Journal of Food Composition and Analysis 121 (2023) 105412

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Influence of fermentation and storage on the content of opium alkaloids in poppy seed yoghurt

Gema Casado-Hidalgo, Sonia Morante-Zarcero, Damián Pérez-Quintanilla, Isabel Sierra *

Departamento de Tecnología Química y Ambiental, E.S.C.E.T, Universidad Rey Juan Carlos, C/ Tulipán s/n, 28933 Móstoles, Madrid, Spain

ARTICLE INFO

Keywords:
Opium alkaloids
Poppy seeds
Yoghurt
Fermentation
Storage
Magnetic solid-phase extraction
Mesostructured silica magnetic composite
β-cyclodextrin
Liquid chromatography-tandem mass spectrometry

ABSTRACT

There is a current trend to make yoghurts with seeds such as poppy seeds to enhance their nutritional benefits. These seeds may be contaminated with opium alkaloids (OAs) present in the own latex of the plant (*Papaver somniferum* L.). Food processing such as heat treatment, grinding or washing may reduce them, but lactic fermentation has not been studied. In this work, an analytical methodology was optimised based on a solid-liquid extraction (SLE) with water, a purification by magnetic solid-phase extraction (MSPE) with a mesostructured silica magnetic composite with β-cyclodextrin (Fe_3O_4 @SiO_2 @mSiO_2 @β-CD) and subsequent analysis by liquid chromatography coupled to a tandem mass detector (HPLC-MS/MS). The methodology was successfully validated in terms of linearity, limits of detection and quantification, matrix effect, precision, recovery tests, and selectivity. Therefore, it was applied to determine OAs in commercial yoghurts, detecting morphine, papaverine and noscapine in all of them but below the limit of quantification. Besides, the effect of fermentation was studied (6 and 18 h) and storage (1 week/ 4ºC), showing a considerable degradation effect (33–80%) in the first hours.

1. Introduction

The seeds of the *Papaver somniferum* L. plant, commonly known as opium poppy, are increasingly being added to different foods, such as bakery products, yoghurts, salads and for making tea and oil (AESAN, Spanish Food Safety and Nutrition Agency, 2020; Carlin et al., 2020; Casado-Hidalgo et al., 2022a; Casado-Hidalgo, et al., 2022b; López et al., 2018; Sproll et al., 2006). This increase in popularity is due to their good nutritional properties, as poppy seeds are a great source of essential fatty acids such as linoleic acid and antioxidant compounds such as vitamin E (Ghafoor et al., 2019; Musa Özcan & Atalay, 2006). The problem with this practice is that although the seeds do not naturally contain opium alkaloids (OAs), they can be contaminated with OAs (morphine, codeine, thebaine, papaverine, noscapine and oripavine) present in the latex of the plant itself due to poor harvesting practices or insect damage (Casado-Hidalgo, et al., 2021a; EFSA, European Food Safety Authority, 2018). This could lead to cases of intoxication and even false positives in drug tests (Lachenmeier et al., 2010).

For this reason, the European Commission published on December 3, 2021, the Regulation (EU) 2021/2142, which came into application on July 1, 2022. This regulation sets maximum levels for morphine equivalents (morphine + 0.2 codeine) for bakery products (1.5 mg/kg) and poppy seeds (20 mg/kg) (Commission Regulation, 2021). It only considers morphine and codeine and does not consider the other OAs as is the case in most of the articles published on OAs (Casado-Hidalgo, et al., 2021b). However, it has been seen that seeds can also be contaminated with other OAs (Casado-Hidalgo, et al., 2021a). Therefore, in 2018 EFSA and the German Federal Institute of Risk Assessment (BfR) claimed new effective analytical methods to quantify all main OAs, because they can be even more toxic (BfR, 2006; EFSA, 2018). In addition, this legislation only includes bakery products, and therefore, health authorities are demanding the study of other food matrices to know the real exposure of consumers. One of the food products with poppy seeds that are being consumed more frequently are yoghurts, which in addition to being commercialized, homemade yoghurts are a widespread practice (AESAN, 2020; EFSA, 2018). To date, to our knowledge, a method for determining OAs in this sample type has not yet been developed and validated.

Furthermore, in previously published articles, the degradation of OAs due to high temperatures during baking has been studied (Vera-Baquero et al., 2022). In fact, 25–100% degradation has been considered to establish legislation for bakery products (Commission Regulation, 2021). Furthermore, in 2014 the European Commission published recommendations for good agricultural and seed processing practices to

* Corresponding author.
E-mail address: isabel.sierra@urjc.es (I. Sierra).

https://doi.org/10.1016/j.jfca.2023.105412
Received 11 March 2023; Received in revised form 12 May 2023; Accepted 19 May 2023
Available online 20 May 2023

reduce the morphine content (European Commission, 2014), and several articles published that washing, grinding and baking treatments can decrease the content of OAs (Carlin et al., 2020; Shetge et al., 2020; Sproll et al., 2006; Vera-Baquero et al., 2022). However, to our knowledge, the influence of fermentation on OAs has not yet been studied. It is important to consider how this process can affect the content of these toxins, as depending on the fermentation conditions and the type of microorganisms used as a starter culture, the content of alkaloids can be affected (Casado et al., 2023). In other non-opium alkaloids, lactic and alcoholic fermentation studies have been carried out, and degradation of the compounds has been observed (De Nijs et al., 2017; Marín-Sáez et al., 2019). It would therefore be interesting to study whether lactic fermentation influences OAs in the yoghurt production process.

Considering that OAs can occur in complex matrices such as yoghurt in low concentrations, sample preparation prior to instrumental analysis plays a significant role in the whole analytical process. For this reason, an adequate sample purification or clean-up treatment is necessary to avoid possible matrix effects of yoghurt thus avoiding erroneous results, and extending the useful life of the equipment (Casado et al., 2020). The most popular technique to purify OAs is solid-phase extraction (SPE) (Casado-Hidalgo, et al., 2022b; Guo et al., 2013; Meos et al., 2017; Stranska et al., 2013). However, the use of the magnetic dispersive SPE version (MSPE) is becoming increasingly popular because is a faster, simpler, and more environmentally friendly miniaturized technique (Casado-Hidalgo, et al., 2022a; Casado-Hidalgo, et al., 2021a; Jiang et al., 2019; Tang et al., 2020; Xu et al., 2019). Until now, Fe_3O_4 particles are the most used as magnetic adsorbents and are usually coated with silica or graphene that can be functionalized with different organic groups or natural polymers (Casado-Hidalgo, et al., 2022a; Casado-Hidalgo, et al., 2021b; Tang et al., 2020; Xu et al., 2019). However, β-cyclodextrin (β-CD) are growing in popularity, because they have a low price, negligible environmental impact and non-toxicity (Gentili, 2020). Structures of CD make possible the formation of inclusion complexes with aliphatic and aromatic non-polar compounds of suitable size by a variety of forces such as hydrogen bonding, hydrophobic and van der Waals interaction (Gentili, 2020; Majd et al., 2021). Thus, it is a potentially effective ligand for interacting with OAs, as demonstrated in our previous work (Casado-Hidalgo, et al., 2023). Where a mesostructured silica magnetic composite with β-cyclodextrin (Fe_3O_4 @SiO_2 @$mSiO_2$ @β-CD) showed successful results with low detection and quantification limits and recovery values between 89% and 94% for all opium alkaloids.

The aim of this work is to study the fermentation and storage effect on opium alkaloids in yoghurts with poppy seeds. To do this, it was developed and validated an efficient, simple, and more respectful of the environment method to quantify six OAs in yoghurts with poppy seeds. The method was based on a solid-liquid extraction (SLE) with water, a purification by MSPE with Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD followed by HPLC-MS/MS analysis. The proposed methodology was applied to a commercial yoghurt with poppy seeds and to homemade yoghurts with poppy seeds prepared and conserved under different conditions to provide data on the content of OAs in the final product to assess intake and to make legislation accordingly.

2. Materials and methods

2.1. Reagents and materials

Standards of morphine, codeine, thebaine and oripavine were obtained from Alcaliber S.A.U. (Madrid, Spain). Noscapine, papaverine, morphine-d3 and codeine-d3 (internal standards, IS) were received from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock standard solutions were prepared at 1 μg/mL in methanol, and working standard solutions were prepared at 1 μg/L in water/ethanol 75/25 (v/v) with 10% formic acid. They were stored in darkness at − 20 °C.

The reagents for the synthesis of the material were: ferric chloride 6-hydrate ($FeCl_3{\cdot}6H_2O$) 99% and ferrous chloride 4-hydrate ($FeCl_2{\cdot}4H_2O$) 99%, which were purchased from Labkem (Barcelona, Spain) and Acros Organics (Geel, Belgium), respectively. Tetraethylorthosilicate (TEOS) 98%, hexadecyltrimethylammonium bromide (CTAB), 3-isocyanatopropyltriethoxysilane 98% and β-cyclodextrin ≥ 97% were purchased from Sigma-Aldrich. Ethanol absolute, formic acid (98%) and ammonia 32%, (w/w), isopropanol, toluene, pyridine, and diethyl ether were of synthesis grade and purchased from Scharlab (Barcelona, Spain). N, N-dimethylformamide (DMF) were purchased from Merk (Darmstadt, Germany). Acetonitrile, methanol, and ethanol used were HPLC-MS quality and were purchased from Scharlab (Barcelona, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). The Nd-Fe-B magnet (5 × 5 × 2 cm) with force 200 kg used in the MSPE procedure was obtained from Superimanes S.L. (Sevilla, Spain).

2.2. Yoghurt samples

To carry out the optimisation of the methodology proposed in this work and, subsequently, to validate it, a natural commercial yoghurt without poppy seeds was used with 2.9 g sugar and 3.8 g fat per 100 g to make controlled contamination. Subsequently, 1% poppy seeds, previously washed and dried and spiked at the concentration set by legislation as the maximum limit (20 mg/kg) were added (Commission Regulation, 2021). To do this, 25 mg of poppy seeds were weighed and spiked and left for 2 h so that the methanol from the standard added had been completely evaporated. Afterwards, 2.475 g of natural yoghurt was added and mixed by vortexing for 10 s and stored in the fridge for 24 h to simulate a commercial yoghurt with poppy seeds.

In addition, a commercial yoghurt sample with poppy seeds was analysed for the presence of OAs to demonstrate the validity of the method developed. Furthermore, homemade yoghurts were made to see the effect of fermentation and storage on the OAs content.

2.2.1. Commercial yoghurt for applied the proposed method

A commercial yoghurt sample with poppy seeds was purchased online from a supermarket in Sevilla (Spain). To obtain a represented sample, four yoghurts of the same batch and brand name were purchased. The ingredients of this commercial yoghurt were milk, cane sugar, poppy seed and lactic ferment. As indicated on the label, the yoghurt contains a total sugar content of 28.1 g of sugar and 5.6 g of fat per 100 g of yoghurt.

2.2.2. Elaboration of homemade yoghurts for study of the fermentation and storage effect

Furthermore, homemade yoghurts with poppy seeds were made to see the effect of the fermentation and storage on the OAs content. To make the homemade yoghurts, as shown in Figs. 1, 1 L of pasteurised whole milk with 3.5% of fat was used, heated to 45 ºC and a packet of a bacterial starter culture (1 g acidophilus yogurt starter culture, Natural Probiotic Selection, Bulgaria) with 2.0×10^{10} UFC, according to the specifications of the manufacturer, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus acidophilus* were added. Once dissolved, 100 mL of the yoghurt mix was distributed into each of the yoghurt containers, and placed in a yoghurt maker at 42 °C. Before adding the yoghurt, 1 g of poppy seeds, previously washed and dried and spiked at 20 mg/kg, was added to these containers. The ratio of 1 g of seeds per 100 g of yoghurt was considered, as commercial yoghurts containing 1% poppy seeds. In addition, as the variable of fermentation time was studied, some yoghurts were in the yoghurt maker at 42 °C for 6 h, and others were for 18 h. After this, the yoghurts were cooled, the pH was measured, and they were frozen until further analysis. An additional test was carried out by storing the yoghurts with a 6-hour fermentation in the refrigerator for 1 week at 4ºC.

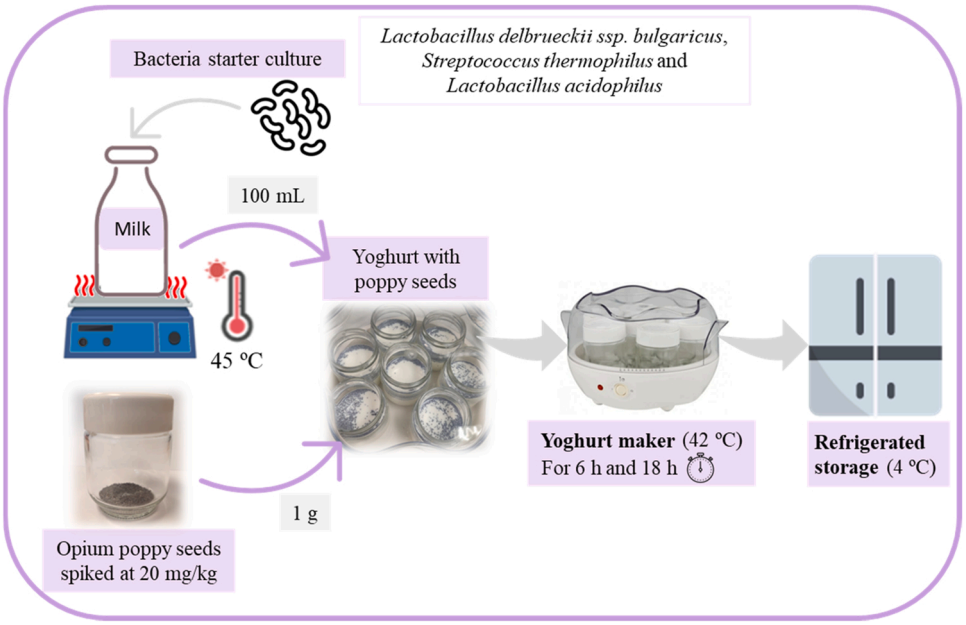

Fig. 1. The process of making homemade poppy seed yoghurts.

2.3. Preparation of the Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD material

The adsorbent material used in the present work was a mesostructured silica magnetic composite with β-cyclodextrin denoted as Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD. For the synthesis and preparation of this material, the protocol previously optimised in our previous work was followed (Casado-Hidalgo, et al., 2023). The material was characterised by performing nitrogen gas adsorption–desorption isotherms with the Brunauer–Emmett–Teller (BET) method to calculate the specific surface areas (S_{BET}) and using the Barrett–Joyner–Halenda (BJH) model to calculate the pore volumes and pore size distributions. In addition, elemental analysis (% N) was performed using a microanalyser Flash 2000 Thermo Fisher Scientific Inc. (Hampton, NH, USA) to determine the degree of functionalisation of β-CD. The results obtained agreed with those obtained in the previous work, showing a S_{BET} of 203 m^2/g, a pore volume of 0.13 cm^3/g, a pore diameter distribution of 20.5 and 39.0 Å and a functionalisation of 0.134 mmol β-CD/g material (Casado-Hidalgo, et al., 2023).

2.4. Optimization of the extraction by SLE and MSPE of OAs from yoghurt with poppy seeds

The analysis methodology developed to quantify OAs in yoghurt with poppy seeds was based on a first SLE, purification by MSPE with Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD material and analysis by HPLC-MS/MS, as shown in Fig. 2. As a starting point, the conditions developed in the previous work were used with the necessary modifications and optimisations for the sample of yoghurt with poppy seeds (Casado-Hidalgo, et al., 2023).

2.4.1. Optimization of SLE

The first step of the analytical methodology was SLE to extract OAs from yoghurt. To achieve an efficient SLE step, the recovery values obtained after the procedure were calculated and compared with those obtained on a simulated sample (a blank yoghurt sample subjected to the same process but spiked just before the HPLC-MS/MS analysis). When choosing the solvent, it was considered that the magnetic adsorbent material functionalised with β-CD has a higher adsorption capacity with analytes dissolved in water (Casado-Hidalgo, et al., 2023). Therefore, the extraction with water at different pH (2, 7 and 9) was compared.

2.4.2. Optimization of MSPE conditions with Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD

First, the MSPE conditions optimised with this adsorbent material for OAs in the previous work were used (Casado-Hidalgo, et al., 2023). However, with the yoghurt sample with poppy seeds, the recovery values obtained were low, especially for morphine, codeine and oripavine. Therefore, both adsorption and recovery values were checked to identify this step that was not effective with this sample and re-optimise it.

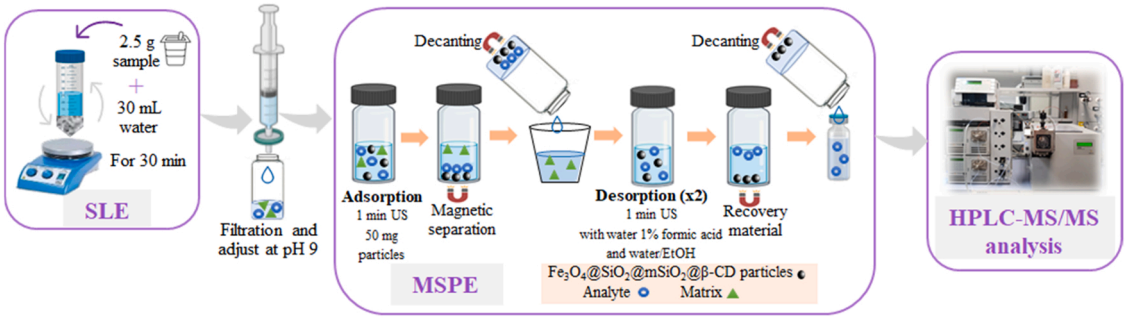

Fig. 2. The proposed methodology to quantify OAs in yoghurts with poppy seeds: extraction (SLE), purification (MSPE with Fe_3O_4 @SiO_2 @$mSiO_2$ @β-CD particles) and analysis (HPLC-MS/MS).

Therefore, to evaluate the load, 2 mL of yoghurt extract with poppy seeds (previously washed and dried to ensure that the seeds did not contain OAs) spiked at 20 mg/kg (maximum legislated limit) was used (Commission Regulation, 2021). This extract was adjusted to pH 9, and 50 mg of Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD particles (previously conditioned with 1 mL of water at pH 9 for 1 min of ultrasound (US)) were added and subjected to 1 min of US for adsorption of the OAs on the material. The supernatant was analysed and compared with a simulated sample (a blank sample subjected to the same process and spiked just before the HPLC-MS/MS analysis).

For the evaluation of the desorption conditions, the process was continued. After adsorption, the supernatant was discarded, and the desorption solvent was added and subjected to US for a given time. The desorption conditions evaluated were the number of consecutive desorption (1 and 2), the time (1, 5 and 10 min), the ratio of ethanol/water with 1% formic acid (30/70 and 70/30) and the percentage of formic acid added (1% and 10%).

2.5. Optimised analysis methodology for quantification of OAs in yoghurt with poppy seeds

First, to perform the SLE step, 2.50 g of yoghurt with poppy seeds were weighed, and 30 mL of water were added for the extraction. It was vortexed for 10 s (Rx^3 Velp Scientifica, Usmate, MB, Italy) and stirred magnetically for 30 min. Afterwards, it was centrifuged (ROTOFIX 32 A Hettich, Tuttlingen, Germany) at 6000 rpm (3992 rcf) for 10 min to recover the supernatant. Then, the extract was filtered through a 0.45 μm nylon filter and adjusted to pH 9 with ammonia to do MSPE step.

For MSPE, 50 mg of Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD particles (previously conditioned with 1 mL of water at pH 9 for 1 min of US) were added and subjected to 1 min of US for adsorption of the OAs on the material. Subsequently, the material with analytes was separated from the solution by an external magnet, and the analytes were desorbed from the magnetic particles in two steps, the first with 1 mL of water with 1% formic acid for 1 min in US and the second, with 1 mL water/ethanol at 50% with 1% formic acid for another minute. Afterwards, 2 mL of these supernatants with the analytes was recovered, and 950 μL with 50 μL of a 1 μg/mL solution of morphine-d3 and codeine-d3 was added and analysed by HPLC-MS/MS.

The analysis of OAs was performed with a Varian 1200/1200 LC (Varian Ibérica, Madrid, España) equipped with a ProStar 410 autosampler (consisting of a 100 μL loop) and two ProStar 210/215 solvent delivery modules and coupled with a 1200 L TQ triple quadrupole mass spectrometer detector with an electrospray ionization (ESI) ion source (data acquisition system was MS Workstation Varian version 6.8). A C18 KromaPhase 100 column (150 × 2.0 mm, 3.5 μm particle size, Scharlab, Barcelona, Spain) with a C18 Kromaphase guard column (10 × 4.0 mm I.D., 5 μm particle size) at 30 °C were used for the chromatographic separation. The injection volume was 10 μL (partial injection), and the flow rate was set at 0.25 mL/min. The mobile phases were water (A) and acetonitrile (B), both with 0.1% of formic acid. The gradient elution was performed as follows: 90 − 30% A (0 −6 min), 30 − 90% A (6 −9 min), and 90% A (9 −11 min) for column re-equilibration (Casado-Hidalgo, et al., 2022a; Casado-Hidalgo, et al., 2022b). Mass spectrometry acquisition was performed with electrospray ionization in the positive mode (ESI+) with the MRM mode (Multiple Reaction Mode) for all analytes (mass peak width Q1 2.5; mass peak width Q3 2.5 and scan width in MRM 0.70 s). N_2 was used as both drying and nebulizer gas. The drying gas was set at 350 °C and 22 psi, and the nebulizer gas was set at 58 psi. The capillary voltage was held at 5000 V and shielded at 600 V. Argon was used as the collision gas set at 1.90 mTorr and detector voltage at 1480 V. Compounds were monitored at cone voltage of 70 V and Table S1 shows the optimal mass spectrum parameters (ion precursor, product ions, ions used for quantification, collision energy, and retention time).

2.6. Method validation

The analysis methodology proposed in the present work was validated to quantify OAs in yoghurt with poppy seeds. The validation was performed in terms of linearity, method detection and quantification limits (MDL, MQL), matrix effect (ME), recovery tests, precision, and selectivity, following the recommendation of the method validation guide for pesticide residues in food and feed shown in the SANTE/11312/2021 document, in European Commission Regulation No. 401/2006, and in the Q2(R1) ICH guidelines (Internation Council for Harmonisation, 2005) (more details in Supporting Information S1). Moreover, the only reference materials known to us are biological samples and only for morphine and codeine. For this reason, validation was carried out with a spiked sample. To spike it to simulate as closely as possible the natural contamination, the following procedure was used: first, poppy seeds washed and dried and then, 25 mg of poppy seeds were weighed and spiked at three known concentrations (100, 200 and 400 μg/kg, representing low, intermediate, and high validation value, respectively) and left for 2 h so that the methanol from the standard added had been completely evaporated. Afterwards, 2.475 g of natural yoghurt was added and mixed by vortexing for 10 s and stored in the fridge for 24 h to simulate a commercial yoghurt with poppy seeds.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 25.0 statistical package (SPSS INC., Chicago, IL, USA) by one-factor ANOVA analysis. Significant differences were considered significant for p values ≤ 0.05.

3. Results and discussion

3.1. Optimization of SLE-MSPE procedure

3.1.1. Conditions of extraction step by SLE

The extraction solvent was optimised at this step of the analysis methodology. In the previous work, the solvent with which the Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD material had the highest adsorption capacity was determined to be water (Casado-Hidalgo, et al., 2023). Therefore, the first assay to extract the OAs from yoghurt was carried out with water to avoid an evaporation step between the SLE and the MSPE, thus shortening the analysis time considerably. To do this, the recovery values obtained with water at different pHs (2, 7 and 9) were evaluated. For this purpose, 30 mL of water (with each pH) was added to 2.5 g of yoghurt and mixed by vortexing for 10 s and stirred magnetically for 30 min. Afterwards, it was centrifuged at 6000 rpm (3992 rcf) for 10 min and filtered for subsequent analysis by HPLC-MS/MS. However, both with water at pH 2 and pH 9, the extract was very turbid and even with several centrifugation cycles, very poor decantation of the solid particles was achieved. Therefore, only the extract made with water at pH 7 could be analysed and adequate recovery values were obtained for all analytes (close to 100%).

3.1.2. Conditions of purification step by MSPE

To determine the efficiency of the load step, 2 mL of extract of yoghurt with poppy seeds (previously washed and dried) spiked at 20 mg/kg (the maximum legislated limit) was used (Commission Regulation, 2021). This extract was adjusted to pH 9 and 50 mg of Fe_3O_4 @SiO_2 @$mSiO_2$ @ β-CD particles (previously conditioned with 1 mL of water at pH 9 for 1 min of US) were added and subjected to 1 min of US for adsorption of the OAs on the material. The supernatant was analysed, and the adsorption capacity values of each analyte were calculated, obtaining good results for all analytes (close to 100%).

Subsequently, the desorption conditions were evaluated by calculating the recovery values for each of the analytes. First, desorption was performed with 2 mL of 50% water/ethanol with 1% formic acid for 1 min in US which were the optimised conditions with standard

solutions in the previous work (Casado-Hidalgo, et al., 2023). As shown in Fig. 3a, the recovery values with the initial conditions were considerably low below 60% for all analytes. Therefore, different assays were performed by modifying one of the variables of the initial conditions. Firstly, it was evaluated whether a longer desorption time in US (5 and 10 min) resulted in the extraction of a greater amount of analytes. As shown in Fig. 3a, no differences were found between 1, 5 and 10 min, which led to the conclusion that the low recovery values were not due to a lack of time. Subsequently, a test of two consecutive 1 min US desorptions was performed to check whether the second desorption was able to desorb what had not been desorbed in the first step. However, as shown in Fig. 3a, a second desorption was not able to desorb all the adsorbed analyte content from the material. Therefore, it was subsequently tested to change the ratio of ethanol and water in the mixture, instead of 50%, a test was carried out with 70/30 and another with 30/70. With a higher proportion of ethanol, adequate recovery values were obtained for the analytes with a more apolar nature (thebaine, papaverine, and noscapine). However, analytes with a more polar nature obtained low recovery values (morphine, codeine, and oripavine). On the other hand, with a lower proportion of ethanol in the mixture, the recovery values increased for the more polar analytes from approximately 30–60% (Fig. 3a). In addition, a test was performed to increase the proportion of formic acid (from 1% to 10% to the 50% ethanol/-water mixture). However, as shown in Fig. 3a, recovery values were not increased compared to those obtained with 1% formic acid.

Therefore, observing the different chemical natures of the OAs in terms of polarity, a two-step desorption was carried out, one aqueous to desorb the more polar analytes and one with a 50% ethanol/water mixture with 1% formic acid. For this, as shown in Fig. 3b, two tests were performed, one with the aqueous desorption step using water with 1% formic acid and the other without acid. Each desorption step was analysed separately to determine the influence of each on the recovery. In the study with unmodified water, hardly any of the analytes were desorbed. However, in the study using the first desorption step with acidified water, 80% was obtained for morphine and codeine, 20% for thebaine, 10% for papaverine and noscapine and 60% for oripavine. In the second step with 50% ethanol/water, approximately 20% was obtained for morphine and codeine, 70% for thebaine, 80% for papaverine, 90% for noscapine and 40% for oripavine. Therefore, adding up the recovery values for each of the desorption stages, approximately 100% was obtained for all the analytes (Fig. 3b). Therefore, once this was obtained, the same study was performed again, but analysing the two desorptions together to confirm the recovery values of the whole process. The results obtained confirmed that performing the desorption in two steps with these solvents was very effective and therefore, it was decided to perform the desorption in this way.

3.2. Method validation

The validation results of the proposed analytical methodology for the quantification of six OAs in yoghurt with poppy seeds samples are shown in Table 1. The calibration lines were obtained with R^2 between 0.999 and 1.000 for all analytes, with a linear range of 0.01–12 mg/kg for thebaine, papaverine and noscapine and of 0.06–12 mg/kg for morphine, codeine and oripavine. The deviation of the back-calculated concentrations of the calibration standards from the true concentrations in the matrix calibration lines were − 17% for oripavine, − 3% for papaverine, 1% for thebaine, 2% for noscapine, 4% for codeine and 13% for morphine. Therefore, these results demonstrated the good linearity of the method, which states good linearity when the deviation of the back-calculated concentrations is $\leq \pm$ 20% (SANTE/11312/2021). Besides, the deviation of the slopes of the calibration lines for three different days (n = 3) was calculated to ensure their reproducibility, obtaining RSDs between 3% and 9% for all analytes.

Regarding the MDL and MQL values were low for all analytes, noscapine 0.9 and 2.9 μg/kg, papaverine 1.6 and 5 μg/kg, thebaine 2.9

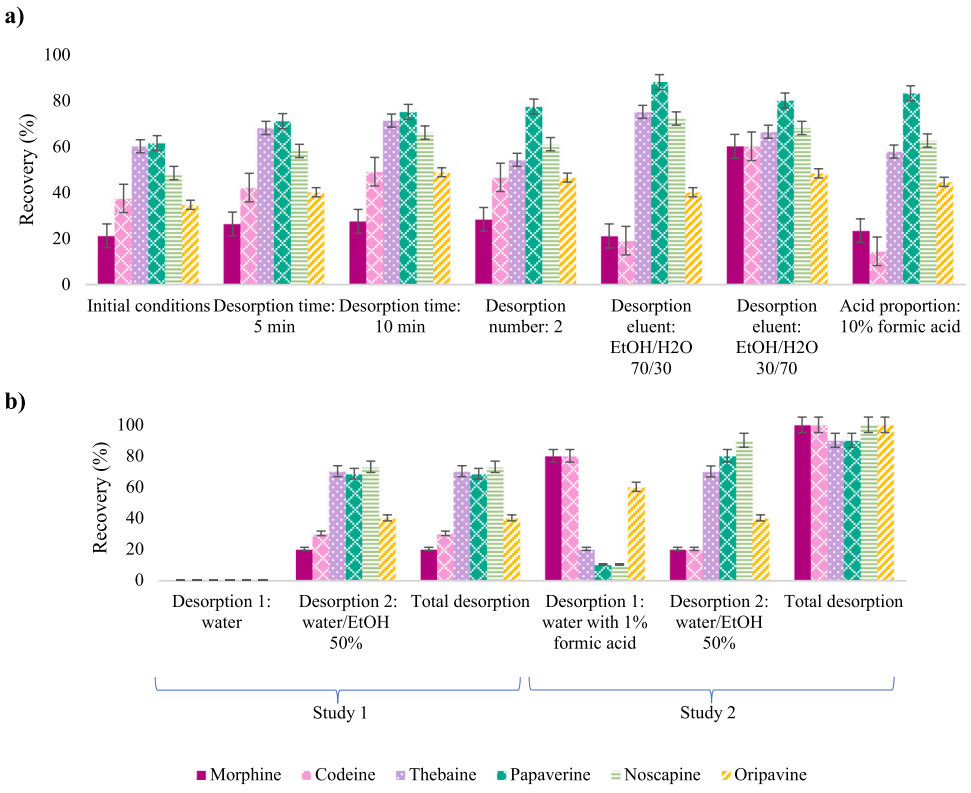

Fig. 3. Optimisation of the desorption conditions of OAs from magnetic particles in the MSPE procedure. (a): recovery values (%) obtain with the initial conditions: desorption with 2 mL of 50% EtOH/H_2O with 1% formic acid for 1 min in US. For the rest of the conditions, one parameter was varied, and the rest were maintained; (b): recovery values (%) obtained with two studies with two consecutive desorption and the sum of each one.

and 9.7 μg/kg, codeine 7 and 23 μg/kg, morphine 4.8 and 16 μg/kg and oripavine was 17 and 58 μg/kg, respectively.

On the other hand, ME was calculated by comparing the slopes of both matrix-matched and solvent-based calibration curves. As shown in Table 1, the ME was negligible because for all analytes the ME values were < +/- 20%. This means that the developed purification procedure was able to eliminate all matrix effects for the six target analytes. Therefore, the quantification could perform with solvent-based calibration curves, which would simplify the analysis.

Recovery tests and precision were evaluated at three different levels of concentration, low (100 μg/kg), medium (200 μg/kg), and high (400 mg/kg). As shown in Table 1, all recovery values were adequate between 70% and 120% between 81% and 104% for all levels and analytes (SANTE/11312/2021). In addition, satisfactory results were obtained for intra-day and inter-day precision at three concentration levels because the RSD values were lower than 20% (SANTE/11312/2021), specifically lower than 11% and 15%, respectively.

Furthermore, the variation of the retention time was ≤ 0.1 min between the chromatograms of the extracted ions obtained for each of the OAs in a standard solution with the extracts of the sample. In addition, the ion ratios of the sample extracts were within ± 30% (relative abundance) of the mean of the standards for each analyte. Therefore, in compliance with the guidelines, the method developed showed good selectivity. In addition, an important point to note is that the proposed methodology is the first method to date developed and validated to analyses OAs in poppy seed yoghurt.

3.3. Application of the proposed methodology to commercial sample of yoghurt with poppy seeds

Once the proposed methodology was validated, its application was confirmed in commercial yoghurts and homemade yoghurts as shown in Table 2. In the case of homemade yoghurts, a calibration line was performed for each condition, i.e., with a fermentation time of 6 h, with 18 h and with one week of refrigerated storage. For this purpose, as shown in Table 2, matrix calibration lines were performed, confirming that the linearity in each of the samples did not vary with respect to that already determined in the previous section (Table 1). In addition, the matrix effect that each of them could have been calculated. In the case of homemade yoghurt, the matrix effect was negligible in any of the conditions studied, as it was lower than + /- 20%. However, commercial yoghurt showed some matrix effect in the case of thebaine and papaverine (−52 and −45%, respectively). This could be since this commercial yoghurt had a higher sugar content compared to the others. On the other hand, recovery values were also calculated on three different samples and adequate validation values between 73 ± 3% and 97 ± 6% were shown for all analytes. These last results confirm that the methodology developed in the present work is efficient for the quantification of OAs in different types of yoghurts.

At the time when the study was carried out, only one yoghurt with poppy seeds could be found on the market, even though many had been available on the market months before. This can be attributed to the recent recommendation that seed suppliers must guarantee a maximum content of morphine equivalents, which may not have been considered by seed manufacturers so far. Therefore, four yoghurts of the same brand were analysed, and two replicates of each yoghurt were made. Morphine, papaverine, and noscapine were detected in all of them, although below the quantification limit of the method. In conclusion, the consumption of this commercial yoghurt does not pose a health risk to the consumer due to its low OAs content.

3.4. Study of fermentation and storage effect

After the elaboration of yoghurts were cooled, their pH was measured, and they were frozen until further analysis by HPLC-MS/MS. The pH of all the yoghurts was measured to check if there were differences between the values obtained at 6 h of fermentation and 18 h and if, in addition, after one week of refrigeration it was also altered. In all cases, the pH values measured were between 3.92 and 4.39, showing no statistically significant differences between the conditions studied. Subsequently, each of the yoghurts was analysed in duplicate, and the mean OAs concentrations obtained in each of the yoghurts (n = 8) were calculated. An additional test was carried out by storing the yoghurts with a 6-hour fermentation in the refrigerator for 1 week at 4ºC to determine whether stored in the refrigerator can reduce the OAs content since although lactic acid bacteria have a slower metabolism at refrigeration temperatures, they are still active and therefore some fermentation is still taking place.

For the analysis of these homemade yoghurts, the analysis methodology proposed in this work was used. The areas obtained were corrected with the signal of the internal standards to give even more validity to the method. For morphine, thebaine, papaverine, noscapine and oripavine, the morphine-d3 standard was used and for codeine the codeine-d3 standard. Subsequently, the areas were interpolated on the matrix calibration line elaborated with the yoghurt at a specific fermentation time (Table 2) and the concentrations were expressed in mg of each of the OAs per 100 g of yoghurt. In addition, considering the concentration at which the seeds were initially spiked, the degradation ratio of each of the yoghurts was calculated using the following formula: 100-(the concentration obtained in each yoghurt/ concentration spiked at the start) x 100. Such as shown in Fig. 4, considerable degradations for all analytes were obtained with lactic fermentation of yoghurt bacteria, showing values up to 80 ± 7% for papaverine, 74 ± 12% for noscapine, 65 ± 13% for thebaine, and somewhat lower for morphine, codeine, and oripavine, being 56 ± 12, 43 ± 10, and 33 ± 6%, respectively. There were no statistically significant differences at 6 and 18 h, so it can be affirmed that the degradation of OAs that originates with the lactic fermentation of yoghurt occurs in the first hours of fermentation, and even if the fermentation lasts more hours, there is no significantly greater degradation. On the other hand, yoghurts made with a 6 h fermentation were stored under refrigeration for one week and analysed. The degradation values obtained did not show statistically significant differences, so the continued fermentation by the lactic acid bacteria under refrigeration does not continue to degrade the amount of OAs in the poppy seed yoghurt. In the case of tropane alkaloids (TAs), the effect of alcoholic fermentation in bread has been evaluated showing between 19% and 65% degradation and an increase of the lower molecular weight TAs (Marín-Sáez et al., 2019). In the case of pyrrolizidine alkaloids (PAs), the effect of lactic fermentation has been evaluated in yoghurt and cheese, and in both cases, a reduction of the initial levels of PAs in the starting milk was observed. In yoghurt, a 27% reduction in total PAs content was determined after 6 h of fermentation at 42 °C. For cheese, a 14% reduction was observed during the cheese-making process (De Nijs et al., 2017).

Regarding the highest quantified mean concentrations (n = 8) of each of the OAs were 0.01 mg/100 g for morphine, 0.012 mg/100 g for codeine, 0.007 mg/100 g for thebaine, 0.008 mg/100 g for papaverine, 0.006 mg/100 g for noscapine and 0.013 mg/100 g for oripavine. Since there are no previous studies in the literature on the possible concentrations of OAs that can be found in poppy seed yoghurts, it is not possible to make a comparison with those obtained in the present study. Therefore, the intake estimation was carried out and compared with the acute dose established. The acute dose of morphine equivalents (morphine + 0.2 x codeine) set by EFSA in 2018 is 10 μg per kg body weight (EFSA, 2018). Thus, for a 20 kg child, the acute dose would be 200 μg, and for a 60 kg adult, it would be 600 μg of morphine equivalents. Therefore, the morphine equivalent amounts quantified in homemade yoghurt are approximately 12.4 μg. Therefore, in order to exceed the recommended acute intake, many yoghurts should be consumed per day (5 in the case of children), as the amount of seeds in the final product is very small (1%). However, since lactic fermentation

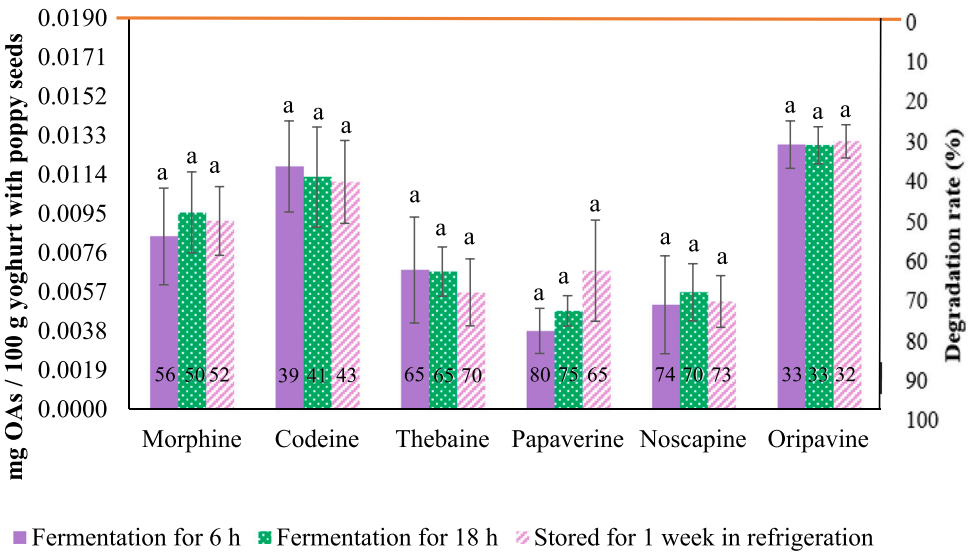

Fig. 4. Mean concentrations (n = 8 for 6 and 18 h and n = 4 for stored for 1 week) of OAs obtained in each yoghurt with their degradation rate (%) for fermentation effect. All of them spiked at 0.019 mg OAs/100 mg yoghurt with poppy seeds. Equal letters mean that there are no statistically significant differences ($p \leq 0.05$).

decreases the amount of OAs in yoghurt, it would be advisable to add poppy seeds before the yoghurt is formed rather than after, especially if the amount added by the consumer is higher.

4. Conclusions

A rapid, simple, and effective analytical methodology was developed for the quantification of 6 opium alkaloids (OAs) in yoghurt with poppy seeds. This methodology was validated in terms of linearity, limits of detection and quantification in the order of ppb, and the purification step with the magnetic material based on mesostructured silica and functionalised with β-CD allowed the matrix effect to be negligible. Recovery tests and precision were adequate at the three levels of validation evaluated, with average recovery values between 92% and 101% for all analytes and with intra-day and inter-day precision values of less than 11% and 15%, respectively. Therefore, the proposed methodology was applied to quantify OAs in a commercial yoghurt sample, detecting morphine, papaverine, and noscapine in all of them but below the limit of quantification. In addition, it was used to evaluate whether the fermentation process has any effect on the OAs content in yoghurt. Two fermentation times (6 and 18 h) were evaluated, and it was left for one week under refrigeration to see if storage would have any effect. The results showed that considerable degradations of all OAs are achieved with lactic fermentation of yoghurt bacteria, showing values up to 80 ± 7% for papaverine, 74 ± 12% for noscapine, 65 ± 13% for thebaine, and somewhat lower for morphine, codeine, and oripavine, being 56 ± 1 2, 43 ± 10, and 33 ± 6%, respectively. Furthermore, this effect is produced in the first hours of fermentation, as no statistically significant differences were shown between 6 and 18 h. Moreover, storage also did not increase the degradation, but was maintained over time. These degradations were only determined with one type of bacterial starter culture strain. An interesting future work could be to see if there are significant differences in degradation after fermentation with different strains. In short, fermentation is a process that allows the reduction of OAs in yoghurt, so it is recommended to add the seeds before fermentation and not to add them directly to the prepared yoghurt to prevent the consumption of this type of food toxins.

Funding

This research was funded by MCIU/AEI/FEDER, UE for project RTI2018-094558-B-I00.

CRediT authorship contribution statement

Gema Casado-Hidalgo: Formal analysis, Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization. **Sonia Morante-Zarcero:** Conceptualization, Visualization, Validation, Methodology, Supervision, Writing – review & editing. **Damián Pérez-Quintanilla:** Conceptualization, Writing – review & editing, Supervision. **Isabel Sierra:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. All authors revised and approved the final manuscript.

Declaration of Competing Interest

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

Data Availability

All data supporting this study are included in the article and Supplemental data.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105412.

References

AESAN (Spanish Food Safety and Nutrition Agency), 2020. Opium alkaloids in poppy seeds. [Online]. Available in: http://www.aecosan.msssi.gob.es/AECOSAN/docs/documentos/seguridad_alimentaria/gestion_riesgos/opio_semillas_adormidera.pdf [access: 20–12-2022].

BfR, German Federal Institute for Risk Assessment, 2006. BfR recommends provisional daily upper intake level and a guidance value for morphine in poppy seeds. BfR Health Assess. 12, 46.

Carlin, M.G., Dean, J.R., Ames, J.M., 2020. Opium alkaloids in harvested and thermally processed poppy seeds. Front. Chem. 8, 737. https://doi.org/10.3389/fchem.2020.00737.

Casado, N., Gañán, J., Morante-Zarcero, S., Sierra, I., 2020. New advanced materials and sorbent-based microextraction techniques as strategies in sample preparation to improve the determination of natural toxins in food samples. Molecules 25, 702. https://doi.org/10.3390/molecules25030702.

Casado, N., Casado-Hidalgo, G., González-Gómez, L., Morante-Zarcero, S., Sierra, I., 2023. Insight into the impact of food processing and culinary preparations on the

stability and content of plant alkaloids considered as natural food contaminants. Appl. Sci. 13, 1704. https://doi.org/10.3390/app13031704.
Casado-Hidalgo, G., Morante-Zarcero, S., Pérez-Quintanilla, D., Sierra, I., 2021a. Opium alkaloids in food products: current and future perspectives. Trends Food Sci. Technol. 108, 92–102. https://doi.org/10.1016/j.tifs.2020.12.013.
Casado-Hidalgo, G., Pérez-Quintanilla, D., Morante-Zarcero, S., Sierra, I., 2021b. Mesostructured silica-coated magnetic nanoparticles to extract six opium alkaloids in poppy seeds prior to ultra-high-performance liquid chromatography-tandem mass spectrometry analysis. Foods 10, 1587. https://doi.org/10.3390/foods10071587.
Casado-Hidalgo, G., Martínez-García, G., Morante-Zarcero, S., Pérez-Quintanilla, D., Sierra, I., 2022a. New validated method for the determination of six opium alkaloids in poppy seed-containing bakery products by high-performance liquid chromatography-tandem mass spectrometry after magnetic solid-phase extraction. J. Agric. Food Chem. 70, 7594–7606. https://doi.org/10.1021/acs.jafc.2c01664.
Casado-Hidalgo, G., Morante-Zarcero, S., Pérez-Quintanilla, D., Sierra, I., 2022b. Pulsed ultrasound-assisted extraction followed by purification with SBA-15 for the control of opium alkaloids in biscuits and sponge cakes. Microchem. J. 183, 108059 https://doi.org/10.1016/j.microc.2022.108059.
Casado-Hidalgo, G., Morante-Zarcero, S., Pérez-Quintanilla, D., Sierra, I., 2023. Magnetic material based on mesostructured silica functionalized with β-cyclodextrin to extract opium alkaloids in poppy seed infusions. Adv. Sample Prep. 6, 100056 https://doi.org/10.1016/j.sampre.2023.100056.
Commission Regulation, (EU) 2021/2142 of 3 December 2021 amending Regulation (EC) No 1881/2006 as regards maximum levels of opium alkaloids in certain foodstuffs. Official Journal of the European Union, 433, 8–10.
De Nijs, M., Mulder, P.P.J., Klijnstra, M.D., Driehuis, F., Hoogenboom, R.L.A.P., 2017. Fate of pyrrolizidine alkaloids during processing of milk of cows treated with ragwort. Food Addit. Contam. Part A 34, 2212–2219. https://doi.org/10.1080/19440049.2017.1364432.
EFSA, 2018. Update of the scientific opinion on opium alkaloids in poppy seeds. EFSA J. *16*. https://doi.org/10.2903/j.efsa.2018.5243.
European Commission, 2014. Commission recommendation 2014/662/EU of 10 September 2014 on good practices to prevent and to reduce the presence of opium alkaloids in poppy seeds and poppy seed products. Off. J. Eur. Union 271, 96–100. https://doi.org/10.2903/j.efsa.2011.2405.
European Commission Regulation No. 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R0401&from=EN [access: 1–10-2022].
European Union. SANTE/11312/2021, 2021. Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed; Accredia: Rome, Italy, 1–57 [Online]. Available online: https://www.eurl-pesticides.eu/userfiles/file/EurlALL/SANTE_11312_2021.pdf [Accessed: 1–10-2022].
Gentili, A., 2020. Cyclodextrin-based sorbents for solid phase extraction. J. Chromatogr. A 1609, 460654. https://doi.org/10.1016/j.chroma.2019.460654.
Ghafoor, K., Özcan, M.M., AL-Juhaimi, F., Babiker, E.E., Fadimu, G.J., 2019. Changes in quality, bioactive compounds, fatty acids, tocopherols, and phenolic composition in oven- and microwave-roasted poppy seeds and oil. LWT 99, 490–496. https://doi.org/10.1016/j.lwt.2018.10.017.
Guo, Q., Zhang, J., Zhao, S., Shao, B., 2013. Determination of five alkaloids of pericarpium papaveris in hot pot broth using ultra-performance liquid chromatography coupled to triple quadruple mass spectrometry. Food Anal. Methods 6, 698–704. https://doi.org/10.1007/s12161-012-9479-2.
Jiang, H.-L., Li, N., Cui, L., Wang, X., Zhao, R.-S., 2019. Recent application of magnetic solid phase extraction for food safety analysis. TrAC Trends Anal. Chem. 120, 115632 https://doi.org/10.1016/j.trac.2019.115632.
Lachenmeier, D.W., Sproll, C., Musshoff, F., 2010. Poppy seed foods and opiate drug testing-where are we today. Ther. Drug Monit. 32, 11–18. https://doi.org/10.1097/FTD.0b013e3181c0eee0.
López, P., Pereboom-de Fauw, D.P.K.H., Mulder, P.P.J., Spanjer, M., de Stoppelaar, J., Mol, H.G.J., de Nijs, M., 2018. Straightforward analytical method to determine opium alkaloids in poppy seeds and bakery products. Food Chem. 242, 443–450. https://doi.org/10.1016/j.foodchem.2017.08.045.
Majd, M., Yazdanpanah, M., Bayatloo, M.R., Nojavan, S., 2021. Recent advances and applications of cyclodextrins in magnetic solid phase extraction. Talanta 229, 122296. https://doi.org/10.1016/j.talanta.2021.122296.
Marín-Sáez, J., Romero-González, R., Garrido Frenich, A., 2019. Degradation of tropane alkaloids in baked bread samples contaminated with Solanaceae seeds. Food Res. Int. 122, 585–592. https://doi.org/10.1016/j.foodres.2019.01.027.
Meos, A., Saks, L., Raal, A., 2017. Content of alkaloids in ornamental *Papaver somniferum* L. cultivars growing in Estonia. Proc. Est. Acad. Sci. 66, 34. https://doi.org/10.3176/proc.2017.1.04.
Musa Özcan, M., Atalay, Ç., 2006. Determination of seed and oil properties of some poppy (*Papaver somniferum L.*) varieties. Grasas Y. Aceites 57, 169–174. https://doi.org/10.3989/gya.2006.v57.i2.33.
Shetge, S.A., Dzakovich, M.P., Cooperstone, J.L., Kleinmeier, D., Redan, B.W., 2020. Concentrations of the opium alkaloids morphine, codeine, and thebaine in poppy seeds are reduced after thermal and washing treatments but are not affected when incorporated in a model baked product. J. Agric. Food Chem. 68, 5241–5248. https://doi.org/10.1021/acs.jafc.0c01681.
Sproll, C., Perz, R.C., Lachenmeier, D.W., 2006. Optimized LC/MS/MS analysis of morphine and codeine in poppy seed and evaluation of their fate during food processing as a basis for risk analysis. J. Agric. Food Chem. 54, 5292–5298. https://doi.org/10.1021/jf0608975.
Stranska, I., Skalicky, M., Novak, J., Matyasova, E., Hejnak, V., 2013. Analysis of selected poppy (Papaver somniferum L.) cultivars: Pharmaceutically important alkaloids. Ind. Crops Prod. 41, 120–126. https://doi.org/10.1016/j.indcrop.2012.04.018.
Tang, T., Cao, S., Xi, C., Li, X., Zhang, L., Wang, G., Chen, Z., 2020. Chitosan functionalized magnetic graphene oxide nanocomposite for the sensitive and effective determination of alkaloids in hotpot. Int. J. Biol. Macromol. 146, 343–352. https://doi.org/10.1016/j.ijbiomac.2019.12.259.
Tietje, C., Brouder, A., 2005. Q2(R1) ICH guidelines (Internation Council for Harmonisation. Handb. Transnatl. Econ. Gov. Regimes 1041–1053. https://doi.org/10.1163/ej.9789004163300.i-1081.897.
Vera-Baquero, F.L., Morante-Zarcero, S., Sierra, I., 2022. Evaluation of thermal degradation of tropane and opium alkaloids in gluten-free corn breadsticks samples contaminated with stramonium seeds and baked with poppy seeds under different conditions. Foods 11, 2196. https://doi.org/10.3390/foods11152196.
Xu, F., Liu, F., Wang, C., Wei, Y., 2019. Amantadine-functionalized magnetic microspheres and stable isotope labelled internal standards for reducing matrix effect in determination of five opium alkaloids by liquid chromatography-quadrupole linear ion trap mass spectrometry. J. Chin. Chem. Soc. 66, 484–492. https://doi.org/10.1002/jccs.201800310.