# Green electromembrane extraction procedure based on biodegradable chitosan films for determination of polyphenolic compounds in food samples: Greenness assessment of the sample preparation approach

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## Abstract

A simple, rapid and environmentally friendly method for the electromembrane extraction of polyphenolic compounds has been developed using chitosan films (60% (w/w) chitosan, 40% (w/w)-Aliquat®336, 10-11 μm thickness) as biopolymeric membrane. In this work for the first time with this type of chitosan-based support, the use of organic solvents has been completely eliminated, which allows considering the proposed methodology as a green solvent-free procedure, as demonstrated by performing analytical greenness metric for sample preparation (AGREEprep). Under optimal experimental conditions (10 mL donor phase, pH 7; 50 μL acceptor phase, pH 9; 100 V applied voltage for 15 min) high enrichment factors (EF ≥ 60) were obtained for all the target analytes. Wide concentration ranges between 52.8 μg $L^{-1}$ and 1000 μg $L^{-1}$, good linearity ($R^2 \geq 0.996$), low limit of detection (15.9-37.1 μg $L^{-1}$), and repeatability (relative standard deviation (RSD) values 4-10%) were achieved. Polyphenolic compounds have been successfully extracted from coffee- and tea-based dietary food supplements in different formats (pills and ampoules). For comparison purposes, target analytes have additionally been determined in green coffee beans and tea.

*Keywords*: C*hitosan; Biopolymeric film; Electromembrane extraction; Polyphenolic compounds; Green chemistry.*

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## 1. Introduction

Within liquid phase microextraction (LPME) techniques, electromembrane extraction (EME) has experienced numerous advances over the years as a sample preparation method, due to its low solvent and sample consumption, in line with the trend towards green chemistry, as well as its high selectivity. In EME, ionized analytes migrate from an aqueous sample to an acceptor solution due to the application of an electric field provided by an external power supply. During electrokinetic migration, the target compounds pass through a supported liquid membrane (SLM), consisting of a water-immiscible and hydrophobic solvent, which is placed on different inert porous supporting materials, mainly polypropylene (PP)-based supports (including hollow fibers or flat sheet membranes) [1]. In addition, similar porous polymers, such as polyvinylidene fluoride (PVDF) or polytetrafluoroethylene (PTFE), have been commonly used [2, 3].

The development of increasingly efficient and selective EME procedures is often a challenge for researchers, and, as a consequence, the support material typically used for the liquid membrane has gained importance in recent years. In this sense, some approaches have been introduced in which the classical PP structure has been chemically modified, such as silver nanometallic-decorated PP hollow fibers [4] or PP hollow fiber reinforced with carbon nanotubes [5], where the porous membrane plays an active role in the extraction process, thus enhancing selectivity.

Furthermore, new materials of different composition have been developed and successfully applied in EME, such as polymer inclusion membranes (PIMs) [6], nanostructured tissues [7], or polyacrylamide gels [8]. In particular, biopolymers have recently become an interesting alternative to commonly used plastic supports. In this regard, agarose-based materials, both films [9, 10] or gel membranes [11-13], are the most

widely used in EME with diverse applications, although other biopolymers, such as tragacanth gel membranes [14], have also been reported for similar purposes.

In addition to those mentioned above, chitosan constitutes a highly versatile biopolysaccharide widely used in multiple fields such as biomedicine, technology or cosmetics [15]. Non-toxicity, bioabsorption, gelation ability, antimicrobial activity and biocompatibility, among others, are some biological and physiochemical properties that make this biopolymer a valuable and advantageous material for different applications [16]. Specially, its thermal stability as well as its mechanical characteristics make chitosan a suitable raw material for the preparation of package structures, films, or membranes for use in a wide variety of fields (environmental, food technology or drug delivery) [17-20].

Within this realm, our research group has been focused on the use of tailor-made chitosan membranes as a new support of SLM in EME procedures. Indeed, the extraction of pharmacologically active compounds, such as non-steroidal anti-inflammatory drugs (NSAIDs), has been successfully carried out from biological samples [21]. Moreover, selective extraction of some antibiotics of veterinary use, i.e., fluoroquinolones, has also been achieved [22]. In both previous studies, the active role of the biopolymeric film during the EME process has been demonstrated. Due to the presence of amine and hydroxyl groups in the glucosamine ring structure of chitosan, hydrogen bonds can be established between the biopolymer and functional groups of the target analytes during the extraction. The existence of these molecular interactions allows a very high selective and targeted extraction of the compounds of interest. This is a key advantage of using chitosan films in EME compared to other proposed materials, which just act as passive supports during the extraction. Thus, the biopolymer actively participates in the extraction process, becoming an essential and highly versatile platform for controlling and improving the EME procedure as required.

On the other hand, polyphenols are an important group of compounds widely known for their antioxidant capacity and plant growth-regulating ability, obtained mainly from natural sources such as plants and fruits [23]. Numerous studies on chitosan-polyphenol interaction have been reported from different points of view. Food industry or biomedicine are some of the frameworks in which this topic is of great interest due to both the high biological activities of polyphenolic compounds (antioxidant, antiviral or antibacterial, among others) and the versatility of chitosan as a natural and biodegradable

polymer [24-26]. Polyphenol-modified chitosan-based materials have been designed for various applications, such as biocoatings, encapsulating agents or bioadsorbents [23, 27-29]. Insights of the chemical interaction occurring between polyphenolic compounds and chitosan have been deeply studied. Evidences on the formation of a stable structure through hydrogen bonding at alkaline media (pH ≥ 9) have been previously reported [30, 31]. Therefore, many phenolic compounds have been used as chitosan crosslinking agents in order to provide additional properties to the biopolymeric film, such as antiviral and antibacterial properties [29, 32-33].

In the present work, the use of a thin chitosan film for the EME of different polyphenolic compounds is proposed. Gallic acid (GAL), chlorogenic acid (CLO), 4-hydroxybenzoic acid (HBA), caffeic acid (CAF), ferulic acid (FER), benzoic acid (BZA), benzenepropanoic acid (BPZ) and cinnamic acid (CIN) have been selected as model analytes for this purpose and, subsequently, successfully extracted from different dietary food products. In a further step of this research, the water immiscibility of the biopolymer has been exploited to develop an EME procedure without using (hazard) organic solvent as SLM. In order to assess the greenness of the proposed method, a novel metric tool called AGREEprep, recently introduced by Wojnowski et al. [34] to evaluate the environmental impact of sample preparation, has been applied for the first time to our knowledge in EME. To this end, a comparison of different miniaturized extraction techniques reported for the determination of phenolic acids in food matrices has been carried out, both in terms of analytical performance and environmental impact.

## 2. Experimental

### 2.1. Chemicals and reagents

All reagents and chemicals used were of analytical grade. Commercial chitosan of 310000-375000 Da molecular weight, obtained from shrimp shells (according to the product specifications), GAL, CLO, HBA, CAF, FER, BZA, BPZ, CIN, acetonitrile, methanol, Aliquat®336 and formic acid were obtained from Fluka-Sigma-Aldrich (Madrid, Spain). Acetic acid and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Working solutions were daily prepared by appropriate dilutions from methanolic stock solutions (400 mg $L^{-1}$) of target analytes. All solutions and dilutions were prepared using Ultrapure water from Milli-Q Plus water purification system (Millipore, Billerica, MA, USA).

## 2.2. EME procedure

EME procedure was carried out using a self-made extraction device developed in previous works (Figure S1) [21]. Aqueous solution of polyphenolic compounds (10 mL, pH 7) was introduced into a 10 mL glass vial serving as the donor phase. The acceptor phase (50 μL, aqueous NaOH solution, pH 9) was placed into a 2 mm i. d. bore glass tube with a chitosan film attached to the bottom. The glass tube filled with the acceptor solution was then inserted into the sample compartment (donor solution) and two platinum electrodes were placed inside the solutions 2 mm apart, both connected to a three-channels Laboratory DC Power Supply (Benchtop Instrument, Pennsylvania, USA) with a programmable voltage in the 1-120 V range. A digital multimeter (3430 4 ½-digit PeakTech®, Ahrensburg, Germany) connected to a personal computer was used for electrical current monitoring and data acquisition during extraction. Optimal extraction conditions were obtained when 100 V DC potential was applied during 15 minutes with constant stirring at 600 rpm, providing average currents of 150-400 mA. After completion of the EME, 20 μL of the acceptor phase were collected with a microsyringe and injected into the HPLC system for further analysis.

In this alternative chitosan-based EME procedure, no organic solvent is required as a SLM, providing a solvent-free EME approach. Biopolymeric film enables physical separation of donor and acceptor solutions, allowing target analytes to cross from one phase to the other during the extraction process.

## 2.3. Chromatographic conditions

Determination of the target compounds was performed using a LabChrom® VWR-Hitachi (Barcelona, Spain) liquid chromatograph, equipped with a L-2200 autosampler (20 μL), a L-7100 quaternary pump and a L-7455 UV/Vis diode array detector (DAD). A Kromasil® 100 Å, C18, 3.5 μm (15 mm×4.6 mm i.d.) (Schrarlab S.L., Barcelona, Spain) was used as guard column. Elution and separation of polyphenolic compounds were carried out on an Eclipse® XDB-C18 3.5 μm (150 mm×3.0 mm i.d) (Agilent Technologies, Little Falls, DE, USA) chromatographic column.

After carrying out the optimization of chromatographic separation, mobile phase consisting of 0.1% (v/v) formic acid aqueous solution (component A) and acetonitrile

(component B) was pumped continuously at flow rate of 0.5 mL $min^{-1}$, applying a gradient elution described in Table S1. For quantitation purposes, the following monitoring wavelengths were used for each compound: 270 nm for GAL, 325 nm for CLO, CAF and FER, 255 nm for HBA, 275 nm for BZA and CIN, and 220 nm for BZP.

## 2.4. Analytical greenness metric tool

AGREEprep open access software [34] was used to assess the greenness of the proposed method and other sample preparation methods previously reported in the literature for the determination of phenolic acids in food matrices for comparative purposes. This metric tool has recently been introduced by Wojnowski et al. to study the environmental impact of the sample preparation stage, as it is a crucial step of great importance in the entire analytical methodology. It is based on the so-called 10 principles of green sample preparation [35] stated as follows: *1*-favor *in situ* sample preparation; *2*-use safer solvents and reagents; *3*-target sustainable, reusable, and renewable materials; *4*-minimize waste; *5*-minimize sample, chemical, and material amounts; *6*-maximize sample throughput; *7*-integrate steps and promote automation; *8*-minimize energy consumption; *9*-choose the greenest possible post-sample preparation configuration for analysis; *10*-ensure safe procedures for the operator. Each criterion is evaluated and scored, and the final result is a pictogram representing the assessment of each criterion together with an overall score at the site on a scale ranging from 0 (considered as a non-environmentally friendly approach) to 1 (the ideal greenest approach). The score is additionally accompanied by an intuitive colour scale from red to green, respectively. Thus, the closer to 1 and the greener the final score is, the more environmentally friendly the procedure is considered.

On the other hand, each criterion is assigned an importance or weight within the total score and can be modified as desired. In this case, default weights defined by the software will be used, which gives the highest weight to criterion 2 (weight 5), followed by criteria 4 and 8 (weight 4), criteria 6 and 10 (weight 3), criteria 3, 5, 7 and 9 (weight 2) and finally, criteria 1 assigned the lowest importance (weight 1).

## 2.5. Real samples

The proposed EME procedure was applied to the separation and subsequent determination of polyphenolic compounds in six different dietary supplements (FS1-FS6) based on green coffee and tea (three of each). Additionally, target analytes were

192 determined in tea leaves (two different samples named TS1 and TS2) and in green coffee
193 beans (*arabica* (CS1) and *robusta* (CS2)) for comparative purposes.
194 Tea samples were purchased from local stores and green coffee beans were kindly
195 provided by a local factory. Dietary supplements in different formats (pills and drinkable
196 ampoules) were also purchased from local shops. The green coffee extract as well as tea
197 content were indicated on the respective product label.
198 Aqueous extracts of tea leaves were obtained by refluxing 2 g of tea samples with
199 hot water (200 mL, 80°C) for 1h. The extract was filtered through a Whatman filter paper
200 (90 mm) and diluted to volume in a 250 mL calibrated flask. Then, the solution was
201 microfiltered (0.22 μm) and aliquots of this solution were diluted appropriately (1:40) for
202 the EME procedure. Aqueous extracts of green coffee beans were obtained in the same
203 way; in this case, 5 g assay portions of ground coffee were necessary. A suitable dilution
204 (1:400) was required before carrying out EME procedure.
205 Sample treatment of coffee and tea food supplements was as follows: the content
206 of 2 pills (or 2 vials in the case of drinkable ampoules) was solved in water and diluted to
207 volume in a 250 mL flask. Once microfiltered (0.22 μm), dilutions of 1:500 and 1:200 for
208 pills (samples FS1, FS3, FS5) and ampoules (samples FS2, FS4, FS6), respectively, were
209 required before EME procedure and sample pH was adjusted to the optimum value.
210
211 **3. Results and discussion**

212 3.1. Preparation and characterization of chitosan-based film

213 Chitosan-based biopolymeric film was synthesized and characterized in a
214 previous work [21]. Nevertheless, for the present research, several modifications have
215 been introduced to improve the synthesis and obtain a thinner membrane. Briefly, the
216 membrane preparation was as follows: 0.1 g of chitosan (310000-375000Da) were
217 dissolved in 25 mL of 1% (v/v) acetic acid, pH was adjusted to 5.0 by the addition of 1M
218 sodium hydroxide and 25 mL of the obtained solution were then added to 0.066 g of
219 Aliquat®336 in a 50 mL volume Falcon® conical tube under continuous stirring. Once
220 homogenized, 20 mL of this solution were poured into glass Petri dishes (90 mm
221 diameter) and subsequently placed in a vacuum stove (35$^{o}$C) until complete evaporation
222 of the solvent. Chitosan films were then washed to prevent membrane swelling during the
223 EME procedure. Water and sodium hydroxide 0.1M were used for the washing step,
224 under gentle agitation on an orbital shaker. Finally, the biopolymeric membrane was

225 introduced again in the vacuum stove until complete dryness. After drying, chitosan films
226 were peeled off the dishes, cut into 5 x 5 mm pieces and glued into glass tubes (2 mm i.
227 d. x 2 cm length) for EME. The chitosan film obtained has a composition of 60% (w/w)
228 chitosan/40% (w/w) Aliquat®336. According to the previous experiments conducted in
229 the above-mentioned work [21], it was demonstrated that Aliquat®336 acts as a
230 plasticizer, providing the membrane with sufficient flexibility to be handled. Further
231 characterization in terms of thickness and homogeneity was carried out by using Scanning
232 Electron Microscopy (SEM) with a Zeiss Auriga (Carl Zeiss Microscopy, Oberkochen,
233 Germany). According to the micrographic images obtained, the chitosan-based films
234 presented an outer side morphology different from the internal one, probably due to the
235 final evaporation of the solvent. Additionally, the thickness of the films was measured
236 from micrographs, being around 10-11 μm (Figure 1a). In this case, the membrane
237 obtained is thinner than the one proposed in previous works [21, 22], which could enable
238 a more efficient passage of the analytes during the EME process. Moreover, a
239 homogeneous distribution of carbon atoms (marked in blue) can be observed throughout
240 the membrane structure (Figure 1b).

241 Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-
242 ATR) was also used to characterize the synthesized films. Figure 2 (green line) shows the
243 obtained spectra from the chitosan membrane by using a Jasco FT/IR-4100 (Jasco
244 Analítica, Madrid, Spain) spectrometer. A wide band can be observed in the 3300 $cm^{-1}$
245 region corresponding to O-H and N-H stretching, which is characteristic of chitosan [23,
246 36]. Typical polysaccharide bands are also observed around 2900 $cm^{-1}$ and 2800 $cm^{-1}$,
247 corresponding to symmetric and asymmetric C-H stretching. Moreover, in the 1800-1300
248 $cm^{-1}$ region several bands appear, being remarkable the presence of a peak at 1641 $cm^{-1}$
249 attributed to residual N-acetyl groups (C=O stretching of amide I and C-N stretching of
250 amide III at 1320 $cm^{-1}$), as well as, N-H bending of amide II at 1530 $cm^{-1}$ and two peaks
251 at 1370 and 1310 $cm^{-1}$, corresponding to $CH_2$ bending and $CH_3$ symmetrical
252 deformations, respectively. The characteristic C-O stretching of the chitosan saccharide
253 structure causes a band at 1060 $cm^{-1}$.

254 3.2. Optimization of EME procedure

255 Different experimental variables governing the development of the extraction
256 procedure were optimized. Both donor and acceptor pH, applied voltage and extraction

time were evaluated. In accordance with previous work [21], volumes of both donor and acceptor phases were fixed at 10 mL and 50 μL, respectively. All assays were carried out using a standard aqueous solution of the target compounds (500 μg $L^{-1}$) as the donor phase.

3.2.1. Influence of applied voltage

Taking into account the importance of the applied voltage on the generated current and, consequently, on the stability of the studied system [37], this parameter was first optimized. Thus, voltages in the range 50-120 V were evaluated, providing a variable electrical current in the range 200-900 μA. The stability of the system was tested for operating currents up to 1000 μA. As can be seen in Figure 3a, voltages in the 100-120 V range provided the best EFs for all compounds, thus both voltages were selected for further experiments.

3.2.2. Influence of pH of acceptor and donor phases

As in all types of electro-assisted extractions, pH of both donor and acceptor solutions constitutes a key experimental factor, since ionic form of the target analytes is often required for an efficient extraction. Thus, according to pKa values of polyphenols (Table S2), pH above 5 is necessary for these compounds to be in their ionic form. Besides, electrolytic generation of $H^+$ and $OH^-$ can occur during EME, affecting the pH of the medium [38]. Therefore, pH of the acceptor phase was first analyzed between 6 and 12 applying both previously selected voltages (100-120 V) for 10 min (for these experiments, pH 6 was set for the donor phase). Above pH 9, the EME system was not stable during the experiments due to the high extraction currents generated (> 1000 μA). For pH 6 to 9, the best results in terms of extraction efficiency, stability and reproducibility, were obtained at pH 9.

On the other hand, pH of donor phase was also studied applying 100 and 120 V (for 10 minutes of extraction time and at fixed pH 9 for acceptor phase). Figure 3b shows the EFs obtained for those experiments when 100 V was applied. It can be observed that for pH>7 EFs decreased for all the analytes or remained practically constant. Similar behavior was observed when the experiments were carrying out applying 120 V. Therefore, pH 7 was set as optimum for the donor phase.

In order to assess the optimal conditions for the extraction, further EME experiments were performed by setting pH 7 in the donor phase and varying the acceptor phase pH between 6 and 9. Best extraction efficiency for all compounds was confirmed at pH 9. Consequently, once the influence of pH was evaluated, pH 9 for acceptor phase and pH 7 for donor phase were set for further experiments.

### 3.2.3. Influence of extraction time

Finally, additional experiments were performed to evaluate the influence of the extraction time in the range of 100-120 V and pH 7 and pH 9 for the donor and acceptor phases, respectively. It was observed (Figure 3c) that the combination of high voltages (>100 V) and times (>15 min) led to a decrease in the extraction efficiency providing a loss of system stability. Furthermore, for extraction times below 5 min no preconcentration of the target analytes in the acceptor phase was observed. Therefore, a voltage of 100 V for 15 min of extraction was set as optimum conditions, providing the following values for the EFs: 131 for GAL, 82 for CLO, 78 for HBA, 75 for CAF, 77 for FER, 118 for BZA, 108 for BZP and 63 for CIN. High preconcentration was obtained with values above 60 for all target analytes. Consequently, it can be stated that chitosan membranes are a good alternative to carry out the EME procedure for polyphenolic compounds.

## 3.3. Characterization of chitosan-based film after EME procedure

The characteristics of the biopolymeric film after performing the proposed EME procedure were also studied. In order to further analyze the physical characteristics of the film, SEM images were obtained once the extraction was completed, where chitosan film appeared slightly thinner (around 8.5-10 μm thickness) after analytes have passed through the membrane (Figure 4a). This could be due to the application of voltage during EME, which leads to a narrowing of the film. On the other hand, film homogeneity does not seem to be altered by the extraction process as shown in Figure 4b.

Additionally, an IR-ATR study was performed. As it can be seen in Figure 2 (blue line), no significant differences can be observed when comparing the spectra of chitosan films before and after the extraction process, and all characteristic peaks previously

described in section 2.2. still remain. This fact might suggest that during the extraction process only hydrogen bonds are formed between chitosan and polyphenolic compounds, which do not cause significant structural changes in the biopolymeric membrane [23]. These results ensure the stability of the membrane during the EME procedure, as its chemical structure remains unaltered, which could allow the reuse of the extraction device, providing an additional advantage of the chitosan biomembrane in EME. Indeed, current studies are focusing on the use of the membrane for consecutive extractions under different conditions.

### 3.4. Validation of the proposed EME method

Analytical performance of the proposed method was carried out by evaluating the figures of merit, which include linearity, precision (repeatability, intermediate precision), sensitivity (LOD, limit of quantitation (LOQ)) and accuracy (effective recovery (ER)). Validation data are depicted in Table 1 and Table 2.

An external calibration was performed applying the proposed procedure, since no matrix effect was observed. Linear ranges between 123.6-1000 µg $L^{-1}$ for GAL, 120.4-1000 µg $L^{-1}$ for CLO, 54.9-1000 µg $L^{-1}$ for HBA, 86.1-1000 µg $L^{-1}$ for CAF, 121.0-1000 µg $L^{-1}$ for FER, 92.6-1000 µg $L^{-1}$ for BZA, 58.7-1000 µg $L^{-1}$ for BZP and 52.8 µg $L^{-1}$ for CIN, as well as % linearity above 97% for all compounds. LOD and LOQ were calculated as three- and ten-times signal-to-noise ratio, respectively [39]. Values between 15.9 µg $L^{-1}$ and 37.1 µg $L^{-1}$ for LOD and between 52.8 µg $L^{-1}$ and 123.6 µg $L^{-1}$ for LOQ were obtained, corresponding to CIN and GAL, respectively.

Repeatability values ranging from 4 to 10% RSD were obtained by applying the EME procedure to fortified food samples at three concentration levels. All measurements were performed on a single day. In the case of intermediate precision, measurements were obtained three days per week during four weeks, providing RSD% ranging from 8 to 13%.

Recovery tests were performed to assess the accuracy of the proposed analytical procedure due to the lack of blank samples. The analyses were carried out by determining (in triplicate) the polyphenolic compounds in non-spiked and spiked samples at three concentration levels. These fortification levels were selected according to the usual levels

of each compound in these kind of samples. Adequate dilution were applied in order to cover the entire linearity range. ER values were calculated as follows:

$$ER\ (\%) = \frac{C_f - C_i}{C_a} x100$$

where $C_f$ is final concentration in the acceptor phase extract, $C_i$ is the initial concentration in the sample and $C_a$ is the concentration added to the sample.

Table 2 shows the corresponding ER% for each fortification level according to the required dilution for each sample. Values ranging from 95.0% to 110.9% were obtained for all samples analyzed. Therefore, it can be stated that the proposed EME procedure is suitable for the extraction of polyphenolic compounds in food samples.

### 3.5. Analysis of food samples

The proposed EME method was applied to the analysis of selected polyphenols in 10 different samples described in section 2.5.

The aqueous extracts obtained were submitted to the EME procedure and the polyphenolic content found in the samples analyzed was as follows: in both coffee samples, only CLO was found, with a higher amount determined in *robusta* coffee (3.81% (w/w)) compared to *arabica* coffee (2.91% (w/w)). In both tea samples, CLO (0.04 and 0.05% (w/w), and GAL (0.09 and 0.11% (w/w)), were determined, whose values are in accordance with those reported in the literature [40, 41].

In the case of food supplements, CLO was determined in all samples in the range 9.15% - 0.06% (w/w). These values are consistent with the fact that these dietary supplements are based on green coffee and tea extracts and are in accordance with the percentage marked on the labeling. Additionally, a high amount of BZA (1.43% (w/w)) was found only in sample FS2. Indeed, the label of this supplement also indicates the presence of raspberry, which would explain the high amount of BZA found in this sample [42].

Representative chromatograms of non-spiked and spiked coffee, food supplements and tea samples are provided in the Supplementary Material (Figures S2-S5). Excellent baselines and a good resolution between peaks are observed in all cases.

### 3.6. Comparison with other microextraction methods

The greenness of the proposed chitosan-based EME procedure has been evaluated and compared with other previously reported microextraction methods for the determination of polyphenols [43-52] by means of AGREEprep metric tool. As can be seen from the assessment results depicted in Figure 5, with the exception of gel electromembrane extraction (G-EME) [52] and the current work, most of the extraction procedures have a final score below 0.5. Among them, vortex-assisted dispersive liquid-liquid microextraction (VA-DLLME) [43] seems to be the least green method with a final score of 0.14, mainly due to the high volumes of hazardous solvents and reagents (criteria 2 and 10) and the excessively long extraction time (criterion 6) with a multitude of consecutive steps (criterion 7) and energy-intensive instrumentation (criterion 8). A higher score ranging from 0.22 to 0.28 is given to methods based on in-vial liquid–liquid microextraction (IV-LLME) [44], dispersive liquid-liquid microextracion (DLLME) [45, 46], ultrasonic-assisted extraction coupled to dispersive liquid-liquid microextraction (UAE-DLLME) [47] and ultrasonic-assisted liquid–liquid microextraction based on deep eutectic solvent (UALLME-DES) [48] for different reasons related to: (i) the subsequent instrumentation used (criterion 9) [44]; (ii) the amount of waste generated (criterion 4) [47]; (iii) the energy demand (criterion 8) and (iv) sample throughput (criterion 6) [45, 46] or (v) to a combination of several factors [48].

On the other hand, the reduction in the amount of sample and reagents and, consequently, waste generated, means that techniques such as hollow-fiber liquid phase microextraction (HF-LPME) [49], solid phase microextraction (SPME) [50] or single-drop microextraction (SDME) [51] score between 0.42 and 0.43 overall. However, despite the reduction in the volume of organic solvents, their use is still not completely eliminated due to the characteristics of the material used (which requires an organic phase as an extractant) or the derivatization step required for subsequent determination by gas chromatography (GC). Furthermore, these techniques use plastic materials such as disposable PP [49] or polyacrylate (PA) [50] fibres, which have a significant environmental impact (criterion 3).

In this sense, the development of solvent-free procedures, as is the case with G-EME [52] and the present method, is a great advantage, since it is also the most important criterion, with an assigned weight of 5 and is therefore the main reason why both

procedures score above 0.5. Moreover, the greenness of sample preparation is greatly enhanced by the use of sustainable and renewable materials, such as biopolymer-based materials (criterion 3). Agarose gels and chitosan films meet this requirement as they are biodegradable and easily available materials from renewable sources. Among both procedures, the proposed method based on chitosan films constitute the greenest approach with a final score of 0.7. Some advantages include the use of a minimal amount of sample (as dilution is required) and, consequently, of waste generated (a few microliters), as well as the number of hazardous labelling chemicals being reduced to one. Moreover, by comparing the analytical performance of both methods as shown in Table 3, it is observed that the proposed EME procedure provides a much higher pre-concentration (5- to 14-fold higher) than G-EME. In addition, one of the main drawbacks of agarose gels lies in the so-called electroosmotic flow (EOF) phenomenon [53], which causes changes in the volumes of the donor or acceptor solutions, depending on its direction. Indeed, particularly in this G-EME work [52], authors observed a decrease in the volume of acceptor phase from 100 to 90 µL under optimal extraction conditions due to this effect. This could introduce instability into the system and is directly related to the extraction efficiency, thus the reduction or even removal of EOF is highly desirable and constitutes, currently, one of the challenges of agarose gels for EME purposes. In contrast, chitosan membranes as thinner biopolymeric films avoid this effect, proving to be stable during EME, as well as maintaining their structure practically constant after extraction.

Finally, a common aspect of all the methods assessed here is that they do not meet criterion 1, which favors *in situ* sample preparation. However, in general, this is hardly fulfilled, and is therefore considered the least important factor with weighting of 1.

## 4. Conclusions

Chitosan films have been successfully used as a membrane for the EME of polyphenolic compounds from different coffee- and tea-based dietary supplements. An important novelty reported in this work is the absence of organic solvent during the extraction process, usually required in this type of methodology. Here, the biopolymeric chitosan membrane acts both as a barrier between the donor and acceptor aqueous solutions, and as an extracting material with active participation in the EME. The AGREEprep metric tool software has been applied for the first time in EME to evaluate

the greenness of the proposed method, proving to be the most environmentally friendly approach for the determination of phenolic acids compared to previous works. In addition to its environmental advantages, the proposed chitosan-based EME method exhibits excellent analytical performance. EME procedure is carried out in 15 minutes providing high preconcentration and sensitivity, wide linearity ranges and low LOD and LOQ values. Moreover, accuracy of the method was demonstrated by the analysis of fortified samples.

Biodegradable chitosan films constitute, therefore, an efficient and attractive alternative as tailor-made membranes for EME purposes compared to commercial or plastic materials traditionally used as supports. Furthermore, this kind of biomembrane might be employed as a versatile platform to build up a sample-specific pretreatment methodology based on target analytes.

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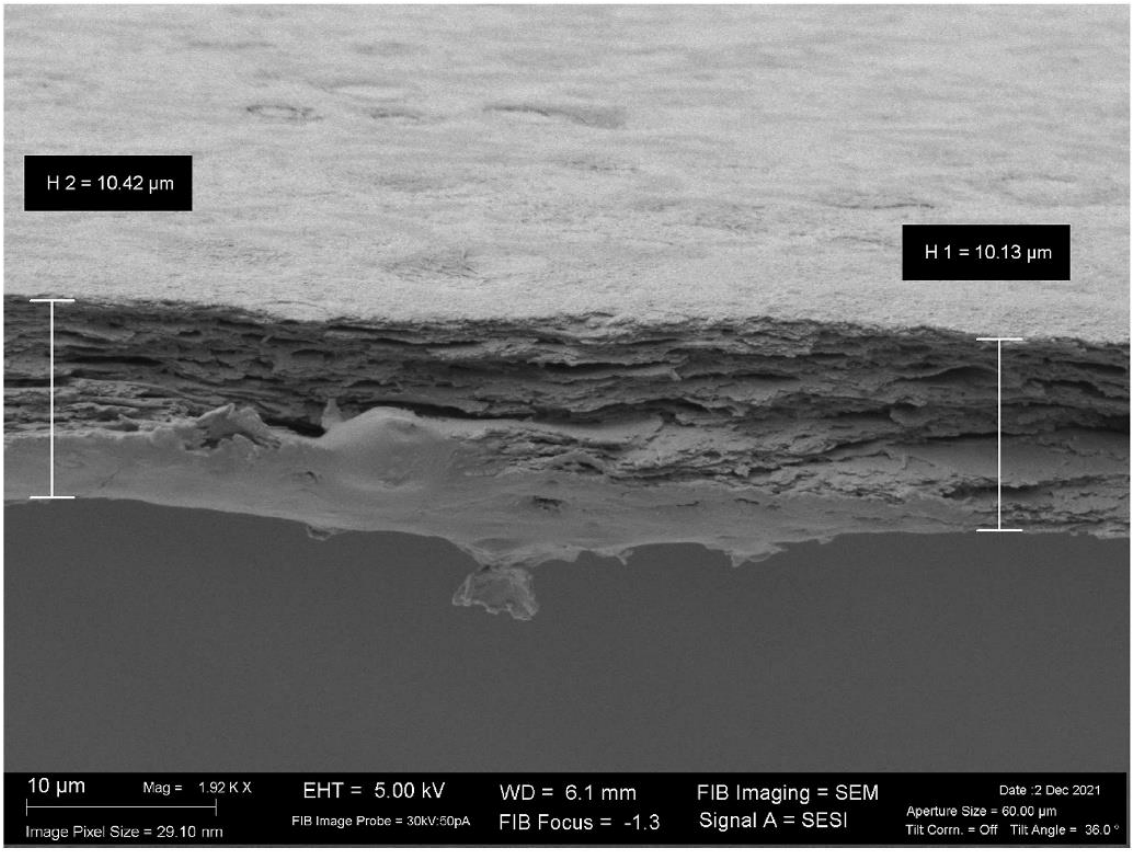
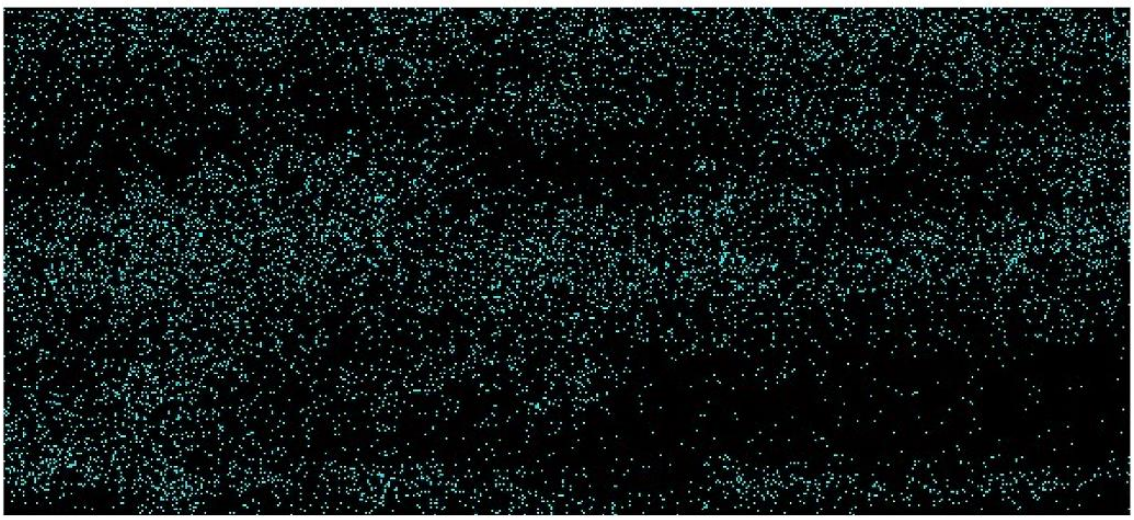
Figure 1. SEM images of a cross-section of chitosan membrane (see text for moredetails). (a) Thickness measurement, (b) Homogeneous distribution of the chitosan membrane structure.

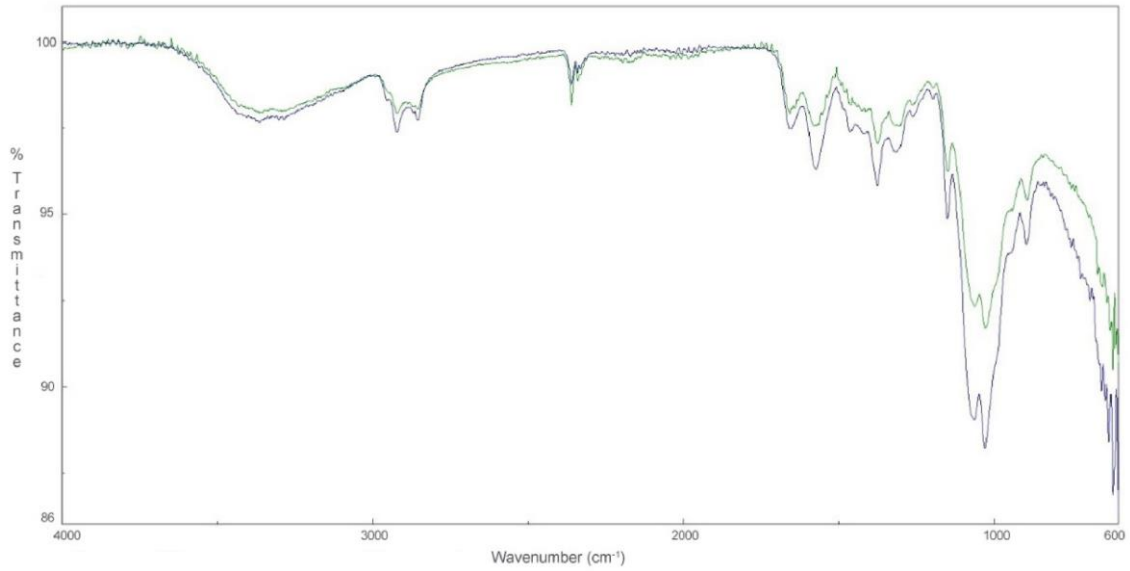



**(a)**

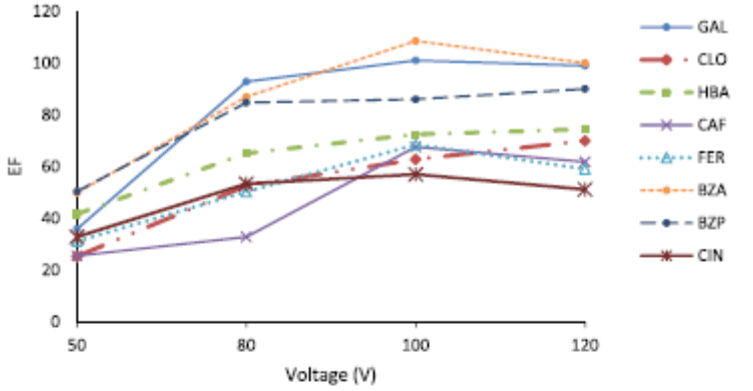


**(b)**

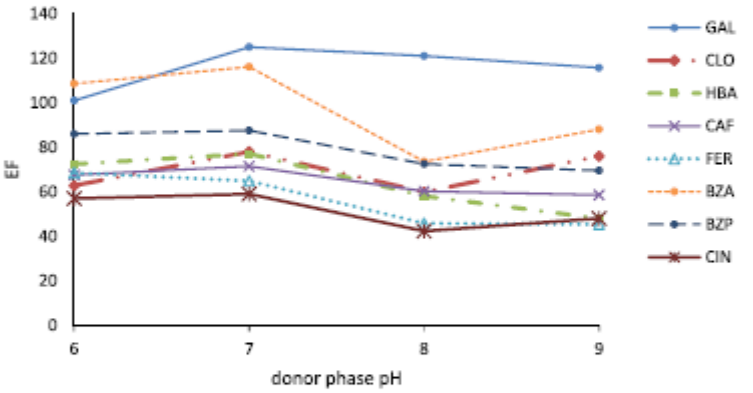
Figure 2. FTIR-ATR spectra of chitosan membrane before EME (green line) and after EME (blue line).



669 Figure 3. Fig. 3. Influence of experimental variables in EME efficiency. (a) Influence of
670 voltage (10 min), (b) Influence of donor phase pH (100 V, 10 min), (c) Influence of
671 extraction time.




(a)




(b)

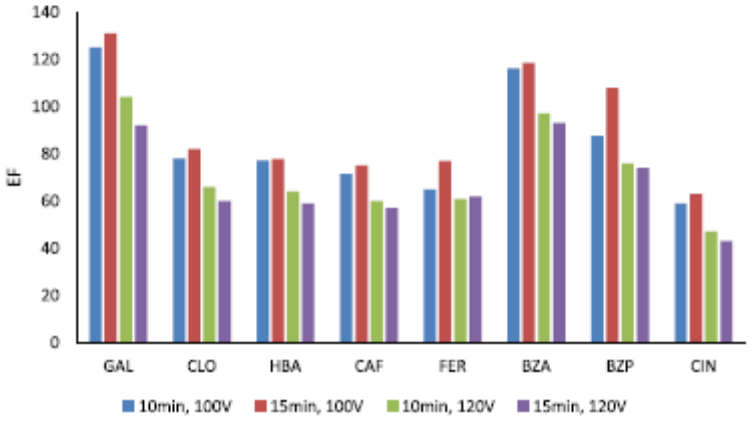



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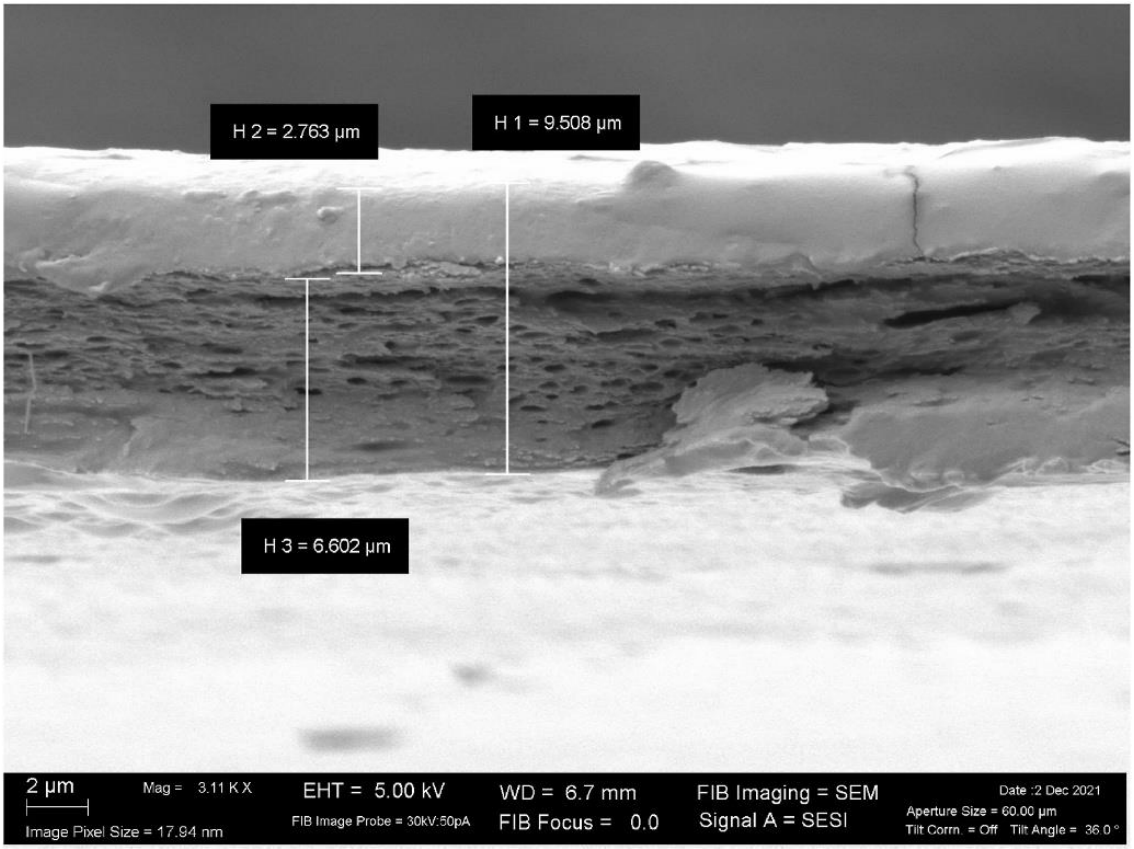
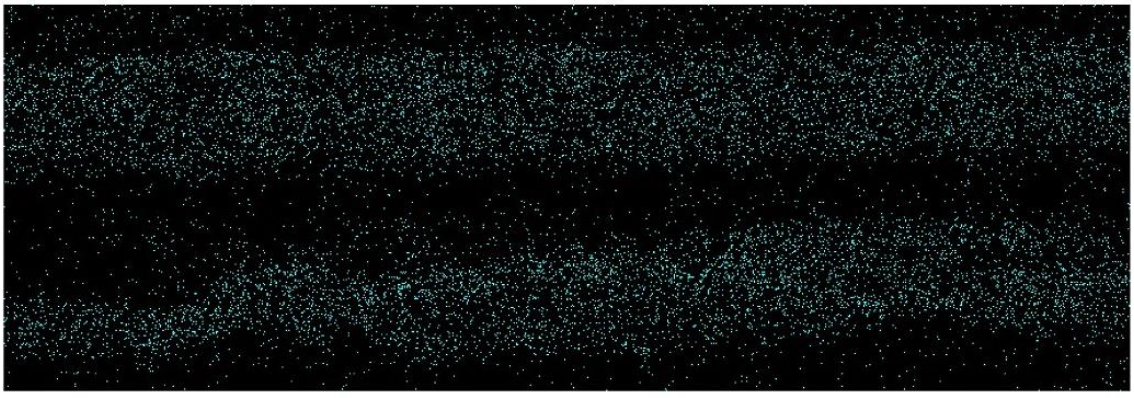
Figure 4. SEM images of a cross-section of chitosan membrane after EME (see text formore details). (a) Thickness measurement, (b) Homogeneous distribution of the chitosan membrane structure.




**(a)**



**(b)**

Figure 5. Assessment results with AGREEprep of the microextraction methods for determination of phenolic acids: (a) VA-DLLME [43]; (b) IV-LLME [44]; (c) DLLME [45]; (d) DLLME [46]; (e) UAE-DLLME [47]; (f) UALLME-DES [48]; (g) HF-LPME [49]; (h) SPME [50]; (i) SDME [51]; (j) G-EME [52]; (k) This work. Full evaluation reports for each procedure are available on the Supplementary Material (Reports S1-S11).

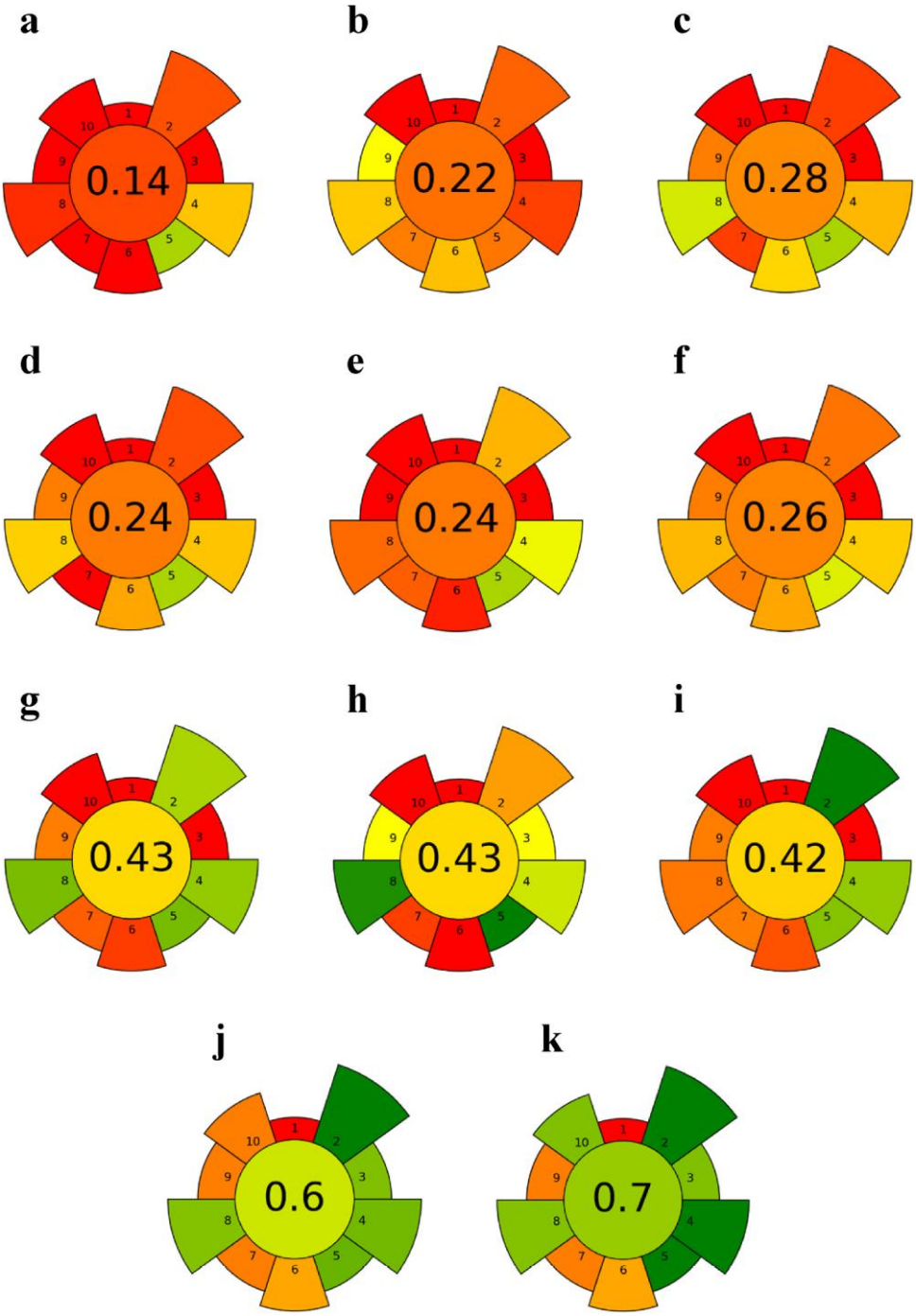


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**Table 1**
Analytical parameters for the proposed EME method.

| Analyte | Linear range (μg $L^{-1}$) | LOD (μg $L^{-1}$) | LOQ (μg $L^{-1}$) | Linearity | |
|---|---|---|---|---|---|
| | | | | (%) | ($R^2$) |
| GAL | 123.6–1000 | 37.1 | 123.6 | 97.7 | 0.9963 |
| CLO | 120.4–1000 | 36.1 | 120.4 | 97.9 | 0.9971 |
| HBA | 54.9–1000 | 16.5 | 54.9 | 98.3 | 0.9966 |
| CAF | 86.1–1000 | 25.8 | 86.1 | 98.1 | 0.9979 |
| FER | 121.0–1000 | 36.3 | 121.0 | 97.5 | 0.9969 |
| BZA | 92.6–1000 | 27.8 | 92.6 | 97.6 | 0.9961 |
| BZP | 58.7–1000 | 17.6 | 58.7 | 98.4 | 0.9973 |
| CIN | 52.8–1000 | 15.9 | 52.8 | 98.5 | 0.9964 |

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**Table 2**
Effective recoveries (%ER) of polyphenols in the analysis of spiked food samples.

| Sample | Dilution | Spiked concentration ($mg\ L^{-1}$) | Effective recovery (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | GAL | CLO | HBA | CAF | FER | BZA | BZP | CIN |
| FS1 | 1:500 | 125 | 95.3 | 95.1 | 96.9 | 97.7 | 98.0 | 99.7 | 96.4 | 99.1 |
| | | 200 | 97.1 | 98.0 | 98.9 | 98.2 | 97.1 | 98.8 | 96.9 | 97.8 |
| | | 300 | 97.7 | 96.2 | 97.4 | 103.2 | 99.1 | 99.8 | 98.1 | 98.6 |
| FS2 | 1:200 | 50 | 99.9 | 97.3 | 107.8 | 98.4 | 99.0 | 110.4 | 98.6 | 102.6 |
| | | 80 | 99.3 | 99.1 | 100.1 | 99.1 | 99.3 | 107.7 | 99.3 | 99.0 |
| | | 120 | 106.3 | 98.1 | 99.5 | 99.9 | 104.2 | 106.4 | 99.8 | 100.9 |
| FS3 | 1:500 | 125 | 95.7 | 95.0 | 97.0 | 98.0 | 97.8 | 99.6 | 98.7 | 100.2 |
| | | 200 | 97.6 | 97.8 | 98.7 | 98.0 | 97.3 | 98.7 | 99.1 | 98.3 |
| | | 300 | 97.3 | 96.9 | 99.1 | 97.4 | 98.6 | 99.9 | 98.3 | 99.2 |
| FS4 | 1:200 | 50 | 100.2 | 97.0 | 101.5 | 98.8 | 98.7 | 103.6 | 98.4 | 101.3 |
| | | 80 | 99.5 | 99.4 | 99.9 | 99.0 | 98.9 | 101.8 | 98.9 | 99.3 |
| | | 120 | 98.8 | 98.4 | 98.1 | 102.1 | 99.9 | 99.4 | 99.7 | 98.8 |
| FS5 | 1:500 | 125 | 95.4 | 95.6 | 96.8 | 97.9 | 98.0 | 99.5 | 98.4 | 99.8 |
| | | 200 | 97.8 | 97.3 | 98.1 | 98.2 | 97.7 | 98.1 | 98.9 | 98.6 |
| | | 300 | 95.9 | 96.6 | 97.4 | 99.1 | 96.7 | 95.3 | 97.1 | 98.4 |
| FS6 | 1:200 | 50 | 100.6 | 97.4 | 99.9 | 98.9 | 98.9 | 102.6 | 98.5 | 100.8 |
| | | 80 | 99.6 | 99.6 | 99.4 | 98.8 | 99.2 | 100.9 | 99.0 | 99.7 |
| | | 120 | 96.1 | 95.4 | 97.9 | 96.7 | 98.2 | 98.7 | 96.9 | 97.8 |
| CS1 | 1:400 | 100 | 99.7 | 100.1 | 98.4 | 96.3 | 98.0 | 97.2 | 96.1 | 99.3 |
| | | 160 | 97.1 | 99.7 | 95.9 | 97.9 | 96.6 | 98.4 | 97.3 | 100.7 |
| | | 240 | 96.8 | 98.8 | 97.2 | 98.0 | 95.9 | 97.0 | 98.4 | 99.0 |
| CS2 | 1:400 | 100 | 99.4 | 97.8 | 96.1 | 98.1 | 97.3 | 96.9 | 95.9 | 98.9 |
| | | 160 | 98.9 | 97.0 | 95.9 | 96.2 | 97.9 | 98.2 | 97.1 | 99.6 |
| | | 240 | 100.4 | 98.4 | 97.3 | 96.1 | 98.2 | 97.0 | 96.9 | 102.1 |
| TS1 | 1:40 | 10 | 108.5 | 99.3 | 97.2 | 99.0 | 99.1 | 97.9 | 98.9 | 98.0 |
| | | 16 | 110.9 | 98.7 | 99.4 | 98.5 | 98.9 | 98.2 | 98.1 | 99.7 |
| | | 24 | 104.1 | 99.9 | 98.7 | 98.8 | 99.4 | 98.0 | 99.3 | 97.9 |
| TS2 | 1:40 | 10 | 100.1 | 99.7 | 98.3 | 96.9 | 99.2 | 97.4 | 99.3 | 98.2 |
| | | 16 | 99.8 | 99.9 | 99.0 | 97.2 | 99.7 | 99.4 | 98.9 | 100.3 |
| | | 24 | 99.1 | 105.1 | 103.3 | 99.8 | 98.8 | 99.9 | 97.4 | 99.0 |

FS: food supplement; CS: coffee sample; TS: tea sample.

**Table 3**
Comparison of the proposed EME method with reported microextraction techniques for the determination of phenolic acids in food matrices.

| Extraction method | Analytes | Extraction organic solvent/device | Extraction conditions | Determination | Extraction efficiency | | LOD ($\mu g\ L^{-1}$) | Matrix | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | EF | R (%) | | | |
| VA-DLLME | GAL, PRO, VAN, SYR, FER | ACN (extractant, 2x493 μL); Ethyl acetate (disperser, 2x1185 μL) | DP: pH 3.92 (HCl)<br>Ultrasound 60min (2 × 30min), centrifuged (2x), vortexed 1min (2 × 30s) | UPLC-QqQ/MS | – | 88.29–95.76 | 0.01–0.3 | Blueberry | [43] |
| IV-LLME | BZA, CAF, CIN, CLO, DHBA, FER, GAL, HBA, *m*-COU, *o*-COU, *p*-COU, SYR, VAN | MeOH:5 mM NaOH (60:40, v/v, extractant, 2x300 μL); *n*-hexane (diluent, 2000 μL) | Vortexed 2min (2 × 1min), centrifuged 10min (2 × 5min), 4000 rpm | CE-UV | – | 88–100 | 17–160 | Vegetable oils | [44] |
| DLLME | CAF, FER, *p*-COU, SYR, VAN | $CHCl_3$ (extractant, 450 μL); $Me_2CO$ (disperser, 750 μL) | DP: pH 2 (HCl), $(NH_4)_2SO_4$ (3 g)<br>Centrifuged 5min, 6000 rpm | HPLC-UV | – | 23.7–94.2 | 1.5–12.0 | Honey | [45] |
| DLLME | CAF, CLO, FER, GAL, *p*-COU, PRO, SYR, VAN | Ethyl acetate (extractant, 1000 μL); ACN (disperser, 500 μL) | Vortexed 20s (2 × 10s), centrifuged 10min (2 × 5min), 5400 *g* | HPLC-UV | – | 76.56–126.87 | 1–10 | Wine | [46] |
| UAE-DLLME | CAF, CIN, CLO, FER, GAL, HBA, NCLO, SYR | UAE: 80% (v/v) EtOH (20 $\mu g mL^{-1}$ DFA[a]), 30min<br>DLLME: *n*-octanol (extractant, 60 μL); EtOH (disperser, 800 μL) | DP: pH 5 (HCl)<br>Ultrasound 10min, centrifuged 5min, 4000 rpm | UPLC-ESI-MS/MS | 162–194 | – | 25.1–103.5 | Fruit juices | [47] |
| UALLME-DES | CAF, CIN, FER | DES: choline chloride/ethylene glycol (1:2) (extractant, 50 μL); *n*-hexane (diluent, 2000 μL) | Ultrasound 5min, 30 °C, centrifuged 10min, 3000 rpm | HPLC-UV | ~40–55 | – | 0.39–0.63 | Vegetable oils | [48] |
| HF-LPME | CAF, CIN, FER, GAL, HBA, *p*-COU, SYR | Hexyl acetate<br>PP fibre | DP: pH 2 ($H_2SO_4$)<br>AP: pH 12 (NaOH), 30% NaCl[b]<br>30min, 1200 rpm | HPLC-UV | 15–408 | – | 0.01–2.0 | Fruit juices | [49] |
| SPME | CAF, FER, GAL, *p*-COU, PRO, SYR, VAN | PA fibre (85 μm) | Pre-derivatization 6min (150 μL MCF in 1 mL ACN/H2O/MeOH/Pyr 14:10:2:3)<br>60min extraction (50 + 10min-desorption), 500 rpm, 25 °C | GC-FID | – | – | 10–1177 | Not applied | [50] |
| SDME | CAF, CIN, HBA, *o*-COU | Hexyl acetate (2.5 μL) | DP: pH 2 (HCl), 0.3 g $mL^{-1}$ NaCl<br>AP: Hexyl acetate[c]<br>20min, 300 rpm, 22 °C<br>Post-derivatization 10min (0.7 μL BSA) | GC-MS | 5–55 | – | 0.6–164 | Fruits and fruit juices | [51] |
| G-EME | BZA, CAF, CIN, DNBA, GAL, HBA, SAL | SLM-free<br>Agarose gel | DP: pH 6 (HCl)<br>AP: pH 12 (NaOH)<br>25 V, 15min, 750 rpm | HPLC-UV | 9.4–26.0 | 26.8–74.4 | 3.0–15.2 | Fruit juices | [52] |
| EME | BZA, BZP, CAF, CIN, CLO, FER, GAL, HBA | SLM-free<br>Chitosan membrane | DP: pH 7<br>AP: pH 9 (NaOH)<br>100 V, 15min, 600 rpm | HPLC-UV | 63–131 | 31.5–65.5 | 15.9–37.1 | Coffee and tea-based food supplements, tea leaves, green coffee beans | This work |

Abbreviations: PRO, protocatechuic acid; VAN, vanillic acid; SYR, syringic acid; DP, donor phase; UPLC-QqQ/MS, ultra performance liquid chromatography–triple quadrupole mass spectrometry; DHBA, 2,4-dihydroxybenzoic acid; *m*-COU, *m*-coumaric acid; *o*-COU, *o*-coumaric acid; *p*-COU, *p*-coumaric acid; CE-UV, capillary electrophoresis with ultraviolet detection; NCLO, neochlorogenic acid; UPLC-ESI-MS/MS, ultrahigh-pressure liquid chromatography-electrospray ionization-tandem mass spectrometry; AP, acceptor phase; MCF, methyl chloroformate; Pyr, pyridine; GC-FID, gas chromatography coupled to a flame-ionization detector; BSA, N,O-bis(tri-methylsilyl)acetamide; GC-MS, gas chromatography-mass spectrometric detection; DNBA, 3,5-dinitro benzoic acid.

[a] DFA: deuterated ferulic acid as IS.

[b] 4-fluorobenzoic acid in MeOH as IS.

[c] Containing 4,4′-dibromobiphenyl as IS.

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