Evaluating the stability of tropane and opium alkaloids during baking in homemade gluten-free poppy seed crackers

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ARTICLE INFO

ABSTRACT

Nowadays, alternative flour to wheat, such as teff and corn are increasingly used in gluten-free bakery products, and seeds, such as poppy seeds (whole or ground) are added for their beneficial nutritional properties. However, these ingredients may be contaminated with natural toxins such as tropane (TAs) and opium (OAs) alkaloids. To reduce toxin exposure, it is essential to assess their thermal degradation during baking. The study aimed to quantify TAs and OAs in doughs before and after baking to evaluate their degradation while preparing homemade gluten-free ground poppy seed crackers. To achieve this, a novel methodology involving solid-liquid extraction (SLE) and solid-phase extraction (SPE) with a bifunctionalized mesostructured silica SBA-15 sorbent (50 mg), followed by UHPLC-MS/MS analysis, was developed and validated. The method validation showed good linearity ($R^2 \geq 0.991$), precision (%RSD ≤17%), accuracy (88–104% for TAs and 79–106% for OAs), and low quantification limits (0.6–1.1 μg/kg for TAs and 0.06–0.46 mg/kg for OAs). The results showed no significant reduction of the alkaloids in the homemade gluten-free ground poppy seed crackers, showing that both families of natural toxins can remain in foods even after intense heat treatments, highlighting the need for accurate monitoring of these natural toxins in processed food.

1. Introduction

The growing demand for innovative and nutritious bakery products has driven the popularity of alternative ingredients such as corn starch and teff flour, especially among consumers with gluten intolerance or wheat allergy (Goldstein & Underhill, 2001; Poole et al., 2006). In addition, some seeds, such as poppy seeds are increasingly used, whole or ground, in bakery products due to their beneficial nutritional properties, such as high levels of fatty acids and dietary fibre (Ghafoor et al., 2019).

However, gluten-free cereals and poppy seeds can be contaminated with natural toxins such as tropane alkaloids (TAs) and opium alkaloids (OAs) (Casado-Hidalgo, Morante-Zarcero, et al., 2021; González-Gómez et al., 2022). For example, TAs like atropine and scopolamine are found in plants belonging to the Solanaceae family that can contaminate cereal crops during harvesting (EFSA, 2011; Mulder et al., 2013). On the other hand, poppy seeds may contain OAs such as morphine, codeine, thebaine, papaverine, noscapine and oripavine due to contamination with the latex of the poppy plant (*Papaver somniferum* L.), which is rich in these compounds (EFSA, 2018).

Consuming food contaminated with TAs and OAs can result in intoxication, adverse effects or even death, particularly with high concentrations of TAs, or false positive drug tests with high concentrations of OAs (EFSA, 2018; EFSA, 2022). Recent notifications in the Rapid Alert System for Food and Feed (RASFF) have highlighted contamination in some cereal-based foods and poppy seeds (RASFF, 2024). To avoid this public health concern, Commission Regulation (EU) 2023/915 sets maximum limits for these contaminants in different food, including a maximum limit between 5 and 15 μg/kg as the sum of scopolamine and atropine in cereals and derived products and 1.5 mg/kg morphine equivalents (morphine + 0.2 × codeine) for OAs in bakery products (European Commission, 2023). For this reason, developing analytical
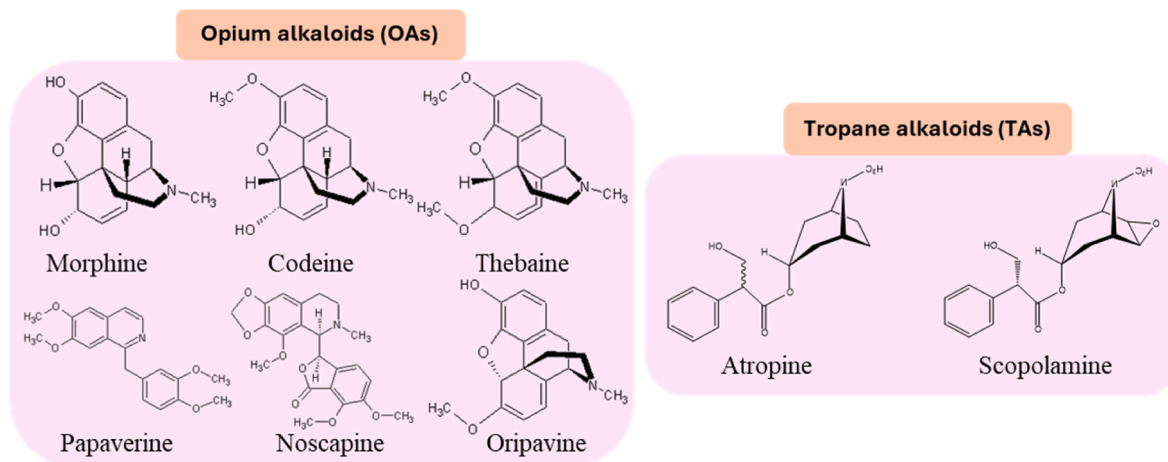

Fig. 1. Chemical structures of the six main OAs and the two TAs.

methodologies that allow the analysis of these families of food toxins is essential to ensure food safety (Vera-Baquero et al., 2024).

On the other hand, certain culinary processes can significantly reduce these toxins through degradation. For example, the high temperatures in baking bakery products can lead to the degradation of both TAs and OAs (Casado et al., 2023). However, particularly for OAs, giving a degradation value can be a tedious task due to the heterogeneous contamination suffered by the poppy seeds that depends on multiple factors, which generates a large dispersion in the concentration data for these toxins (Casado-Hidalgo, Pérez-Quintanilla, et al., 2021; López et al., 2018). Moreover, the wide variation in the results observed could also be due to inadequate control of critical parameters in the baking, such as time or temperature (Casado et al., 2023). Consequently, contradictory findings exist in the literature for both toxin families. Some studies suggest significant degradation of TAs at high temperatures (Kaltner, 2022; Marín-Sáez et al., 2019), while others argue for their stability (Friedman & Levin, 1989). Similarly, there are conflicting views on the influence of temperature on OAs. Some studies report complete degradation at high temperatures, especially when the seeds are used as toppings (Carlin et al., 2020; Vera-Baquero et al., 2022), while others suggest that OAs remain highly stable even after hours of exposure to elevated temperatures (Shetge et al., 2020; Shetge & Redan, 2022). Regarding grinding, the European Commission (EC) reported in 2014 morphine degradation between 25 and 34% (European Commission, 2014). However, other studies suggest that some grinding methods, which extract fat from the seeds, have a protective effect on the degradation of morphine (Casado-Hidalgo et al., 2024). Therefore, combining effective grinding conditions with other thermal processes like baking could enhance the degradation efficiency of OAs.

Considering that these analytes are present in complex matrices such as bakery products in low concentrations, a proper sample preconcentration and clean-up process before chromatographic analysis plays a crucial role in the analytical process. Ensuring clean extracts and minimizing interferences is essential to facilitate the interpretation of results and to maintain the lifetime of the UHPLC column. Solid-phase extraction (SPE) is the most conventional technique for these purposes (Wang et al., 2022). The choice of sorbent material is crucial, with ongoing efforts focused on creating more efficient materials that support miniaturization and ensure environmentally friendly (Andrade-Eiroa et al., 2016). Bare mesostructured silicas like Santa Barbara Amorphous-15 (SBA-15) have gained prominence due to their ordered structure, controlled pore size, larger pore area and volume, enabling functionalization with different ligands for targeted analyte interaction. These ligands can vary in nature and can even be used in mixed mode, meaning they can have double functionalization (Wang et al., 2022).

In this sense, bifunctionalized SBA-15 with a reversed-phase/strong cation-exchange mixed-mode retention mechanism was successfully applied and evaluated as SPE sorbent for the simultaneous extraction of 26 veterinary drug residues in meat samples (Casado et al., 2017). However, to the best of our knowledge, bifuncionalized mesostructured silicas have not been evaluated as SPE sorbents in the determination of other contaminants like alkaloids in foods. Therefore, it would be interesting to explore the application of bifunctionalized silica sorbents that provide ionic and polar interactions to favour the retention of TAs and OAs during food analysis.

Therefore, this work aimed to evaluate the effect of baking on the degradation of two TAs (atropine and scopolamine) and six OAs (morphine, codeine, thebaine, papaverine, noscapine, and oripavine) in naturally contaminated homemade gluten-free ground poppy seed crackers prepared with corn starch or teff flour. For this, a methodology was developed and validated to control these alkaloids in raw dough and baked crackers to minimize quantification errors. The method consisted of solid-liquid extraction (SLE) and SPE purification with bifunctionalized SBA-15 silica with sulphonic acid and cyano groups (denoted as SBA-15-SO$_3$H-CN), followed by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS).

2. Materials and methods

2.1. Reagents and materials

The OAs and TAs standards used were of high-purity grade 98%. Morphine, codeine, thebaine and oripavine were supplied by Alcaliber S.A.U. (Madrid, Spain) while noscapine, papaverine, scopolamine hydrobromide and atropine were sourced from Sigma-Aldrich (Sigma-Aldrich, Zwijndrecht, The Netherlands). The structures of both analyte families studied are shown in Fig. 1. Individual stock standard solutions were prepared at a concentration of 1000 mg/L in methanol (MeOH). An intermediate mixed standard solution was then prepared at a concentration of 10 mg/L in MeOH. All standard working solutions were stored at −20 °C in darkness.

Tetraethylorthosilicate (TEOS) at 98% purity and poly(ethylene glycol)-block-poly (propylene glycol)-block-poly(ethylene glycol) (EO20PO70EO20, Pluronic 123, P123) were acquired from Sigma-Aldrich (St. Louis, MO, USA). HCl 37%, acetic acid (HAc), Hydrogen peroxide (H$_2$O$_2$, 30% *v/v*), HPLC-MS grade acetonitrile (ACN) and MeOH, sodium hydroxide (NaOH) and ammonia solution (32% *v/v*) were sourced from Scharlab (Barcelona, Spain). (3-mercaptopropyl) triethoxysilane (MPTES) at 94% purity was obtained from Alfa Aesar (Karlsruhe, Germany). 3-cyanopropyltriethoxysilane (CNPTES) at 98% was acquired from Sigma-Aldrich (St. Louis, MO, USA). NaCl was purchased from Panreac Química (Castellar del Vallès, Barcelona, Spain).
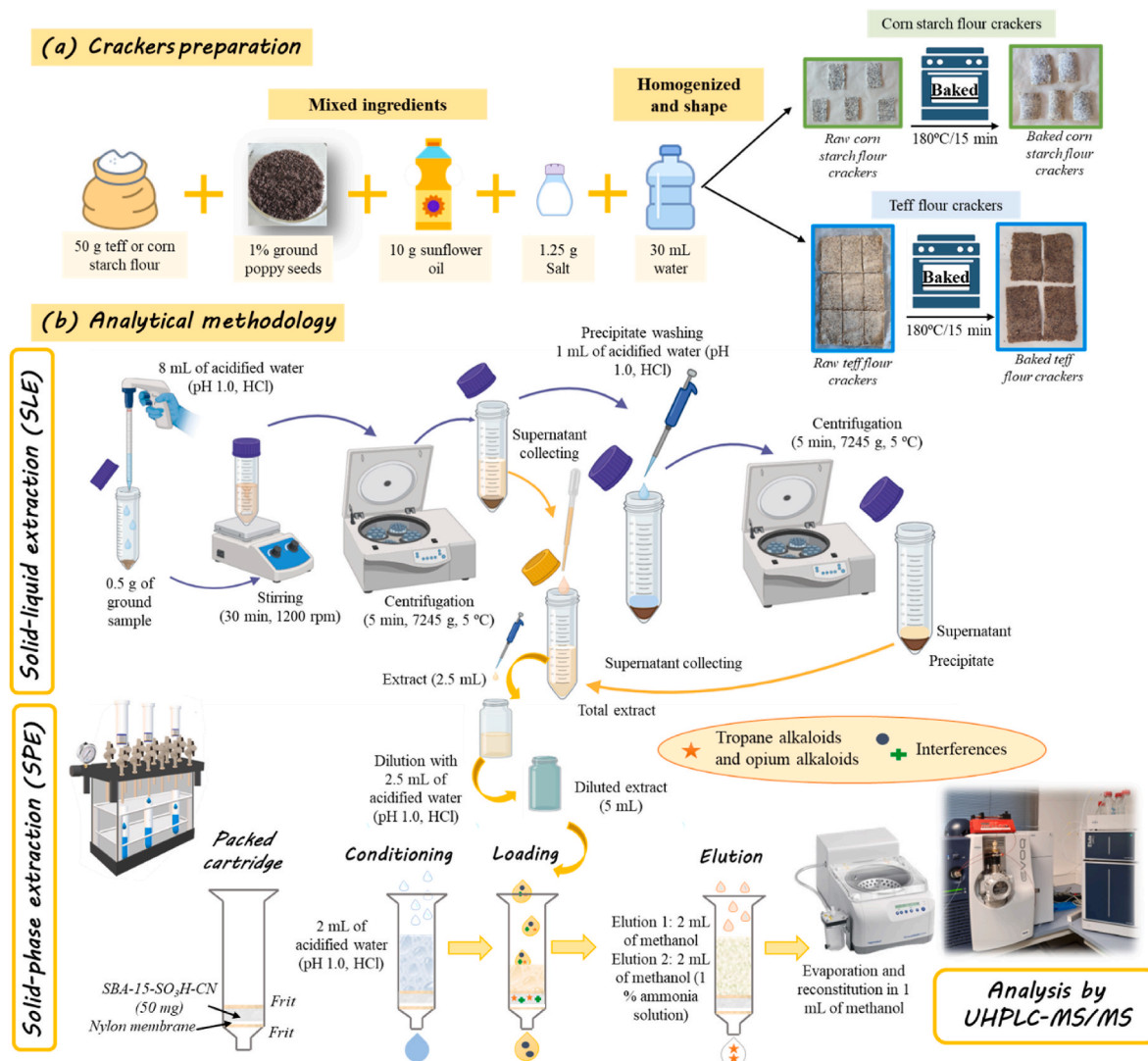
Fig. 2. Schematic representation of (a) cracker preparation and (b) optimized analytical methodology based on solid-liquid extraction (SLE) followed by solid-phase extraction (SPE) prior to ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS).

Formic acid (HCOOH) of HPLC-MS grade was from Fluka (Busch, Switzerland). Nylon filter membranes (0.45 μm), polyethylene frits (0.20 μm), 3 mL empty syringes, and nylon syringes filters (0.45 μm) were sourced from Scharlab (Barcelona, Spain). Ultra-pure water with a resistance of 18.2 MW cm was procured from a Millipore Milli-Q-System (Billerica, MA, USA). Water acidified with 1.1% HCl (pH 1.0) was prepared as the extraction solvent for the TAs and OAs.

Commercial crackers with poppy seeds used for method optimization were purchased from local supermarkets in Madrid (Spain). Organic whole teff flour, corn starch and poppy seeds purchased in supermarkets in Madrid (Spain) were used to prepare the homemade cracker samples. Poppy seeds were ground with a poppy seed mill (Poppy Seed Mill, Westmark, Elspe, Germany) to obtain a powder with a small particle size and no fatty appearance.

2.2. Homemade gluten-free ground poppy seed crackers elaboration

For the preparation of the crackers, such as shown in Fig. 2a, 50 g of teff flour or corn starch, 10 g of sunflower oil, 1.25 g of salt, 9 g of ground poppy seed (1g/100g, *w/w*) and 30 mL of water were mixed. The dough was then rolled out to a thickness of 1 mm, and the crackers were cut into rectangular shapes (5 cm long x 3 cm wide) and weighed (approximately 3 g) to obtain the fresh weight of each cracker. Three

batches of teff or cornstarch dough were prepared to ensure good sampling. Finally, they were baked for 15 min at $180 \pm 5\,°C$ in a Candy fan-assisted electric oven (Madrid, Spain). To control the temperature, a T-type probe coupled to a TESTO model 926 digital thermometer (Madrid, Spain) was inserted in the centre of the samples. They were then allowed to cool to room temperature and weighed to evaluate the weight loss (WL) of the samples after baking. Before analysis, the baked cracker samples were crushed with a basic analytical mill A11 (IKA, Staufen, Germany) and sieved. To calculate the amount of OAs and TAs, the samples were analyzed according to the protocol described in section 2.5. Results obtained for baked crackers were normalized by expressing them considering water loss by evaporation during baking according to the following formula = [actual concentration measured in baked crackers (mg/kg) × (100 − WL)]/100.

2.3. Synthesis and characterization of SBA-15-SO$_3$H-CN material

SBA-15 was prepared according to the method outlined by Zhao et al. (Zhao et al., 1998). Once SBA-15 was synthesized, it was functionalized with sulfonic acid groups following the work of our group (González-Gómez et al., 2020). Initially, 2.5 g of SBA-15 was dissolved in 250 mL of 0.1 mol/L HCl aqueous solution, and MPTES was added in a molar ratio of 0.10 MPTES/SiO$_2$. After stirring for 7 h at room
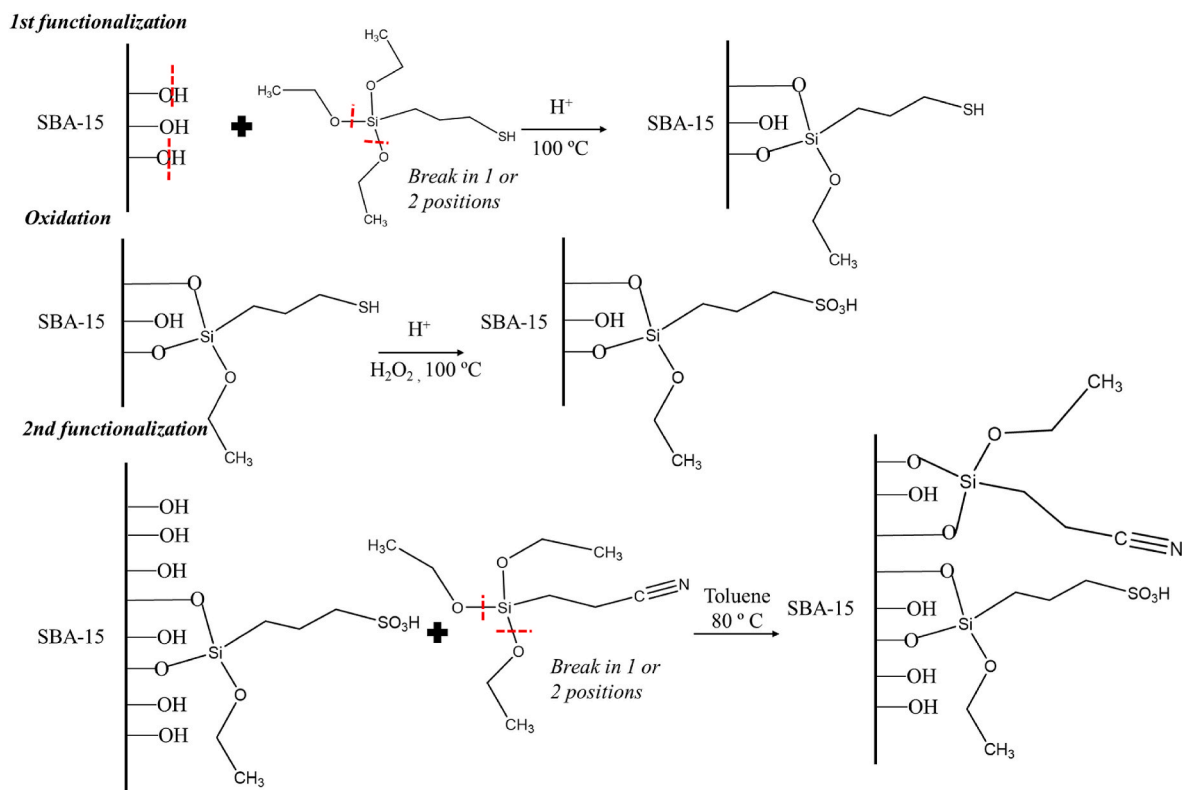
Fig. 3. Schematic representation of the synthesis of bifunctionalized SBA-15 silica with sulphonic acid and cyano groups (SBA-15-SO$_3$H-CN).

temperature, the mixtures were transferred to an autoclave and kept at 100 °C for 24 h. The resulting solids were filtered, washed with water, and dried overnight at 50 °C in an oven. Subsequently, the silicas were suspended in 335 g of 2 mol/L HCl, and 11.4 g of H$_2$O$_2$ (30% v/v) were added. After stirring for 5 min at room temperature, the mixtures were transferred to an autoclave and maintained at 100 °C for 6 h. The SBA-15-SO$_3$H were recovered by filtration, washed with water and dried standing to start the next stage. For bifunctionalization, 2.5 g of SBA-15-SO$_3$H was mixed with 100 mL of toluene and stirred in a stirring plate at room temperature for 2 min at 250 rpm. Subsequently, CNPTES was added in a weight ratio of 0.2 CNPTES/SBA-15-SO$_3$H. Then, the mixture was stirred at 80 °C for 24 h. The SBA-15-SO$_3$H-CN were recovered by filtration, washed with toluene, ethanol, and diethyl ether, and dried at 70 °C in an oven for 24 h. A schematic of the synthesis is shown in Fig. 3. To confirm the correct functionalization, the synthesized materials were characterized by N$_2$ gas adsorption-desorption isotherms, Fourier-Transform Infrared (FT-IR) spectroscopy and elemental analysis. Details of the equipment and conditions can be found in Supporting Information S1.

2.4. Trials for optimizing analytical methodology

Firstly, an optimization was carried out with standard working solutions prepared in different solvents to obtain the best performance of the method. The chosen solvents were acidified water (pH 1 and 3) and MeOH/water with 0.1% HCOOH (50/50, v/v). To perform this test, 50 mg of the SBA-15-SO$_3$H-CN material was packaged to miniaturize the method (this amount of sorbent was fixed for all optimization tests) and the SPE protocol was carried out (n = 3), the cartridge was conditioned with 2 mL of the solvent used during loading. Next, 5 mL of a standard working solution with 2 μg/L of atropine and scopolamine, and 300 μg/L of morphine, codeine, thebaine, papaverine, noscapine, and oripavine were loaded into the cartridge. These concentrations were selected by the maximum residue limits (MRLs) set by the Commission Regulation

(EU) 2023/915 for bakery products (European Commission, 2023). Afterwards, the analytes were eluted with 2 mL of MeOH followed by 2 mL of MeOH containing 1 % ammonia solution. The eluate was collected and directly injected into the UHPLC-MS/MS. To calculate the recovery, one cartridge was loaded with the solvent, and the target analytes were added after the elution step (simulated sample) and then injected. The recovery values were determined by comparing the peak areas obtained for the analytes in both cases.

The same tests were then carried out using commercial cracker samples. The samples were first subjected to SLE, for which 1 g was mixed with 5 mL of the extraction solvents (shaken at 1200 rpm for 15 min, followed by centrifugation at 7245g for 10 min at 5 °C). Afterwards, the extract was filtered and loaded into the SPE cartridge previously conditioned with the appropriate solvent. The analytes were then eluted as in the previous study. To evaluate the recovery of the protocol, three cracker samples were spiked before starting the SLE and one sample was spiked at the end of the SLE-SPE protocol. Additional tests on the SLE were performed to improve the results and then, with these conditions SPE, was tested again. Finally, the final conditions were confirmed with the samples prepared according to section 2.2.

2.5. Optimized SLE-SPE protocol

0.5 g of ground sample was weighed into a 50 mL Falcon™ tube, and 8 mL of acidified water (pH 1.0, HCl) was added. The mixture was stirred on a shaker plate (1200 rpm) for 30 min at room temperature and then centrifuged using a centrifuge Digicen 21 R from Ortoalresa (Madrid, Spain) at 7245 g for 5 min at 5 °C. The supernatant was collected, and the precipitate was washed with 1 mL of acidified water (pH 1.0, HCl). The mixture was again centrifuged with the same centrifuge program as the previous one. The supernatant obtained was combined with the previous one and filtered through a 0.45 μm nylon filter, then 2.5 mL of the filtered extract was diluted with another 2.5 mL of acidified water (pH 1.0, HCl), and the sample was subjected to the SPE
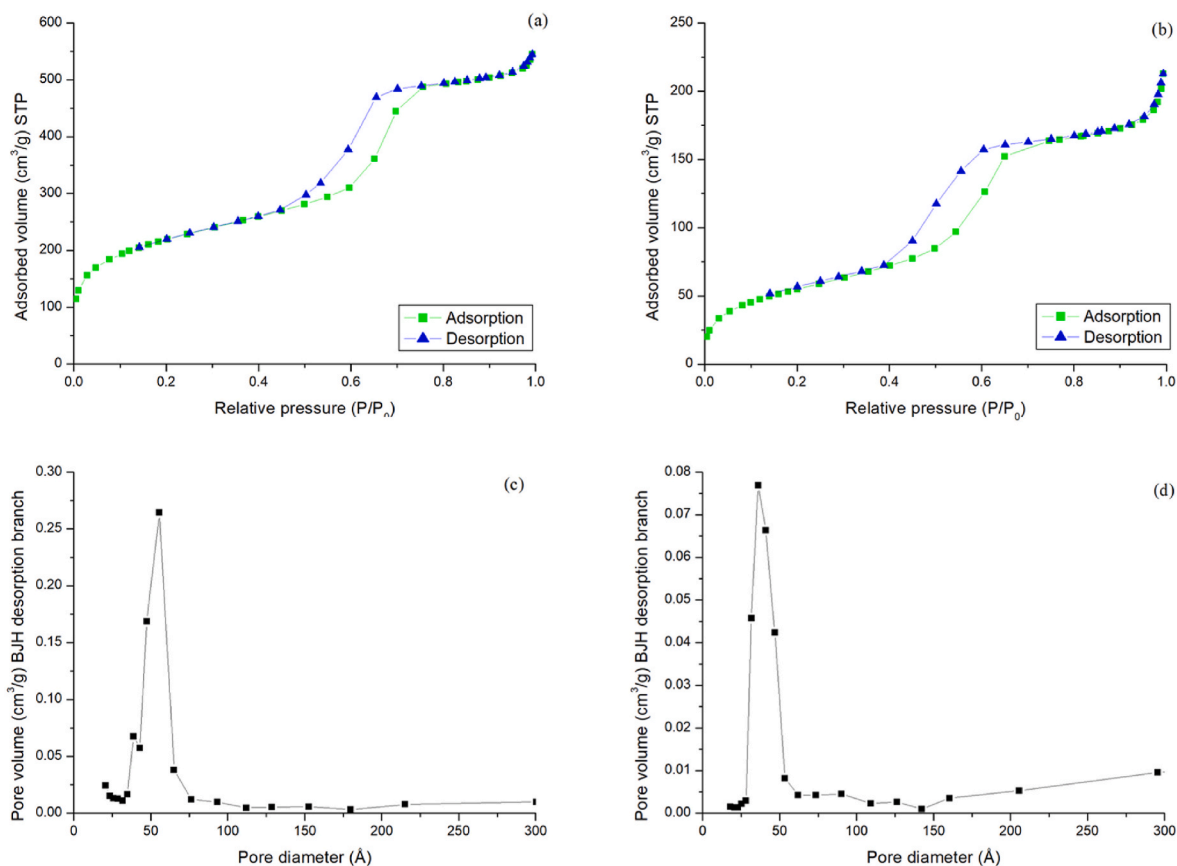
Fig. 4. N$_2$ adsorption-desorption isotherms of: (a) SBA-15, (b) SBA-15-SO$_3$H-CN; Pore size distribution of: (c) SBA-15, (d) SBA-15-SO$_3$H-CN.

purification step.

First, empty SPE cartridges (3 mL) were filled with 50 mg of SBA-15-SO$_3$H-CN material and sealed with polyethylene frits at both ends. Additionally, a nylon membrane with a pore size of 0.45 μm was inserted at the bottom of the sorbent bed to prevent material loss during the SPE protocol. Next, the cartridges were conditioned with 2 mL of water at pH 1.0 (HCl) at a flow rate of approximately 1 mL/min, using a Scharlab ExtraVac® solid extraction vacuum manifold (12-port) connected to a vacuum pump at 10 psi. Subsequently, the diluted sample (5 mL) was loaded. Finally, the target analytes were eluted using 2 mL of MeOH, followed by 2 mL of MeOH containing 1 % ammonia solution. The eluates were evaporated to dryness using an Eppendorf® concentrator Plus from Eppendorf SE (Hamburg, Germany) and redissolved in 1 mL of MeOH for analysis in the UHPLC-MS/MS. Fig. 2b summarizes the optimized protocol.

2.6. UHPLC-MS/MS analysis

The quantification was performed with an Elute HPLC system (Bruker Daltonics Inc, Billerica, MA, USA) equipped with an Elute HPLC HPG 1300 pump with two pairs of individually controlled linear drive pump heads coupled in series, an Elute Autosampler maintained at 10 °C (with a loop of 100 μL) coupled to a triple quadrupole tandem mass spectrometer detector (EVOQ® Elite, Bruker) with a heated electrospray ionization ion source (HESI). Chromatographic separation was performed on an Intensity Solo 2 C18 column (100 mm × 2.1 mm × 2 μm, Bruker). The injection volume was 5 μL (partial injection), and the flow rate of the mobile phase was constant at 0.4 mL/min during the analysis. The mobile phases were water (mobile phase A) and acetonitrile (mobile phase B), with 0.1% HCOOH. A linear gradient from 95% mobile phase A to 30% mobile phase A over 3.5 min, followed by an increase to 95% mobile phase A over 0.2 min, that was maintained until 5 min was

utilized. The equilibration time between injections was 1 min. Fig. S1 shows the separation of the analytes. The detection was optimized by direct infusion of the standard working solution in MeOH/water (60/40, v/v) with 0.1% HCOOH of 1 mg/L of each analyte. For this, a syringe pump at 5 μL/min of flow rate was used. The mass spectrometer was in HESI+ with multiple reaction monitoring (MRM) and a window time of ±0.5 min using a cone temperature and gas pressure of 300 °C and 0,137895 MPa, N$_2$ as drying gas at 350 °C and 0,27579 MPa, Ar as nebulizer gas at 0,413685 MPa and collision gas at 2 mTorr, ion spray voltage of 3000 V, collect delay at 0.5 min and detector voltage at 1.65 V. Optimal parameters for the analysis of each analyte are shown in Table S1.

2.7. Instrumental and method validation

Firstly, the instrumental validation was performed by analysing standard working in the UHPLC-MS/MS to determine the linear range, detection and quantification limits (LOD and LOQ), repeatability and reproducibility. Then, the method based on a SLE followed SPE and UHPLC–MS/MS was validated with samples of raw dough and baked crackers (prepared with teff flour and ground poppy seeds). For this purpose, SANTE/11312/2021 document and AOAC International Guidelines for Dietary Supplements and Botanicals were followed to evaluate linearity, quantification, and detection method limits (MQL and MDL), matrix effect (ME), accuracy, precision, and selectivity, as there are currently no official regulations on analytical performance requirements for OAs or TAs in food (AOAC, 2017; SANTE, 2021). For more details, see Supporting Information S2. Additionally, for raw dough and baked crackers prepared with corn starch and ground poppy seed, only accuracy (n = 6) at the intermediate concentration level and linearity calibrations were evaluated.
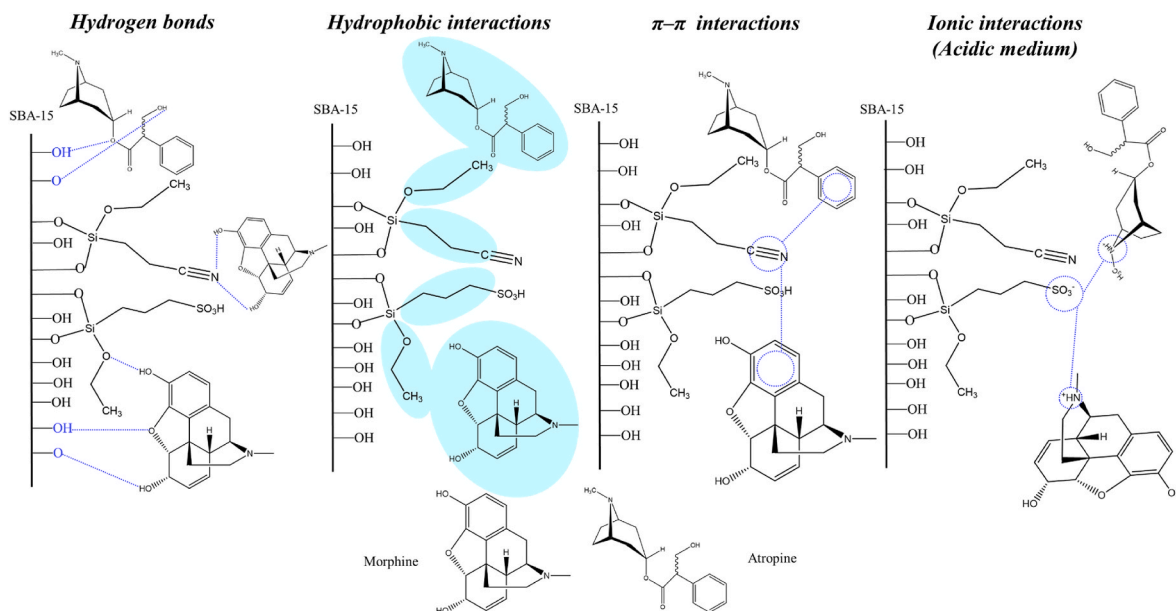
Fig. 5. Different types of interaction that can occur between TAs and OAs with the sorbent (SBA-15-SO₃H-CN) used for solid-phase extraction.

2.8. Statistical analysis

To perform the statistical analysis, the SPSS 25.0 statistical package (SPSS INC., Chicago, IL, USA) was used. A Student's t-test with two tails and a 95% confidence interval was carried out. Differences were considered significant for p values ≤ 0.05 and different letters were used to indicate this.

3. Results and discussion

3.1. Characterization of SBA-15-SO₃H-CN material

The N$_2$ adsorption-desorption isotherms and pore size distribution for ordered mesostructured silicas prepared are shown in Fig. 4. As can be seen, both the SBA-15 (Fig. 4a) and the bifunctionalized SBA-15-SO₃H-CN (Fig. 4b) isotherms were type IV according to IUPAC categorization with narrow hysteresis loop typical of mesoporous solids. Both materials displayed an H1 hysteresis loop, indicative of uniform cylindrical pores. The Barret-Joyner-Halenda (BJH) method was used to calculate the pore size distribution of mesostructured silicas, resulting in a narrow distribution. This provided evidence of uniform mesoporosity within the framework (as shown in Fig. 4c and d). Physical parameters of nitrogen isotherms, such as the Brunauer-Emmett-Teller surface area (S$_{BET}$), total pore volume, and average pore diameter for the mesostructured silicas are presented in Table S2. As shown, after functionalization, a considerable decrease in the S$_{BET}$, pore volume and average BJH pore diameter was observed, which can be attributed to the presence of functional groups on the silica surface.

Table 1

Recovery percentages (% ± SD) for tropane (10 µg/kg) and opium alkaloids (1500 µg/kg) in commercial crackers using SBA-15-SO₃H-CN material as sorbent in solid-phase extraction (SPE).

Sample amount	Extraction solvent (5 mL)	Recovery percentages (% ± SD), n = 3[a]							
		Atropine	Scopolamine	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
1 g	Aqueous solution of HCl (pH 1)	83 ± 3	34 ± 15	41 ± 6	53 ± 16	111 ± 33	53 ± 11	46 ± 10	58 ± 10
	Aqueous solution of HCl (pH 3)	99 ± 36	29 ± 1	47 ± 2	73 ± 3	96 ± 13	15 ± 1	24 ± 1	62 ± 16
	MeOH/water with 0.1% formic acid (50/50 v/v)	3 ± 1	0 ± 0	13 ± 6	38 ± 1	101 ± 9	21 ± 5	17 ± 2	34 ± 1
0.5 g	Aqueous solution of HCl (pH 1)	81 ± 6	67 ± 10	72 ± 9	84 ± 1	85 ± 12	76 ± 9	79 ± 9	84 ± 5

[a] SLE: 1 or 0.5 g 5 mL put in contact with 5 mL of the tested solvents, shaken at 1200 rpm for 15 min and centrifuged for 10 min at 7245 g and at 5 °C. The extract obtained was filtered and loaded directly into the SPE cartridge; SPE conditions: For conditioning, 2 mL of the solvent used in the load. For loading: 5 mL of filtered extract. For elution, 2 mL of MeOH followed by 2 mL of MeOH containing 1 % ammonia solution.

Table 2

Recovery values (%) obtained applying the SLE and SPE, loading the homemade cracker extract spiked at 10 µg/kg for tropane alkaloids and 1500 µg/kg for opium alkaloids and diluted[a].

Sample	Recovery percentages (% ± SD), n = 3[a]							
	Atropine	Scopolamine	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
Raw teff dough with ground poppy seeds	114 ± 2	85 ± 8	85 ± 3	104 ± 9	102 ± 12	82 ± 1	82 ± 5	101 ± 13
Baked teff cracker with ground poppy seeds	97 ± 4	81 ± 7	80 ± 4	88 ± 6	98 ± 6	86 ± 7	81 ± 5	85 ± 1

[a] SLE conditions: 0.5 g of sample mixed with 8 mL of acidified water (pH 1.0, HCl) and shaken at 1200 rpm for 15 min, then the sample was centrifuged for 10 min at 7245 g and at 5 °C. The supernatant was collected, and the precipitate was washed (1 mL with the previous solvent). The mixture was again centrifuged. The supernatant obtained was combined with the previous one. 2.5 mL of the filtered extract was diluted with another 2.5 mL of aqueous solution of HCl (pH 1). The extract was directly loaded into SPE. SPE conditions - For conditioning, 2 mL of the solvent used in the load; for loading, 5 mL of filtered extract; for elution, 2 mL of MeOH followed by 2 mL of MeOH containing 1 % ammonia solution.

Table 3
Linearity, limits of the method and matrix effect for tropane alkaloids (TAs) and opium alkaloids (OAs) in the samples studied.

Analyte	Matrix	Linear Range[a]	Matrix Matched Calibration (R^2)	MDL[b]	MQL[c]	ME[d] (%)
Atropine	Raw teff dough	0.2–2.8	$1.0 \times 10^4 x + 1.5 \times 10^4$ (0.993)	0.39	0.86	−60
	Baked teff crackers	0.1–2.8	$1.3 \times 10^4 x + 7.8 \times 10^3$ (1.000)	0.20	0.71	−41
	Raw corn starch dough	0.2–2.8	$1.3 \times 10^4 x + 7.0 \times 10^3$ (0.993)	0.32	1.1	−49
	Baked corn starch crackers	0.1–2.8	$7.3 \times 10^3 x + 1.4 \times 10^3$ (0.998)	0.16	0.54	−71
Scopolamine	Raw teff dough	0.1–2.8	$1.4 \times 10^3 x + 1.3 \times 10^2$ (1.000)	0.18	0.60	−64
	Baked teff crackers	0.1–2.8	$1.0 \times 10^3 x − 3.4 \times 10^1$ (0.991)	0.23	0.74	−74
	Raw corn starch dough	0.1–2.8	$1.2 \times 10^3 x + 3.7 \times 10^1$ (0.998)	0.22	0.72	−68
	Baked corn starch crackers	0.1–2.8	$8.2 \times 10^2 x + 8.9 \times 10^1$ (0.998)	0.22	0.76	−79
Morphine	Raw teff dough	0.1–0.4	$1.1 \times 10^6 x + 2.2 \times 10^4$ (0.998)	0.12	0.41	−69
	Baked teff crackers	0.1–0.4	$7.9 \times 10^5 x − 1.1 \times 10^4$ (0.993)	0.06	0.21	−78
	Raw corn starch dough	0.1–0.4	$1.1 \times 10^6 x - 1.5 \times 10^4$ (0.992)	0.14	0.46	−69
	Baked corn starch crackers	0.1–0.4	$7.1 \times 10^5 x - 8.6 \times 10^3$ (0.999)	0.11	0.38	−80
Codeine	Raw teff dough	0.02–0.4	$2.4 \times 10^6 x + 1.0 \times 10^4$ (0.995)	0.01	0.04	−6
	Baked teff crackers	0.02–0.4	$1.8 \times 10^6 x - 7.8 \times 10^3$ (0.998)	0.03	0.10	−27
	Raw corn starch dough	0.02–0.4	$2.2 \times 10^6 x + 1.9 \times 10^4$ (0.993)	0.04	0.14	−14
	Baked corn starch crackers	0.02–0.4	$1.5 \times 10^6 x + 9.5 \times 10^3$ (0.998)	0.03	0.11	−42
Thebaine	Raw teff dough	0.02–0.4	$1.4 \times 10^7 x + 4.0 \times 10^5$ (0.999)	0.04	0.14	−41
	Baked teff crackers	0.02–0.4	$1.6 \times 10^7 x + 4.1 \times 10^5$ (1.000)	0.04	0.13	−33
	Raw corn starch dough	0.02–0.4	$1.4 \times 10^7 x − 5.5 \times 10^4$ (0.999)	0.04	0.12	−41
	Baked corn starch crackers	0.02–0.4	$1.0 \times 10^7 x + 2.8 \times 10^5$ (0.995)	0.02	0.08	−56
Papaverine	Raw teff dough	0.02–0.4	$2.6 \times 10^7 x + 6.2 \times 10^5$ (0.996)	0.04	0.12	15
	Baked teff crackers	0.02–0.4	$2.2 \times 10^7 x + 4.9 \times 10^5$ (0.999)	0.03	0.10	−2
	Raw corn starch dough	0.02–0.4	$2.1 \times 10^7 x + 2.5 \times 10^5$ (0.997)	0.03	0.09	−9
	Baked corn starch crackers	0.02–0.4	$2.0 \times 10^7 x + 4.3 \times 10^5$ (0.999)	0.03	0.09	−11
Noscapine	Raw teff dough	0.02–0.4	$3.5 \times 10^7 x + 7.4 \times 10^5$ (0.996)	0.03	0.08	0
	Baked teff crackers	0.02–0.4	$3.5 \times 10^7 x + 3.0 \times 10^5$ (0.999)	0.03	0.08	3
	Raw corn starch dough	0.02–0.4	$3.1 \times 10^7 x + 4.5 \times 10^5$ (0.995)	0.02	0.08	−10
	Baked corn starch crackers	0.02–0.4	$3.0 \times 10^7 x + 6.9 \times 10^5$ (0.994)	0.02	0.06	−14
Oripavine	Raw teff dough	0.02–0.4	$2.2 \times 10^6 x + 2.2 \times 10^4$ (0.994)	0.02	0.06	−11
	Baked teff crackers	0.02–0.4	$1.6 \times 10^6 x + 2.0 \times 10^4$ (0.998)	0.03	0.10	−32
	Raw corn starch dough	0.02–0.4	$2.0 \times 10^6 x − 3.8 \times 10^4$ (1.000)	0.04	0.12	−16
	Baked corn starch crackers	0.02–0.4	$1.5 \times 10^6 x + 2.8 \times 10^4$ (0.998)	0.03	0.11	−38

[a] Linear range for TAs (0.1–2.8 ng/mL) and OAs (0.02–0.4 mg/L) corresponds in weight/weight to 0.7–20 µg/kg and 0.15–3 mg/kg, respectively according to the validated analytical methodology.

[b] MDL: Method detection limit expressed in µg/kg for TAs and mg/kg for OAs.

[c] MQL: Method quantification limit expressed in µg/kg for TAs and mg/kg for OAs.

[d] ME: Matrix effect. Calculated by comparing the slopes of the matrix-matched calibration curve with the slopes of the solvent calibration curve (See Table S5) with the formula ((matrix-matched slope/solvent-based slope - 1) × 100).

The successful incorporation of the functional groups in functionalized SBA-15 mesostructured silicas was confirmed by FT-IR (Fig. S2). The FT-IR spectrum of SBA-15-SO₃H-CN (Fig. S2b) exhibited two peaks at 2950 cm^{-1} (asymmetrical stretching, CH_2) and 2850 cm^{-1} (symmetrical stretching, CH_3), confirming the existence of organic groups in the pores of the silica. The presence of the cyano groups is evidenced by the band at 2248 cm^{-1}, attributed to the C ≡ N stretching vibration. Additionally, bands at 650 cm^{-1} and 1409 cm^{-1}, related to the C-S stretching mode, confirm the presence of sulfonic groups. Furthermore, a band observed at 1730 cm^{-1} can be attributed to the S-H vibration of possibly unoxidized thiol groups. At the same time, common bands appear in both spectra (Figs. S2a and b) at 450 cm^{-1} corresponding to the tetrahedral vibration of SiO_4, and 800 cm^{-1} to the symmetrical vibration of SiO_4 (Si-O-Si). On the other hand, a large band between 1000 and 1260 cm^{-1} assigned to the asymmetric vibrations (Si-O-Si) and at 3400 cm^{-1} corresponding to the free silanol groups (Wahab et al., 2004; Valle-Vigón et al., 2012). The functional groups attached to the mesostructured silica (L_o) were estimated from the sulphur and nitrogen content calculated by elemental analysis (Table S2). As for % S and % N, the presence of 1.0 mmol/g and 0.8 mmol/g of S and N, respectively, was estimated in the bifunctionalized material (SBA-15-SO₃H-CN).

3.2. Optimization of the sample preparation protocol

In this paper, a SLE protocol combined with SPE was optimized by applying SBA-15-SO₃H-CN material to purify and preconcentrate the analytes. The first test was carried out with standard working solutions prepared in different solvents (as indicated in section 2.4). These solvents were selected based on the polarity of the analytes since both TAs and OAs are highly soluble in acidic aqueous solutions (Casado-Hidalgo, Morante-Zarcero, et al., 2021; Dräger, 2022). Papaverine and noscapine are less polar than the rest, so a mixture of MeOH/water with 0.1% HCOOH (50/50, v/v) was also evaluated. The results obtained are shown in Table S3. It seems that both at pH 1 and 3 the analytes are retained correctly since their recoveries are around 100%. On the other hand, with MeOH/water with 0.1% HCOOH (50/50, v/v), atropine was not completely retained, due to the use of MeOH in the load solvent, as its recovery percentage decreases to 60% (Table S3). The bifunctionalization of the bare SBA-15 with sulphonic acid and cyano groups allows the simultaneous and efficient extraction of both families of analytes such as OAs and TAs, by including different interaction mechanisms. For example, acidic sulfonic groups (SO₃H) allow cation exchange interactions favoured by the acidic pH of the solvent used with OAs and TAs, while the inclusion of cyano groups allows for interactions of the π electrons of the aromatic rings of the target analytes with the π electrons of the covalent triple bond of the cyano group. Other types of interactions can also occur, such as hydrophobic interactions between non-polar alkyl chains and non-polar parts of analytes or, H-bonding of the free silanols of the silica or the analytes and the free N-electron pairs of the cyano group. In addition, the double functionalization avoids analyte competition for active sites and allows for numerous interactions, and consequently the use of minimal amounts of sorbent with excellent extraction performance. Fig. 5 shows the types of interactions described.

Given the good results obtained with standard working solutions, similar studies were carried out on commercial crackers. The results obtained are shown in Table 1. In general, worse results were found for most of the analytes due to the interferences generated by other sample

Table 4
Accuracy and precision of the proposed method for the analysis of tropane (TAs) and opium (OAs) alkaloids in a representative homemade cracker prepared with teff flour and ground poppy seeds.

Analyte	Spiked level[a] (ng/mL for TAs or mg/L for OAs)	Raw teff dough with ground poppy seeds			Baked teff cracker with ground poppy seeds		
		Accuracy (recovery % ± SD)[b]	Intra-day precision (% RSD)[c]	Inter-day precision (% RSD)[d]	Accuracy (recovery % ± SD)[b]	Intra-day precision (% RSD)[c]	Inter-day precision (% RSD)[d]
Atropine	0.1	89 ± 5	12	10	104 ± 6	7	11
	1.4	91 ± 7	16	16	93 ± 4	14	8
	2.8	91 ± 10	14	11	91 ± 10	16	6
Scopolamine	0.1	107 ± 9	8	5	101 ± 10	15	15
	1.4	88 ± 8	5	12	86 ± 6	6	6
	2.8	88 ± 9	13	15	91 ± 9	14	17
Morphine	0.02	83 ± 6	8	17	92 ± 8	8	8
	0.2	86 ± 3	5	13	83 ± 3	4	10
	0.4	87 ± 3	4	11	89 ± 7	15	5
Codeine	0.02	103 ± 5	9	15	99 ± 7	11	7
	0.2	90 ± 11	13	17	95 ± 8	12	6
	0.4	95 ± 6	11	8	106 ± 6	14	2
Thebaine	0.02	92 ± 5	6	7	101 ± 6	8	9
	0.2	94 ± 11	14	16	90 ± 8	10	4
	0.4	93 ± 6	16	8	97 ± 8	14	2
Papaverine	0.02	84 ± 6	3	5	79 ± 7	4	10
	0.2	86 ± 11	16	7	82 ± 5	8	9
	0.4	89 ± 6	10	9	87 ± 8	10	11
Noscapine	0.02	91 ± 3	3	4	90 ± 4	7	4
	0.2	90 ± 8	16	6	91 ± 6	7	10
	0.4	96 ± 8	11	14	100 ± 6	11	5
Oripavine	0.02	98 ± 6	10	8	95 ± 3	9	7
	0.2	90 ± 9	17	10	97 ± 8	10	6
	0.4	93 ± 6	13	7	105 ± 5	16	9

[a] Spiked level expressed in kg of sample is 0.7 µg/kg (low level), 10 µg/kg (medium level) and 20 µg/kg (high level) ng/kg for TAs and 0.15 mg/kg (low level), 1.5 mg/kg (medium level) and 3 mg/kg (high level) mg/kg for OAs.
[b] Accuracy (n = 6).
[c] Intra-day precision (n = 6, in one day).
[d] Inter-day precision (n = 9, in three different days).

components, which prevented good retention of the analytes on the sorbent. To improve the recoveries, the sample amount was reduced from 1 to 0.5 g, and only acidified water (pH 1.0, HCl) was tested as extraction solvent as it showed better recoveries than MeOH/water with 0.1% HCOOH (50/50, *v/v*) (Table 1) and a cleaner extract than the acidified water solvent (pH 3, HCl). In this case, using acidified water (pH 1.0, HCl) in the SPE loading step, the recoveries improved significantly for all analytes with values between 67 and 81% for TAs and 72–85% for OAs (Table 1). However, some recoveries were still slightly low for some analytes, such as morphine (67%) and scopolamine (72%) (Table 1). Therefore, it was tested to increase the volume in the SLE to 8 mL using acidified water (pH 1.0, HCl), including 1 mL to wash the precipitate, to avoid losses, and injected directly. In turn, the extract obtained using 5 mL was injected for comparative purposes. This study confirmed the increase of the recoveries by increasing the solvent volume (8 mL + 1 mL to wash the precipitate), with values between 93 and 104 % for TAs and 85–112 % for OAs, improving recoveries of morphine (92 ± 5 %) and scopolamine (104 ± 1 %). While slightly lower recoveries would be obtained with 5 mL, between 81 and 88 % for TAs and 74–106 % for OAs. This study demonstrated that a greater volume in SLE allows a greater extraction of the target analytes. Subsequently, SPE was again performed after the optimized SLE protocol, but similar results to those shown in Table 1 were obtained (65–90 % for all analytes). Based on these results and to reduce the amount of matrix components that are introduced in the cartridge, the extract was diluted before passing it through the SPE. In this case, recoveries above 80 % were confirmed for all analytes.

Finally, these optimized conditions were applied to homemade cracker samples to assess the effectiveness of the sample preparation protocol. The results, shown in Table 2, demonstrate recoveries between 85 and 114 % for TAs and 82–101 % for OAs in raw teff dough with ground poppy seeds and between 81 and 97% for TAs and 80–98 % for OAs in baked teff crackers with ground poppy seeds. These findings confirm the method's reliability in both raw and baked matrices.

3.3. Instrumental and method validation

The instrumental validation showed two linear ranges at low and high concentrations with good linearity coefficients and RSD% between three days. Low LOD and LOQ and good precision for repeatability and reproducibility. For more details, see Supplementary Information S3.

Regarding method validation for both the raw dough and baked cracker prepared with teff flour or corn starch and ground poppy seeds (Tables 3 and 4), good linear regression was observed for TAs and OAs, with $R^2 \geq 0.991$ (Table 3), as shown by the criteria established in the validation guides (AOAC, 2017; SANTE, 2021). Matrix-matched calibration curves were prepared on different days, and % RSD was calculated between slopes. Low dispersion was found for the 4 samples evaluated. Specifically, between 4 and 14% for raw teff dough, between 0.1 and 10% for baked teff crackers, between 3 and 14% for raw corn starch dough and between 0.2 and 6% for baked corn starch crackers in both TAs and OAs. Observing the ME calculated in the 4 samples, all analytes showed a large negative ME except papaverine and noscapine, and only in raw dough samples, codeine and oripavine. Values exceeding ±20% are considered a slight negative ME according to the SANTE guide (SANTE, 2021). Analytes and samples that did not present ME can be quantified using solvent calibration curves without the need to use the matrix-matched calibration curves. On the other hand, low limits were found. MQL was between 0.6 and 1.1 µg/kg for TAs and between 0.06 and 0.46 mg/kg for OAs. Hence, the proposed method exhibits values for TAs below the thresholds outlined in Commission Recommendation 2015/976 (European Union, 2015) and following Commission Regulation 2023/2783 (Commission Implementing Regulation, 2023), which suggests that the MQL should preferably be below 2 µg/kg for cereal-based finished food products such as crackers. Regarding OAs, MQLs for OAs in bakery products must be equal to or
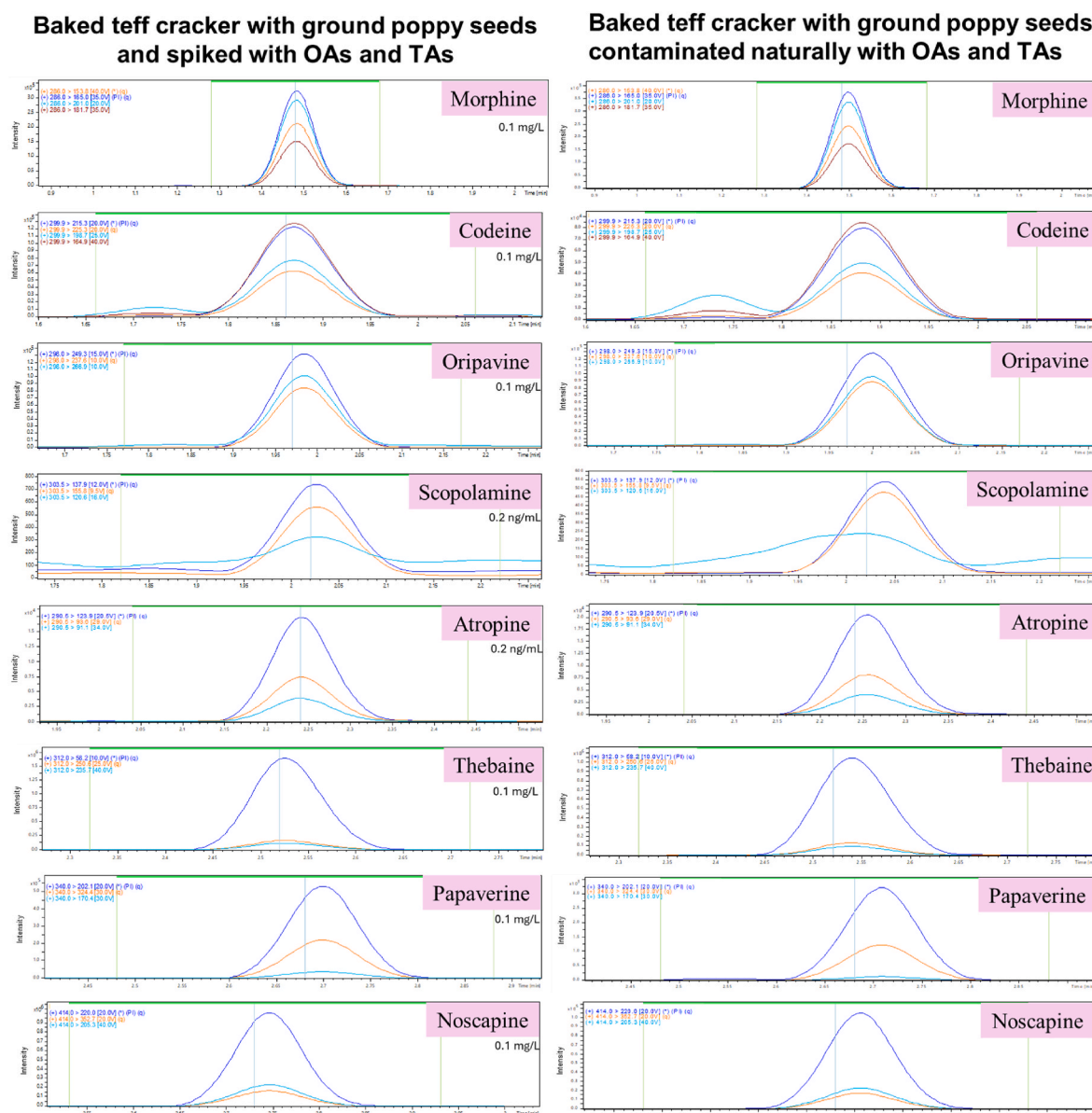
Fig. 6. Comparison between the extracted ion chromatograms of a baked teff cracker sample with ground poppy seeds spiked with OAs and TAs and a naturally contaminated sample to evaluate the selectivity.

less than 500 µg/kg (Commission Implementing Regulation, 2023). This underscores the method's excellent sensitivity for both TAs and OAs.

To evaluate selectivity, contaminated, and spiked samples at similar concentrations were compared to confirm the absence of interfering peaks at the retention times of TAs and OAs, with a standard deviation of less than or equal to ±0.1 min (Fig. 6). Additionally, ion transition ratios were checked using unit mass resolution MS/MS in contaminated samples and compared to spiked samples (Fig. S3). The deviation was found to be less than 30% (relative abundance), indicating that the method's selectivity is considered adequate (SANTE, 2021).

Finally, accuracy and precision were evaluated at three levels, 0.7, 10 and 20 µg/kg for TAs and 0.15, 1.5 and 3 mg/kg for OAs. Table 4 shows the recovery percentages for accuracy at the three validated levels and for raw dough and baked crackers made with teff flour and ground poppy seeds. The recoveries ranged between 88 and 104% for the raw and baked sample in the case of TAs, and between 79 and 106% for the OAs in both samples, which falls within the range accepted by the validation guidelines (70%–120%). On the other hand, the intermediate level was evaluated in the raw dough and baked corn starch crackers

with ground poppy seeds showing recoveries of 87 ± 10% and 85 ± 11% for atropine, 115 ± 6% and 96 ± 9% for scopolamine, 93 ± 15% and 99 ± 13% for morphine, 83 ± 11% and 90 ± 10% for codeine, 89 ± 13% and 85 ± 10% for thebaine, 100 ± 12% and 84 ± 1% for papaverine, 102 ± 13% and 87 ± 16% for noscapine, and 91 ± 13% and 103 ± 6% for oripavine, respectively. Therefore, TAs and OAs exhibited good percentages at the three levels studied. Consequently, precision showed good RSD % at the three levels evaluated (Table 4), with values ≤ 17% for all analytes in intra- and inter-day precision and both matrices studied, complying with the values recommended by the validation guides (RSD ≤20%).

3.4. Study of baking effect on the degradation of TAs and OAs in homemade crackers

After validating the methodology, it was used to assess the impact of baking on the concentrations of TAs and OAs. Six replicates of teff and corn starch crackers with ground poppy seeds (raw dough and baked) were prepared and analyzed. Fig. S4 shows chromatograms of one

Table 5
Mean concentration (mg/kg) ± standard deviation (SD) of atropine and scopolamine (tropane alkaloids, TAs) and morphine, codeine, thebaine, papaverine, noscapine and oripavine (opium alkaloids, OAs) in raw doughs and baked crackers made with teff flour or corn starch, both with ground poppy seeds.

Analyte	Teff flour crackers with ground poppy seeds (mg/kg ± SD)		Corn starch crackers with ground poppy seeds (mg/kg ± SD)	
	Baked crackers[a]	Raw doughs	Baked crackers[a]	Raw doughs
Atropine	0.033 ± 0.007[a]	0.028 ± 0.004[a]	0.007 ± 0.001[a]	0.010 ± 0.003[a]
Scopolamine	0.018 ± 0.003[a]	0.016 ± 0.003[a]	0 ± 0[a]	0 ± 0[a]
Morphine	9 ± 1[a]	11 ± 2[a]	10 ± 1[a]	12 ± 1[a]
Codeine	1.6 ± 0.3[a]	1.1 ± 0.2[a]	1 ± 0[a]	1.2 ± 0.4[a]
Thebaine	3.3 ± 0.7[a]	2.1 ± 0.5[a]	4 ± 1[a]	2 ± 1[a]
Papaverine	< MQL	< MQL	< MQL	< MQL
Noscapine	< MQL	< MQL	< MQL	< MQL
Oripavine	3 ± 1[a]	2.2 ± 0.4[a]	3 ± 1[a]	1.7 ± 0.3[a]

Same letters ([a]) in the same row mean that there is not a statistically significant difference ($p \leq 0.05$) with Student t between the results obtained on raw and baked samples of each type of cracker.

[a] Results obtained for baked cracker were normalized by expressing them considering water loss by evaporation during baking according to the following formula = [actual concentration measured in baked crackers (mg/kg) × (100 − WL)]/100. Weight loss (WL) for teff crackers 35 ± 1 % and for corn starch crackers 36 ± 3 %.

contaminated sample of raw dough and baked cracker made with teff flour and ground poppy seeds. To compare the raw and baked results, these six replicates were weighed before and after baking to evaluate weight loss, obtaining a weight loss of 35 ± 1% in the case of the teff cracker samples and 36 ± 3% in the case of the corn starch cracker samples. All eight target analytes were found in the raw dough samples, showing contamination of the raw materials (teff flour or corn starch and poppy seeds) and allowing a real evaluation of the effect of baking. Table 5 compiles the mean results obtained with their SD, and the normalized values considering the weight loss after baking (see section 2.2). These results showed that there is not a statistically significant difference between the concentration obtained in raw doughs and those obtained after baking. Therefore, the analytes did not show any decrease or degradation effect, so it was concluded that at 180 °C for 15 min they were stable and did not degrade. It should be noted that it would be interesting to evaluate the metabolic pathways, formation, and transformation into other compounds within the TA and OA families to understand their toxicity. Many of these analytes can transform into others within the family during baking, as observed in previous works evaluating cooking and baking (Avula et al., 2023; Marín-Sáez et al., 2019). In this case, it is not possible to evaluate the formation and transformation into other compounds, since under the thermal conditions of time and temperature studied in this work, no degradation was observed in any of the analytes studied. Therefore, it is unlikely that the studied analytes are transformed into other compounds. However, further investigation into the degradation of alkaloids is crucial during different kinds of pre-treatments and processing by performing high-resolution mass spectrometry-based analysis (HRMS). It should be noted that despite all the points mentioned to address the study of possible degradation products, it is necessary to study not only other conditions and types of baking (traditional oven, air oven, air fryer, etc.) but also other types of crackers or cookies, made with different raw materials such as buckwheat flour, which are also susceptible to contamination with TAs. This could pose a limitation in the validity of the developed method that should be confirmed, given that the method has been validated for one type of crackers made with two types of flour (cornstarch and teff) and ground poppy seeds, and changes in the matrix could result in variations in sensitivity and mainly in the ME, which would produce errors in the final quantification and therefore in the conclusions drawn.

Regarding the results found in the bibliography, contradictory results have been obtained, with some authors confirming its high stability against high temperatures for prolonged periods (Friedman & Levin, 1989; Shetge et al., 2020) and other authors confirming its potential degradation (European Commission, 2014; Marín-Sáez et al., 2019; Sproll, Perz, & Buschmann, 2007; Vera-Baquero et al., 2022). Some reasons for these differences could also be related to how the comparison is carried out before and after baking (Kuntz et al., 2021). Some studies have been performed after spiking the samples with standards so that the initial concentration is known (Marín-Sáez et al., 2019; Vera-Baquero et al., 2022). However, to be more realistic, it is more convenient to carry out these studies with naturally contaminated samples (López et al., 2018). In addition, it has been shown in some studies that the stability of natural toxins, in particular TAs, is higher in their natural form than when the sample is spiked (Torrents-Masoliver et al., 2024). Therefore, the most appropriate way is to determine the concentration in the same raw and subsequently baked dough. On the other hand, another aspect to note is that European Commission recommendations to mitigate opium alkaloids have included grinding and baking to reduce around 80–95% of these toxins (European Commission, 2014). However, it has been seen that neither grinding by itself (Casado-Hidalgo et al., 2024) nor its combination with high temperatures seems to show considerable degradation of OAs during processing.

4. Conclusions

A new method using a bifunctionalized mesostructured silica material (SBA-15-SO$_3$H-CN) was developed and validated to study the degradation of OAs and TAs during baking in homemade crackers. Samples were made using teff flour, corn starch, and ground poppy seeds, which were naturally contaminated with TAs and OAs. These samples were analyzed before and after baking to evaluate the degradation process in a real way. The results showed that the target alkaloids remained stable after baking at 180 °C for 15 min. This indicates that both natural toxins can persist in food even after intense thermal processing, emphasizing the importance of accurately monitoring naturally contaminated samples before and after baking to assess potential degradation.

CRediT authorship contribution statement

Lorena González-Gómez: Writing – original draft, Validation, Investigation, Formal analysis. **Gema Casado-Hidalgo:** Writing – original draft, Investigation, Formal analysis. **Judith Gañán:** Software, Investigation, Formal analysis. **Damián Pérez-Quintanilla:** Software, Investigation, Formal analysis. **Sonia Morante-Zarcero:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Isabel Sierra:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.117080.

Data availability

Data will be made available on request.

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