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Fast and green methodology based on miniaturised liquid–liquid extraction with a hydrophobic natural deep eutectic solvent of opium alkaloids from poppy seed beverages

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A R T I C L E I N F O

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A B S T R A C T

Currently, the development of environmentally friendly analytical methodologies is a challenge for analytical chemistry. Therefore, the search for solvents to extract analytes of interest from complex matrices has progressed considerably in recent years. In the present work, 15 hydrophobic natural deep eutectic solvents (HNADES) consisting of a mixture of different donors and acceptors in different ratios have been prepared and compared to evaluate the most efficient extraction of opium alkaloids (OA) from fortified water samples. After selecting the most effective one consisting in a mixture of thymol and camphor in the ratio 1:1 ([Thy]:[Cam] 1:1), a rapid analytical methodology to determine OA in poppy seed beverages was optimised and validated for the first time. It was based on a miniaturised liquid–liquid extraction (m-LLE) with 0.5 mL of sample and 0.5 mL of HNADES by vortexing for 1 min, followed by 1 min centrifugation and subsequent analysis by high-performance liquid chromatography with diode array detector (HPLC-DAD). The validation was successful, showing extraction efficiencies between 91 and 107 % for all analytes, low limits of quantification (0.1 μg/mL for all analytes, except for morphine, 0.4 μg/mL) and precision values below 15 %. In addition, the greenness of the method was evaluated, obtaining a 0.79 on the AGREEprep metric scale. Therefore, it was demonstrated that the methodology developed to analyse OAs in poppy seed beverage was efficient, fast, simple and environmentally friendly.

1. Introduction

In recent years, Green Analytical Chemistry (GAC) has rapidly evolved to solve pressing demands concerning the reduction on the use of harmful reagents, their effects on the environment and the safety of operators [1]. These items are also major features to be considered in the context of Green Sample Preparation (GSP) [2]. Other aspects, like miniaturisation of the process, reduction of the manual handling of extracts, as well as the consumption of energy are also considered.

In chemical analysis, the extraction of analytes from the real samples and their subsequent purification are the steps that generates the most significant volumes of chemical waste. For this reason, the use of new alternative green solvents has become an important issue in GSP [2]. In this context, deep eutectic solvents (DESs) have emerged as a new environmentally friendly solvents to replace hazardous volatile organic solvents of fossil origin [3,4], with beneficial features for the environment and humans [5–7]. These mixtures can be prepared by mixing two pure components, including quaternary ammonium salt as an hydrogen bond acceptor (HBA) with metal halides (type I DESs), hydrated metal halides (type II), or organic hydrogen bond donors (HBD, type III), as well as combining metal chlorides with different HBDs (type IV) [4,8]. Later on, a new type of DESs, known as type V, were introduced. Type V DESs are prepared by combining non-ionic HBDs and HBAs. However, despite the undeniable potential of DESs, there is still controversy over their toxicity. For this reason, research switched to natural deep eutectic solvents (NADESs) [9]. NADESs are formed by primary metabolites, such as organic acids, amino acids, sugars, alcohols and amines [10], are characterised by their higher biodegradability, reduced environmental

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impact and biocompatibility [11], and belong to this category.

Type I-IV DESs had a hydrophilic behaviour, which limited their application for the extraction of analytes in aqueous samples. However, this problem was solved with the preparation of hydrophobic NADESs in 2015 (HNADESs) [4,12–14], where the dominant driving forces might be based on π-π interactions [15]. One aspect to consider is that some of these HNADESs may have slight solubility and subsequent cross-contamination of water when used as extractants for the removal of contaminants from water and wastewater [16].

Another priority in the context of GSP is to generate the minimum amount of waste and use the smallest possible amount of sample. Miniaturised liquid–liquid extraction (m-LLE), which uses minimal solvent and sample volumes, allows greener methodologies, particularly when combined with NADES as the extractant [17–20], a feature that can be evaluated using the Analytical Greening Metric for Sample Preparation (AGREEprep) [21] a tool designed to assess the sustainability of analytical methods. This tool provides a visual representation of the greening of sample preparation, represented by a pictogram with an inner circle showing the overall score and a color-coded system from red to green. The evaluation criteria are based on ten principles of GSP, such as using safe solvents and reagents, minimizing waste and maximizing sample throughput.

The seeds of the *Papaver somniferum* L. plant, commonly known as the opium poppy, are being increasingly utilized for human consumption due to their nutritional benefits [22]. One of the ways in which they can be consumed is in the form of poppy seed beverages. These drinks are made by soaking poppy seeds in water for several hours, then crushing and straining them to make a poppy seed beverage, which is commonly in Eastern Europe. The problem with this practice is that the commercially available seeds can be contaminated with high concentrations of opium alkaloids (OAs) [23–25], which can lead to serious cases of intoxication or even false positive drug tests [26–29]. Although some analytical methodologies have been reported for the determination of OAs in poppy seeds [24], to our knowledge, no analytical methodology has been developed to quantify OAs in poppy seed beverages yet. In fact, there is a demand from health authorities to develop and validate effective analytical methodologies for this type of sample [30]. New analytical methodologies should preferably include all the OAs that may be present (i.e., morphine, codeine, thebaine, papaverine, noscapine and oripavine) and not just morphine and codeine, which are the only ones included in the legislation due to the lack of data [23,31]. In this way, assess the convenience of amending the legislation in relation to these other OAs.

Therefore, the aim of the present work was to develop an environmentally friendly analytical methodology to determine OAs in poppy seed beverages using HNADES to perform the extraction for the first time. Thereby, the extraction efficiencies of 15 HNADESs for extracting OAs from spiked water samples were compared and a m-LLE procedure was optimised with the most efficient HNADES using HPLC-DAD for the determination or quantification in the extracts.

2. Experimental section

2.1. Materials and reagents

Compounds used to prepare HNADESs were thymol 98.5 % (CAS: 89-83-8) [Thy], camphor (Cam) 96 % (CAS: 76-22-2) [Cam], DL-menthol 99 % (CAS: 89-78-1) [Men], cinnamyl alcohol 98 % (CAS: 104-54-1) [Cin], phenol 100 % (CAS: 108-95-2) [Phe], fenchyl alcohol 96 % (CAS: 1632-73-1) [Fen], lactic acid solution 85 % (CAS: 50-21-5) [Lac], all purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). For HPLC mobile phase preparation, acetonitrile (AcN) and trifluoroacetic acid (TFA) from Scharlab (Barcelona, Spain) and ultrapure water (resistivity 18.2 MΩ cm) from a Milli-Q water purification system (Millipore, Billerica, MA, USA) were used.

Standards of morphine, codeine and thebaine were obtained from Alcaliber S.A.U. (Madrid, Spain). Noscapine and papaverine were purchased from Sigma-Aldrich. Individual stock solutions were prepared in methanol with concentrations of 1000 μg/mL of each of the OAs. The working standard solutions were prepared by serial dilution of the stock solution with water to 50 μg/mL. All solutions were stored protected from light at −20 °C.

Five different brands of poppy seeds (between PS-01 and PS-05) were acquired from supermarkets in Madrid (Spain) (see Table S1). The packaging of these seeds highlighted their suitability for diverse culinary uses, including adding them to cakes, breads, salads, creams, and more.

2.2. Poppy seed beverage preparation with different poppy seeds

The poppy seed beverage was made according to the traditional recipe. For this purpose, 100 g of poppy seeds were taken and soaked overnight in 100 mL of water. The seeds were then ground to a paste using a mill (A11 Basic analytical mill, IKA, Staufen, Germany). After grinding, the seeds were filtered through a cloth filter commonly used to produce vegetable drink. Finally, the filtered liquid was collected and made up to 1000 mL with water.

2.3. Preparation of different HNADESs

For the preparation of the different HNADESs, [Thy], [Men], [Phe] and [Cin] were taken as HBA and [Cam], [Men], [Cin], [Fen] and [Lac] as HBD in different molar ratios. The different combinations resulted in a total of 15 HNADESs (Table 1). Each of them were prepared following the heating method described by Dai et al. [32] with slight modifications.

Briefly, the corresponding components were weighed in the appropriate molar ratio (Table 1) and the mixture of solids was heated at 60 °C with magnetic stirring until a homogeneous, clear liquid was obtained. All tested HNADESs remained stable after preparation.

2.4. Evaluation of the extraction efficiency of the selected HNADESs for m-LLE of OAs from aqueous media

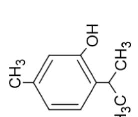
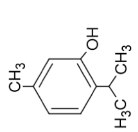
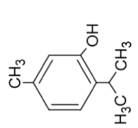
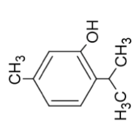
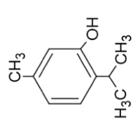
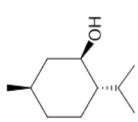
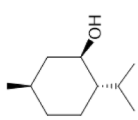
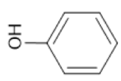
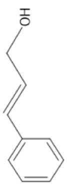
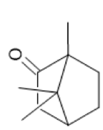
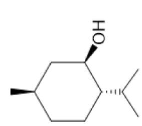
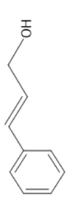
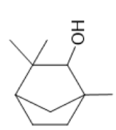
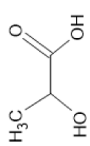
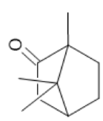
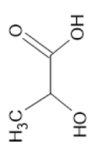
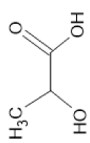
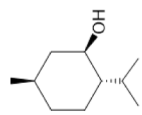
The efficiency of the 15 HNADESs tested for the extraction of six investigated OAs was evaluated by m-LLE using Milli-Q water samples spiked at the 2 μg/mL level. In brief, 0.5 mL of spiked water were mixed with 0.5 mL of the evaluated HNADES by vortexing (Rx^3 Velp Scientifica, Usmate, MB, Italy) for 1 min. Subsequently, the phases were separated by centrifugation at 8000 rpm for 1 min in a mini centrifuge model 2507/14 (Nahita Blue, Auxilab, Spain). It is important to highlight that the injection of HNADESs into a chromatographic system can generate problems associated to the viscosity of some of these solvents, which can affect the the stationary phase and promote interactions with the mobile phase, thus resulting in modifications on the resolution and retention times of the analytes, as well as possible interference with detectors such as UV–Vis [4]. To overcome these problems, a suitable HNADES that, preferably, does not affect the instrumental determination. Otherwise, the chromatographic conditions must be reoptimized. To avoid this shortcoming during method developed, the selection of the most effective HNADES among the 15 evaluated was carried out by analysing the aqueous part of the extraction system. By this way, 0.3 mL of the aqueous lower phase was taken for subsequent analyte determination by HPLC-DAD. Thereby, the extraction efficiency (EE) of each of the analytes was evaluated according to the following formula:

$$EE(\%) = 100 - \left(\frac{A_{final}}{A_{initial}}\right)x100$$

Where EE (%) is the extraction efficiency; A_{final} is the area obtained in the aqueous fraction after m-LLE; $A_{initial}$ is the area obtained at the initial concentration in the water samples spiked at 2 μg/mL.

Once the most efficient HNADES was selected, the instrumental

Table 1
Compilation of each of the 15 HNADESs prepared in this work from the pure components and the corresponding molar ratios.

HBA	 Thymol	 Thymol	 Thymol	 Thymol	 Thymol	 Menthol	 Menthol	 Phenol	 Cinnamyl alcohol
HBD	 Camphor	 Menthol	 Cinnamyl alcohol	 Fenchyl alcohol	 Lactic acid	 Camphor	 Lactic acid	 Lactic acid	 Menthol
Name	[Thy]:[Cam]	[Thy]:[Men]	[Thy]:[Cin]	[Thy]:[Fen]	[Thy]:[Lac]	[Men]:[Cam]	[Men]:[Lac]	[Phe]:[Lac]	[Cin]:[Men]
Molar ratio	1:1, 3:2, 7:3	1:1, 1:2, 1:3, 2:1	1:1	1:1	1:3.1	1:1	1:1, 1:2	2:1	1:1
Ref.	[37]	[13]	–	–	[38]	–	–	[38]	[14]

HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; Ref: Reference.

analytical conditions were re-optimised for this particular extractant. In the subsequent experiments, the HNADES phase was directly injected in the HPLC-DAD instrument (Section 2.7).

2.5. Characterisation by ATR-IR of HNADESs that showed higher OAs extraction capacity in aqueous media

Attenuated Total Reflection Fourier-Transform Infrared (ATR-FT-IR) spectra were recorded with a Spotlight 200i (Perkin Elmer, Shelton, USA) spectrometer in the region 4000–500 cm^{-1} to identify the functional groups of each of the components and to check possible band shifts to confirm the formation of HNADESs. The measurements were done with around vacuum-dried 1 mg of the corresponding HNADES or component at room temperature in the transmittance mode with 64 scans per spectrum at a resolution of 4 cm^{-1}.

2.6. Optimised m-LLE procedure of OAs from poppy seed beverage

The different experimental parameters affecting the EE were optimised using a one-at-a-time approach and spiked water as test sample. Firstly, to optimise the m-LLE procedure, different vortex extraction times (30 s and 1 min) and sample:HNADES ratios were tested, keeping the volume of sample at 0.5 mL and changing that of HNADES (0.1, 0.25 and 0.5 mL), as well as keeping the volume of HNADES at 0.5 mL and that of the sample (0.75 and 1 mL). To carry out this optimisation, a 0.3 mL aliquot of the HNADES part with the extracted analytes was taken and analysed by HPLC-DAD. To carry out the analysis, a washing step was added at the end of the chromatographic method to remove the remains of HNADES retained on the column and to ensure reproducibility between injections.

Once the m-LLE conditions were optimised, the proposed methodology was applied to extract OAs from poppy seed beverage. For this purpose, 0.5 mL of sample and 0.5 mL of [Thy]:[Cam] 1:1 were mixed by vortexing for 1 min. Subsequently, the phases were separated by centrifugation for 1 min and 0.3 mL of the HNADES (upper phase) was separated for subsequent analysis by HPLC-DAD.

2.7. Analysis of OAs by HPLC-DAD

The analysis of OAs was carried out with an Agilent 1260 Infinity II HPLC-DAD HPLC system (Agilent Technologies, Madrid, Spain) equipped with an automatic pump (G7104C 1260 Infinity II Flexible Pump), a thermostated autosampler (G7167A 1260 Multisampler), a thermostated column compartment (G7116A 1260 Multicolumn Thermostat) and a DAD detector (G7117C 1260 DAD HS). Data analysis was performed with the Agilent OpenLab CDS ChemStation Edition Software. Separation was carried out with an InfinityLab Poroshell 120 EC-C18 column (3.0 mm × 150 mm, 2.7 μm particle size) at 30 °C coupled to a pre-column, InfinityLab Poroshell EC-C18.

The mobile phase, flow rate and detection wavelengths for the analysis were selected according to a previous work of our group with slight modifications [33]. Briefly, a mixture of solvent A (Milli-Q water with 0.1 % TFA) and solvent B (AcN) was used as the mobile phase at a flow rate of 1.0 mL/min. The temperature of the column was maintained at 30 °C and the autosampler tray at 4 °C. Detection was performed at 212 nm (morphine, codeine, oripavine, thebaine and noscapine), and at 240 nm (papaverine).

Parameters such as mobile phase composition, washing and conditioning of the column between injections, and the injection volume were optimised to achieve appropriate separation of OAs from remaining HNADESs in the analysed aqueous phase, as well as reproducible injections. The optimised gradient elution was as follows: 0–1 min 2 % solvent B, 1–5 min 35 % solvent B, 5–6 min 40 % solvent B, 6–6.30 min 90 % solvent B, 6.30–12 min 90 % solvent B to ensure complete HNADES removal, 12–12.10 min 2 % solvent B and 3.90 min more to equilibrate the column. The total analysis time was 16 min. The volume of sample injected was 10 μL.

2.8. Method validation

To carry out the validation of the developed method, the criteria described in SANTE/11312/2021 for pesticides, in EC Regulation 401/2006 and in the ICH Q2(R1) guidelines [34–36] were followed, as there is no official regulation on analytical performance requirements for the quantification of OAs in food or other matrices. Method validation involved the evaluation of the linear dynamic range (LDR), method limits of detection and quantification (MDL, MQL), matrix effect (ME), EE and intra-day and inter-day precision. For this purpose, the validation was performed with a spiked blank poppy seed beverage at three known concentration levels: low (L, 0.5 μg/mL), medium (M, 2 μg/mL), and high (H, 10 μg/mL), selected according to the maximum limit of OAs established by legislation [31] in poppy seeds and estimated in poppy seed beverage with the seeds and water ratios used following the traditional recipe.

The LDR was assessed using matrix-matched calibration curves prepared over three consecutive days. These curves were generated using samples supplemented at six known concentration levels within the evaluated linear range. For this purpose, blank sample were spiked with an aliquot of a standard solution containing the target alkaloids according to the desired concentration level of the calibration curve and then, they were subjected to the optimised m-LLE procedure. Additionally, the precision of the LDR was estimated by calculating the relative standard deviation (RSD) of the slopes obtained on three different days.

The sensitivity of the method for the investigated analytes was determined through the MDL and MQL of the OAs from the analysis of the lowest concentration analysed (0.1 or 0.5 μg/mL), which were estimated as the minimum concentration yielding a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Matrix effects (ME) were determined by comparing the slopes of the calibration equations obtained from matrix-matched with those of solvent-based calibration curves, calculating:

$$ME(\%)_{OA} = \left(1 - \frac{SlopeMatrix - matched}{SlopeSolvent - based}\right)x100$$

The lower the resulting absolute value, the lower the ME and, according to the guidelines, the ME is considered negligible when it is lower than ±20 %.

The EE was calculated with 3 replicates in 3 different days (n = 9) assessed by comparing the concentrations obtained after interpolating the areas obtained from a poppy seed beverage spiked to a known concentration and subjected to the proposed m-LLE procedure in the matrix calibration line, with the concentrations to which these samples were spiked.

On the other hand, the method precision was evaluated in terms of intra-day and inter-day precision, using the same validation levels indicated above. For intra-day precision (expressed as RSD%), six samples were extracted and analysed in triplicate spiked with the OAs at the corresponding validation ($n = 6$) on the same day. The inter-day precision (also expressed as RSD%) was calculated by three samples spiked each day for three days (9 samples) and analysed in triplicate ($n = 9$). According to the validation guidelines, the RSD values for these precision parameters should be ≤20 %.

2.9. Statistical analysis

The SPSS 25.0 statistical package (SPSS INC., Chicago, Il, USA) was used for the statistical analysis of the obtained results. For this purpose, a two-tailed Student's *t*-test with a 95 % confidence interval was performed. Differences were considered significant for p-values ≤0.05 and different letters were used to indicate this.

3. Results and discussion

3.1. Evaluation of the extraction efficiency of selected HNADESs for m-LLE of OAs in aqueous media

A preliminary selection of HNADESs with potential to extract OAs was carried out on the base of previous publications dealing with the analysis of aqueous matrices. Criteria for solvent selection included their solubility in water and their structural similarity to OAs, but also their environmental friendliness, readily availability, easy to synthesize, biodegradability, selectivity and effectiveness as extraction solvents [13,14,37,38]. As none of the pre-selected HNADESs were previously used to extract OAs, different combinations between donors and acceptors at different molar ratios were evaluated (Table 1). After preparation of the 15 tested HNADESs, their behaviour in aqueous media was evaluated by m-LLE.

As shown in Fig. S1, [Men]:[Lac] 1:1 and 1:2 were instable in aqueous medium due to polymerisation of lactic acid forming polylactic acid. HNADESs [Phe]:[Lac] 2:1 and [Thy]:[Lac] 1:3.1 were either completely or partially dissolved in the aqueous medium (Fig. S1). So, they was discarded for this application.

For the remaining 11 HNADESs, as shown in section 2.4., the EE was calculated. As shown in Table 2, [Thy]:[Cam] 1:1 showed the highest EEs, with values above 96 % for all the analytes, the chemical structure of which is shown in Fig. 1. This may be because the interactions between OAs and HNADES are mainly hydrophobic (with the apolar parts of the analytes) and electrostatic of π–π type (with the aromatic rings) (Fig. 1). With this HNADES and this ratio, a balance between these two interactions is achieved for efficient extraction. As hydrophobicity decreases, both by increasing the thymol fraction in this HNADES and with other HNADES such as [Thy]:[Cin] 1:1, worse results are shown for the more polar analytes (morphine and oripavine) (Table 2). This may be because when performing m-LLE in a polar medium such as water, HNADES can form hydrogen bonds with the water instead of with the analytes, resulting in lower extraction, especially for the more polar analytes. Even if hydrophobic interactions are further decreased, as is the case with [Cin]:[Men] 1:1, the extraction of all analytes decreases (Table 2). On the other hand, decreasing π–π type interactions such as with [Thy]:[Men], [Thy]:[Fen] and [Cam]:[Men] with respect to those with [Thy]:[Cam] 1:1 also decreases the extraction (Table 2). Therefore, [Thy]:[Cam] 1:1 was selected to carry out the m-LLE of the samples.

In brief, the best results for the extraction of OAs in aqueous media were [Thy]:[Cam] in its three molar ratios (1:1, 3:2 and 7:3) and [Thy]:[Men] 1:1 and 1:2, as they showed EE% values higher than 50 % for all the analytes.

3.2. Characterisation by ATR-IR of HNADESs that showed higher OAs extraction efficiency in aqueous media

The most used method to demonstrate the correct formation of HNADESs is to carry out ATR-IR characterisation of the individual components and of the corresponding HNADES to determine the presence of hydrogen bonds in the eutectic mixture. The absorption profile of the mixtures is usually similar to that of the individual components. However, the presence of hydrogen bonds changes the force constant of the donor and acceptor functional groups and, consequently, their stretching vibrational frequencies are shifted [39]. Thus, ATR-IR of the individual components and of the HNADESs formed at the different molar fractions that gave the best results. The selection criterion was that in the m-LLE assays at least an EE (%) of 50 % was obtained for each of the analytes evaluated.

As shown in Fig. 2a, in the case of the evaluated [Thy]:[Cam]-based HNADESs, there was a change in the –OH band of thymol from 3207.7 cm^{-1} to 3400.2 cm^{-1} at the three tested molar ratios, which agrees with that described by Makoś et al. [37]. As shown in Fig. 2b, in the case of [Thy]:[Men]-based HNADESs, the spectrum of menthol shows the –OH

Table 2
Extraction efficiency and standard deviation (%) obtained of each of OAs with the different HNADESs after performing m-LLE of water spiked with 2 μg/mL of OAs (n = 3).

HNADES and Molar ratio	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
[Thy]:[Cam] 1:1	98 ± 2	97 ± 2	99 ± 1	99 ± 3	96 ± 1	98 ± 4
[Thy]:[Cam] 3:2	54 ± 1	91 ± 11	99 ± 7	99 ± 16	66 ± 2	96 ± 3
[Thy]:[Cam] 7:3	92 ± 2	94 ± 2	96 ± 1	97 ± 3	64 ± 2	51 ± 1
[Thy]:[Men] 1:1	63 ± 1	93 ± 1	100 ± 4	100 ± 1	100 ± 3	97 ± 1
[Thy]:[Men] 1:2	61 ± 2	93 ± 2	99 ± 7	100 ± 3	99 ± 5	97 ± 5
[Thy]:[Men] 2:1	16 ± 5	5 ± 7	85 ± 2	99 ± 11	99 ± 4	15 ± 3
[Thy]:[Men] 1:3	24 ± 5	43 ± 1	95 ± 5	100 ± 4	60 ± 2	76 ± 1
[Thy]:[Cin] 1:1	28 ± 7	64 ± 3	99 ± 3	100 ± 3	100 ± 3	85 ± 4
[Thy]:[Fen] 1:1	23 ± 2	40 ± 7	95 ± 10	100 ± 5	100 ± 1	61 ± 3
[Cam]:[Men] 1:1	57 ± 9	60 ± 3	84 ± 1	83 ± 2	61 ± 4	36 ± 1
[Cin]:[Men] 1:1	12 ± 2	9 ± 5	33 ± 2	90 ± 3	57 ± 3	15 ± 5

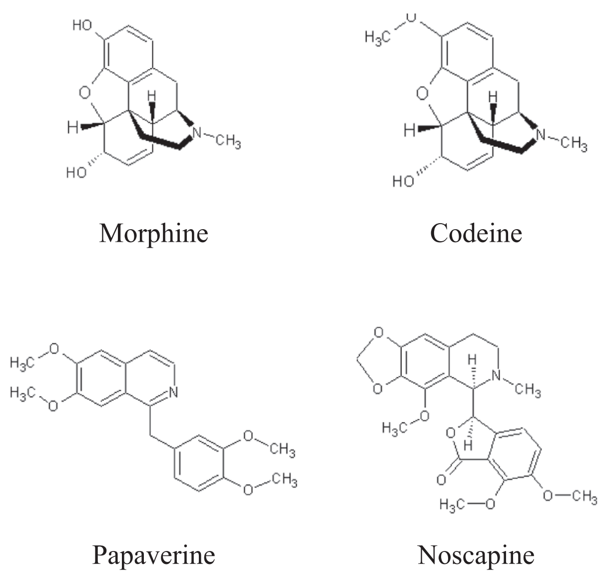


Fig. 1. Chemical structures of the six main opium alkaloids in contaminated poppy seeds.

stretching vibration peak at 3261 cm^{-1} and that of thymol at 3188 cm^{-1}. When the corresponding eutectic mixtures were formed, these bands shift to 3335 cm^{-1}, in agreement with that reported by Cherniakova et al. [40]. Therefore, based on the results obtained, it can be concluded that hydrogen bonds between thymol and camphor and between thymol and menthol were formed at all evaluated molar ratios.

Therefore, considering the correct formation of each of them, HNADES was finally selected as it showed a higher extraction capacity for OAs in aqueous media, being [Thy]:[Cam] 1:1 with an EE(%) above 96 % for all analytes (Table 2).

3.3. Optimization of OAs analysis with [Thy]:[Cam] 1:1 by HPLC-DAD

For the chromatographic separation and analysis of the six investigated OAs, different conditions were tested based on previous work [33]. Parameters such as mobile phase composition, column washing and conditioning between injections, and injection volume were optimized to achieve adequate separation of OAs among them, from the [Thy]:[Cam] 1:1 peak, and to eliminate the remaining HNADES in the analytical system in between injections to ensure an adequate reproducibility for the proposed method.

The mobile phases tested were composed of water as phase A and acetonitrile or methanol as phase B, both with and without TFA. The best separation and resolution among the analytes was obtained using water with 0.1 % TFA as phase A and acetonitrile as phase B. The initially used elution gradient started with 0–1 min 2 % solvent B, 1–5 min 35 % solvent B, 5–6 min 40 % solvent B, 6–6.30 min 2 % solvent B, and an additional 1 min to equilibrate the column. However, it was observed that, after several injections, a lack of reproducibility became apparent (i.e. the retention times of the analytes were not fully maintained), and the detector started to register a persistent saturated signal during the whole analysis run. These effects were attributed to the [Thy]:[Cam] 1:1 that remained in the chromatographic system since, when successive washing injections were carried out, the initial signal and the retention times were recovered. Therefore, to reduce the analysis time and improve the efficiency of the method, and addition step was added to the HPLC gradient program. This step involved an increase in the proportion of organic phase during few minutes in order to remove the [Thy]:[Cam] 1:1 remaining in the instrument. By this way, from 6.30 to 12 min 90 % solvent B (acetonitrile) was added and, then, from 12 to 12.10 min mobile phase was changed to initial conditions (2 % solvent B), which were maintained during 3.90 min to equilibrate the column. Thus, the total analysis time was 16 min. Under these conditions, a reproducible method was optimized and no variation of the retention times of the analytes or interferences were observed with successive injections.

Different injection volumes (5, 10, and 15 μL) were also tested, and 10 μL were selected to achieve lower quantification limits while preventing peak broadening and splitting.

The proposed methodology yielded retention times of 3.6 min for morphine, 4.4 min for codeine, 4.6 min for oripavine, 5.7 min for thebaine, 6.0 min for papaverine, and 6.1 min for noscapine. Meanwhile, the [Thy]:[Cam] 1:1 eluted completely separated from the analytes at the final part of the chromatogram (Fig. 3). As can be observed in this

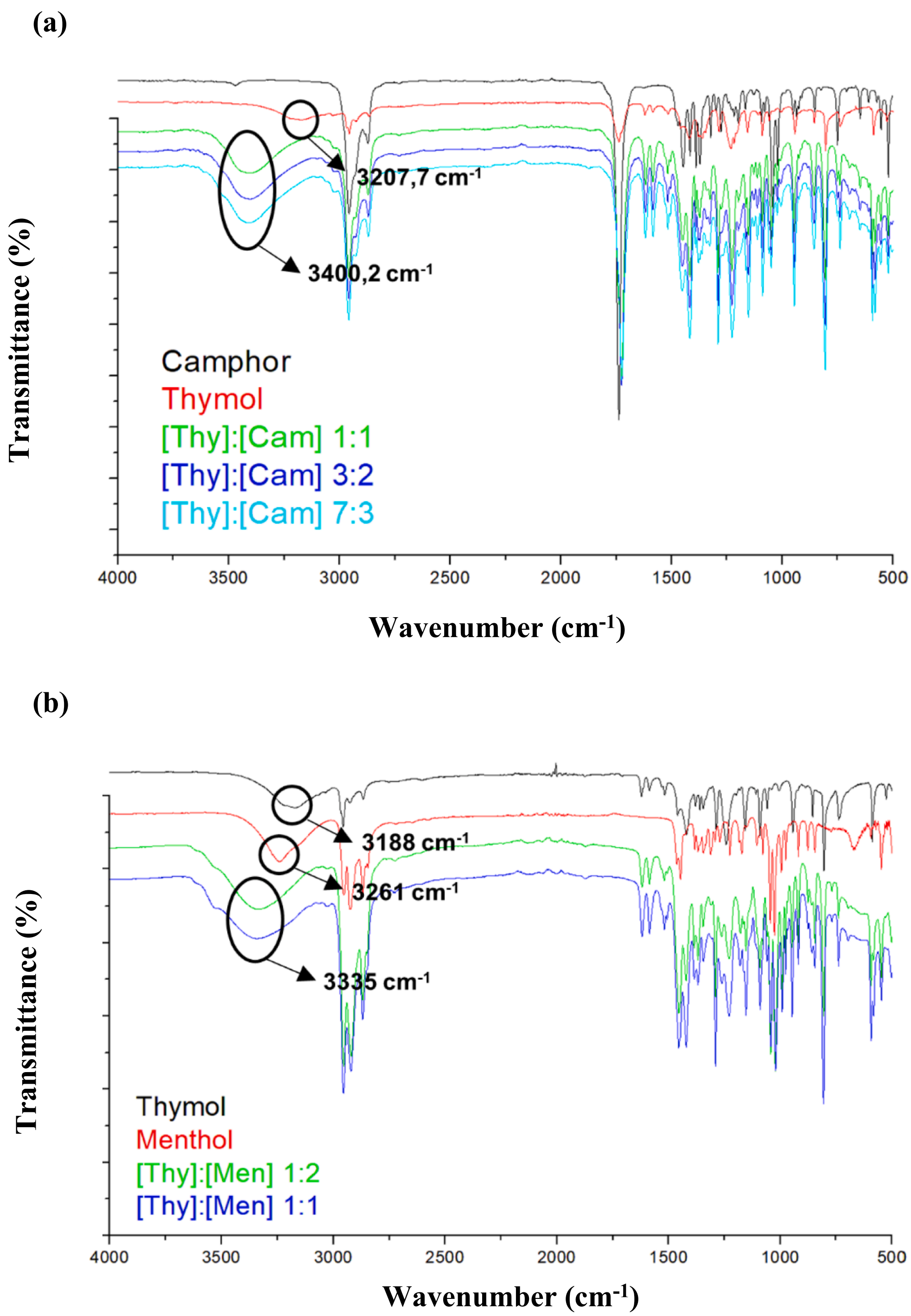


Fig. 2. ATR-IR spectra of the pure components and of selected HNADESs prepared with (a) [Thy]:[Cam] and (b) [Thy]:[Men] in different molar ratios.

figure, the analytes showed some tailing in the chromatogram (Fig. 3b), which could be associated to a higher elution power of the HNADES compared to that of the LC mobile phase.

3.4. Optimization of m-LLE procedure with [Thy]:[Cam] 1:1

To optimise the m-LLE procedure using [Thy]:[Cam] 1:1, different vortex extraction times (30 s and 1 min) were tested. For this purpose, m-LLE of 0.5 mL of a sample spiked with 2 μg/mL OAs was performed using 0.5 mL of [Thy]:[Cam] 1:1. Fig. 4 shows that 1 min of extraction time provided higher signals for all test analytes than 30 s. Therefore, the time selected for further m-LLE optimization was 1 min.

Then, different sample:HNADES ratios were evaluated as described in Section 2.6, keeping the volume of sample at 0.5 mL and changing that of HNADES (0.1, 0.25 and 0.5 mL), as well as keeping the volume of HNADES at 0.5 mL and that of the sample (0.75 and 1 mL). As a result, it was found that a correct separation was only achieved by centrifuging after vortex extraction with the 0.5 mL sample:0.5 mL HNADES ratio. With the rest of the proportions, a correct separation of the extract and HNADES was not achieved, so it was not possible to take an aliquot for analysis in the HPLC-DAD and these tests were discarded.

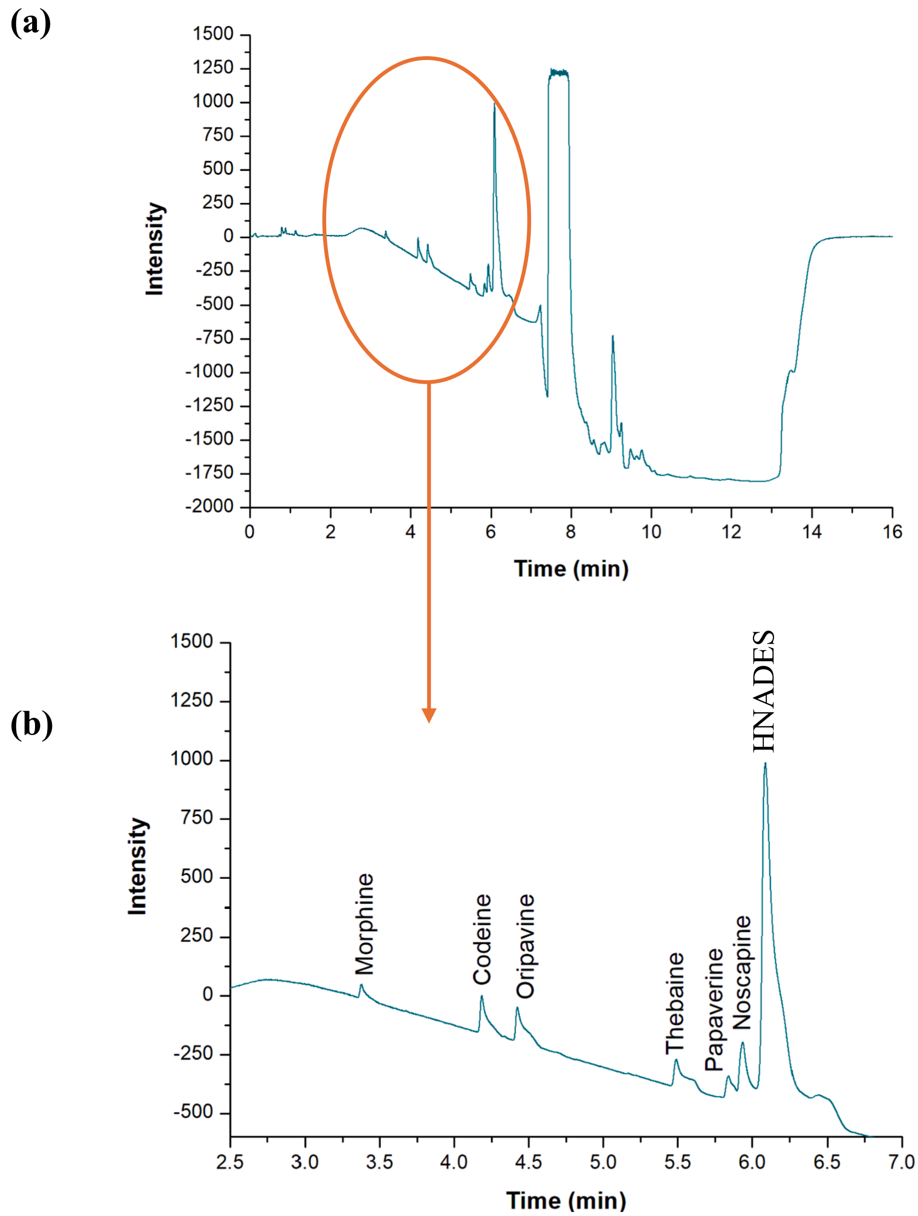


Fig. 3. Chromatographic separation obtained in the optimized conditions: (a) complete and (b) zoom showing the separation of OAs and the HNADES after m-LLE with [Thy]:[Cam] 1:1 of a sample spiked with 2 μg/mL of OAs. Separation was carried out with an InfinityLab Poroshell 120 EC-C18 column (3.0 mm × 150 mm, 2.7 μm particle size) at 30 °C coupled to a pre-column, InfinityLab Poroshell EC-C18. Solvent A (Milli-Q water with 0.1 % TFA) and solvent B (AcN). Gradient elution was: 0–1 min 2 % solvent B, 1–5 min 35 % solvent B, 5–6 min 40 % solvent B, 6–6.30 min 90 % solvent B, 6.30–12 min 90 % solvent B, 12–12.10 min 2 % solvent B and 3.90 min more to equilibrate the column. DAD at 212 nm for all OAs.

3.5. Method validation

The validation results of the proposed method for the quantification of OAs in poppy seed beverage samples are shown in Table 3. Calibration lines with an R^2 higher than 0.992 were obtained for all analytes in the evaluated range of 0.1–10 or 0.5–10 μg/mL, depending on the OA considered. Additionally, the deviation of the slopes of the calibration lines over different days ($n = 3$) was calculated to determine the precision, obtaining RSDs between 1 and 7 % for all analytes.

The MDL and MQL values were low enough to determine the maximum values legislated of OAs in the poppy seed beverage samples, with MDL and MQL for morphine being 0.1 μg/mL and 0.4 μg/mL, respectively, and 0.03 μg/mL and 0.1 μg/mL for the rest of the analytes (Table 3).

On the other hand, the ME was calculated by comparing the slopes of the matrix and solvent-based calibration curves. As shown in Table 3, negligible MEs were observed for all the tested analytes (<±20 %). This means that the poppy seed beverage matrix did not interfere in either the m-LLE or HPLC-DAD analysis for any of the analytes determined.

Additionally, as shown in Table 3, satisfactory results were obtained for the EEs, with values above 91 % for all analytes at the three concentration levels evaluated.

Furthermore, intra-day and inter-day precision at the three studied concentration levels were satisfactory, as RSD values were lower than 15 % in all cases (Table 3).

Thus, all these validation parameters demonstrated that the developed methodology was effective and valid for application to real samples of poppy seed beverages to ensure consumer safety.

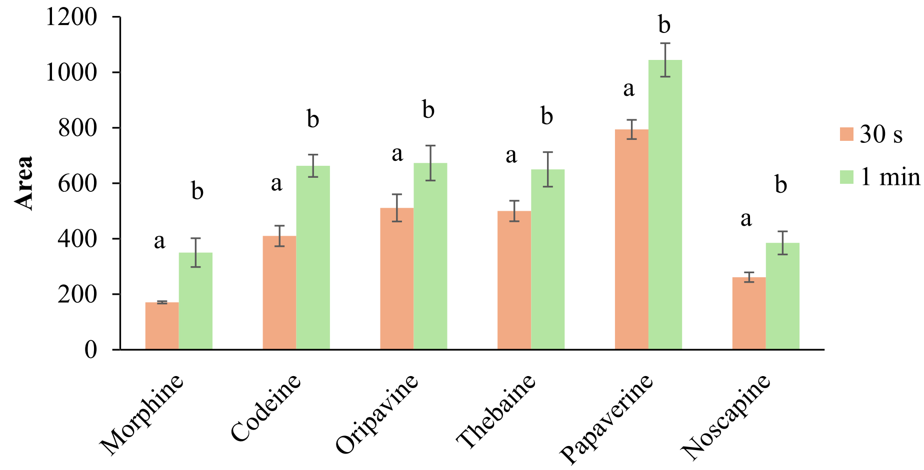


Fig. 4. Chromatographic pico areas obtained after m-LLE of 0.5 mL of a sample spiked with 2 μg/mL OAs and 0.5 mL of [Thy]:[Cam] 1:1 at different vortexing times (30 s and 1 min). Different letters (a and b) indicate statistically significant differences for p-values ≤0.05.

Table 3
Validation parameters of the proposed HNADES-m-LLE and HPLC-DAD methodology for the quantification of OAs in poppy seed beverages.

Analytes	LDR (μg/mL)	Matrix matched calibration curve (R^2)	MDL (μg/mL)	MQL (μg/mL)	ME (%)	Extraction efficiency (%)	Precision (RSD %)	
							Intra-day (n = 6)	Inter-day (n = 9)
Morphine	0.5–10	y = 42.8x + 5.1 (0.992)	0.1	0.4	10	98^{L} 105^{M} 97^{H}	12^{L} 3^{M} 7^{H}	15^{L} 5^{M} 10^{H}
Codeine	0.1–10	y = 125.2x + 7.3 (0.996)	0.03	0.1	−2	103^{L} 97^{M} 95^{H}	7^{L} 5^{M} 6^{H}	11^{L} 12^{M} 8^{H}
Oripavine	0.1–10	y = 129.3x + 57.3 (0.995)	0.03	0.1	−7	91^{L} 107^{M} 98^{H}	10^{L} 5^{M} 5^{H}	15^{L} 11^{M} 6^{H}
Thebaine	0.1–10	y = 146.3x − 2.3(0.993)	0.03	0.1	13	92^{L} 107^{M} 104^{H}	13^{L} 2^{M} 6^{H}	15^{L} 11^{M} 7^{H}
Papaverine	0.1–10	y = 314.7x − 100.4 (0.995)	0.03	0.1	−3	106^{L} 94^{M} 91^{H}	9^{L} 2^{M} 12^{H}	12^{L} 8^{M} 12^{H}
Noscapine	0.1–10	y = 54.5x + 35.6 (0.994)	0.03	0.1	3	93^{L} 98^{M} 102^{H}	10^{L} 7^{M} 9^{H}	7^{L} 11^{M} 10^{H}

LDR: linear dynamic range expressed in μg/mL; MDL: method detection limit; MQL: method quantification limit; ME: matrix effect; Extraction efficiency and precision were obtained by spiking samples at three known concentration levels: low (L, 0.5 μg/mL), medium (M, 2 μg/mL) and high (H, 10 μg/mL). Extraction efficiency was calculated with 3 replicates in 3 different days (n = 9).

3.6. Comparison of this analysis methodology with other previously published methodologies for the analysis of OAs

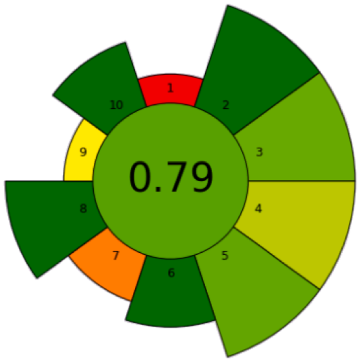
In comparison with previously published methodologies for analyzing OAs, it is important to highlight that this is the first method developed specifically for the analysis of OAs in poppy seed beverages. This is a crucial step, as the EFSA is advocating for the study of new food samples to better understand the actual exposure to all potential OAs present, which will help inform future legislation [23]. In addition, one of the most important advantages of the methodology proposed in this work is that the extraction of the target analytes was carried out using an m-LLE so efficient that sample preparation required only 2 min. Therefore, this makes the methodology developed faster and simpler than others previously published, whose sample preparation time can reach between one and several hours. Such is the case of the work of Sproll et al. (2006) who used two hours to extract poppy seed OAs by solid–liquid extraction (SLE) [41] or the work of Xu et al. (2018) who required almost 20 min for LLE and subsequent purification by magnetic solid phase extraction (MSPE) in hot pot [42].

On the other hand, the use of a HNADES makes the sample preparation completely environmentally friendly, eliminating the use of reagents harmful to the environment and the health of the analyst, as had been used until now. In addition, its potential characteristics mean that the volumes required to make a complete extraction are lower than those used with conventional organic solvents. An example is the work of Acevska et al. (2012) where they use 5 mL of methanol as the extraction solvent [43]. Guo et al. (2013) used 20 mL of hydrochloric acid solution and 10 mL of petroleum ether [44]. Another work is that of Yamaguchi et al. (2011) where 10 mL of isobutanol:dichloromethane 10:90, v/v is used [45].

In short, the present methodology presents several advantages that make it very effective for the analysis of this family of natural toxins and specifically in beverages made with poppy seeds whose matrix has not been previously analysed.

3.7. Greenness analysis of the developed HNADES m-LLE methodology

The AGREEprep metric [21] was used to evaluate the greenness of the analytical methodology developed in this work. Each criterion was scored between 0 and 1, with 1 being optimal green performance (see Fig. 5). The proposed method achieved an excellent score of 0.79 points due to the limited sample and solvent consumption, being only 0.5 mL of



Criterion	Input	Reasoning for input	Weight	Score
1	Ex situ	Sample preparation performed in the analytical chemistry laboratory	1	0.00
2	0	HNADES is a green solvent	5	1.00
3	>75%	All reagents used (HNADES) are green	5	0.75
4	1	Sum of residues (sample and HNADES)	5	0.63
5	0.5	Amount of poppy seed beverage used per analysis	5	0.77
6	144	Extraction time: 1 min; Centrifugation time: 1 min; Time to take the aliquot to analyse: 1 min. (6 samples at the same time)	3	1.00
7	2 steps or fewer/ manual systems	Step 1: vortexing Step 2: centrifugation	2	0.25
8	0.68 W per sample	Vortex (15 W × 0.017 h) + Centrifuge (25 W × 0.017 h) = 0.68 W	5	1.00
9	HPLC-DAD	Liquid chromatography, gas chromatography with quadrupole detection, etc.	1	0.50
10	0 hazards	None of the components of HNADES is considered hazardous	3	1.00

Fig. 5. Greenness of the proposed HNADES-m-LLE method for OAs determination evaluated using the AGREEprep metric.

each, the miniaturization of the sample processing technique, the use of a totally environmentally friendly extraction solvent as it is a HNADES and the significant reduction in extraction time, needing only 1 min of vortexing for extraction and another minute of centrifugation for separation, which allowed this methodology besides being very fast and simple, to have a low cost of energy consumption (Fig. 5). For all these reasons, this methodology is not only fast, simple and effective for the analysis of OAs in poppy seed beverage samples, it is also environmentally friendly.

3.8. Application to quantify OAs in real samples of poppy seed beverages

The optimised m-LLE-based sample preparation method was applied to analyse OAs in five poppy seed beverages prepared from different poppy seeds available in supermarkets (Table S1). These are mostly from ecological cultivation and come from outside the European Union, mainly from Turkey, with various indications of use, such as infusions or to add to different foods. It should be noted that some were purchased before the entry into force of the relevant legislation (PS-01 and PS-02), while others were purchased after (PS-03, PS-04 and PS-05).

Results obtained are summarised in Table 4. At least one of the investigated OAs was found at quantifiable levels in all the analysed samples, with the only exception of sample PSB-03, where none of them was detected. Regarding the concentrations obtained, samples PSB-01 and PSB-02, made with seeds purchased before the current legislation came into force (Table S1), presented higher concentrations of OAs than the rest, in particular for morphine. It should be mentioned that the rest of the evaluated samples were prepared with seeds purchased after the legislation came into force [31]. Current legislations set maximum limits for OAs in seeds but not for this type of beverages. However, considering the amount of seeds and water used to prepare the beverages analysed in this study (100 g poppy seeds in 1 L of water) and that, as already demonstrated, grinding of the seeds does not affect the concentration of OAs in the seeds [46], the concentration determined in samples PSB-01 and PSB-02 would indicate that the poppy seeds used to prepare these beverages would exceed the maximum limit established by the legislation of 20 mg/kg morphine equivalents [31].

4. Conclusions

A fast, efficient, and environmentally friendly HNADES m-LLE methodology for monitoring OAs in poppy seed beverages has been successfully developed for first time. A total of 15 HNADESs were initially prepared for their potential to interact with OAs in aqueous samples. Four of them, generally formed by lactic acid, were discarded because of their instability in water. The remaining eleven were evaluated by performing m-LLE, obtaining that the HNADES with the highest EE(%) was [Thy]:[Cam] 1:1, with values higher than 91 % for all the analytes. The optimised HNADES m-LLE methodology required only 0.5 mL of both the beverage sample and [Thy]:[Cam] 1:1, with a short processing time of 1 min vortexing and 1 min centrifugation, yielding a total 2 min sample treatment time. The methodology was validated, offering excellent results in terms of MQL, precision and extraction efficiency. In addition, its environmental impact was assessed, confirming its green credentials. The optimised method was then applied to the analysis of beverages prepared using five commercially available poppy seeds, demonstrating that samples purchased after the legislation came into force showed lower and safer concentrations for the consumer. Therefore, the present work demonstrated that HNADES-based extraction is a viable and efficient alternative to conventional procedures, offering advantages such as being more efficient, selective, faster, easier to handle, and environmentally friendly.

Novelty statement

This research introduces a groundbreaking and sustainable approach for the extraction of opium alkaloids (OAs) from poppy seed beverages, employing miniaturized liquid–liquid extraction (m-LLE) with hydrophobic natural deep eutectic solvents (HNADES). This is the first time such a methodology has been applied to this type of food matrix, filling a

Table 4
Mean concentrations obtained for the investigated OAs in the five poppy seed beverages expressed in μg/mL of beverage ± standard deviation (SD) (n = 3).

Sample code	Morphine	Codeine	Oripavine	Thebaine	Papaverine	Noscapine
PSB-01	4 ± 1	0.1 ± 0.1	ND	0.3 ± 0.1	ND	ND
PSB-02	3 ± 2	0.4 ± 0.1	0.19 ± 0.01	0.9 ± 0.1	ND	0.7 ± 0.4
PSB-03	ND	ND	ND	ND	ND	ND
PSB-04	ND	ND	ND	<MQL	ND	ND
PSB-05	ND	ND	0.399 ± 0.001	0.18 ± 0.02	ND	ND

PSB: poppy seed beverage (made from 100 g poppy seeds in 1 L of water); ND: no detected; <MQL: below to method quantification limit.

critical gap in the current analytical techniques for OA detection. The novelty of this work lies in the use of [Thymol]:[Camphor] 1:1, a highly efficient HNADES, which achieved extraction efficiencies exceeding 96 % for all analytes, a remarkable improvement over existing methods.

The optimized procedure requires only 0.5 mL of the beverage sample and 0.5 mL of the HNADES, combined with a brief 1-minute vortex and 1-minute centrifugation, making the method exceptionally fast and resource-efficient. In addition to reducing the extraction time and solvent volumes, this approach aligns with green chemistry principles, contributing to a lower environmental impact compared to conventional extraction techniques.

A key aspect of this work is its validation for precision, quantitation limits, and extraction efficiency, ensuring the robustness and reproducibility of the method. Furthermore, the green metrics, including solvent reduction and minimal energy use, confirm its environmental sustainability. The methodology was successfully applied to the analysis of commercially available poppy seed beverages, offering practical insights into the levels of OAs present in products both before and after new regulatory changes.

Importantly, the study revealed that beverages made from poppy seeds purchased after recent legislation came into force contained lower and safer OA concentrations, directly addressing public health concerns. This highlights the relevance and timeliness of the developed method, which not only meets the current demands for rapid, reliable, and sustainable analytical techniques but also provides an effective tool for food safety monitoring.

In summary, this research represents a significant advancement in the field of green analytical chemistry, offering a fast, efficient, and eco-friendly alternative for the analysis of opium alkaloids in poppy seed beverages. The innovative application of HNADES in this context, coupled with its excellent performance and minimal environmental footprint, underscores the method's potential for broader application in food safety and environmental monitoring.

CRediT authorship contribution statement

Gema Casado-Hidalgo: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Rubén Cordo:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Damián Pérez-Quintanilla:** Writing – review & editing, Supervision, Resources. **Sonia Morante-Zarcero:** Writing – review & editing, Supervision, Resources, Conceptualization. **Belén Gómara:** Writing – review & editing, Funding acquisition. **Lourdes Ramos:** Writing – review & editing, Funding acquisition. **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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.111954.

Data availability

Data will be made available on request.

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