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Pulsed ultrasound-assisted extraction followed by purification with SBA-15 for the control of opium alkaloids in biscuits and sponge cakes

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ABSTRACT

Food products containing poppy seeds are increasingly consumed. These seeds may be contaminated with opium alkaloids (OAs) from the latex of the plant (*Papaver somniferum* L.), which may present a health hazard to the consumer. Therefore, the aim of this work was to develop an efficient, fast and environmentally friendly methodology to control OAs in biscuits and sponge cake products by analysis with liquid chromatography coupled to a triple quadrupole tandem mass detector (HPLC-MS/MS). For this purpose, the ultrasound-assisted extraction (UAE) step was optimised using a 5-variable full factorial design at two levels, obtaining lower solvent volume and time than with other classical methods. Then, a solid phase extraction (SPE) was used to remove matrix effects. A commercial material (HLB) and two silica synthesised materials (HMS and SBA-15) were evaluated and optimised, selecting SBA-15 (50 mg). Finally, the method was validated and applied to real samples, showing morphine concentrations in 5 of 7 products but below the maximum permitted limit.

1. Introduction

Nowadays, the consumption of food with *Papaver somniferum* L. (opium poppy) seeds, such as bakery products (bread, biscuits and sponge cake), salads, yoghurts, teas and oils, is increasing, due in large part to their nutritional value [1–5]. However, it is reported that the seeds used may be contaminated with opium alkaloids (OAs; i.e. morphine, codeine, thebaine, papaverine, noscapine and oripavine) from the latex of this plant [3–7] and therefore even foods with poppy seeds [8]. Consumption of contaminated food can cause false positive drug tests and intoxication in consumers [9,10]. As a control measure, Regulation (EU) 2021/2142 was published in December 2021, establishing maximum morphine equivalent limits (morphine + 0.2 × codeine) in seeds and bakery products [11]. However, this legislation only includes morphine and codeine, and health authorities are demanding further studies to regulate the other OAs, which can be even more toxic [10]. Therefore, in order to know consumer exposure and to legislate accordingly, there is a need to develop an efficient, rapid and environmentally friendly analytical methodology to analyse the six main OAs in biscuits and sponge cakes with poppy seeds and to validate for the first time for this type of samples [1].

Sample preparation takes a crucial role in the analysis of organic contaminants in food samples, as these compounds are often present in very low concentrations and their analytical determination is strongly influenced by the matrix components because they are very complex matrices. Extraction methods for opium alkaloids have been described in the literature, such as liquid–liquid extraction, using high solvent volumes and extraction time, and being less environmentally friendly. For example, López et al. used 100 mL of AcN/water/formic acid, 80/19/1, v/v/v/v and 1 h of extraction for each sample, [4] and Sproll et al. [5] used 30 mL of methanol 0.1 % acetic acid for 1 h of extraction.

To reduce the extraction time and the volumes of solvent used, ultrasound-assisted extraction (UAE) may be applied, which is one of the most exploited modern extraction techniques in the last years for its many advantages [12–14]. However, to our knowledge, none of them has used in OAs extraction yet. Due to the cavitation effect of the ultrasounds, this technique is a simple, cheap and efficient extraction method that allows obtaining high extraction yields with minimum damage to structural and molecular properties of interest compounds, reduces solvents use, and assures a better penetration of the extracted matrices [15,16]. The efficiency of UAE is strongly affected by several factors, including solvent volume, acid proportion, extraction time, amplitude and mode of sonication (continuous or pulsed). Optimisation of these parameters is considered essential to maximise extraction yield. To carry out the optimization and to measure the interaction between factors the response surface methodology (RSM) experimental design is

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applied in this study [17]. RSM is a powerful tool to evaluate multiple factors and their interactions simultaneously, being a set of sophisticated mathematical and statistical techniques, helpful in developing, improving and optimizing processes by establishing empirical models [12]. RSM is based on fitting a polynomial equation to experimental data, which, in turn, must describe the behaviour of the data set and make statistical predictions [18].

In addition to efficient extraction, performing a purification step before analysis is necessary to eliminate possible matrix effects of biscuits and sponge cakes that can lead to erroneous results or further damage to equipment. For this purpose, solid-phase extraction (SPE) with different types of commercial sorbents such as diatomaceous earth [19], Chem Elute column [20], Clean Screen ® DAU [21], Oasis ® MCX and Oasis ® HLB [22], has been used in OAs. The Oasis ® HLB is a universal polymeric adsorbent with hydrophilic-lipophilic balance developed to extract a wide range of acidic, basic and neutral compounds from various matrices using a simple and generic protocol. In addition, more and more efforts are being made to synthesise and produce sorbent materials instead of using commercial ones because of their lower cost and greater control of the desired textural characteristics. Due to the chemical nature of OAs and in consideration of their interactions with the –OH groups of other mesostructured silica based materials [7], the application of unfunctionalised SBA-15 (Santa Barbara Amorphous-15) and HMS (hexagonal mesoporous silica) materials in the purification step can be evaluated, avoiding functionalisation steps with the consequent cost of reagents and time. This materials are increasingly being used as sorbent materials in other compounds due to the advantages in terms of their ordered structure with controlled pore size, large surface area and pore volume [23–26].

The aim of this work was to develop and validate an efficient, fast and environmentally friendly methodology for quantification of the six main OAs in samples of biscuits and sponge cake using a UAE-SPE sample preparation protocol previous to the analysis by liquid chromatography coupled to a triple quadrupole tandem mass detector (HPLC-MS/MS).

2. Materials and methods

2.1. Reagents and materials

Supel ™-Select HLB SPE cartridges (30 mg/1 mL) were purchased in Sigma-Aldrich (St. Louis, MO, USA), 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, P123, MW = 5800 g/mol) and dodecylamine (DDA) 98 % (M = 185.36) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid 37 % was acquired from Scharlab (Barcelona, Spain). Standards of morphine, codeine, thebaine and oripavine were received from Alcaliber S.A.U. (Madrid, Spain). Noscapine, papaverine, morphine-d_3 and codeine-d_3 (internal standards, IS) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock standard solutions were prepared at 1000 μg/mL in methanol and working standard solutions were prepared at 1 μg/mL in water/acetonitrile 90/10 (v/v) with 0.1 % formic acid. All of these were stored in darkness at −20 °C. Acetic acid and ammonia 32 % (w/w) were purchased from Scharlab (Barcelona, Spain). Acetonitrile and methanol used were HPLC-MS quality and were purchased from Scharlab (Barcelona, Spain). Polyethylene frits (0.20 μm) and nylon filter membranes (0.45 μm) were obtained from Scharlab (Barcelona, Spain). Formic acid 99 % Optima™ LC-MS grade was from Fisher Chemical (Madrid, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

SBA-15 and HMS materials were prepared and characterized according to previous works [27,28] (see Supplementary Information S1, for preparation details). The surface area of SBA-15 and HMS was 702 and 778 m^2/g, the pore volume was 0.74 and 0.99 cm^3/g and the pore distribution was 56.2 and 26 Å, respectively.

2.2. Commercial samples for analysis

Five different brands of biscuits (B-1 to B-5) and two sponge cakes (SC-1 and SC-2) with poppy seeds were purchased from supermarkets in Madrid and Valencia (Spain). The poppy seed content of these products was in the range of 4 to 13 %. To obtain a representative and homogeneous sample with small particle size, each packet was ground with a manual mortar so as not to grind the poppy seeds and reduce OAs levels. To facilitate grinding, sponge cake samples were frozen with liquid nitrogen and all the samples were passed through a 18 mesh sieve (1 mm).

2.3. Optimised analysis methodology for quantification of opium alkaloids in biscuits and sponge cakes

The analysis methodology developed was based, as shown in Fig. 1, on a first sample pre-treatment step, a subsequent UAE, purification by SPE and analysis by HPLC-MS/MS.

Then, the ground, sieved and homogenised sample was exposed to acoustic waves under controlled UAE conditions (according to the experimental design) by Bandelin Sonoplus 529 (Amplichron ®-System, Bandelin, Berlin, Germany) with MS 73 probe with a diameter of 13 mm. Therefore, for extraction by UAE, 2.5 g of sample were mixed with 10 mL of methanol with 1 % formic acid for 30 s in the vortex (Rx^3 Velp Scientifica, Usmate, MB, Italy). Then, the mixture was exposed to 75 % amplitude for 5 min in pulse mode in a 50 mL falcon tube immersed in a glass with crushed ice to prevent heating. After the UAE, the mixture was centrifuged at 6000 rpm (3992 rcf) for 5 min to recover the supernatant (ROTOFIX 32A 204 Hettich, Tuttlingen, Germany). Then, the SPE procedure was performed using 50 mg of SBA-15. For this purpose, a conditioning stage was first carried out with 2 mL of methanol/water 0.1 % acetic acid, loading with 1 mL of the extract diluted 1/10 with methanol/water 1 % acetic acid, drying under vacuum for 3 min and elution with 2 mL of methanol with 10 % ammonia. Subsequently evaporated on a vacuum line to dryness, reconstituted with 50 μL of a 0.1 μg/mL dilution of each of the internal standards (morphine-d3 and codeine-d3) and 150 μL of water/AcN (90/10, v/v) with 0.1 % formic acid and for subsequent analysis by HPLC-MS/MS.

For the chromatographic analysis of OAs in biscuits and sponge cakes, a Varian 1200/1200 LC (Varian Ibérica, Madrid, España) with a ProStar 410 autosampler (100 μL loop) coupled to a triple quadrupole tandem mass spectrometer detector (1200 L TQ) with electrospray ionisation (ESI) ion source was used (data acquisition system was MS Workstation Varian version 6.8). Chromatographic separation was performed like our previous work [8] using a C_{18} Kromaphase 100 column (150 × 2.0 mm, 3.5 μm particle size, Scharlab, Barcelona, Spain) at 30 °C. The injection volume was 10 μL (partial injection) and the flow rate was set at 0.25 mL/min in a gradient elution of water (A) and acetonitrile (B), both with 0.1 % of formic acid as follows: 90–30 % A (0–6 min), 30–90 % A (6–9 min) and 90 % A (9–11 min) for column re-equilibration. Mass spectrometry acquisition was with electrospray ionization in positive mode (ESI+) with MRM mode like our previous work [8]. N_2 was used as drying (at 350 °C and 22 psi) and nebulizer gas (at 58 psi). The capillary voltage was 5000 V and shield 600 V. Argon at 1.90 mTorr was collision gas and detector voltage 1480 V. Mass peak width Q_1 2.5, mass speak width Q_3 2.5 and scan width in MRM 0.5 s. Compounds were monitored at cone voltage at 72 V. Table S1 shows the optimal mass spectrum parameters.

2.4. Optimisation of the extraction and purification methodology of opium alkaloids from biscuits and sponge cakes

For the optimisation of the sample preparation, first, the parameters of the SPE purification step were optimised to determine the recovery values of this step. For this purpose, the two mesostructured silicas were

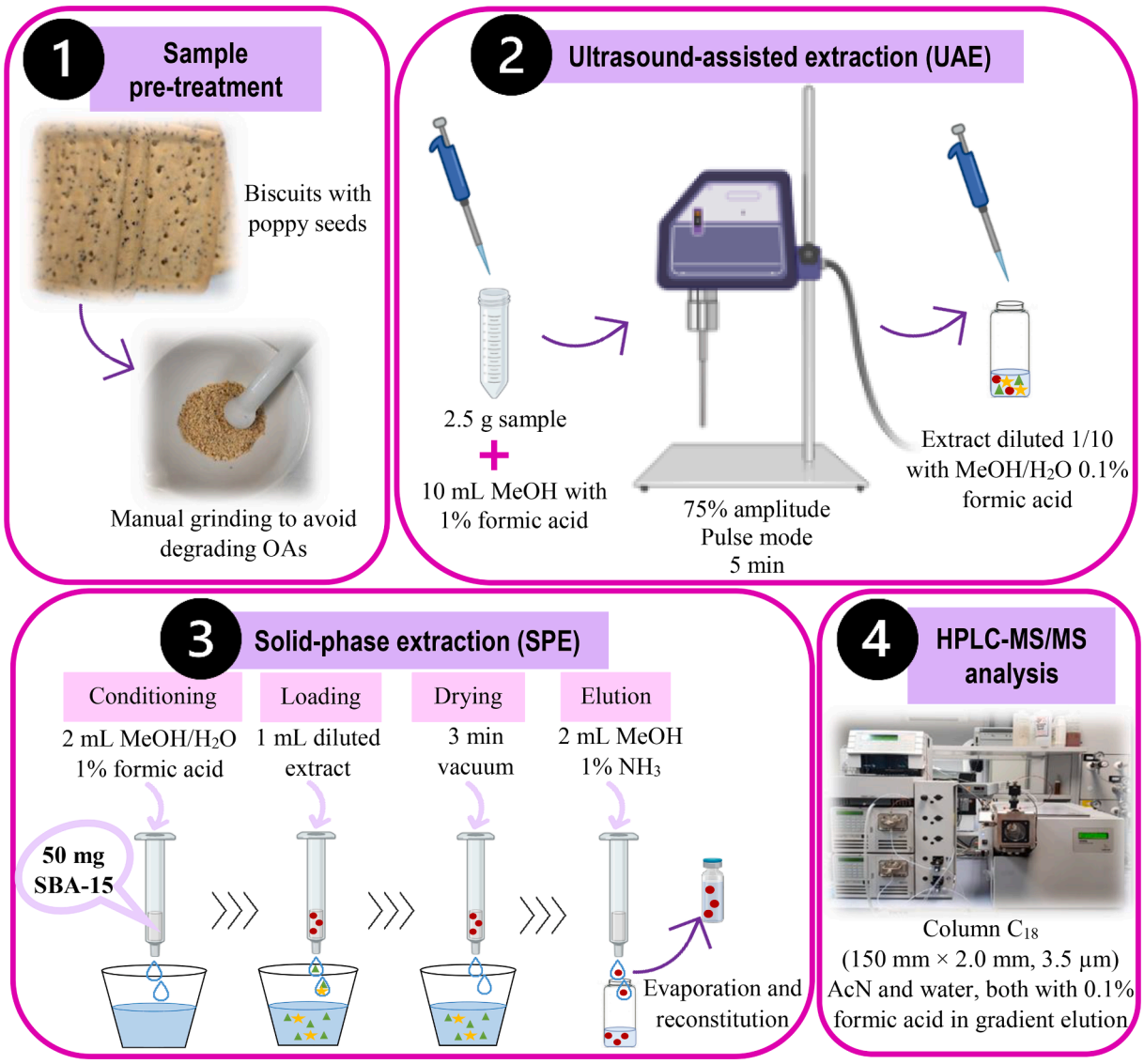

Fig. 1. Diagram of the proposed UAE-SPE methodology to quantify OAs in biscuits and sponge cakes.

evaluated with 80 µg/L solutions of each of the OAs and the one that showed the highest adsorption of the opioids was selected. To calculate the adsorptions of each material, the solvent remaining after the loading step in the cartridge was analysed by HPLC-MS/MS. Subsequently, the elution step of the analytes was optimised. After optimisation of the SPE with standards, the sample clean-up step was optimised. For this, a sample extract (sample B-1) was spiked just before the purification process with a known concentration (80 µg/L) to calculate the recovery values of each of the analytes in this step. To spike the extracts, surface contamination of poppy seeds was simulated by adding the corresponding aliquot, homogenizing it with the vortex for 10 s and allowing it to stand at room temperature for 30 min for evaporation of the solvent (MeOH). After a small re-optimisation with the sample extract, the sample extraction was optimised with UAE. For this purpose, sample B-1 was spiked with 3 mg/kg of each of the analytes to calculate the recovery values for the complete proposed UAE-SPE methodology.

2.4.1. Sorbent material selection and preliminary optimisation of the SPE with standards

To select the best sorbent to be used in this work in the purification stage, the procedure was optimised with standards to compare and choose the most efficient one.

First, the adsorption capacity and retention values of the commercial cartridge (HLB) under study were evaluated. For this, the method recommended by the manufacturer was followed, which was based on conditioning with methanol followed by water, loading of the dissolved analytes in an aqueous medium and subsequent elution in AcN/MeOH, 50/50 (v/v). Subsequently, the conditions were optimised with standards. In addition, the necessary conditions for the SBA-15 and HMS silicas were optimised. To this, their adsorption capacity was evaluated in the loading stage with the extraction solvents used in previous work [7,8]. Therefore, two loading solvents were evaluated (methanol/water (50/50, v/v) and methanol with 0.1 % acetic acid). Firstly, the elution solvent was ethyl acetate/methanol (50/50, v/v) with 10 % ammonia, similar to previous work with mesoporous silica [7] but with a less volatile organic solvent to avoid excessive evaporation during the SPE process. Once the sorbent material and the loading solvent had been selected, the elution solvent was optimised. To do this, different solvent mixtures were evaluated (ethyl acetate/methanol (50/50, v/v) with 10 % ammonia, methanol, acetonitrile, methanol and acetonitrile with 0.1 % ammonia). In addition, the ammonia proportion (0.1, 1 and 10 %) and volume of eluent (1 or 2 mL) were also studied. All studies were performed in triplicate with a first conditioning step with 2 mL of the same solvent as the loading, loading with 2 mL of an 80 µg/L standard solution

of each of the OAs and a vacuum drying step for 3 min prior to elution. To do this, a Supelco Visiprep SPE vacuum manifold 12 port model (Sigma Aldrich, St. Louis, MO, USA) connected to a vacuum pump at 7.6 psi. 3 mL polypropylene empty cartridges (length of 65 mm and i.d. 10 mm) were packed with 50 mg of each silica type and plugged with polyethylene frits at both ends. In addition, a pore size nylon filter membrane (0.45 μm) was inserted at the bottom of the material bed to prevent the material lost during sample loading.

2.4.2. Purification of sample extracts with selected cartridge

Once the SPE procedure was optimised with standards, the recovery of the purification step was determined with 2 mL of the sample extract obtained after the UAE and spiked with a high validation level of OA at 3 mg/kg (double the established maximum limit) [11]. However, due to the variation of the pH of the extract with the sample and the need to add a proportion of water to the extract to improve the retention of the analytes in the cartridges, a 1/10 dilution of the extract with 50 % methanol/water with 1 % acetic acid was performed and the loading volume was modified (1 mL).

2.4.3. Experimental design and statistical analysis of UAE

For both screening and optimisation of the UAE parameters, different experimental design methodologies were employed sequentially. First, a five-factor two-level full factorial design (2^5) was applied to determine the effect of the volume of solvent used (A), the proportion of acid added (B), the extraction time (C), the amplitude of sonication (D) and the mode of sonication (E) on the recovery of opium alkaloids. Each factor was examined at the two most promising levels, as shown in Table S2. The election of the levels of each independent variable was based on preliminary experiments and previous related research. Following the results obtained, the critical factors influencing both pulsed and continuous extraction modes were confirmed. Solvent volume and extraction time were the two most important independent factors selected based on the preliminary experiments, the optimal levels of which were determined for each extraction mode by RSM. The experimental design, the analysis of the results and the predicted responses were carried out with Statgraphics Centurion software (version 19.3.03).

2.5. Method validation

Since there is currently no official regulation on analytical performance requirements for OAs in food or feed, the method validation was done in terms of linearity, method detection and quantification limits (MDL, MQL), matrix effect (ME), accuracy, precision and selectivity, following the criteria described in the SANTE/12682/2019 document, in regulation EC No 401/2006, and in the Q2(R1) ICH guidelines (Internation Council for Harmonisation, 2005), [29–31]. Moreover, the only reference materials to our knowledge are biological samples and only for morphine and codeine. For this reason, the validation was carry out with a spiked sample (S-1). Accordingly, linearity was assessed with matrix-matched calibration curves prepared on three consecutive days. All these curves were prepared for sample B-1 at six known concentration levels within the linear range evaluated. For this purpose, the sample extracts obtained after the UAE-SPE procedure were spiked with an aliquot of a standard solution containing the target alkaloids according to the desired concentration level of the calibration curve. In addition, quantification of morphine and codeine by means of isotope labelled IS correction was carried out. To do this, 50 μL of 0.1 μg/mL of each IS were added to each point of the matrix-matched calibration curves. The criteria for good linearity involve values $\leq \pm 20$ % for the deviation of the back-calculated concentrations of the calibration standards from the true concentrations [29,30]. Matrix effects were determined by comparing the slopes of the calibration equations obtained from both matrix-matched and solvent-based calibration curves (both expressed in the same units μg/mL), calculating (the ratio slope matrix-matched/slope solvent-based -1) $\times$ 100 for each analyte. The ME is lower when closer to 0 %, and according to the guideless the ME is negligible when is lower than ± 20 %. Positive values greater than 20 % indicate signal enhancement, and negative values indicate signal suppression. However, when the signal suppression or enhancement is higher than this margin of 20 %, matrix effects must be considered in calibration. The sensitivity of the method for each sample was determined through the MDLs and MQLs of the OAs from the analysis of the lowest concentration analysed (0.01 or 0.001 μg/mL), which were estimated as the minimum concentration yielding a signal-to-noise ratio (S/N) of 3 or 10, respectively [31]. The recovery assays were assessed by comparing the areas obtained for samples spiked ($n = 6$) with a known concentration of analytes and subjected to the UAE-SPE procedure with those areas obtained for simulated samples (samples spiked at the same concentration but at the end of the procedure prior to their chromatographic analysis). The recovery assays were performed by spiking the samples at three concentration levels of 3 mg/kg (high value), 1.5 mg/kg (medium value) and 0.75 mg/kg (low value), according to the Regulation (EU) 2021/2142 was published in December 2021, establishing maximum morphine equivalent limits in bakery products at 1.5 mg/kg [11]. The recovery values should be between 70 and 120 %. On the other hand, the method precision was evaluated in terms of repeatability and reproducibility, using the same validation levels (low and high) as for the accuracy. For repeatability (expressed as RSD%), a sample spiked with the OAs at the corresponding validation level was consecutively carried out six times ($n = 6$) on the same day. The reproducibility (also expressed as RSD %) was calculated by the analysis of three replicates of a sample (spiked with the analytes at the corresponding validation level), which were carried out in triplicate over three different days ($n = 9$). According to the validation guidelines, the RSD values for these precision parameters should be $\leq$20 %. The selectivity of the method was determined by comparing the spectra of the different analytes obtained from standard solutions with the spectra obtained in the samples. It was considered satisfactory when the variation in the spectra was few than ± 30 % and the retention time of the target analytes was within the interval of ± 2.5 % [29].

3. Results and discussion

3.1. Optimisation of the extraction and purification methodology

3.1.1. Optimisation of the purification by SPE

3.1.1.1. Sorbent material selection and preliminary optimisation of the SPE with standards. To select the best material in this work to use in the purification step, the adsorption capacity (%) and recovery values (%) of the commercial cartridge (Supel ^{TM}-Select HLB SPE) and two synthesised silicas (SBA-15 and HMS) were evaluated.

Firstly, the commercial HLB cartridge was evaluated for the purification step with standard solutions of each of the opium alkaloids at a concentration of 30 μg/mL. For this, the method recommended by the manufacturer was followed. The results obtained were not favourable as the recoveries obtained did not exceed 70 % for any of the analytes, so the conditions were optimised. To evaluate both loading and elution, adsorptions (%) and recoveries (%) were calculated by analysing the solvent after passing through the cartridge in each of the stages. In the case of adsorptions (%), variable results were obtained with water, especially in the case of morphine, which showed an RSD of 32 %. Therefore, it was tried to add small percentages of organic solvent, methanol and acetonitrile (both at 1 and 10 %), but the adsorptions of morphine, codeine and oripavine were lower than 30 % in all cases. Subsequently, acidification with 1 % formic acid was tested, and although all analytes showed good adsorption, morphine only showed 19 %. The next step was to test a basic medium, which, according to the manufacturer's recommended method, for basic polar compounds that

are difficult to retain, adjusting the pH two units above the pKa of that analyte would aid retention on the cartridge. Therefore, to improve the adsorption of morphine, which was the most difficult analyte to retain, a 25 mM ammonium formate buffer adjusted with ammonia at pH 12 was tested, since morphine shows a pKa around 9. However, only 50 % adsorption was achieved in the case of morphine and oripavine. To calculate the recovery values, the adsorption solvent was maintained with water, and the elution solvent was modified. Different conditions were tested with 100 % methanol, acidified at 1 % with formic acid and with different percentages of acetonitrile (1, 10, 20 and 30 %). And with all these conditions good recoveries were obtained (around 85 %). However, the recoveries were not satisfactory in the case of morphine, the highest being 60 ± 20 % with MeOH with 1 % AcN. Therefore, the retention of morphine in the cartridge was not reproducible and in some replicates was very low due to its high polarity, so the use of this cartridge in the SPE stage was discarded.

Secondly, the purification step was optimized with the synthesized SBA-15 and HMS cartridges. To do this, the loading step was with the extraction solvents used in previous work [7,8]. Therefore, 2 mL of 80 μg/L of each of the opium alkaloids were tested in two different solvents (methanol/water (50/50, v/v) and methanol with 0.1 % acetic acid) with 50 mg of each material. The elution solvent for this assay was 2 mL of ethyl acetate/methanol (50/50, v/v) with 10 % ammonia, similar to previous work with mesoporous silica material [7] but with a less volatile organic solvent to avoid excessive evaporation during the SPE process. The results obtained from this assay are shown in Fig. 2. Regarding the adsorption values (%), those of the SBA-15 were higher than those of the HMS with the two loading solvents, being the highest with methanol 0.1 % formic acid with adsorptions between 93 and 100 % of all the analytes. Regarding the recovery values obtained, they were low in all cases, especially for morphine, where the maximum was 39 % and oripavine 35 %. Therefore, SBA-15 was selected over HMS because of its higher adsorption capacity for opium alkaloids and methanol with 0.1 % acetic acid as a solvent in the loading stage. Once the sorbent material and the loading solvent had been selected, the elution solvent was optimised to improve the low values obtained in the previous assay. To do this, different solvent mixtures were evaluated (methanol, acetonitrile, methanol and acetonitrile with 0.1 % ammonia). As shown in Figure S1, the highest recovery values were obtained by eluting with methanol with 0.1 % ammonia, ranging from 66 to 89 % for all analytes. Once the elution solvent was selected, different ammonia proportions to elution solvent (0.1, 1 and 10 %) were tested. As shown in Figure S2, the recovery values were higher with 1 % ammonia, showing values between 91 and 95 % for all analytes. At 10 %, some values decreased, especially for oripavine, which dropped to 69 %. In addition, the elution solvent volume was decreased from 2 to 1 mL, but lower recovery values of 30–50 % were obtained for all analytes.

3.1.1.2. Purification of sample extract with SBA-15 material. Once the SPE procedure was optimised with SBA-15 material with standards, the recovery of the purification step was determined with 2 mL of the sample extract obtained after the UAE and spiked with the high validation level (80 μg/L) of each OA.

The recovery results obtained with the sample extract were significantly lower than those obtained with standards, being around 20 % for papaverine and noscapine, 40 % for morphine and oripavine and 60 % for codeine and thebaine. After these low results, the solvent was analysed after the loading step in the cartridge to identify where the problem was, in the loading or in the elution. After calculating the adsorptions, it was found that the problem was at this stage, as the adsorptions were like the previous recovery values. This could be due to the change in pH of the methanol solution 0.1 % acetic acid in the sample, so it was decided to acidify the extract with a higher proportion of acetic acid (1 %). After further acidification, higher recovery values were obtained (for morphine and oripavine close to 90 %, for codeine and thebaine close to 80 % and papaverine and noscapine around 50 %). These results showed that pH adjustment is a key aspect for OAs to interact effectively with the SBA-15 material. Furthermore, to improve the interaction of papaverine and noscapine with the material, a proportion of water was added to the extract as these two compounds show

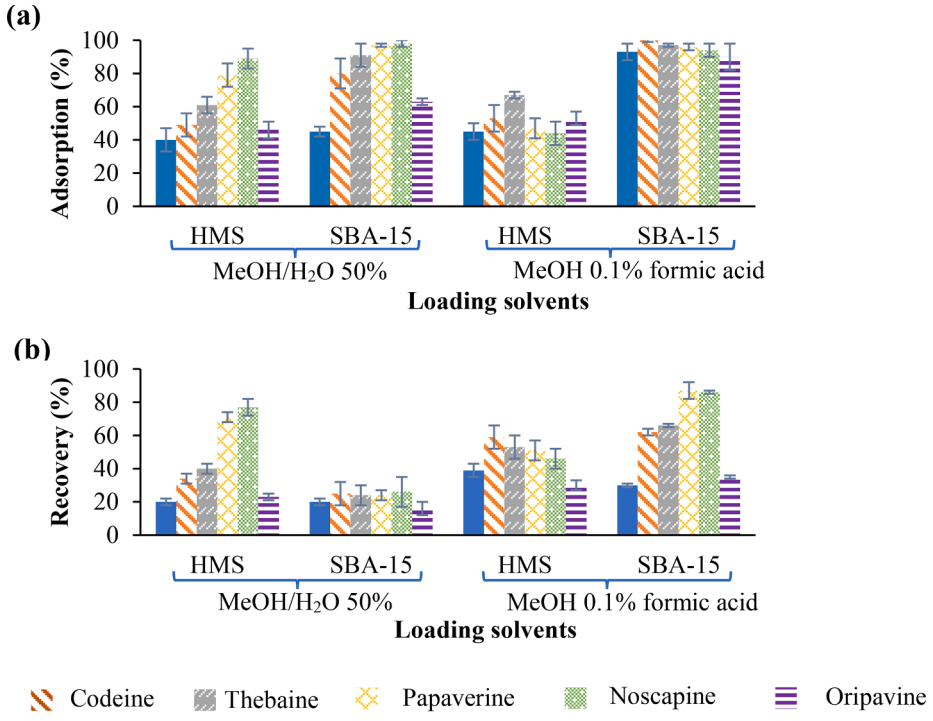

Fig. 2. Comparison between adsorption (a) and recovery (b) values (%) obtained with 50 mg of HMS and SBA-15 materials with 2 mL of 30 μg/L of opium alkaloids in two different loading solvents (MeOH/H_2O at 50 % and MeOH with 0.1 % formic acid) and using 2 mL of ethyl acetate/methanol at 50 % with 10 % ammonia for elution.

the lowest water solubility (0.013 and 0.18 g/L, respectively), in contrast to morphine and oripavine, which are the most water soluble (10.20 and 0.87 g/L, respectively) [1]. Therefore, the extract was diluted 1/10 with a 50 % methanol/water mixture with 1 % formic acid, and the recovery values obtained were 74 % for morphine, 87 % for codeine, 97 % for thebaine, 99 % for papaverine, 96 % for noscapine and 80 % for oripavine.

Subsequently, to ensure the correct loading of the analytes in the material in the case of obtaining a more concentrated extract with the UAE (instead of 10 mL, with 5 mL), the same test was performed at a higher concentration (150 μg/L of each OA). The recovery results obtained were lower (around 60 % for morphine, codeine and oripavine and around 75 % for thebaine, papaverine and noscapine), and it was determined that the adsorptions were like these recovery results, so it was considered that the 50 mg of material used might be saturating. To avoid increasing the amount of material, it was decided to pass 1 mL of the diluted extract through the cartridge instead of 2 mL and the results obtained were again satisfactory (75 % for morphine and oripavine, 86 % codeine, 94 % for thebaine, 105 % noscapine and 107 % papaverine).

3.1.2. Optimisation of UAE

3.1.2.1. Evaluation of UAE variables influencing extraction efficiency, their main effects and statistical analysis. A screening of the variables was done to estimate the influence of the different independent variables and to identify the factors that have significant effects on the dependent variables. To do this, the experimental factorial design methodology was used, specifically a screening design. Therefore, five possible independent variables that affect the process of UAE: solvent volume (A), acid proportion (B), extraction time (C), sonication amplitude (D) and sonication mode (E) were investigated using a five-factor two-level full factorial design (2^5) and the dependent variables were the recovery values of each of the analytes: morphine (Y_1), codeine (Y_2), thebaine (Y_3), papaverine (Y_4), noscapine (Y_5) and oripavine (Y_6). Each factor was examined at the two most promising levels based on preliminary experiments and previous related research (Table S2).

The results obtained from the experimental design are shown in Table S3. It should be noted that for each condition, extraction was performed in triplicate, and the responses presented in Table S3 are the mean values of the three replicates ± standard deviation (SD). Under the set conditions, the experimental values for each of the responses ranged from 23 ± 1 to 75 ± 5 % for morphine, 26 ± 1 to 88 ± 1 % for codeine, 46 ± 2 to 97 ± 2 % for thebaine, 50 ± 1 to 101 ± 8 % for papaverine, 56 ± 1 to 104 ± 2 % for noscapine and 26 ± 1 to 82 ± 2 % for oripavine.

To determine the effects of each variable and the possible interactions between them, the Pareto Chart was plotted, as it shows the absolute values of the standardised effects from the largest effect to the smallest effect and allows to determine whether a factor has a positive or negative effect on the response. In addition, the chart also plots a reference line indicating which effects are statistically significant. Therefore, as shown in Fig. 3, it was confirmed that all independent variables studied individually showed a positive effect on all analytes, that is the recovery values (%) of all analytes increased with increasing solvent volume, acid ratio, extraction time, sonication amplitude and, in addition, the pulsed mode also showed a higher performance than the continuous mode.

All variables showed statistically significant differences individually for all analytes, except for sonication mode, which only showed statistically significant differences for morphine and acid proportion for codeine and papaverine. Furthermore, the Pareto Charts (Fig. 3) revealed

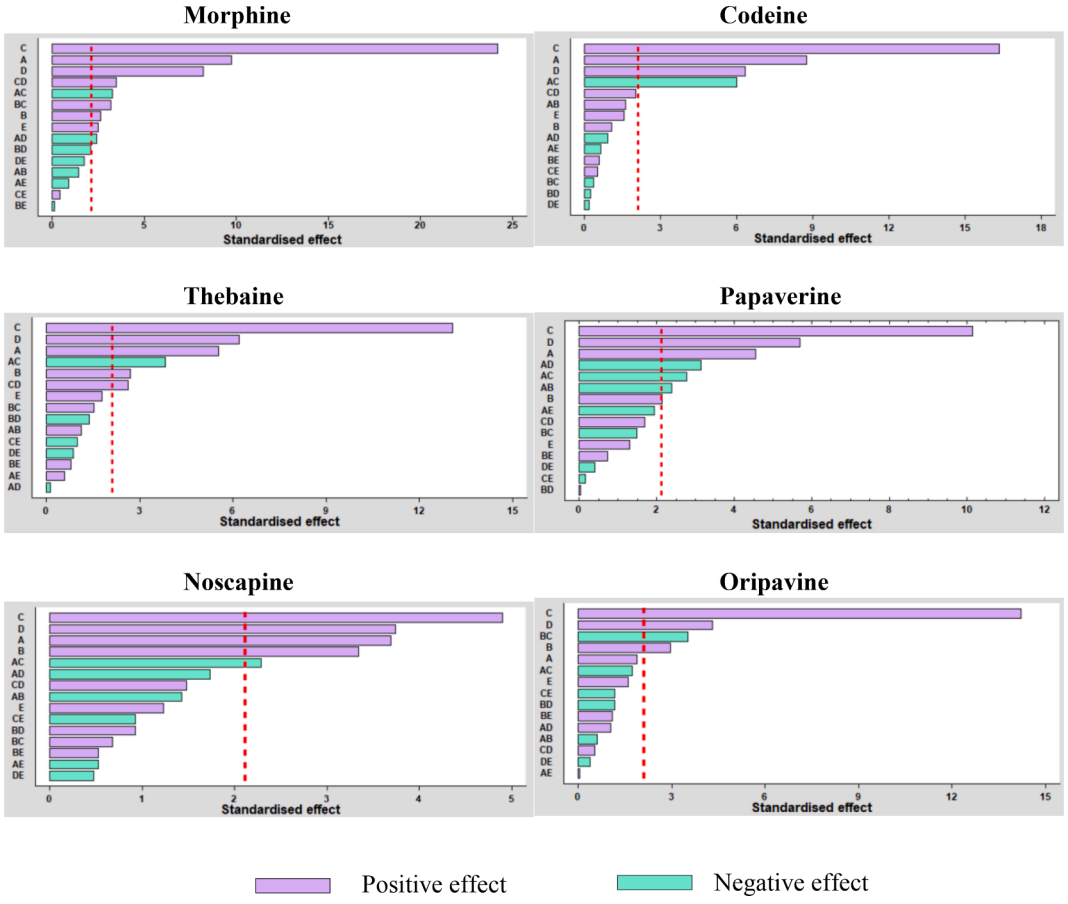

Fig. 3. Response from full factorial design 2^5 by the Pareto Chart of the standardised effect of each of the responses (recovery of each analyte), showing the five factors: (A) solvent volume (mL), (B) acid proportion (%), (C) extraction time (min), (D) sonication amplitude (%), (E) sonication mode to UAE optimisation.

that extraction time (C) was the parameter that most influenced the response of all analytes, showing a much higher standardised effect than the rest of the factors.

The fact that an increase in the solvent has a positive effect on the extraction yield may be due to the fact that a small solvent-solid ratio may lead to an increase in the viscosity of the solvent, decreasing the diffusion of the analytes through the extraction medium, or that saturation may be reached early, and the extraction process cannot be completed [32]. This factor was the second most influential in the extraction of all OAs except morphine and codeine which was the third and in the case of oripavine showed non-significant differences.

A higher acid proportion having a positive effect on the extraction may be since a more acidic pH of the medium increases the solubility of the analytes and thus increases the extraction yield. A longer extraction time may increase the extraction yield, but in the case of the use of UAE it may exhibit the opposite effect when the extraction time is longer than optimal due to the possibility of degradation due to heat generation, which may lead to degradation of the compounds [12]. In sonication mode, there were no significant differences except for morphine where pulsed mode showed a higher performance. Other studies claim the advantages of pulsed mode over continuous mode due to lower energy input, better temperature control, and reduced ultrasound probe tip erosion [33].

Regarding the interactions between the variables that showed statistically significant differences, the interactions of solvent volume (A) with acid proportion (AB) in papaverine, with amplitude (AD) in morphine and papaverine and with time (AC) in all the analytes except oripavine were the most significant. Other statistically significant interactions shown by time (C) were with acid proportion (BC) in morphine and oripavine and with amplitude (CD) in morphine and thebaine.

3.1.2.2. Optimization of UAE parameters based on an RSM approach. After performing the statistical analysis of the full factorial design of the 5 variables at 2 levels, a final optimisation of the solvent volume (A) and extraction time (C) was considered necessary. These variables were the most influential in the efficiency of the extraction, and the aim of the present work was to try to reduce these two variables as much as possible to achieve the most environmentally friendly and fastest possible methodology. Therefore, the critical variables (A, C) were studied in detail (A: 5, 7.5 and 10 mL, C: 1, 3 and 5 min) both in continuous and pulsed mode. The acid proportion and amplitude of the sonication were set at their previously determined optimal values, acid proportion 1 % and amplitude 75 %, as these were the levels that showed a significantly higher extraction performance. The final optimisation procedure was based on an RSM approach, and the experimental design, as well as the results obtained for the continuous and pulsed modes are presented in Tables S4 and S5 of the Supplementary Material, respectively.

The statistical parameters obtained from the statistical analysis (ANOVA) (p-values, R^2, R^2adj., R^2pred.) were compiled in Table S6 and indicated that the resulting quadratic models have very high predictability and could be used in RSM to optimise the extraction procedure by maximising the recovery values of each of the analytes. The statistical analysis also confirmed the significant terms of the response surface quadratic models obtained ($p < 0.05$). The variables with the highest impact on extraction performance using continuous ultrasound-assisted extraction (CUAE) were the linear terms of solvent volume (A) and extraction time (C), followed by the interaction term between the two critical variables (AC) and the quadratic term of extraction time (*C*2). In the case of pulsed ultrasound-assisted extraction (PUAE), the results indicated that the first order linear term of the extraction time (C) was the most significant, followed by its quadratic term (*C*2) and the linear term of the extraction volume (A) and the interaction between the two variables (AC). Once again, extraction time proved to be the most critical factor affecting the recovery of all analytes. In addition, these results were introduced into a multiple regression analysis to generate the mathematical models shown in Table S6, which adequately describe the empirical relationship between the two variables examined and the responses of each of the analytes with both extraction modes (CUAE and PUAE). Furthermore, to visualise the relationship between the independent and dependent variables, response surface and contour plots were generated based on the acquired polynomial equations, as shown in Figure S3. In the case of CUAE, the recoveries increased with increasing solvent volume in the same way as they increased with increasing extraction time. Furthermore, it is evident that the mutual interaction between solvent volume and extraction time was very significant due to the elliptical contour plot obtained for most of the analytes. In particular, the different shapes of the contour plots indicate whether the mutual interactions between the studied variables are significant [12]. In the case of PUAE, increasing the solvent volume showed few increases in yield and a more significant increase in extraction time (Figure S3).

Numerical optimisations were performed to determine the optimal level of each independent variable. For some analytes, the optimal value for the extraction volume variable was closer to 5 mL, while for codeine, thebaine and papaverine the optimal level was 10 mL, the same as for the extraction time, where some analytes needed 5 min to obtain the maximum recovery (morphine, thebaine, papaverine and noscapine). Therefore, the optimal conditions selected for optimal recovery of all OAs were 10 mL of solvent for 5 min of extraction.

To verify the reliability of the response surface models for the quantitative predictions, the results experimentally obtained were compared to the results predicted with the mathematical equations with the estimated optimal conditions. As shown in Table S7, the experimental results were very similar to the predicted values. Thus, the effectiveness and validity of the response surface models to reflect the response values and to be able to determine the best extraction conditions are confirmed.

3.2. Method validation

The results of the validation of the proposed UAE-SPE-HPLC-MS/MS method for the quantification of six AOs in biscuit and cake samples are shown in Table 1. The calibration lines were obtained with R^2 between 0.998 and 0.999 for all analytes, and the deviation of the back-calculated concentrations of the calibration standards with respect to the real concentrations in the matrix calibration lines was between −12 and −17 for all analytes, demonstrating the good linearity of the method as they are $\leq \pm 20$ % values according to the reference document [29]. In addition, the deviation of the slopes of the calibration lines for different days ($n = 3$) was calculated to ensure reproducibility, obtaining RSDs between 3 and 16 %.

Regarding the MDL and MQL values obtained, they were low for all analytes: 1 and 2 μg/kg for papaverine, 1 and 3 μg/kg for noscapine, 1 and 4 μg/kg for codeine and thebaine, 3 and 10 μg/kg for morphine and 6 and 20 μg/kg for oripavine, respectively.

On the other hand, the ME was calculated for both the complete methodology and the methodology without the purification step, to determine the need to introduce this step in the methodology. For this purpose, the slopes of both matrix and solvent calibration curves were compared. As shown in Table 1, the ME of the complete methodology was negligible, as all values were within +/− 20 %. However, the ME of the methodology without the purification step showed increased signal enhancement values for papaverine and noscapine (25 and 43 %). On the other hand, for oripavine, a ME of decreased signal (−51 %) was shown and its MDL and MQL were also affected, being worse. This means that the developed purification procedure was able to eliminate all possible matrix effects for all six target analytes. As no matrix effect is observed, solvent calibration lines could be used in the analysis of samples to quantify which would simplify the analysis.

Table 1
Validation parameters of the UAE-SPE-HPLC-MS/MS method for the quantification of six OAs in biscuits and sponge cakes.

Analytes	Linear range (mg/L)	Matrix-matched calibration (R^2) [a]	MDL (μg/kg) [b]	MQL (μg/kg) [c]	ME [d]	Accuracy [e]		Precision	
						Recovery (% ± SD)	Mean recovery (% ± SD)	Intra-Day Precision (RSD %)	Inter-Day Precision (RSD %)
Morphine	0.005–1	$y = 6.7 \times 10^7x + 3.3 \times 10^5$ (0.999)	3	10	9	77 ± 2^L 77 ± 4^M 82 ± 5^H	79 ± 4	2^L 5^M 6^H	3^L 5^M 7^H
Codeine	0.001–1	$y = 9.9 \times 10^7x + 7.1 \times 10^5$ (0.998)	1	4	15	90 ± 4^L 88 ± 9^M 93 ± 4^H	90 ± 6	4^L 10^M 4^H	5^L 11^M 5^H
Thebaine	0.001–1	$y = 9.3 \times 10^8x + 7.1 \times 10^6$ (0.998)	1	4	5	97 ± 6^L 97 ± 5^M 100 ± 3^H	98 ± 5	6^L 5^M 3^H	8^L 7^M 4^H
Papaverine	0.001–1	$y = 1.4 \times 10^9x + 1.6 \times 10^7$ (0.998)	1	2	16	101 ± 2^L 103 ± 3^M 100 ± 4^H	101 ± 3	2^L 2^M 4^H	3^L 3^M 5^H
Noscapine	0.001–1	$y = 1.5 \times 10^9x + 2.2 \times 10^7$ (0.998)	1	3	16	97 ± 2^L 98 ± 3^M 99 ± 2^H	98 ± 2	2^L 3^M 2^H	4^L 5^M 3^H
Oripavine	0.005–1	$y = 9.6 \times 10^7x + 1.0 \times 10^6$ (0.998)	6	20	4	78 ± 7^L 86 ± 4^M 95 ± 4^H	86 ± 5	9^L 5^M 4^H	9^L 6^M 7^H

The linear range expressed in μg/kg is 20–4000 in the case of morphine and oripavine and 4–4000 in codeine, thebaine, papaverine and noscapine [a] The calibration line is in the units: μg/mL; [b] MDL: method detection limit; [c] MQL: method quantification limit; [d] ME: matrix effect (dividing the purified matrix slope by the solvent slope); [e] Accuracy and precision were obtained by spiking samples at three concentration levels: low (L, 0.75 mg/kg), medium (M, 1.5 mg/kg) and high (H, 3 mg/kg).

Accuracy and precision were evaluated at three different concentration levels, low (0.75 mg/kg), medium (1.5 mg/kg) and high (3 mg/kg), showing adequate recovery values, between 77 and 103 % (Table 1). On the other hand, as shown in Table 1, satisfactory results were obtained for intra-day and inter-day precision at the three concentration levels, since the RSD values were lower than 20 %, the lowest value is 11 %. Furthermore, as shown in Figure S4, a good selectivity of the method was obtained. The chromatograms of the extracted ions obtained for each of the OAs in a standard solution were compared with the sample extracts. It was found that the variation of the t_R was ≤0.1 min, and the ion ratios of the sample extracts were within ±30 % (relative abundance) of the mean of the standards for each analyte.

3.3. Comparison of the proposed UAE-SPE method with previously methods for OAs

The most significant advantages of the proposed method over previously published methods were the lower solvent volume and extraction time optimised. This allowed the development of a faster, cheaper and, above all, more environmentally friendly methodology [34]. In this method, only 10 mL and 5 min are enough to achieve complete extraction. However, in previously published works, when not using the UAE and opting for simple SLE, up to 100 mL of AcN/water/formic acid, 80/19/1, v/v/v/v and 1 h of extraction are used for each sample, as in the work of López et al. [4]. Another example with lower solvent volume but with the same extraction time is the work of Sproll et al. [5] which used 30 mL of methanol 0.1 % acetic acid for 1 h of extraction or our previous work on seeds with 30 mL of MeOH/water, 50/50 (v/v) for 1 h [7] or that on breadsticks and sliced bread with 20 mL of MeOH 0.1 % acetic acid for 1 h [8].

In addition to the advantages of efficient extraction, the advantages of the purification step are also important. As has been shown in this work, these samples have a considerable matrix effect that can lead to an increase in the signal of two analytes (papaverine and noscapine) and a decrease in another (oripavine). However, the purification step was able to completely eliminate all matrix effects using a similar or even lower amount of sorbent than in other studies (50 mg) and lower solvent volume in the process (1 mL to load and 2 mL to elution) [7,22,35]. Most of the previously published studies have not performed this purification step and have therefore obtained really matrix effects such as López et al. [4] with + 30 % or have not even been evaluated [3,5]. In addition, there are some works that even with the purification step, considerable matrix effects are obtained, usually signal decrease effects such as that of Tang et al. [36] with −60 % in some analytes and in Casado-Hidalgo et al. [7] with −69 % in oripavine. Other works show a mixture of signal increase/decrease, such as the work of Guo et al. [22] where some analytes have a ME of −39 % and others of +101 %.

It is also interesting to note the economic advantages of using a synthesised material rather than commercial materials that are often considerably more expensive [22]. In addition, the synthesis of SBA-15 is very simple and uses reagents that are cheap and give a high synthesis yield in only 3 days. This is very different from other syntheses that are more complex, longer and more costly after functionalisation steps with specific organic molecules [35,36]. Therefore, the methodology presented in this work is simpler, faster, cheaper and more environmentally friendly than any of the previously published methodologies to quantify OAs in food samples.

3.4. Application of the proposed method to commercial samples of biscuits and sponge cake

As the validation of all parameters was successful (section 3.2) according to the reference documents [29–31], the proposed methodology was applied to the analysis of seven commercial samples, five of them biscuits and two sponge cakes.

The solvent calibration line could be used for quantification, which would simplify and reduce the cost of routine analysis of this type of sample. This is because the SPE purification step is able to remove all matrix effects and the recovery values are within the desired range. However, to confirm that similar values were obtained, the signals were corrected with the recovery values obtained at the low level of the validation (Table 1). Subsequently, the signal was corrected with the internal standards and interpolated in the calibration line of the matrix of internal standards. Only the two internal standards of morphine-d3 and codeine-d3 were purchased as these are the compounds that are legislated [11]. So, to signal correct morphine-d3 was used for morphine and codeine-d3 was used for codeine and thebaine due to their similar characteristics like pKa and water solubility [1].

The samples analysed showed low levels of opium alkaloid contamination as shown in Table 2. Especially, oripavine could only be detected in sample SC-1, noscapine was detected in all samples except B-4 and SC-2 and papaverine was detected in all samples, but below the

Table 2
Occurrence of opium alkaloids in the commercial samples analysed (n = 3).

Sample [a]		B-1	B-2	B-3	B-4	B-5	SC-1	SC-2
Poppy seeds amount (%) [b]		5	4	5	Not specified	4	Not specified	13
Range of occurrence (mg/kg) ± SD	Morphine	0.013 ± 0.006	ND	0.010 ± 0.008	ND	0.010 ± 0.002	0.03 ± 0.01	0.153 ± 0.009
	Codeine	ND	ND	ND	ND	ND	<MQL	0.004 ± 0.002
	Thebaine	ND	ND	ND	ND	ND	<MQL	0.015 ± 0.003
	Papaverine	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
	Noscapine	<MQL	<MQL	<MQL	ND	<MQL	<MQL	ND
	Oripavine	ND	ND	ND	ND	ND	<MQL	ND
Morphine equivalents (mg/kg) [c]		0.013	ND	0.010	ND	0.010	0.03	0.154

[a] B: biscuits; SC: sponge cakes; [b] Poppy seed content was obtained from the ingredients list on the product packages; [c] maximum limit of morphine equivalents in bakery products of 1.5 mg/kg in Commission Regulation (EU) 2021/2142; ND: not detected; <MQL: lower than method quantification limit but higher than method detection limit.

MQL in all cases. Codeine and thebaine were detected in SC-1 and were quantified in sample SC-2, which showed 0.004 ± 0.02 mg/kg of codeine and 0.015 ± 0.003 mg/kg of thebaine. On the other hand, morphine concentrations were found in all samples except B-2 and B-4. However, these results were low and in all cases below the legislated maximum limit for bakery products (1.5 mg/kg of morphine equivalents) [11]. In sample B-1 it was 0.013 ± 0.006 mg/kg, in B-3 0.010 ± 0.008 mg/kg, in B-5 0.010 ± 0.002 mg/kg, in SC-1 0.03 ± 0.01 mg/kg and in SC-2 0.153 ± 0.009 mg/kg. Therefore, the most contaminated sample was SC-2, which coincides with a higher proportion of seeds (13 %) than the rest of the samples.

These low concentrations could be due to the heat treatment the seeds have undergone in the baking of biscuits and sponge cakes as stated in some previous studies and EU recommendations [3–5,37–40]. In addition, as demonstrated in recent work by Vera-Baquero et al. the location of the seeds in the sample could also influence the resulting opium content [40]. For example, in sample SC-1 all seeds were located on the surface of the product, so degradation due to baking temperature could be higher as they are more exposed and can reach higher temperatures. However, in general, all the biscuits studied are small and thin and the dough might not protect the seeds as much, therefore no differences are seen between SC-1 where the seeds are located on the surface and the rest of the biscuits where they are added in the dough. This aspect might be more relevant in the bakery products studied in the previous work [8], where differences were shown between breadsticks where the seeds were on the surface and sliced bread where the seeds were in the dough and higher opium concentrations were found (with two samples exceeding the maximum limit). However, this aspect should be further studied to determine how temperature really influences these compounds and whether it really allows them to degrade and therefore to be safe samples for the consumer.

4. Conclusions

A fast, efficient and environmentally friendly method for the control of opium alkaloids in biscuit and sponge cake samples has been developed. For this purpose, UAE was used for the first time in OAs, which allowed using lower solvent volumes and extraction time than with other classical methods. This was followed by a purification step using SBA-15 in SPE as it showed more efficiency than HMS silica and the commercial HLB sorbent, eliminating all matrix effects. After successful validation, the method was shown to have low MDL and MQL and favourable recovery values (79–101 %). The method was applied to seven commercial samples of biscuits and sponge cakes, obtaining morphine concentrations in five of the samples below the established maximum limit (1.5 mg/kg morphine equivalents). The rest of the analytes were detected below the limit of quantification except in one of the samples where the proportion of seeds was higher (13 %) than in the rest of the products (around 5 %).

CRediT authorship contribution statement

Gema Casado-Hidalgo: Methodology, Validation, Investigation, Writing – original draft, Visualization. **Sonia Morante-Zarcero:** Conceptualization, Writing – review & editing, Supervision. **Damián Pérez-Quintanilla:** Conceptualization, Writing – review & editing, Supervision. **Isabel Sierra:** Conceptualization, 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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2022.108059.

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