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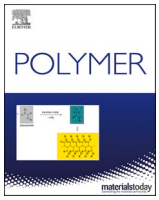

Determination of atropine and scopolamine in honey using a miniaturized polymer-based solid-phase extraction protocol prior to the analysis by HPLC-MS/MS

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ABSTRACT

Two homopolymers have been prepared employing methacrylic acid and 4-vynylpiridine as functional monomers (p-MAA, p-4-VP) through an easy and quick precipitation polymerization method for application as sorbents in solid-phase extraction (SPE) to determine atropine and scopolamine in honey. The optimized SPE conditions were as follows: 25 mg of p-MAA, 4 mL of sample loading volume (diluted honey 1:10 with water), 4 mL of elution solvent (methanol/water with 1 % formic acid, 80/20, v/v). The extracts were analyzed by HPLC-MS/MS. The cartridges were reusable for forty cycles demonstrating an environmentally friendly approach. The methodology was validated in terms of linearity, accuracy, precision, selectivity, matrix effect and sensibility, highlighting the absence of matrix effect. The miniaturized polymer-based SPE was successfully applied to fifteen honeys, showing concentrations up to 7.23 ng/g in the most contaminated honey. All quantified honey samples (5 in total) were of multifloral type.

1. Introduction

In recent years, food safety has gained increasing attention, and this quality parameter can be threatened by the presence of environmental contaminants, drugs, natural toxins, biological contaminants, or the improper use of additives in food. Specifically, natural toxins belong to a group of chemicals synthesized by living organisms such as animals or plants. These substances suppose a risk to humans when they are ingested and therefore require careful consideration in food safety assessments. The family of natural toxins includes tropane alkaloids (TAs), which constitute a group of over 200 compounds produced as secondary metabolites by a wide variety of plant families such as Solanaceae, Brassicaceae, Convolvulaceae, and Erythroxylaceae. The two most representative compounds in this group are atropine and scopolamine, and some of the foods susceptible to containing these toxics are cereals (millet, sorghum, buckwheat), teas, and herbal infusions, among others [1]. TAs can be introduced into the food chain through various pathways, including cross-contamination with TA-producing plants, horizontal transfer through the soil, or transfer facilitated by certain insects such as bees [2,3]. The ingestion of these toxins can lead to toxic effects on the peripheral nervous system, such as mydriasis, dry mouth, tachycardia, or urinary retention. Additionally, these toxins can induce effects on the central nervous system, including delirium, hallucinations, muscle spasms, and, in extreme cases, can result in death [4]. For these reasons, TAs are regulated to ensure the monitoring and control of these toxins in specific foods such as cereals, baby food, and herbal infusions [5]. Although most studies focus on analyzing TAs in plant-based foods, it is necessary to investigate animal-based foods as well. Some of these products, such as meat or milk, may contain TAs due to the consumption of feeds contaminated with these toxins by animals [6]. Furthermore, honey is an animal product that generates interest due to the beneficial compounds that it contains, but it is important be aware of the contaminants that may be present in this product [7]. For example, TAs could be found in all parts of different TA-producing plants, such as

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leaves, seeds, roots and flowers. These toxins have been detected in the floral nectar and pollen of various species, and therefore, they can be present in honey [8,9]. However, as these compounds are not currently regulated in honey and to ensure the health of the population, especially honey consumers, it is necessary to develop sensitive, selective, and sustainable methodologies that allow for the determination of these toxins. Actually, the current trend focuses on the development of "greener" methodologies and analyst now consider the environmental impact produced by method development, aiming to replace polluting methodologies with more sustainable alternatives [10,11]. In line with this concept, the field of Green Analytical Chemistry (GAC) has emerged, and analytical chemists also considered that their procedures should be as sustainable as possible. Twelve principles have been proposed based on the direct analysis of the sample without pretreatment, the miniaturization and automatization of processes, and the use of non-toxic reagents to ensure operator safety, among other factors [12]. Nowak et al. (2021) introduced the concept of White Analytical Chemistry (WAC), which integrates the principles of GAC with the idea that a method must also be useful and effective [13]. While the concept of eliminating the sample preparation stage, as proposed by the authors, may be ideal, it is often impractical in many cases due to the complexity of certain samples, such as foods, and the low concentrations at which certain analytes are present. Sample preparation remains a crucial step in analytical chemistry, and current trends involve the development of methods with fewer steps, shorter analysis times, miniaturization and automatization of processes and, the synthesis of new materials for their application as sorbents instead of conventional extraction procedures. These aspects are all encompassed under the term Green Sample Preparation (GSP) [14].

Solid phase extraction (SPE) is a widely used technique for sample preparation due to its simplicity and flexibility [15,16]. New materials employed as sorbents for SPE can be useful in the sample preparation step for the selective recognition of the analyte to be determined. Some of these materials include the polymeric-based materials, silica-based materials, or the magnetic nanoparticles [17–19]. They are employed due to their advanced physicochemical properties, which contribute to increase the selectivity and sensitivity in analytical methods [20]. Polymeric-based materials are extensively utilized as sorbents due to their porosity, high selectivity, reusability, chemical resistance against acids and bases, and the possibility of being functionalized, coupled with mechanical strength and these materials have been applied over the years and this field is continuously expanding. They can be used for numerous applications, including the extraction of certain compounds in environmental, food or biological samples, and commercial polymers or homemade polymers can be applied [21–24]. Polymeric-based materials can be synthesized through a diversity of routes, such as bulk polymerization, which was the primary approach. However, this route often results in irregular particle sizes, as the material needs to be ground before the synthesis procedure. Polymers produced using the precipitation method, on the other hand, have a regular diameter, providing an advantage over those synthesized through bulk polymerization [25]. Regarding the classification of the polymers, if the polymerization is carried out using only one type of monomer, the resulting polymer is referred as a homopolymer. Conversely, if the polymerization involves more than one type of monomer, the material is referred to as a copolymer [26]. Methacrylic acid (MAA) is one of the most commonly used monomers for polymerization due to its functionality, flexibility, durability, robustness, and excellent binding efficacy with a wide variety of compounds. Additionally, 4-vynilpyridine (4-VP) is widely employed as polymeric sorbent in numerous applications [27,28]. These characteristics make polymers a promising alternative for use as sorbents in SPE.

Currently, only five studies have been conducted with the objective of developing methods to analyze TAs in honey samples [8,9,29–31]. For the extraction of the TAs and the purification of the sample, authors used methodologies based on SPE and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) or a simple solid-liquid extraction with methanol and formic acid without purification step. With all this in mind, the aim of this study was to synthesize a simple and efficient polymer-based material as sorbent for SPE to extract atropine and scopolamine in honey prior to their analysis by HPLC-MS/MS, which allowed the development of a sustainable methodology that involved the use of the minimum amount of sorbent and its reuse.

2. Materials and methods

2.1. Solvents, materials and standard solutions

Scopolamine hydrobromide (≥98 %) and atropine (≥99 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard solution of the analytes (1000 mg/L) were prepared in amber vials by diluting 1 mg of each one in 1 mL of methanol (MeOH). A solution containing the two analytes (1 mg/L) was prepared in MeOH by appropriate dilution of the standard solution. All of them were stored at −18 °C in darkness.

MeOH LC-MS grade and ACN LC-MS grade were acquired from Scharlab (Barcelona, Spain). A Millipore Milli-Q system (Billerica, MA, USA) was used to obtained ultrapure water (H_2O) (18.2 MΩ cm). Formic acid (FA) LC-MS grade was purchased from Fischer Scientific (Loughborough, UK). Nylon syringe filters (0.45 μm, 0.23 mm) were acquired from Mervilab (Madrid, Spain), empty SPE cartridges (3 mL) and polyethylene frits were purchased from Scharlab (Barcelona, Spain).

4-Vinylpyridine (4-VP, 95 %) was acquired from Acros Organics at it was stored at less than −15 °C. Methacrylic acid (MAA, 99 %) was purchased from Sigma Aldrich. 2,2-azobis(2-methylpropionitrile) (AIBN, 96 %) were acquired from Sigma Aldrich and ethylene dimethacrylate (EGDMA, 98 %) were purchased from Acros Organics and they were stored at 4 °C.

2.2. Synthesis of the polymers

Two different homopolymers were synthesized: the first containing 4-VP (denoted as p-4-VP), and the other one containing MAA (denoted as p-MAA) as functional monomers. Initially, 6 mmol of each functional monomer were dissolved in 25 mL of ACN. The mixture was uniformly dispersed with sonication for 2 min, followed by an incubation period of 30 min at room temperature. Then, 45 mg of AIBN (initiator of the polymerization) and 20 mmol of EGDMA (cross-linker) were added to the mixture, and it was sonicated for another 2 min. Subsequently, it was bubbled with nitrogen for 7 min to remove the oxygen present in the solution. Finally, the polymerization was carried out at 60 °C for 24 h in a silicon bath with stirring (350 rpm). The obtained solid polymer was washed with acetone, followed by a drying period at 40 °C for 12 h.

2.3. Characterization of the polymers

The polymers were characterized through a scanning electron microscope study, the determination of the nitrogen gas adsorption-desorption isotherms, and X-ray diffraction. The surface morphology was examined by a Scanning Electron Microscope (SEM) EM-30AX Plus COXEM from JASCO (COXEM, Korea). Before SEM analysis, the samples were coated with Au using a SPT-20 sputter coater. The samples were mounted in a metal stub using a sticky carbon disc and they were coated with 50 nm of gold for 300 s at 50 mA. The prepared samples were then observed under SEM at an accelerated voltage of 20 kV and a magnification between 70 and 100,000 times. Measurements of isotherms were carried out using a Micrometrics analyser (ASAP 2020, Micrometrics, Norcross, Georgia, USA). The method selected to calculate the surface specific area was the Brunauer-Emmett-Teller (S_{BET}) and the method used to obtain the pore size distribution was the Baret-Joyner-Halenda (BJH). Previously to this analysis, 0.2 g of material was dried under vacuum. Characterization X-ray diffraction (XRD) patterns of the polymers were obtained on a Philips Diffractometer model PW3040/00

X'Pert MPD/MRDat 45 kV and 40 mA, using Cu Kα radiation (α = 1.5418°A).

2.4. Honey samples

Fifteen honey samples were analyzed, ten of them were purchased from local markets, and the others were directly obtained from honeycombs in different farms of Spain. Ten of these samples were multifloral type, and the other five samples were monofloral honeys including rosemary, sunflower, eucalyptus and orange blossom (see Table S1). The samples had different origin countries such as Spain, Ukraine, Bulgaria, Brazil, China, Argentina, Cuba and Uruguay.

2.5. Optimized sample preparation procedure

0.4 g of homogeneous honey (±0.0001 g) was weighed, and it was dissolved with 4 mL of H_2O. This mixture was stirred for 5 min at room temperature to obtain a homogeneous solution of the sample. To carry out the purification, a polymer-based SPE procedure was optimized. For this, different studies were carried out to determine the optimized conditions of the extraction procedure, such as the type and amount of sorbent, the loading and elution solvent, and their volumes. The optimized protocol can be summarized as follows: 25 mg of p-MAA were packed between two frits into 3 mL empty SPE cartridge, which was disposed on a Supelco Visiprep SPE vacuum manifold 12 port model (Sigma Aldrich, St. Louis, MO, USA) connected to a vacuum pump at 10 psi. The SPE sorbent was conditioned with 2 mL of MeOH, followed by 2 mL of H_2O. Then, the diluted honey sample was loaded, and 4 mL of MeOH/H_2O (1 % FA) 80/20 (v/v) were used as elution solvent. The eluate was evaporated in a vacuum line, reconstituted in 0.5 mL of MeOH, so a preconcentration factor of 8 was obtained. Finally, it was filtered using a 0.45 μm nylon filter before the HPLC-MS/MS analysis (Fig. 1).

2.6. HPLC-MS/MS analysis

The purified extracts were analyzed by an HPLC system coupled to a triple quadrupole (QqQ) tandem mass spectrometer detector (1200/1200 LC-MS/MS, Varian, Ibérica, Madrid, Spain) with a data acquisition system MS Workstation (version 6.3). The HPLC contained two modules (Prostar 210/215), an autosampler with a 100 μL loop (Prostar 410) and a column heater section. The chromatographic separation was performed at 30 °C using a reverse C18 Kromaphase 100 column (150 mm × 2.0 mm, 3.5 μm particle size) coupled to a C18 Kromaphase guard column (10 mm × 4.0 mm, 5 μm particle size) that were purchased from Scharlab (Barcelona, Spain). The separation was carried out following the method developed by González-Gómez et al. (2021) using a mobile phase gradient elution with ACN with 0.1 % of FA (solvent A) and H_2O with 0.1 % of FA (solvent B) as follows: the gradient starts with 90 % of B, from 90 to 30 % in 10 min, from 30 to 90 % in 1 min and finally 90 % for 4 min constituting a total run time of 15 min with a flow rate of 0.25 mL/min and the injection volume was 10 μL [32].

The parameters used for the mass spectrometry detection were set as follows: electrospray ionization interface (ESI) in positive ion mode, the ion spray voltage age was 5000 V for capillary and 600 V for shield, the drying gas (N_2) was at 22 psi (350 °C), the nebulizer gas (N_2) pressure was at 58 psi and a voltage of 1480 V, and the collision gas (Ar) was at 1.9 mTorr. Multiple reaction monitoring (MRM) scan mode was used for the analytes (scan width 0.7, mass peak width Q_1 2.5; Q_3 2.5) and the mass spectrum parameters were obtained by direct infusion of individual standard solution of the scopolamine and the atropine (10 mg/L) with a flow rate of 20 μL/min. For atropine the precursor ion was 290.1 m/z, and the products ions were 124.1, 93.0 and 90.9 m/z with a collision energy of 20.5, 29.0 and 34.0 V, respectively and the product ion selected for the quantification was 124.1 m/z. For scopolamine the precursor ion was 304.1 m/z, and the products ions were 156.0, 138.1 and 121.0 m/z with a collision energy of 9.5, 12.0 and 16.0 V respectively and the product ion selected for the quantification was 138.1 m/z. Fig. S1 shows the extracted ion chromatogram (EIC) of a standard solution (10 ng/mL) of atropine (Fig. S1a) and scopolamine (Fig. S1b) and their mass spectrum.

2.7. Analytical validation of the methodology

The proposed polymer-based SPE-HPLC-MS/MS procedure was validated in terms of accuracy, precision, linearity, selectivity, matrix effect (ME), method detection (MDL) and quantification (MQL) limits. The analytical parameters were evaluated following the

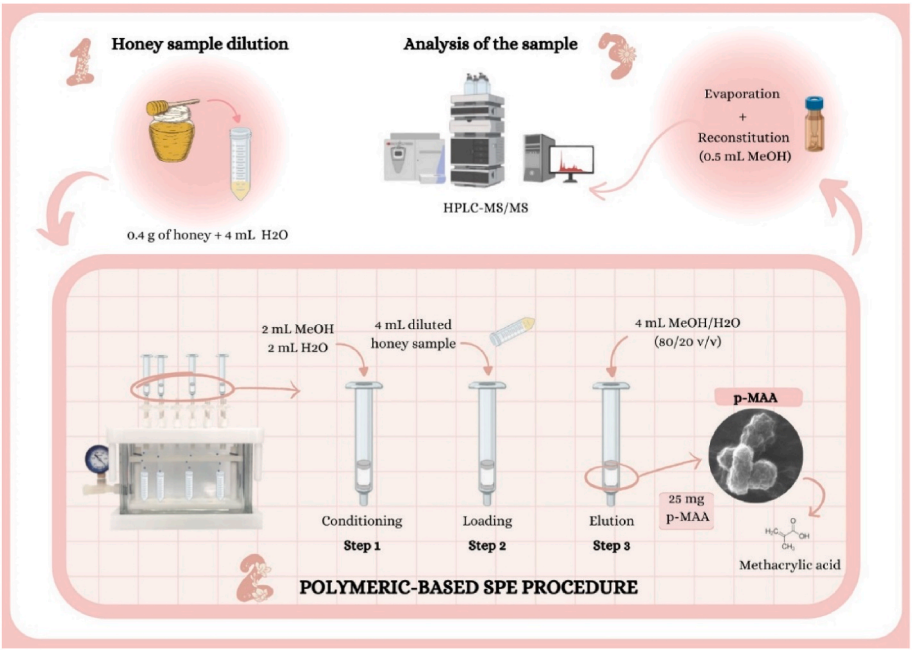

Fig. 1. Polymer-based SPE-HPLC-MS/MS developed methodology.

recommendations of Guidance SANTE/11312/2021 for pesticides [33]. Three validation levels were selected to evaluate the feasibility of the method. The low level was choose since it corresponds to the minimum concentration capable of quantifying with the method developed (0.5 ng/mL corresponding to 0.625 ng/g for the atropine and 1.5 ng/mL corresponding to 1.875 ng/g for the scopolamine). The intermediate level was selected according to the TAs levels found in honeys previously analyzed by other authors (40 ng/mL corresponding to 50 ng/g). For the high level, eight times the intermediate level was chosen (320 ng/mL corresponding to 400 ng/g). The linearity of the method was evaluated by matrix calibration in a range of 0.625–400 ng/g for the atropine and 1.875–400 ng/g for the scopolamine by spiking extracts of a blank honey sample with standard solutions at different concentrations of the target contaminants. According to the validation guide, the linear coefficient of determination (R^2) should be close to 1. On the other hand, a calibration curve with standard solutions was prepared, and ME (%) was estimated as: (slope matrix-matched/slope solvent-based calibration) x 100. A percentage lower than 100 % means that the signal of the analyte is suppressed by the matrix, and when is higher than 100 % means a signal enhancement. If the percentage is between 80 and 120 %, the ME can be ignored, but otherwise it should be considered for the quantitative measurement of the target analytes. The selectivity is related to the spectra of the sample extracts, and it is considered satisfactory when there is a variation in the ion ratio of less than ±30 % and if the retention time of the analytes does not vary more than ±2.5 %. The sensitivity of the method is related to the MDL and the MQL which were calculated based on the signal-to-noise ratios (S/N) provided by the HPLC-MS/MS from the extracted ion chromatograms of the multifloral and monofloral honeys at the low concentration level of the matrix-matched curves. Consequently, the concentration corresponding to a S/N of 3 represented the MDL, while the concentration corresponding to a S/N of 10 denoted the MQL, both expressed in ng of TA per g of honey.

The accuracy of the method was evaluated at three levels of concentration in terms of recovery, for which the area obtained by doping a honey sample and subjecting to the extraction process was compared with the area of a simulated sample that was doped after the extraction process previously to the chromatographic analysis. The results were expressed as the recovery obtained from nine samples in different days (n = 9) and the percentages must be between 70 % and 120 % according to the selected validation guide. The precision was also evaluated at the same levels of validation (high, intermediate and low) in terms of repeatability (intra-day precision) and reproducibility (inter-day precision). The intra-day precision was evaluated by analysing on the same day six replicate extracts (n = 6) of a honey sample (multifloral and monofloral types), while the inter-day precision was evaluated by analysing three replicates honey extracts the sample obtained over three different days (n = 9). The results were expressed in terms of relative standard deviation (RSD, %) and the values should be below the 20 %.

3. Results and discussion

3.1. Structural characterization of the polymers

The p-MAA and p-4-VP polymers were synthesized quickly and easily, requiring very little volume of solvents. This could be an advantage compared to other more expensive and laborious methods of polymeric synthesis, such as MIPs which requires more time due to the need of mortar and template extraction [34,35]. To determine the morphology of the polymers, SEM images were obtained. Fig. 2a and b show that the p-4-VP has a quasi-spherical form, while the p-MAA exhibits a sponge-type morphology.

The N_2 adsorption-desorption isotherms for the polymers synthesized are shown in Fig. 3a and b. As can be seen, both showed similar type IV isotherms with a H3 hysteresis loop according to the International Union of Pure and Applied Chemistry (IUPAC) classification.

The p-4-VP exhibits a high S_{BET} (252 m^2/g) and pore volume (0.97 cm^3/g) compared to the p-MAA (80 m^2/g and 0.18 cm^3/g, respectively) as shown in Table 1. In both polymers, pore sizes were lower than 20 Å indicating that they can be considered microporous materials.

The XRD patterns of the p-4-VP and p-MAA (Fig. 4) reveal a broad diffraction peak at 16° indicating an amorphous structure.

3.2. Optimization of the polymer-based SPE procedure

3.2.1. Study of type and amount of polymers as sorbents in different conditions

Initially, the two synthesized polymers (p-MAA and p-4-VP) were employed as sorbent for SPE process to assess their efficiency in extracting atropine and scopolamine from honey samples. In terms of interactions between the functional monomers and the target analytes, p-MAA contains MAA, which possesses hydroxyl groups capable of forming hydrogen and ionic bonds with the tropane alkaloids. On the other hands, p-4-VP contains aromatic rings that can interact with the analytes through π- π bonds [36]. Various studies were conducted with both homemade polymers, using different amounts, to determine which one yields better results. Additionally, three different loading solvents at varying concentration levels were tested to assess in which medium occurs higher retention of the analytes, as shown in Table 2.

To conduct this experiment, SPE empty cartridges were packed with 50 and 25 mg of p-MAA and p-4-VP. The study involved three concentration levels, by loading 2 mL of standard solutions containing atropine and scopolamine at concentrations of 10, 25 and 50 ng/mL in H_2O, MeOH and ACN. Subsequently, following a method described in the literature [37], elution was performed using 2 mL of a MeOH/H_2O (1 % FA) 60/40 (v/v) solution. In case of p-4-VP, using 50 mg of the polymer resulted in the sorbent adopting a rubbery texture, preventing the solvent from passing through. When the study was conducted with 25 mg of material, satisfactory recoveries were achieved for ACN and H_2O; however, handling proved challenging, leading to difficulties in

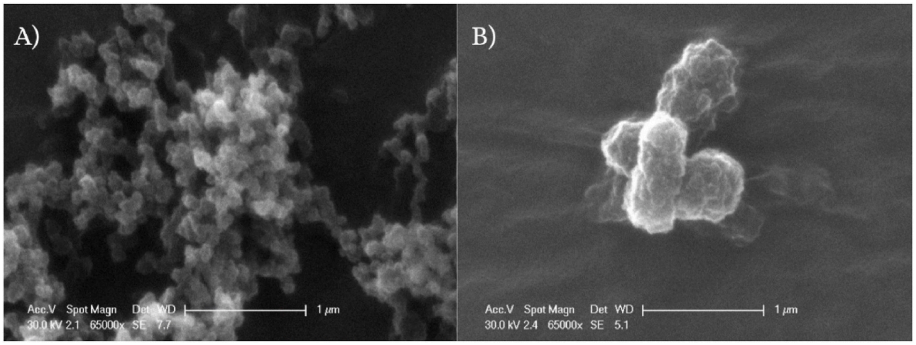

Fig. 2. A) SEM image for the p-4-VP. **B)** SEM image for the p-MAA.

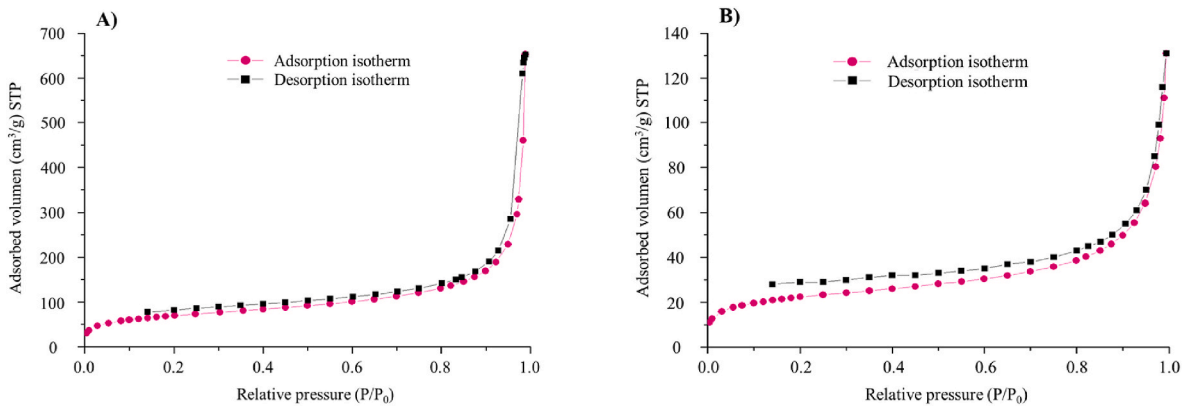

Fig. 3. A) N_2 adsorption-desorption isotherms for the p-4-VP. **B)** N_2 adsorption-desorption isotherms for the p-MAA.

Table 1
Textural properties and morphology of the synthesized polymers.

Polymer	S^a_{BET} (m^2/g)	Pore Volume (cm^3/g)	Pore Size (Å)	Morphology
p-4-VP	252	0.97	<20	Quasi-spherical
p-MAA	80	0.18	<20	Sponge-type

controlling the extraction process flow and prolonging assay duration for hours. Due to these issues and the high standard deviation observed in the results with the p-4-VP, this material was excluded from further consideration, and the p-MAA was selected as the optimal choice for the remaining tests. As shown in Table 2, in the case of p-MAA, assays could be carried out with 50 and 25 mg, since this polymer allowed better handling. The results were very similar in both cases, prompting the decision to proceed with the minimum amount of material (25 mg) for subsequent studies. Comparing the three solvents tested, it was found that MeOH provided the least favorable results, leading to its exclusion from consideration. Finally, additional assays were performed with honey samples to observe the material's behavior under these conditions. Up to this point, the most effective loading solvents were found to be H_2O and ACN. However, following a solubility test with the sample in these two solvents and considering the principles of GSP, ACN was discarded. This decision was influenced by the fact that honey is not soluble in ACN, and it is a less environmentally friendly solvent. Therefore, H_2O was selected as the loading solvent for the subsequently studies. Fig. S2 shows the recovery percentages obtained by spiking 2 mL of honey at three concentrations levels (10, 25 and 50 ng/mL). For atropine, recoveries ranged between 91 and 109 %, while for scopolamine, percentages fell between 78 and 81 %. As a result, the optimal conditions obtained in the preliminary studies were 25 mg of p-MAA as sorbent for cartridges packaging and H_2O as the loading solvent.

3.2.2. *Optimization of loading and elution volume*

Subsequently, loading and elution volumes of the SPE process were evaluated trying to preconcentrate the analytes in the sample as much as possible. Different assays were carried out loading 2, 4, 6 and 8 mL of diluted honey sample spiked at a concentration of 10 ng/mL with atropine and scopolamine and the analytes were eluted with the same loading volume with a MeOH/H_2O (1 % FA) 60/40 (v/v) solution. Fig. 5a shows that the highest recovery percentages were obtained by loading 2 and 4 mL of the diluted sample. Conversely, for 6 and 8 mL, the recoveries of the SPE process decreased. For this reason, a sample loading volume of 4 mL was selected, as larger volumes enable the loading of a greater amount of analyte. On the other hand, an elution volume study was performed so that, 2, 3 and 4 mL of MeOH/H_2O (1 % FA) 60/40 (v/v) solution was used to elute the target analytes. As shows Fig. 5b, the best results were obtained with 4 mL, so this elution volume was selected as optimal. This fact can be considered a disadvantage of the SPE protocol since it is not possible to preconcentrate the extract. For this reason, the eluate was evaporated and reconstituted in 0.5 mL of MeOH, so a preconcentration factor of 8 was obtained.

3.2.3. *Optimization of elution solvent*

Finally, the optimum elution solvent for the extraction process was evaluated. Based on the literature, five solvents were selected to carry out this study: MeOH/H_2O (1 % FA) 60/40 (v/v), MeOH (1% FA), MeOH/H_2O (1 % FA) 80/20 (v/v), MeOH/H_2O (2 % FA) 80/20 (v/v)

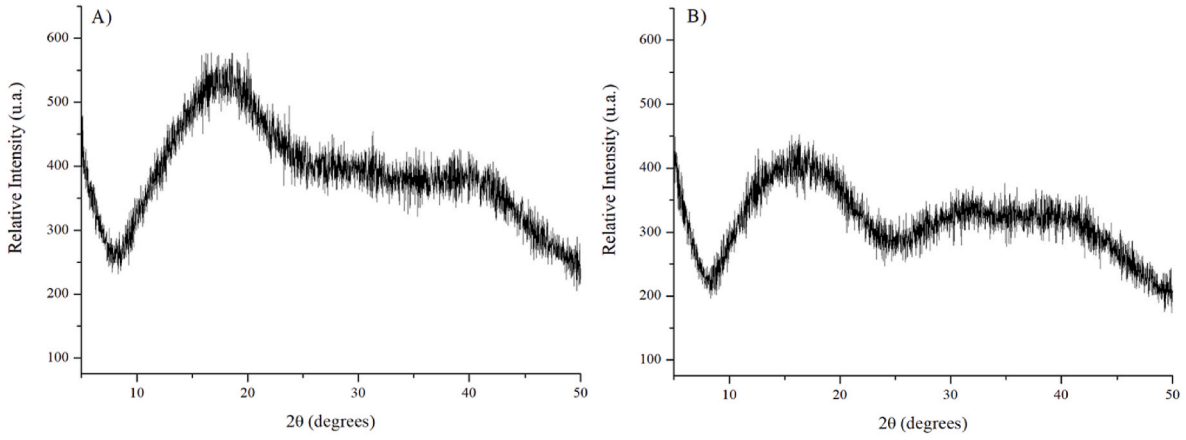

Fig. 4. A) XRD of p-4-VP. **B)** XRD of p-MAA.

Table 2
Recoveries obtained for atropine and scopolamine after carrying out the SPE process with 50 and 25 mg of p-MAA or p-4-VP by loading 2 mL of a standard solution (10, 25 and 50 ng/L) in MeOH, ACN and H_2O eluting with 2 mL of MeOH/H_2O (1% FA) 60/40 (v/v).

Standard solution		Atropine (Recovery, % ± SD)				Scopolamine (Recovery, % ± SD)			
		p-MAA		p-4-VP		p-MAA		p-4-VP	
		50 mg	25 mg	50 mg	25 mg	50 mg	25 mg	50 mg	25 mg
MeOH	10 ng/L	73 ± 1	65 ± 1	–	56 ± 4	75 ± 1	55 ± 6	–	45 ± 6
	25 ng/L	66 ± 5	54 ± 8	–	45 ± 5	64 ± 1	52 ± 5	–	52 ± 8
	50 ng/L	57 ± 7	52 ± 6	–	56 ± 6	56 ± 7	44 ± 6	–	45 ± 12
ACN	10 ng/L	101 ± 3	81 ± 8	–	81 ± 1	97 ± 1	91 ± 31	–	76 ± 33
	25 ng/L	102 ± 8	79 ± 1	–	80 ± 14	92 ± 8	75 ± 3	–	65 ± 21
	50 ng/L	80 ± 6	76 ± 7	–	78 ± 24	72 ± 10	76 ± 11	–	76 ± 5
H_2O	10 ng/L	98 ± 7	82 ± 2	–	85 ± 3	112 ± 14	97 ± 1	–	74 ± 10
	25 ng/L	86 ± 6	74 ± 12	–	78 ± 6	80 ± 5	88 ± 13	–	84 ± 4
	50 ng/L	86 ± 5	96 ± 10	–	81 ± 14	96 ± 2	102 ± 8	–	80 ± 6

[a]SD: standard deviation.

and pure MeOH. Fig. 6 indicates that the least favorable results were obtained with MeOH and MeOH (1% FA), while the best results were obtained with solvents containing a mixture of MeOH and H_2O. Comparing the other three elution, the solvent with the highest proportion of H_2O (MeOH/H_2O (1 % FA) 60/40 (v/v)) was excluded to optimize the sample preparation time, because the evaporation of a solvent with a higher proportion of H_2O requires more time and energy. Since similar results were observed with MeOH/H_2O (1 % FA) 80/20 (v/v) and MeOH/H_2O (2 % FA) 80/20 (v/v), the first solvent was selected because its lower proportion of acid may contribute to extending the useful life of the chromatographic column. Additionally, this choice reduces consumption of contaminant reagents, aligning with GSP practices.

3.3. Reusability study and evaluation of the polymer-based SPE procedure as a sustainable methodology

The reuse of solid sorbents is a fundamental task in the GAC. In SPE process, this parameter poses a challenge due to the possible decrease in the efficiency of the extraction step when cartridges are reused. In this sense, a study was carried out to demonstrate the reusability of the synthesized polymer. Under the optimal polymer-based SPE conditions (see section 2.5), a cartridge packed with 25 mg of p-MAA was employed for forty successive extractions of honey samples. The recoveries from these studies are presented in Fig. 7, with five extractions conducted each week (n = 5) over eight weeks. It was observed that the material could be reused at least forty times without any significant decrease in recovery, which is a positive advantage over the use of other conventional sorbents that cannot be reused as many times, making the process more economical and sustainable.

To assess if the methodology aligns with the principles of the GSP, the AGREEprep tool designed for the evaluation of analytical sample preparation greenness was applied. This metric tool evaluates the sample preparation stage following the ten principles of the GSP which are:

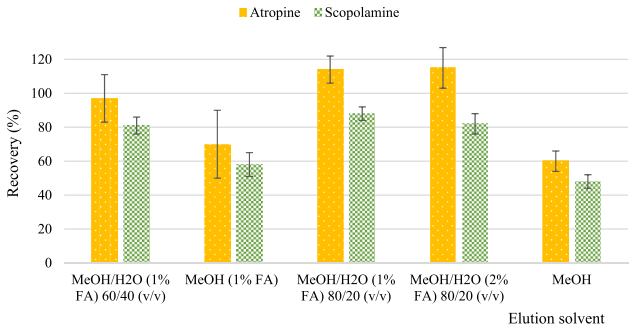

Fig. 6. Recoveries obtained for atropine and scopolamine using a honey sample diluted in H_2O (0.4:4, w/v) spiked with 10 ng/mL of both TAs after carrying out the SPE process with 25 mg of p-MAA using 4 mL of different elution solvents.

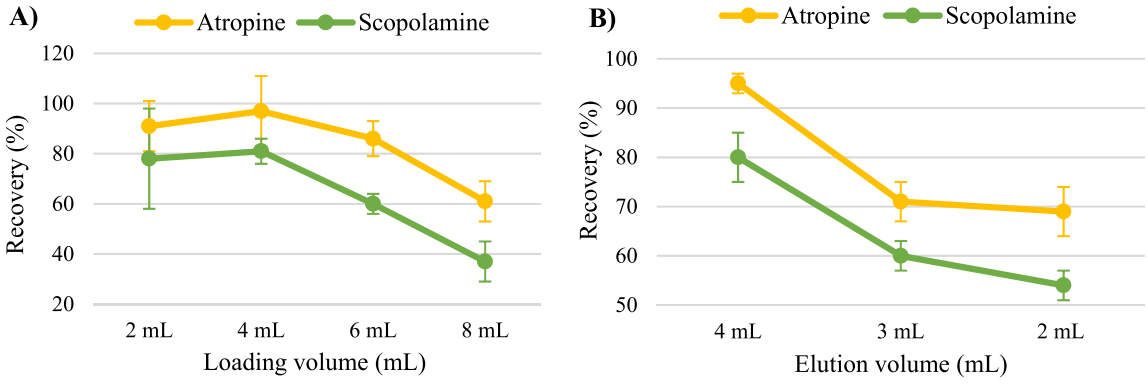

Fig. 5. A) Recoveries obtained for atropine and scopolamine using a honey sample diluted in H_2O (0.4:4, w/v) spiked with 10 ng/mL of both TAs after carrying out the SPE process with 25 mg of p-MAA at different loading volume, eluting with the same volume as the load volume. **B)** Recoveries obtained for atropine and scopolamine using a honey sample diluted in H_2O (0.4:4, w/v) spiked with 10 ng/mL of both TAs after carrying out the SPE process with 25 mg of p-MAA at different elution volumes.

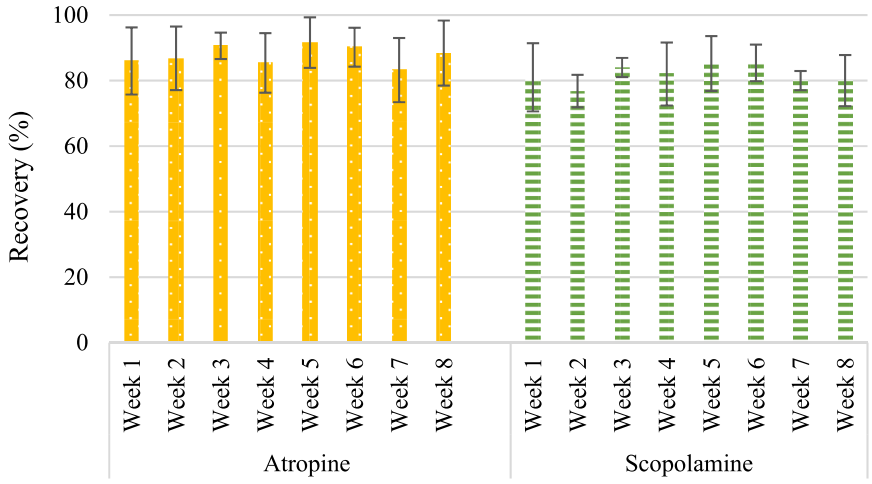

Fig. 7. Recoveries obtained for atropine and scopolamine per week (n = 5 × 8 weeks) to evaluate the reusability of the cartridge after carrying out the SPE process with 25 mg p-MAA under the optimized conditions (loading diluted honey sample in H_2O (0.4:4, w/v) spiked at 10 ng/mL with a standard solution of both TAs and eluting with 4 mL of MeOH/H_2O (1 % FA) 80/20 (v/v)).

1) the sample preparation placement, 2) the use of hazardous materials and the sustainable, 3) the renewability and reusability materials, 4) the waste of the methods, 5) the size of the sample, 6) the sample throughput, 7) the integration and automation, 8) the energy consumption, 9) the post-sample preparation analysis and 10) the operator's safety. As it is shown in Fig. 8, according with this tool, the methodology developed is "green" highlighting aspects such as the use of sustainable and reusable materials, the relatively small sample size (0.4 g), high sample throughput (up to 36 samples per hour), and operator's safety, since only MeOH is used as a potential hazard for the analyst. However, the two principal aspects to improve were the sample preparation placement, as the GSP aims to encourage the *in-situ* analysis, and the post-sample preparation configuration for analysis since the use of MS/MS detectors (more energy-intensive) and chromatographic techniques significantly low the overall assessment of greenness.

As can be seen in Fig. 8, the score obtained for our procedure was 0.59 (greater than 0.5), so it is considered a green method of analysis (green color in the middle of the clock-like pictogram).

3.4. Method validation

Two samples (one monofloral honey and one multifloral honey) were chosen for the validation of the proposed methodology to evaluate two types of honey samples in this work. Table 3 shows the linearity, matrix-matched calibration curves, MDL, MQL and ME of the method for each analyte (atropine and scopolamine).

The linearity of the extraction procedure was evaluated between 0.625 and 400 ng/g in case of atropine and 1.875–400 ng/g in case of scopolamine. The matrix-matched calibration curves showed good linear regression with R^2 up to 0.991 in both cases. Regarding the ME for the atropine, it was obtained a 118 % for the multifloral honey and 100 % for the monofloral, while for scopolamine, the ME was 106 % for the

Criterion	Input	Justification for input	Weight	Score
Sample preparation placement	On site	Although the sample preparation is performed in the laboratory, it could be possible to perform on site because of the versatility and flexibility of the SPE procedure	1	0.33
Hazardous materials	0.5	0.5 mL of MeOH per sample	5	0.47
Sustainability, renewability, and reusability of materials	> 75 % of reagents and materials are sustainable or renewable	Water is the solvents most used in this methodology what implies more than the 75 % of sustainable and renewable materials. SPE cartridges are not sustainable, but they can be used several times (more than 40 times per cartridge)	2	0.75
Waste	0.9	0.00625 g of p-MAA (each cartridge can be reused 40 times: 0.025 g/40 = 0.00625 g), 0.5 mL of MeOH and 0.4 g of sample	4	0.65
Size economy of the sample	0.4	Amount of honey sample (g) used	2	0.80
Sample throughput	36	10 min per polymeric-based extraction procedure and the possibility of carry out 6 samples at the same time, so 36 samples can be extracted in one hour	3	0.84
Integration and automation	2 steps, manual system	SPE extraction and evaporation of the extract	2	0.50
Energy consumption	81.6 Wh per sample	SPE extraction (245 W) for 10 minutes and evaporation of the sample (245 W) for 10 minutes	4	0.47
Post-sample preparation configuration for analysis	Liquid chromatography	HPLC-QqQ-MS/MS	2	0.25
Operator's safety	1 hazard	Used of MeOH	3	0.75

Fig. 8. AGREEprep test for the polymer-based SPE-HPLC-MS/MS proposed.

Table 3
Linearity, matrix-matched calibration, limits of detection and quantification and matrix effects of the polymer-based SPE-HPLC-MS/MS procedure in diluted honey samples.

Analyte	Sample	Linearity (ng/g)	Matrix-matched calibration (R^2)	MDL[a] (ng/g)	MQL[b] (ng/g)	ME[c] (%)
Atropine	H-3 (Multifloral honey)	0.625–400	$y = 1.5 \times 10^5 x - 2 \times 10^4$ (0.991)	0.19	0.625	118
	H-1 (Monofloral honey)	0.625–400	$y = 1.3 \times 10^5 x - 1 \times 10^4$ (0.995)	0.19	0.625	100
Scopolamine	H-3 (Multifloral honey)	1.875–400	$y = 4.9 \times 10^4 x - 3.9 \times 10^4$ (0.991)	0.56	1.875	106
	H-1 (Monofloral honey)	1.875–400	$y = 3.6 \times 10^4 x - 1.1 \times 10^4$ (0.996)	0.56	1.875	81

[a] MDL: method detection limit.
[b] MQL: method quantification limit.
[c] ME: matrix effect = (slope matrix-matched/slope solvent-based) × 100.

multifloral honey and 81% for the monofloral honey. Therefore, as the results obtained for both samples and analytes did not exceed the ±20 % marked by the validation guide, it is not necessary to consider the ME to quantify the target analytes in the honey samples, so external standard calibration curves could be used which simplifies the procedure of sample quantification. The proposed method shows MQL of 0.625 and 1.875 ng/g for atropine and scopolamine, respectively, and MDL of 0.19 and 0.56 ng/g for atropine and scopolamine, respectively. The selectivity of the method was evaluated verifying that the retention time of the analytes in the sample extracts corresponds to the time of the standard in the matrix-matched calibration. In addition, it was checked that the ion transition ratios in contaminated samples did not deviate more that 30 % (relative abundance) in comparation with the spiked samples as shows Fig. S1.

The accuracy and the precision of the method were evaluated at three concentration levels with the two blank honey samples mentioned before. The results, as shown in Table 4, indicate good accuracy at all the concentration levels, with recovery percentages between 86 and 92 % (multifloral honey) and between 89 and 95 % (monofloral honey) for the atropine and 71 and 85 % (multifloral honey) and between 72 and 85 % (monofloral honey) for the scopolamine. Additionally, precision also showed satisfactory results since there were obtained RSD ≤6 % for atropine and scopolamine for the intra-day precision and ≤3 % for atropine and scopolamine for the inter-day precision.

Table 4
Accuracy and precision of the polymer-based SPE-HPLC-MS/MS procedure in diluted honey samples.

Analyte	Sample	Spiked level (ng/g)	Accuracy (recovery % ± SD)	Intra-day precision (% RSD)	Inter-day precision (% RSD)
Atropine	H-3 (Multifloral honey)	0.625[a]	92 ± 2	3	2
		50 [b]	87 ± 1	4	1
		400[c]	86 ± 1	5	1
	H-1 (Monofloral honey)	0.625[a]	91 ± 1	4	1
		50 [b]	89 ± 2	6	3
		400[c]	95 ± 4	3	2
Scopolamine	H-3 (Multifloral honey)	1.875[a]	87 ± 1	4	1
		50 [b]	86 ± 1	5	1
		400[c]	71 ± 1	3	1
	H-1 (Monofloral honey)	1.875[a]	72 ± 2	4	1
		50 [b]	82 ± 3	4	2
		400[c]	85 ± 2	3	2

Recovery: intra-day precision: six replicate extracts (n = 6) analyzed on the same day of a diluted honey sample (multifloral and monofloral) spiked with the analytes at a known concentration level; Inter-day precision: three replicates extract of a diluted honey sample (multifloral and monofloral) analyzed throughout three different days (n = 9) and spiked with the analytes at a known concentration level. [a] Low spiked level (0.625 ng/g for atropine and 1.875 ng/g for scopolamine); [b] Medium spiked level (50 ng/g); [c] High spiked level (400 ng/g).

3.5. Comparison with other methodologies

Only five works focused on TAs analysis in honey have been currently published (Table 5). Regarding the extraction procedure, Casado et al. (2024) analyzed atropine and scopolamine (besides twenty-one pyrrolizidine alkaloids, PAs) in seven honey samples using a miniaturized μ-SPEed® protocol with commercial polymeric cartridges (PS-DVB). The same alkaloids were analyzed by Kowalcyck et al. (2022) in twenty-nine honey samples, who developed an analytical procedure based on mixed-mode cation exchange SPE. In both works, a dissolution of the sample in acidic medium (sulfuric acid) was need, previously to the purification step. Romera-Torres et al. (2020) analyzed nine TAs in nineteen honey samples by performing a first solid-liquid extraction (SLE) with MeOH/H_2O/FA (75/25/0.4, v/v/v), followed by a clean-up step with graphitized black carbon and magnesium sulphate. Thomson et al. (2020) used a protocol based on a SLE with ACN for the determination of atropine and scopolamine in twenty-three honey samples. Finally, Martinello et al. (2017) conducted a study to determine nine PAs, atropine and scopolamine in fourteen commercial honey samples using a QuEChERS methodology. As can be see, in most of this works prior to the purification step, reagents that are not environmentally friendly, such us the sulfuric acid, MeOH or ACN are used (Table 5). On the other hand, in the work of Thomson et al. (2020) a purification step was not considered, which could be hazardous for the chromatographic column. In addition, precision also showed satisfactory results since there were obtained RSD ≤6 % for atropine and scopolamine for the intra-day precision and ≤3 % for atropine and scopolamine for the inter-day precision. In our work, we introduced a polymer-based SPE to remove interfering compounds and to extend the column lifetime. In addition, before the SPE step, the honey sample was only diluted with H_2O, without the use of organic solvents and acid conditions. One notable advantage of our method is the potential for material reuse, contributing to its sustainability.

Regarding the validation parameters (Table 5), it can be concluded that the MDL and MQL obtained in this work are similar to those reported by previous studies, except the MQL of Romera-Torres et al. (2020) which was notably high (20 ng/g). On the other hand, our methodology has the advantage that ME was negligible, whereas in other studies, significant negative or positive ME was found.

3.6. Real samples application

To demonstrate the applicability of the developed and validated method, fifteen honey sample (multifloral and monofloral) were analyzed. Each sample was extracted in triplicate, and the extract was injected three times in the HPLC-MS/MS. The target TAs were quantified using the matrix-matched calibrate curves.

Table 6 shows the results of the analyzed samples expressed in ng of analyte (atropine or scopolamine) per g of honey.

As can be seen, only two samples (13.3 % of the total) did not present atropine and scopolamine and eight samples (53.3 %) presented at least one of the two target analytes below the MQL. The other five remaining

Table 5
Comparison of proposed methodology with other approaches for TAs analysis in honey samples.

Sample preparation and analysis	Analyte	Accuracy (Recovery, %)	Repeatability (RSD, %)	Reproducibility (RSD, %)	MDL (ng/g)	MQL (ng/g)	ME (%)	Ref.
10 g of honey in 20 mL H_2SO_4 (0.05 M) + 1 g zinc dust SPE (mixed-mode cation exchange cartridges) LC-MS	AT[a] SC[b]	88.6–102.2 83.9–102.5	<10.4 <9.4	<10.3 <9.4	0.11 0.15	0.36 0.49	127 105	[8]
5 g of honey + 10 mL sodium acetate solution + 10 mL ACN LLE HILIC-MS/MS	AT[a] SC[b]	86.9–102.7 88.7–106.1	<5.0 <6.2	<4.2 <5.0	0.002 0.003	0.01 0.01	– –	[9]
0.5 g of honey in 2.5 mL H_2SO_4 (0.05 M) μSPEed® (PS/DVB) UHPLC-MS/MS	AT[a] SC[b]	89–97 81–89	<10 <8	<15 <15	0.3 0.3	1.0 1.0	97 98	[23]
1.5 g of honey in 10 mL H_2SO_4 (0.1 M) + 0.5 g zinc dust. QuEChERS (4.9 g $MgSO_4$ + 1 g trisodium citrate dehydrate + 0.5 g disodium hydrogen citrate sesquihydrate + 1 g NaCl + 150 mg PSA) LC-HRMS	AT[a] SC[b]	100.9–103.7 96–108.6	<2.7 <15.1	<3.5 <15.6	0.1 0.2	0.5 0.5	105 102	[24]
2.5 g of honey in 10 mL MeOH/H_2O/FA (75/25/0.4, v/v/v/) QuEChERS (0.3 g $MgSO_4$ + 50 mg GBC) LC-HRMS	AT[a] SC[b]	85–103 116–120	<8.0 <8.3	<18.1 <19.7	– –	20 20	63 51	[25]
0.4 g of honey + 4 mL of H_2O Polymeric-based SPE (homemade cartridges) HPLC-MS/MS	AT[a] SC[b]	86–95 71–87	<6 <5	<3 <2	0.19 0.56	0.625 1.875	100–118 81–106	This work

[a] AT: atropine.
[b] SC: scopolamine.

Table 6
Atropine and scopolamine content in different honey samples analyzed with the polymer-based SPE-HPLC-MS/MS procedure.

Sample[a]	Atropine (ng/g ± SD)	Scopolamine (ng/g ± SD)	Total of TAs[b] (ng/g ± SD)
H-1	ND	ND	–
H-2	< MQL	ND	< MQL
H-3	ND	ND	–
H-4	3.24 ± 0.01	ND	3.24 ± 0.01
H-5	< MQL	ND	< MQL
H-6	3.7 ± 0.3	< MQL	3.7 ± 0.3
H-7	1.4 ± 0.2	ND	1.4 ± 0.2
H-8	< MQL	4 ± 1	4 ± 1
H-9	< MQL	ND	< MQL
H-10	< MQL	< MQL	< MQL
H-11	< MQL	< MQL	< MQL
H-12	1.7 ± 0.4	5.53 ± 0.09	7.2 ± 0.5
H-13	< MQL	ND	< MQL
H-14	< MQL	< MQL	< MQL
H-15	< MQL	< MQL	< MQL

[a] Each sample was extracted in triplicate and the extract was injected three times in the HPLC-MS/MS.
[b] Sum of atropine and scopolamine.

samples (33.3 %) could be quantified since they exceeded the MQL. Fig. 9 shows the EIC for atropine and scopolamine in a blank and contaminated sample, one with atropine (Fig. 9c) and another with scopolamine (Fig. 9d).

H-4 and H-7 samples presented contamination with atropine at 3.24 ± 0.01 and 1.4 ± 0.2 ng/g respectively. In H-8 sample contamination with scopolamine was found at a concentration of 4 ± 1 ng/g. H-6 sample was contaminated with 3.7 ± 0.3 ng/g of atropine, whereas scopolamine was under the MQL. Finally, one sample showed contamination with the both analytes, H-12, that presented a total of 7.2 ± 0.5 ng/g of TAs (1.7 ± 0.4 and 5.53 ± 0.09 ng/g of atropine and scopolamine respectively). All the positive honeys quantified were of the multifloral type, as expected. This honey type is obtained by bees that pollinates a variety of different flowers, reflecting the diverse flora present in the bee's foraging area. Consequently, the potential contamination due to TA-producing plants may be higher in this kind of honey. Regarding the five contaminated samples which exceeded the MQL, the 60 % of them were not commercial (honey directly collected from honeycombs in small family farms), whereas the other 40 % were commercial products. In other studies, concentrations of TAs (atropine and scopolamine) were found in a range between 0.012 and 27 ng/g. Martinello et al. (2017), observed that nine of the samples analyzed showed atropine levels ranging from 1.4 to 3.8 ng/g, but none of the samples were contaminated with scopolamine. Casado et al. (2024) found atropine in all the honey samples analyzed (3.7–18.6 ng/g) and only one sample with scopolamine. On the other hand, Romera-Torres et al. (2020) found only one sample contaminated with 27 ng/g of scopolamine, while atropine was not detected in any of the samples. Thompson et al. (2020) observed that one sample was contaminated with both TAs at a concentration of 0.012 ng/g for both analytes. Finally, Kowalcyck et al. (2022) did not find any TAs in the samples analyzed.

There is no regulation that established the maximum limits for TAs in honey, but the European Food and Safety Authority (EFSA) established an acute reference dose (ARfD) for the sum of atropine and scopolamine of 0.016 μg/kg of body weight (b.w.). Assuming a consumption of 1 tablespoon (21 g) per day of the most contaminated honey analyzed (H-12), the amount of TAs ingested would be of 0.15 μg. Considering a 60 kg person the maximum amount would be 0.96 μg, so the quantity ingested would be 6.4 times lower that the ARfD limit, which indicates that the risk of consumption of this type of products is relatively low. However, for a one-year-old baby (around 10 kg), that intake can be concerning. In that respect, it is important to develop analytical methods to identify this type of toxins in foods and to carry our toxicological studies to ensure the safety of the most vulnerable consumers (e.g., children).

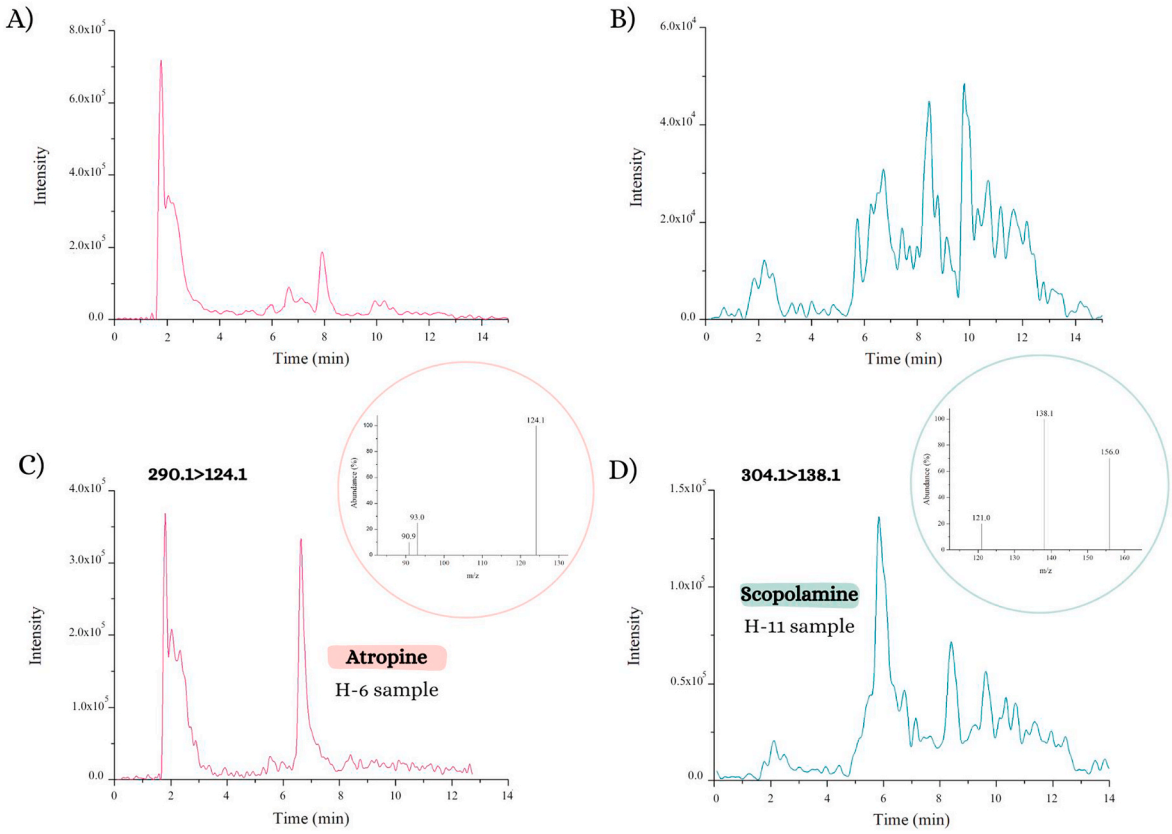

Fig. 9. A) Extracted ion chromatograms of atropine in a blank sample. **B)** Extracted ion chromatograms of scopolamine in a blank sample. **C)** Extracted ion chromatograms and mass spectrum of atropine in a contaminated sample. **D)** Extracted ion chromatograms and mass spectrum of scopolamine in a contaminated sample.

4. Conclusions

In this work, two homopolymers (4-p-VP and p-MAA) were synthesized for their application as SPE sorbents for the extraction of atropine and scopolamine in honey samples. While the p-4-VP did not yield good extraction efficiency, the p-MAA provided good recovery percentages using only 25 mg of the synthesized material. The green methodology developed, evidenced by the good reusability of the material and the low use of hazardous reagents, was successfully validated and applied to the quantification of TAs in fifteen honeys by HPLC-MS/MS. Contamination of atropine was found in thirteen samples and scopolamine in seven samples. The highest concentration of TAs was found in a multifloral honey with a sum of both TAs of 7.23 ng/g. These results confirm that analytical data should be collected on occurrence of TAs in honey to estimate the dietary exposure of consumers and to perform a risk assessment by the safety authorities.

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CRediT authorship contribution statement

Begoña Fernández-Pintor: Writing – original draft, Validation, Methodology, Formal analysis. **Gema Paniagua:** Writing – review & editing, Methodology, Data curation. **Judith Gañán:** Writing – review & editing, Methodology, Data curation. **Sonia Morante-Zarcero:** Writing – review & editing, Supervision, Methodology, Data curation. **Rosa María Garcinuño:** Writing – review & editing, Data curation. **Pilar Fernández:** Writing – review & editing, Funding acquisition. **Isabel Sierra:** Writing – review & editing, Supervision, Funding acquisition.

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

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.polymer.2024.126904.

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