material containing accessible functional groups more uniformly arranged inside the porous channels and without pore blocking with the organic ligands [23].

To confirm that the ligands were correctly anchored to the silicas synthesised by (co) route, ^{29}Si PDA-MAS-NMR and ^{13}CP-MAS-NMR analyses were carried out. Fig. 3c and 3d shows the ^{29}Si PDA-MAS-NMR spectrums of SBA-15-SO_3^-(co) and HMS-SO_3^-(co), respectively. Both shows the two main peaks appear at − 109 and − 102 ppm, which are assigned to Q4 framework silica sites ($(SiO)_4Si$) and Q3 silanol sites ($(SiO)_3SiOH$), respectively. The spectrums also shows a shoulder at −91 ppm attributed to the Q2 peak ($(SiO)_2Si(OH)_2$). Two other peaks that appear in both at −67 and −58 ppm, which are assigned to T2 ($(SiO)_2SiOH$–R) and T3 ($(SiO)_3Si$-R) sites, respectively, which verify the attachment of the organic groups to the silica. Fig. 2e and 2f shows the ^{13}CP-MAS-NMR spectrums of SBA-15-SO_3^-(co) and HMS-SO_3^-(co), respectively, showing important information about the groups anchored on the materials surface that confirms its functionalization. As can be seen, in both appear four signals at 10.4, 22.4, 52.7 and 63.9 ppm corresponding to the three carbon atoms related to the propyl sulfonic group, numbered in the figure as (6), (7) and (8). The signal due to methylene (2) of the ethoxy group appears at 57 ppm and the signal of the methyl group (1) at 16.7 ppm. Also, additional signals appear in the spectra which according to other authors can be assigned to carbon atoms (3), (4) and (5), confirming the presence of thiol groups anchored to the pore walls and indicating that part of the -SH groups could not be oxidised [17,24]. These results provide evidence that the functionalization of these materials was successful. In both spectrums there are also some peaks that do not correspond to the ligand, which according to Margolese et al. [24] may be peaks of residual species of block copolymers, especially in SBA-15-SO_3^-(co).

Fig. 3g and 3 h shows the IR spectrum for SBA-15 and SBA-15-SO_3^-(co) and HMS and HMS-SO_3^-(co), respectively. The presence of the sulfonic groups is confirmed in both with the identification of the C-H stretching vibration (propyl chains) in the 2950–2850 cm^{-1} region. Furthermore, the band at 650 cm^{-1} relates to the C–S stretching mode vibrations and the presence of band at 1409 cm^{-1} was attributed to the S = O vibrations. In addition, in all the spectra common bands appear with wave numbers at 450 cm^{-1} corresponding to the tetrahedral vibration of SiO_4, at 800 cm^{-1} corresponding to the symmetric vibration (Si-O-Si), a large band between 1000 and 1260 cm^{-1} assigned to the asymmetric vibrations (Si-O-Si) and at 3400 cm^{-1} corresponding to the free silanols groups (Si-O-H) [20,25].

TEM micrographs showed well-ordered arrangements of hexagonal pores of the perpendicular channels with uniform sizes for SBA-15-SO_3^-(g) and SBA-15-SO_3^-(co) (Fig. S3a and S3c). In contrast, Fig. S3b and

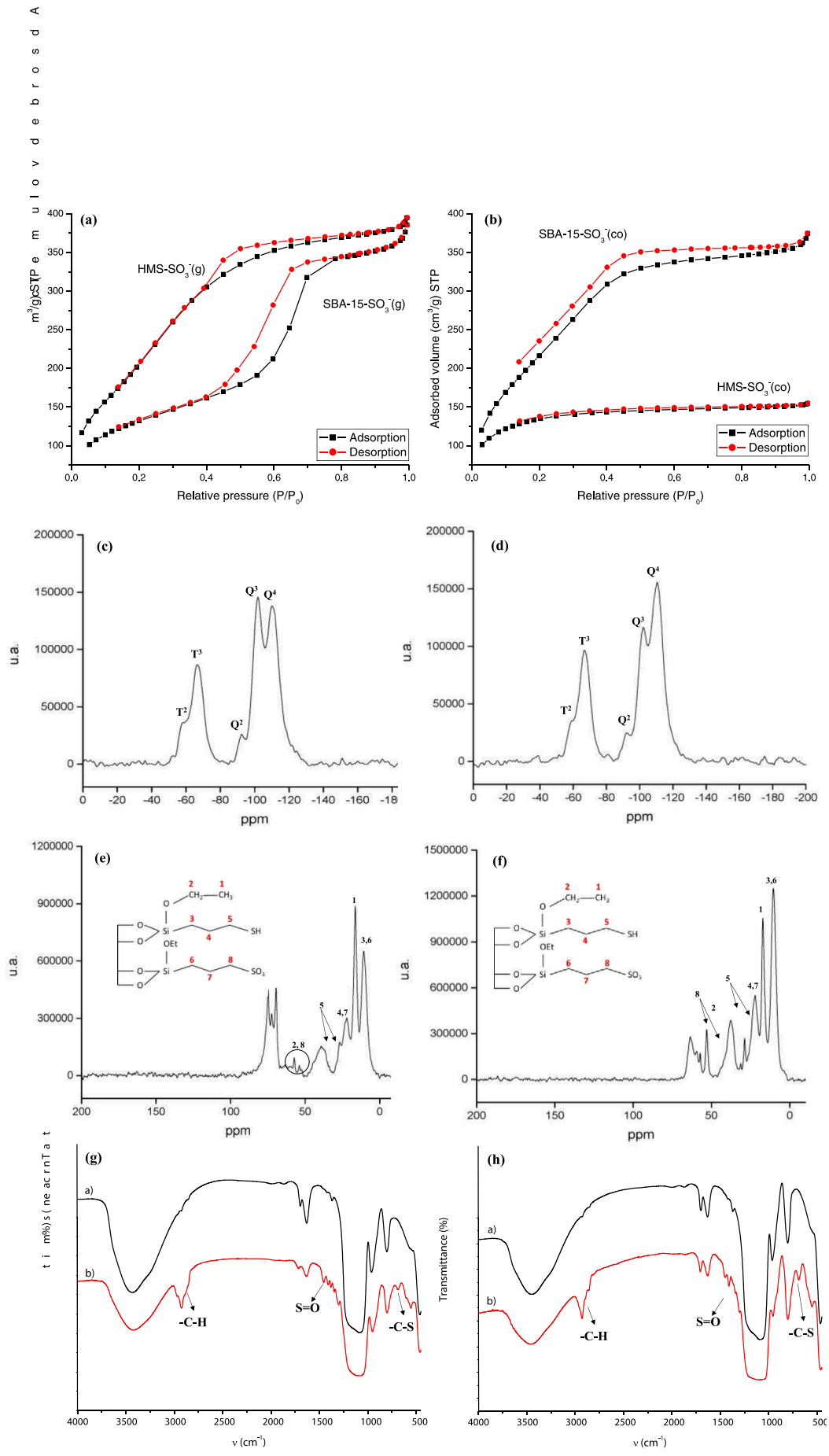

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Fig. 3. Nitrogen adsorption–desorption isotherms of bare and functionalized: (a) SBA-15-SO_3^-(g) and HMS-SO_3^-(g), (b) SBA-15-SO_3^-(co) and HMS-SO_3^-(co) materials. ^{29}Si PDA-MAS-NRM spectra of: (c) SBA-15-SO_3^-(co) and (d) HMS-SO_3^-(co). ^{13}C CP-MSD-NMR spectra of: (e) SBA-15-SO_3^-(co) and (f) HMS-SO_3^-(co). FT-IR spectra of: (g) SBA-15 and SBA-15-SO3-(co) and (h) HMS and HMS-SO3-(co).

S3d showed irregularly aligned mesopores throughout the material (wormhole-like pore arrangement) for HMS-SO_3^-(g) and HMS-SO_3^-(co).

3.2. Evaluation of chiral columns and optimization of chiral HPLC-MS/MS analysis

Liquid chromatography is the most widely used technique for chiral analysis due to its advantages and, usually, the best enantiomeric selectivity can be achieved with a suitable CSP and an adequate mobile phase. Polysaccharide-based CSPs are the most widely used packing materials for the chromatographic separation of enantiomers at both analytical and preparative levels [26]. Considering that the CSP is crucial for chiral separation, three types of CSPs based on amylose or cellulose polysaccharides with different types of ligands were evaluated for the enantioseparation of (+)- and (−)-hyoscyamine. Figure S1 shows structures of the Chiralpak® AD-H, Lux® Cellulose-1 and Chiralpak® AY-3 CSPs evaluated. Three chiral columns were tested in NP, PO and RP elution modes to obtain the best chiral separation with an optimum flow rate, in order to reduce the solvent consumption and achieve quicker analysis times.

In NP elution mode, mobile phases composed by Hex with *i*-PrOH, EtOH or MeOH as organic modifier and DEA as basic additive were tested. In PO phase elution mode EtOH, MeOH, ACN or *i*-PrOH were tested as mobile phase with different basic additives and, finally, in RP elution mode, a mobile phase composed by H_2O/ACN in different proportions and TFA as additive were tested. Table 1 summarises the most important results obtained for enantioseparation of atropine in terms of retention time for the second enantiomer (t_{r2}), retention factors (k_1 and k_2) and chiral resolution (R_s). All studies have been performed according to the specifications recommended by the column suppliers in terms of mobile phase composition, maximum flow rate and recommended pressure to maximise the lifetime of the columns.

In PO elution modes, alcohols (EtOH, MeOH, or *i*-PrOH) or ACN are used, alone or in combination, with and without different basic additives. The PO mode reduce analysis time, is compatible with MS detection and has the advantage of using mobile phase in which analyte is more soluble [27]. Table 1 shows chromatographic results obtained with three CSP in more representative mobile phase tested at a flow rate of 1 mL/min and room temperature. It has been demonstrated that the addition of basic or acidic additives may be necessary to improve the R_s and peak shapes of analytes, and in the case of chiral separations, they can increase enantioselectivity, minimising peak broadening due to unwanted interactions between polar solutes and stationary phase [28]. For this reason, the effect of the addition of amines (DEA and TEA) in the mobile phase was studied. As shown in Fig. 4a and Table 1, using Chiralpak® AY-3 as CSP and a mobile phase containing EtOH, a single peak ($t_r = 1.943$ min, $k = 1.09$) was obtained while the addition of 0.1 % DEA in EtOH produced two narrow peaks of (+)- and (−)-hyoscyamine with a chiral R_s of 4.30 ($k_1 = 1.29$ and $k_2 = 1.47$). With Lux® Cellulose-1 using the same mobile phase (EtOH with 0.1 % DEA) a partial chiral resolution of the enantiomers was achieved ($R_s = 1.42$) (see Fig. 4b, Table 1) and when Chiralpak® AD-H was used as CSP under the same conditions, separation of the enantiomers could not be achieved (Table 1). When this amine was used as additive in mobile phase composed by 100 % MeOH, ACN or *i*-PrOH (Table 1) atropine enantiomers only could be resolved with Lux® Cellulose-1 as CSP and MeOH with 0.1 % DEA as mobile phase, although the R_s does not reach baseline ($R_s = 1.57$) as shown in Fig. 4c. Taking into account the recommendations of the column manufacturers the effect of the basic additive in the mobile phase (TEA) was investigated. When 0.1 % TEA was added in a mobile phase composed by EtOH or MeOH, no R_s was obtained with any of the CSPs tested (Table 1). As it is known, the concentration and type of organic modifier in the mobile phase has an important effect on the chiral separation by competing with the analyte for the specific adsorption sites of the CSP modifying the enantioselectivity, the effect of the addition of *i*-PrOH or ACN in the mobile phase composed by MeOH was studied (Table 1). The addition of 10 % *i*-PrOH and 20 % ACN resulted in a decrease in t_r in all cases, except in the Chiralpak® AY-3 column, where t_r increased with the addition of *i*-PrOH. However, a $R_s = 0$ was observed in all cases, except for the Lux® Cellulose-1 column, where $R_s = 1.04$ (Table 1).

In NP elution mode, the mobile phase is typically based on mixtures of a nonpolar solvent as Hex with an alcohol of low molecular weight such as *i*-PrOH, EtOH and MeOH as organic modifier. The type of

Table 1
Results obtained for the chiral separation of (+)- and (−)-hyoscyamine in terms of retention time of the second enantiomer, retention factors and chiral resolution using different chiral stationary phases in polar organic, normal and reversed phase elution mode by HPLC-DAD.

Mobile phase	Chiralpak® AY-3				Chiralpak® AD-H				Lux® Cellulose-1			
	t_{r2}^a	k_1^b	k_2^c	R_S^d	t_{r2}^a	k_1^b	k_2^c	R_S^d	t_{r2}^a	k_1^b	k_2^c	R_S^d
Polar organic phase												
EtOH	1.943	1.09	1.09	0	–				–			
EtOH, 0.1 % DEA	2.606	1.29	1.47	4.30	5.558	1.73	1.73	0	3.600	1.17	1.23	1.42
EtOH, 0.1 % TEA	2.062	1.17	1.17	0	5.117	1.57	1.57	0	4.856	2.79	2.79	0
MeOH, 0.1 % DEA	2.171	1.16	1.16	0	5.010	1.57	1.57	0	3.755	1.20	1.27	1.57
MeOH, 0.1 % TEA	2.589	1.17	1.17	0	5.748	1.44	1.44	0	3.643	1.24	1.24	0
MeOH/ACN (80/20), 0.1 % DEA	2.042	1.11	1.11	0	4.206	1.33	1.33	0	3.420	1.10	1.10	0
MeOH/*i*-PrOH (90/10), 0.1 % DEA	2.963	1.34	1.34	0	4.578	1.43	1.43	0	3.619	1.18	1.24	1.04
ACN, 0.1 % DEA	1.873	2.12	2.12	0	–				3.012	5.98	5.98	0
i-PrOH, 0.1 % DEA	2.402	1.08	1.08	0	–				5.767	3.30	3.30	0
Normal phase												
Hex/*i*-PrOH (90/10), 0.1 % DEA	3.600	1.92	1.92	0	4.790	1.57	1.57	0	4.516	1.82	1.82	0
Hex/EtOH (90/10), 0.1 % DEA	3.718	1.89	1.98	1.13	7.297	2.20	2.20	0	7.054	2.05	2.05	0
Hex/MeOH (90/10), 0.1 % DEA	2.727	1.17	1.17	0	5.313	2.07	2.07	0	6.823	1.44	1.44	0
Reversed phase												
H_2O/ACN (55/45), 0.1 % TFA	2.019	1.16	1.24	1.25	3.006	2.85	2.85	0	2.359	1.47	1.47	0
H_2O/ACN (65/35), 0.1 % TFA	2.033	1.15	1.21	0.93	2.097	1.20	1.20	0	4.099	1.38	2.97	2.73

Chromatographic conditions: flow rate 1 mL/min and ambient temperature. – no tested. n-Hex, hexane; *i*-PrOH, isopropanol; EtOH, ethanol; MeOH, methanol; ACN, acetonitrile; DEA, diethylamine; TEA, triethylamine; TFA, trifluoroacetic acid. [a] t_{r2} = Retention time of second enantiomer (min), [b] k_1 = Retention factor of first enantiomer, [c] k_2 = Retention factor of second enantiomer, [d] R_S = Chiral resolution.

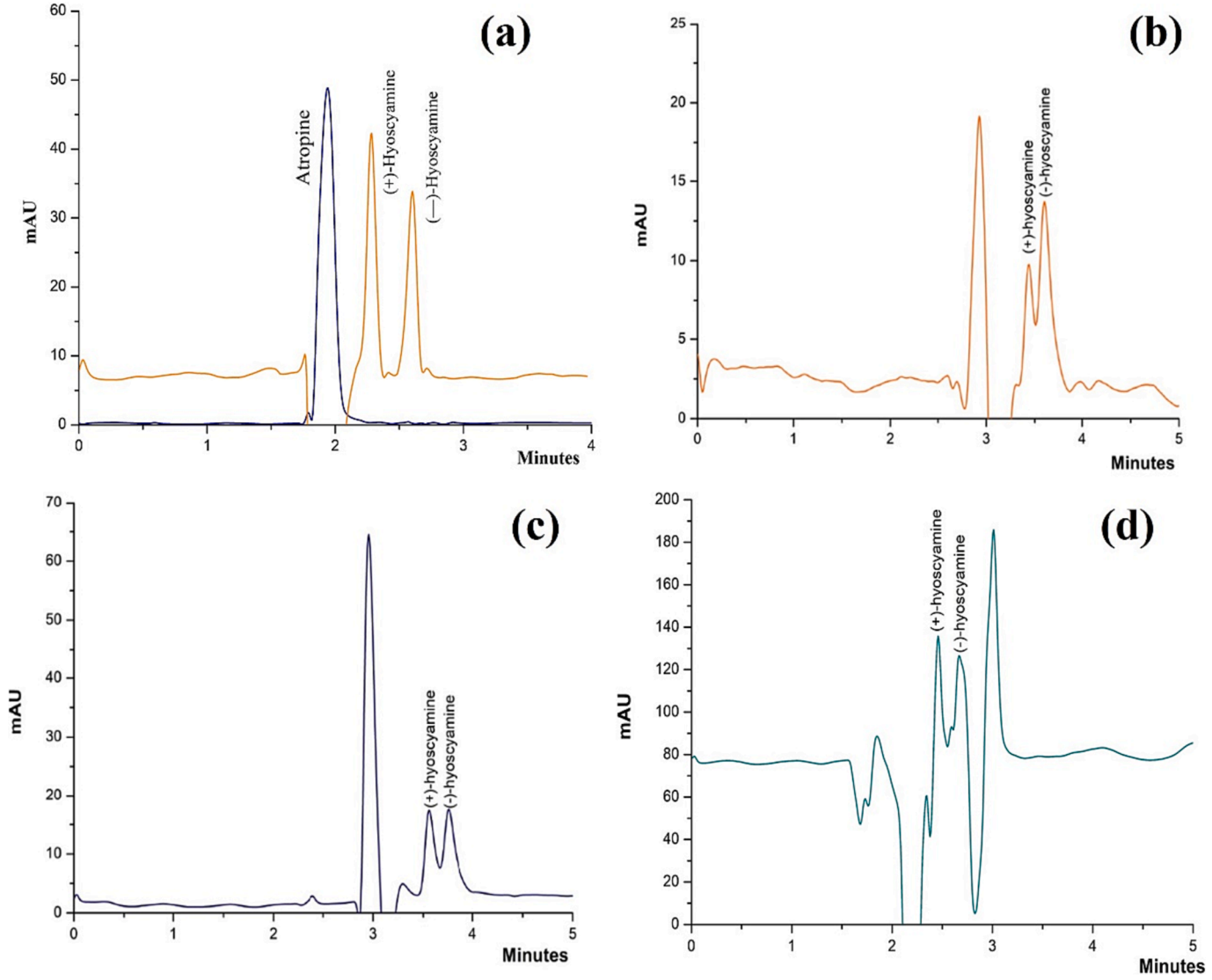

Fig. 4. Chiral chromatographic separation of atropine enantiomers by HPLC-DAD in: (a) Chiralpak® AY-3 with ethanol (blue) or ethanol with 0.1 % diethylamine (yellow) as mobile phase. (b) Lux® Cellulose-1 with ethanol with 0.1 % diethylamine as mobile phase. (c) Lux® Cellulose-1 with methanol with 0.1 % diethylamine as mobile phase. (d) Chiralpak® AY-3 with hexane/ethanol (90/10, v/v) with 0.1 % diethylamine as mobile phase. Flow rate 1 mL/min, room temperature, $\lambda = 230$ nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

organic modifier can significantly affect the chiral separation and the variation of the alcohol content in the mobile phase is frequently used as an effective modulator of the eluotropic power to adjust the retention of the analytes [29]. In addition, polar basic additives can mask underivatized silanols and reduce peak tailing [30]. Table 1 shows the R_s values obtained with mobile phases consisting of mixtures of Hex with *i*-PrOH, EtOH and MeOH in 90/10 (v/v) and 0.1 % DEA at a flow rate of 1 mL/min at room temperature. As can be seen, all experiments carried out with Chiralpak® AD-H and Lux® Cellulose-1 columns failed to separate the atropine enantiomers. On the other hand, when using the Chiralpak® AY-3 column, separation of atropine enantiomers was only possible when EtOH was used as organic modifier obtaining a chiral R_s of 1.13 ($k_1 = 1.89$ and $k_2 = 1.98$) as shown in Fig. 4d.

Finally, RP elution mode was studied using a mobile phase composed by H_2O/ACN in different proportions and 0.1 % of TFA as acid additive since this is perfectly compatible with MS detection and as these conditions were used in the separation of TAs in a previous work [31] (Table 1). The addition of water has an unpredictable effect on enantioseparation because can promote a strong solvation of the chiral selector and affect of all other interactions with enantiomers such as the hydrogen bonding directly involved in the chiral separation [32]. Chiralpak® AD-H was not suitable for the chiral separation of atropine under the conditions tested (Table 1). Using Chiralpak® AY-3, an increase in R_s to 1.25 was observed with increasing ACN concentration. In contrast, with the Lux® Cellulose-1 column, the opposite situation occurred and if ACN was reduced to 35 %, the $R_s = 2.73$ ($t_{r2} = 4.09$ min).

Employing Chiralpak® AY-3 column and EtOH, as a green organic solvent for the mobile phase in PO elution mode, with 0.1 % of DEA, other experimental parameters that can influence on chiral separation were evaluated. First, the concentration of DEA in the mobile phase was tested. Increasing the DEA concentration from 0.02 to 0.2 % did not yield significant changes, neither in the chiral R_s nor in the t_r. Based on the results, a concentration of 0.1 % DEA was chosen, as it resulted in improved peak shape and reduced width. The influence of temperature on the chiral R_s of atropine enantiomers was also studied since this parameter can improve R_s in any analytes and in others can lead to a loss in column efficiency [33]. In our study, an increase in column temperature from 20 to 30 °C decreases the retention time of the enantiomers (but no change in resolution) and slightly reduce the peak width, so it was decided to work at 30 °C. The flow rate of the mobile phase in

chromatography has a significant influence on the separation of compounds. A higher flow rate often leads to shorter retention times but can negatively affect the resolution between chromatographic peaks. In contrast, a lower flow rate often prolongs retention times and, under certain circumstances, improves R_s. Based on these considerations, this parameter was optimized studying flow rates in the range of 0.3 to 1 mL/min. It was observed that the best results were achieved at a flow rate of 0.8 mL/min, which reduced the analysis time to 5 min without compromising the Rs. This adjustment provided an efficient optimisation between speed of analysis and quality of chromatographic

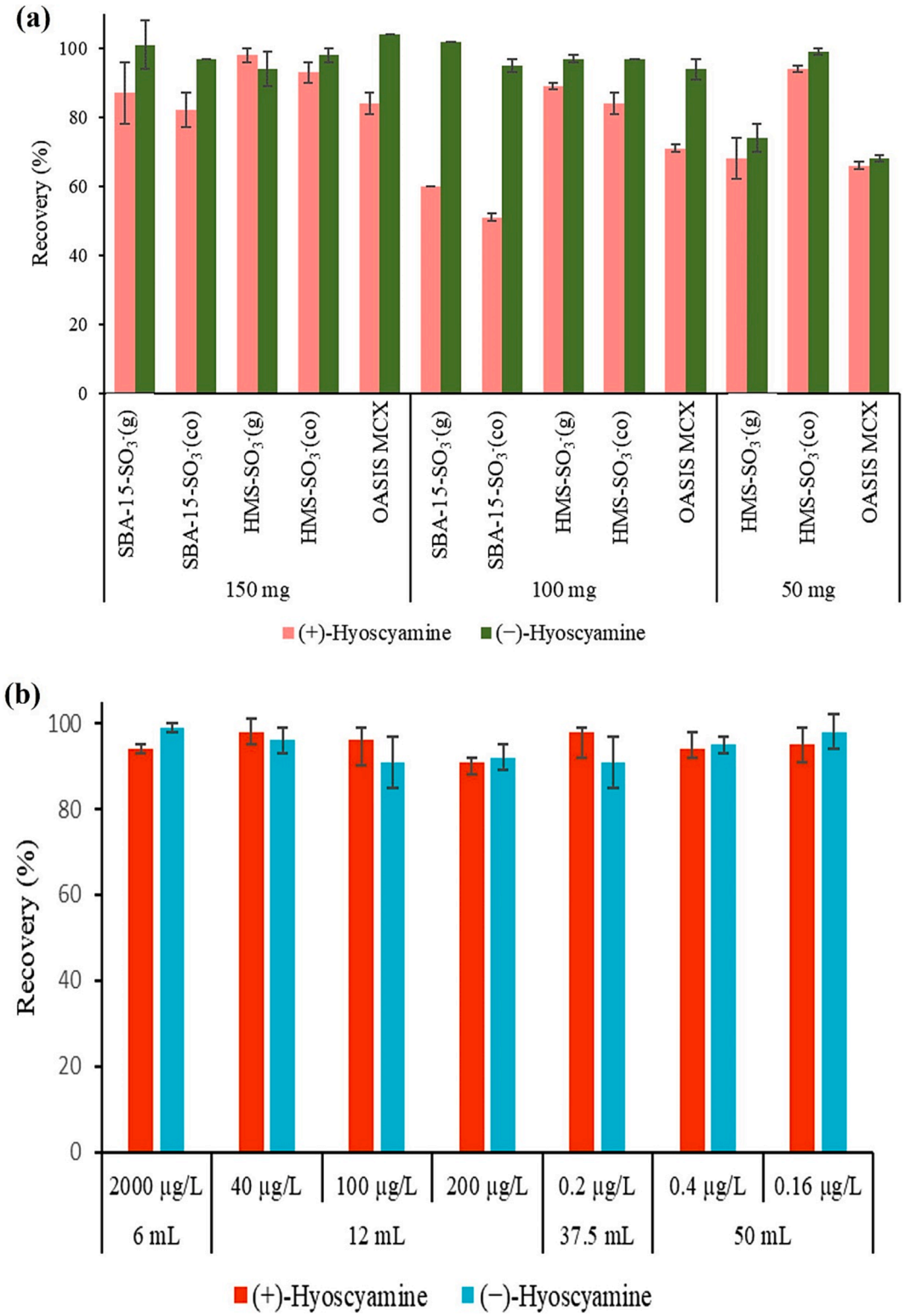

Fig. 5. (a) Recovery of (+)-hioscyamine and (−)-hioscyamine with the proposed SPE protocol using different amounts of the synthesised mesostructured silicas compared to the OASIS® MCX commercial material. (b) Recovery of atropine enantiomers under different loading volumes and analyte concentration in the SPE process.

separation.

Finally, to improve the sensitivity of the method the chiral chromatographic conditions were adapted to an HPLC-MS/MS system. The mobile phase was kept as EtOH, but since DEA can suppress the analyte response in MS/MS (ESI +) [33], its concentration was reduced to 0.05 % after confirming that separation was not lost (Fig. S4). The final flow rate of isocratic mobile phase (EtOH with 0.05 % DEA) was 0.6 mL/min with an analysis time of 6.5 min, a column temperature of 30 °C, and an injection volume of 10 μL (partial injection mode). Under these optimized conditions, $R_s = 1.59$ and $t_{r1} = 4.397$ min and $t_{r2} = 5.057$ min were obtained for (+)-hyoscyamine and (−)-hyoscyamine, respectively. The elution order of atropine enantiomers was studied by injecting a racemic mixture of atropine and a reference standard of (−)-hyoscyamine at a concentration of 100 μg/L. The order of elution observed was (+)-hyoscyamine/(−)-hyoscyamine as show Fig. S4.

There are few studies on the chiral separation of hyoscyamine enantiomers in food samples by HPLC-MS/MS. Jandric et al. [34] developed a method for determination of TAs in grains and seeds (wheat, rye, maize, soybean, linseed) with a Chirobiotic® V column in RP. The analysis time was 15 min and the flow rate was 1 mL/min but failed to achieve enantioselective separation of (+)- and (−)-hyoscyamine with sufficient sensitivity. More recently, Marín-Sáez et al. [35] evaluated two chiral columns Chirobiotic® V and Chiralpak® AY-3 for the separation of (−)- and (+)-hyoscyamine in *Stramonium* and *Brugmansia* seeds and in contaminated buckwheat. When the ChiralPak® AY-3 column was used, the best chiral resolution was achieved using EtOH with 0.1 % diethanolamine as mobile phase in isocratic elution mode for 10 min at a flow rate of 0.4 mL/min. According to these previous works, the chromatographic separation developed in our study have some advantages as analysis time was reduced to 6.5 min using a lower concentration of additive in the mobile phase (0.05 %).

3.3. Evaluation of mesostructured silicas as sorbent for SPE

The sample preparation stage is a crucial step in the analysis of complex samples such as food to obtain clean extracts, free of matrix interferences that can affect results and damage both the chromatographic columns and the MS/MS detector [13]. Based on previous work carried out by our group, the SCX-SPE was tested due to the excellent results obtained [18]. For this reason, four types of mesostructured silicas functionalised with sulphonic groups were evaluated and compared with the commonly used commercial sorbent OASIS® MCX.

SCX sorbents contain surface groups that are negatively charged over the entire pH range (e.g., acid functional group, such as sulfonic acid). Then, the material surface interacts with the oppositely charged target analytes via electrostatic (ionic) interactions. As was indicated in the 2.5 section, for hyoscyamine enantiomers analysis, it was necessary to prepare the infusion sample so that it exhibits the lowest ionic strength possible and has a pH that results in the enantiomers being negatively charged (pH 2.5). Interferences were removed from the sorbent with a washing step (MeOH/water/formic acid), while leaving the hyoscyamine enantiomers retained on the SCX sorbent. Finally, for eluting the hyoscyamine enantiomers, the pH was adjusted (MeOH containing ammonia) so that the analyte is neutralized and eluted from the SPE sorbent.

Fig. 5a shows the recoveries of (+)- and (−)-hyoscyamine, obtained for the different materials and amounts evaluated as SPE sorbents. As can be seen, when the cartridges were packed with 150 mg of material, recoveries of more than 80 % for (+)-hyoscyamine and more than 90 % for (−)-hyoscyamine were obtained using the mesostructured silicas as sorbents. When these results were compared with those obtained with the OASIS® MCX, using the same conditions, similar recoveries (>80 %) were obtained for both enantiomers. In view of these good results, further studies were carried out to optimise the amount of material to be used and reduce it. When 100 mg was tested, a decrease in the recovery of (+)-hyoscyamine was observed (below 60 %) with SBA-15-SO_3^-(g) and SBA-15-SO_3^-(co) sorbents. In the case of HMS-SO_3^-(g) and OASIS® MCX, when the amount of packed sorbent was lowered to 50 mg, the recoveries were reduced below 70 %. However, the recoveries with HMS-SO_3^-(co) were 94 % and 99 % for (+)- and (−)-hyoscyamine, respectively. Bearing in mind these results, cartridges packed with 50 mg HMS-SO_3^-(co) were selected for SPE in the present study. The good recoveries obtained with only 50 mg of HMS-SO_3^-(co) sorbent could be attributed to the higher L_o of this material, compared to the HMS-SO_3^-(g). In addition, the small particle size of HMS-SO_3^-(co) sorbent, with pseudo-spherical morphology, provides better access to the wormlike-framework confined mesopores in extraction processes, as well as a more homogeneous packaging of SPE cartridges, compared to SBA-15-SO_3^-(co) sorbent which helps to achieve best extraction efficiency. This fact was previously evidenced by our group using HMS functionalized with C18 groups for extraction of drug residues from food samples [16].

Then, additional experiments were carried out to evaluate the effect of sample loading volume on recovery, at different concentrations of atropine (Fig. 4b). These studies were carried out with the I-SE-B2 sample doped at a high concentration of atropine (2000 μg/L) using 50 mg of HMS-SO_3^-(co) as sorbent. Initially, a 6 mL loading volume was investigated. Despite the high concentration, recoveries for both enantiomers reached values close to 100 %. Based on these favourable results, the loading volume was increased, and the concentration of the target analytes reduced. A volume of 12 mL of the I-SE-B2 doped with atropine to a final concentration of 200, 100 and 40 μg/L was evaluated. As can be seen in the Fig. 4b (Table S4), recovery values higher than 90 % were obtained for both enantiomers. Subsequently, a loading volume of 37.5 mL, as was recommended in the study of Mulder et al. [8], and an atropine concentration of 0.20 μg/L (which is the maximum concentration allowed by Regulation (EU) 2023/915 for plant infusions (liquid), as sum of atropine and scopolamine [5,8]) was studied. Under these conditions, good recoveries of 98 % for (+)-hyoscyamine and 91 % for (−)-hyoscyamine were obtained. Finally, higher volume (50 mL) with 0.16 μg/L of atropine (below the maximum established in Regulation (EU) 2023/915) was evaluated and good recovery values of 95 % and 98 % of each enantiomer were obtained (Fig. 4b, Table S4). Considering these results, 50 mg HMS-SO_3^-(co) sorbent and 50 mL loading volume were established as the best conditions resulting in a pre-concentration factor of 167. Finally, a study of the reuse of the HMS-SO_3^-(co) synthetized as sorbent for SPE of hyoscyamine enantiomers was carried out, and the results confirmed that it can be reused at least 5 times (recovery 96 ± 5 after 5 cycles).

3.3.1. Evaluation of the racemisation process during SPE procedure

To our knowledge, there are few studies on the atropine racemisation, as it is a complex process that can occur during the isolation from plant material but also during certain metabolic changes in the living plant. This was evidenced by the presence of almost pure (−)-hyoscyamine in young plant organs, 100(−)/0(+) ratio, while in older organs higher amounts of the (+)- enantiomer are found [1]. It has been observed that in solutions with pH 3, racemisation does not appear to occur, with only slight hydrolysis. However, in solutions with pH higher than 3 and elevated temperatures, racemisation is triggered [35,36]. In a specific study by Marín-Sáez et al. [35] enantiomerization process of atropine was studied in Solanaceae seed samples at different conditions of temperature (30 to 80 °C) and pH (3 to 9), observing that racemization occurs at high pH when high temperature is applied for long periods of time [35].

In this context, our next studies were focused to evaluate the racemisation that might occur during the SPE process under the optimized conditions. The racemisation rates during purification using HMS-SO_3^-(co) as sorbent are presented in Table S5. Initially, an atropine standard solution was injected to confirm the 50(−)/50(+) ratio of both enantiomers. Subsequently, the atropine standard solution was added to 50 mL of I-SE-B2 sample to a final concentration of 0.1 μg/L of each

enantiomer, then pH was adjusted to 2.5 and the optimised SPE protocol was applied. The results confirmed that there are no statistically significant differences in the proportion of each enantiomer after del SPE (with a 49(−)/51(+) ratio). On the other hand, similar experiments were carried out to estimate enantiomerization rate of (−)- to (+)-hyoscyamine by adding a (−)-hyoscyamine standard solution to 50 mL of different baby herbal infusions (I-SE-B2, I-DP-3 and I-DP-5, see Table S5). Firstly, the (−)-hyoscyamine standard solution was injected to verify that no significant racemization occurred due to separation conditions used in the HPLC-MS/MS (due to mobile phase composition), obtaining a ratio 95(−)/5(+). Then, the effect of the SPE process on the racemization was evaluated. I-SE-B2 samples (prepared with 2.5 and 5 g dissolved in 50 and 75 mL of water, respectively, according to the manufacturer's recommendations), and I-DP-3 and I-DP-5 samples (prepared with 1.5 g teabag infused in 200 mL of water) were used. A mean 92.5(−)/7.5(+) ratio was observed for instant infusions, whereas for herbal infusions from dried plants a mean ratio of 80.5(−)/19.5(+) was achieved, indicating that some racemization of (−)- to (+)-hyoscyamine occurs during the SPE process. This transformation is attributed to the basic pH used for the elution of the target analytes because similar results were observed when extracts were spiked with the (−)-hyoscyamine standard solution after the SPE elution step. These results are supported by previous studies indicating that racemisation occurs at basic pH [35]. Bearing in mind these results, to quantify the amount of each enantiomer in the samples analysed, a correction factor was applied to the data obtained depending on the type of herbal infusion.

3.4. Method validation

For the instrumental validation of the HPLC-MS/MS analysis, an atropine standard solution was used, and the results are presented in Table S6. A linear range of 10 to 500 μg/L was established for both enantiomers, with coefficient of determination (R^2) values above 0.999. In addition, LOD and LOQ of 3 and 10 μg/L, respectively, were obtained for both enantiomers. On the other hand, the reliability of the developed SPE-HPLC-MS/MS method was performed by evaluating parameters such as linearity, accuracy, precision, selectivity, MDL, MQL and ME, following the recommendations described in SANTE/11312/2021, ICH Harmonised Tripartite Guideline [21,22]. The I-SE-B2 sample was used for method validation with baby herbal infusion prepared from soluble plant extracts. In the case of teabag products, I-DP-4 sample was used. Matrix matched calibration curves showed good linear regression with R^2 greater than 0.99 for both enantiomers (Table 2 and Table S7). The method demonstrated excellent selectivity, with the ion ratio deviations in sample no exceeding ± 30 % when compared to the mass spectra derived from standard solutions. Additionally, the retention time for all analytes fell within the range of ± 0.1 min. The method was linear in the range 14.0 to 100 μg/L for (+)-hyoscyamine and 15.0 to 100 μg/L for (−)-hyoscyamine. The MDL were 4.4 μg/L and 4.6 μg/L for (+)-hyoscyamine and (−)-hyoscyamine, respectively. The MQL were 14.8 μg/L and 15.3 μg/L for (+)-hyoscyamine and (−)-hyoscyamine, respectively (Table 2), that corresponds to 0.089 μg/L and 0.092 μg/L in the infusion sample (preconcentration factor 167). These limits are lower than the maximum content of tropane alkaloids (sum of atropine and scopolamine) set for liquid herbal infusions (0.2 ng/mL), published in the Commission Regulation (EU) 2023/915 [5]. The ME was 83 % for both enantiomers in I-SE-B2 sample (Table 2) and 104 % for both enantiomers in I-DP-4 sample (Table S7), so no significant ME was found since ME does not exceed ± 20 %. Satisfactory average recoveries were obtained for the three validation levels, 92 % and 93 % for (+)-hyoscyamine and (−)-hyoscyamine, respectively in I-SE-B2 sample (Table 2). In I-DP-4 sample recovery values of 90 % for (+)-hyoscyamine and 88 % for (−)-hyoscyamine at a concentration of 0.1 μg/L were obtained. On the other hand, precision also showed satisfactory results with RSD ≤ 8 % for both enantiomers.

3.5. Sample analysis

Finally, the feasibility of the developed method was applied to the analysis of different baby herbal infusions mainly used to help with sleep, calm digestion, soothe the throat, soothe irritation, or contribute to the relaxation of babies. The results of the samples analysed with the proposed SPE-HPLC-MS/MS methodology (Table S8) show that three of the infusions studied were contaminated with (−)-hyoscyamine. The current legislation regulating the maximum content of TAs in certain foodstuffs sets a maximum content of 0.20 ng/mL for plant infusions (liquid) (sum of scopolamine and atropine, without differentiating enantiomers), as there are few studies in foodstuffs in which enantioseparation of atropine has been achieved [5].

Of the three batches of instant infusions analysed, two were contaminated. Batch-1 had a concentration < MQL for (+)-hyoscyamine and 0.075 μg/L of (−)-hyoscyamine and Batch-3 had a higher concentration of both enantiomers (Fig. 6), 0.09 μg/L for (+)-hyoscyamine and 0.21 μg/L of (−)-hyoscyamine (ratio 70(-)/30(+)), with the sum of both enantiomers being 0.30 μg/L, exceeding the 0.20 ng/L level set by the legislation. According to the EC Regulation [5], as these three batches were legally placed on the market before 1 September 2022, they may remain on the market until their date of minimum durability (that is 2025). In the case of infusions prepared from the dried plants in teabag format, hyoscyamine was detected in one sample but both enantiomers were below the MQL. According to the findings of Marín-Sáez et al. [35], it is not expected that the racemization occurred during the infusion preparation, taken into consideration that pH of the infusions was

Table 2
Validation parameters of SPE-HPLC-MS/MS method proposed for determination of (+)-hyoscyamine and (−)-hyoscyamine in a representative sample of baby herbal infusion (I-SE-B2).

Analyte	Linear range (μg/L)*	Matrix-matched calibration (R^2)	Accuracy		Precision		MDL (μg/L) *	MQL (μg/L) *	ME (%)
			Recovery (% ± sd)	Mean recovery (% ± sd)	Repeatability (RSD %)	Reproducibility (RSD %)			
(+)-Hyoscyamine	14.0–100	$y = 2.62{\cdot}10^5x + 2.00{\cdot}10^6$ (0.996)	88 ± 7^a 90 ± 7^b 97 ± 2^c	92 ± 3	8^a 8^b 3^c	8^a 7^b 2^c	4.4	14.8	83
(−)-Hyoscyamine	15.0–100	$y = 2.59{\cdot}10^5x + 2.00{\cdot}10^6$ (0.996)	89 ± 5^a 94 ± 3^b 97 ± 4^c	93 ± 1	6^a 3^b 4^c	7^a 4^b 4^c	4.6	15.3	83

*MDL: method detection limit and MQL: method quantification limit estimated as 3 and 10 times the signal/noise ratio, respectively (concentrations in the sample extract after the SPE process). MQL corresponds to 0.089 μg/L for (+)- and 0.092 μg/L for (−)- enantiomers in the infusion sample according to the validated analytical methodology (preconcentration factor = 166.7). Matrix effect (ME) = (slope matrix-matched calibration/slope solvent calibration) x 100. Accuracy (mean recovery obtained from six samples, n = 6) and precision were obtained by spiking samples at three known concentration levels. Repeatability: three replicate samples injected in triplicate in the same day (n = 9) on the same day; Reproducibility: three replicate samples injected in triplicate throughout three different days (n = 9). [a] Low spiked level (0.08 μg/L), [b] Medium spiked level (0.1 μg/L), [c] High spiked level (0.2 μg/L) of each enantiomer.

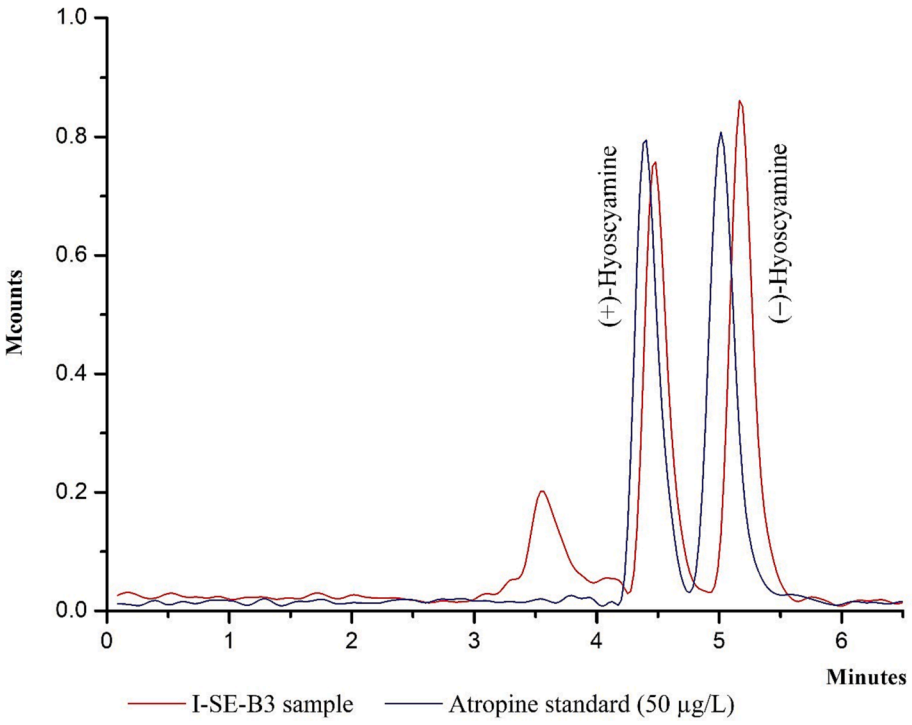

Fig. 6. Extracted ion chromatogram for a contaminated baby herbal infusion (I-SE-B3) compared with a 50 µg/L standard solution of atropine (*m/z* 290.1 > 124.0).

between 5.3 and 6 and the preparation was done with warm water (for instant infusions) or with hot water (5 min for teabag infusions). For this reason, a possible explanation is that an unequal mixture of the two enantiomers, whose proportions range from 100(-)/0(+) (in young plants) to 51(−)/49(+) [37], was present in the TAs-containing weeds co-harvested with the infusion plants. On the other hand, the results of this study are in line with those of a previous one [38], where 33 samples of tea and herbal infusions brewed at 97 °C for 5 min were analysed. The study revealed that 64 % of the samples analysed contained TAs [38], exceeding the maximum limit for liquid herbal teas (0.2 ng/mL). In addition, it was demonstrated the resistance of these compounds to high temperatures and short times, highlight the significant influence of the infusion preparation on the extraction of these compounds [38]. Considering these previous results and those found in the present work, it can be concluded that it is important to assess the (−)- and (+)-hyoscyamine content in the infusions (liquid) instead of in the dried herbs to avoid an overestimation of the exposure to (−)-hyoscyamine.

4. Conclusions

In this work, a chiral HPLC-MS/MS method was developed for the determination of (−)- and (+)-hyoscyamine that provides very good resolution for both enantiomers in only 6.5 min, using a mobile phase based on environmentally friendly ethanol with very low concentration of diethylamine as additive. The sulfonic HMS mesostructured silica synthesized by co-condensation has proven to be a good SCX-SPE sorbent, with a very high degree of functionalization and adequate textural properties. Under optimized conditions, recoveries close to 100 % were achieved for both enantiomers, using a minimal amount of material (50 mg that can be reused at least 5 times without significant deterioration of its original preconcentration capacity) that meets GAC requirements. The limit of quantification of the method was lower than the maximum content of tropane alkaloids established for liquid herbal infusions, so it was successfully applied for the determination of hyoscyamine enantiomers in eight baby herbal infusion samples. An unequal mixture of the two enantiomers was found in the contaminated samples, which confirmed the importance of evaluating the content of each enantiomer to avoid overestimation of (−)-hyoscyamine exposure in children.

CRediT authorship contribution statement

Fernando L. Vera-Baquero: Writing – original draft, Visualization, Validation, Methodology, Investigation. **Judith Gañán:** Writing – original draft, Methodology. **Sonia Morante-Zarcero:** Writing – review & editing, Supervision, Conceptualization. **Isabel Sierra:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

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