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Fibreglass membrane chemically modified with amino-functionalised SBA-15 and its application in solid-phase extraction to determine macrolide antibiotics in eggs

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ABSTRACT

Macrolides are a group of high-spectrum antibiotics used to treat animal ailments, sometimes these drugs can appear as residues in animal foods such as eggs, which must be controlled as required by the European Union. Therefore, in this paper a methodology based on solid–liquid extraction followed on the application of a new membrane-solid-phase extraction (membrane-SPE) was developed and validated for the extraction and purification of five macrolide antibiotics such as spiramycin (SPI), erythromycin (ERY), roxithromycin (ROX), josamycin (JOS) and tylosin (TYL), in egg samples for their analysis by high-performance liquid chromatography coupled to mass spectrometry analysis (HPLC-MS/MS). The newly synthesised membrane-based sorbent consisted of a fibreglass membrane chemically modified with silica SBA-15 type functionalised with amino groups (FGM-SBA-15-NH_2). The optimal conditions for membrane-SPE on eggs were determined using a three-factor, three-level Box Behnken design (BBD). The conditions obtained were by loading with 4 mL of water and eluting with 3 × 2.5 mL of MeOH with ammonia solution (0.5 %, *v/v*). The methodology was validated according to Regulation (EU) 2021/808 showing good selectivity, linearity ($R^2 \geq 0.993$), low limits (MQL 1.1–2.1 ng/g, MDL 0.3–0.6 ng/g), acceptable decision limit for confirmation (CCα), good accuracy (recoveries 85–100 %) and an adequate precision (RSD ≤ 16 %). The FGM-SBA-15-NH_2 proved to be reproducible in different batches and reusable around 70 times. Finally, the validated method was successfully applied to fourteen egg samples detecting SPI in one hen egg sample and JOS and ROX in one quail egg sample. The proposed methodology introduces a groundbreaking membrane-SPE approach, addressing limitations in conventional SPE methods. The use of functionalised silica to chemically modify FGM significantly improves its textural properties, allowing the simultaneous extraction and purification of macrolide antibiotics from egg samples.

1. Introduction

Macrolides are a group of bacteriostatic antibiotics discovered in the 50 s and used over the years to treat respiratory and intestinal infections in livestock and poultry, as they are effective against a wide range of bacteria, including Gram-positive and some Gram-negative bacteria [1,2]. This family of antibiotics produced by *Streptomyces* species contains a large macrocyclic lactone ring to which one or more sugars are attached [3,4]. The most commonly used macrolides antibiotics contain 14, 15 or 16 carbon atoms in the lactone ring, such as erythromycin (ERY), roxithromycin (ROX), spiramycin (SPI), josamycin (JOS), or tylosin (TYL) [4]. The concern about these drugs is that their excessive or incorrect use in livestock and poultry may lead to residues in animal foodstuffs that can cause undesirable effects in humans, such as the development of allergies, resistant bacteria, etc. [2,4,5]. In fact, these antibiotics have been found in various food matrices such as eggs [6–10], milk and dairy products [11,12], honey [11] muscle tissues and meat [13,14]. For this reason, starting from the end of 2009, the

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European Union (EU) has implemented Regulation 37/2010 [15], which establishes maximum residue limits (MRLs) for pharmacologically active substances. This includes macrolide antibiotics present in matrices of animal origin, such as muscle, fat, liver, kidney, milk, and eggs.

The poultry industry is increasingly in demand [16], specially eggs as a source of high biological value protein, which makes it easier to spread poultry diseases on farms due to the excessive number of animals. This leads to an increased use of antibiotics in poultry, such as macrolides [17]. Also, these antibiotics can be used for prophylactic purposes or as growth promoters, although this practice was banned in 2003 [16,18]. TYL and ERY are specially used in the poultry industry because TYL is used to treat mycoplasma diseases or ERY is used for the treatment of arthritis caused by *Staphylococcus aureus* [17]. Therefore, these two macrolides were regulated in egg matrices, with a maximum residue limit set at 200 ng/g for TYL and at 150 ng/g for ERY [15].

High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) has been the main analytical technique used for macrolide determination in egg samples [6,19,20], as recommended by Regulation (EU) 2021/808 [21]. For sample preparation, dilution [22], protein precipitation with heat [23] and other protocols based on solid–liquid extraction (SLE) [6,19], ultrasonic extraction, solid-phase extraction (SPE) [7,19,20], matrix solid-phase dispersion (MSPD) [19], QuEChERS and modified QuEChERS procedure [10,19] or ultrasonic assisted extraction (UAE) combined with SPE [24] have been developed. However, in the last years, new materials, such as materials based on carbon, silica, magnetic nanoparticles or molecular printing polymers, are being highly applied in the preparation of food samples to improve the analysis of organic contaminants. They are mainly used in packed form as sorbents in cartridges for SPE, showing important advantages in comparison with commercial sorbents [25]. In recent years, new membrane-based sorbents have been developed as an alternative to the conventional SPE cartridges, such as molecularly imprinted membranes (MIMs) [26,27], carbon-based membranes exclusively made of carbon nanomaterials and polysaccharide membranes that contain dispersed carbon nanomaterials [28]. For the time being, methodologies applying MIMs have mainly been developed for other families of antibiotics, such as β-lactams [29], quinolones [30], sulfonamides [31] or glycopeptide antibiotics [32]. For example, Zhao et al. [30] prepared MIMs by modifying printed nanocomposites with the quinolone norfloxacin on regenerated cellulose membranes. Nevertheless, only one work has been focused on MIMs applied for the selective extraction of ERY and SPI in milk [33]. An alternative to the previously developed membrane-based sorbents could involve modifying fibreglass membranes (FGMs) with functionalized silica. In this sense, mesostructured silica such as MCM-41 have already been successfully applied as sorbent in SPE for the extraction and purification of macrolides in honey and bovine milk [11]. However, the issue with packed silicas is that it can be challenging to reuse the cartridge. Between protocols, when the sorbent dries out, problems may arise, such as the development of preferential channels that decrease recovery rates, preventing cartridge reuse and generating waste. Nevertheless, the inclusion of mesostructured silicas in FGMs can enhance the membrane's textural properties, resulting in benefits like increased surface area and pore volume [25]. Additionally, this integration helps mitigate conventional issues associated with SPE, such as cartridge clogging and the formation of preferential channels. As well as the introduction of organic ligands may confer a more efficient and simultaneous extraction of different macrolide antibiotics from samples. However, to the best of our knowledge, there are no described modified FGMs for the extraction of contaminants from food samples.

Accordingly, this work aimed to chemically modified a FGM with SBA-15 mesostructured silica functionalised with amino (NH_2) groups. This modified membrane was designed for use as a novel membrane-based sorbent, specifically for the simultaneous extraction and purification of different macrolide antibiotics from egg samples. The membrane-SPE protocol employing the SBA-15-NH_2-functionalised membrane (named FGM-SBA-15- NH_2) for the extraction and purification of SPI, ERY, ROX, JOS and TYL was optimised using a Box-Behnken response surface design. Subsequently, the analysis of these five antibiotics was carried out by HPLC-MS/MS with a triple quadrupole analyser. This work suggests a new membrane-based sorbent and a new membrane-SPE device with remarkable advantages compared to conventional SPE cartridges.

2. Materials and methods

2.1. Chemicals and reagents

Five macrolide antibiotics were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibiotics were SPI, ERY, ROX, JOS and TYL, all standard analytical grade. Stock standards solutions (1000 mg/L) were prepared by diluting 10 mg of each macrolide antibiotic in acetonitrile (ACN) and stored at −20 °C in darkness. Standard working solutions containing both antibiotics were prepared at the desired concentrations in methanol (MeOH) and stored in the dark in the freezer (−20 °C).

Solvents LC-MS grade such as ACN, MeOH and ethanol (EtOH) were purchased from Scharlab (Barcelona, Spain). Formic acid (FA, 99 % Optima™) LC-MS grade used as an additive for mobile phases was obtained from Fisher Chemical (Madrid, Spain). Reagents such as tetraethylorthosilicate (TEOS, 98 %, 208.3 g/mol, 0.93 g/mL), poly(ethylene − glycol) − *block* − poly (propylene − glycol) − *block*-poly (ethylene − glycol) (EO20PO70EO20, Pluronic 123, 5800 g/mol, 1.019 g/mL), 3-aminopropyl-triethoxysilane (≥98 %, 221.37 g/mol, 0.946 g/mL), (3-chloropropyl)triethoxysilane (95 %, 240.70 g/mol, 1.000 g/mL) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (37 %, 36.45 g/mol, 1.19 g/mL), acetic acid (HAc) and solvents used in the synthesis such as toluene, EtOH, acetone and diethyl ether were purchased from Scharlab (Barcelona, Spain). Ultra-pure hydrophilic FGM (25 mm, 1 μm) and swinnex filter holder (polypropylene device and silicone seal) were purchased from Análisis Vínicos (Tomelloso, Spain). Scharlab ExtraVac® vacuum manifold (12-port model) used for swinnex filter holder coupling were obtained from Scharlab (Barcelona, Spain). Nylon syringe filters (0.45 μm, 13 mm) used to filter the egg samples were obtained from Mervilab (Madrid, Spain). The Milli-Q H_2O used in this work was obtained from a Millipore Milli-Q-System (Billerica, MA, USA). The resistivity of Milli-Q H_2O was 18.2 MΩ cm.

2.2. Samples

For this study, ten different types of chicken eggs and four different quail eggs were collected for the analysis. Samples were purchased from local markets in Madrid (Spain). Also, samples were collected from smallholdings in Madrid, Toledo and Cáceres (Spain). Table S1 (Supplementary material) shows additional information for the samples analysed. All samples were stored in a refrigerator at 5 °C until analysis. Each sample was analysed in triplicate.

2.3. Chemical modification of the membrane

The chemical modification of the hydrophilic FGM was carried out in three steps, as shown in Fig. 1.

2.3.1. Silanisation of the FGM

First, a FGM was dried for 2 h in a vacuum line in a sand bath at 60 °C. After 2 h, the silinisation agent was added. For this purpose, a 5 % (*v*/*v*) solution of 3-chloropropyltriethoxysilane in dry toluene was added and the reaction started at 80 °C with magnetic agitation (200 rpm for 48 h). Then, the solvent used in the synthesis was removed and silanised FGM was washed twice with dry toluene and acetone and dried.

2.3.2. Synthesis of SBA-15 and functionalisation with amino groups

The synthesis of SBA-15 was prepared following the work of Zhao

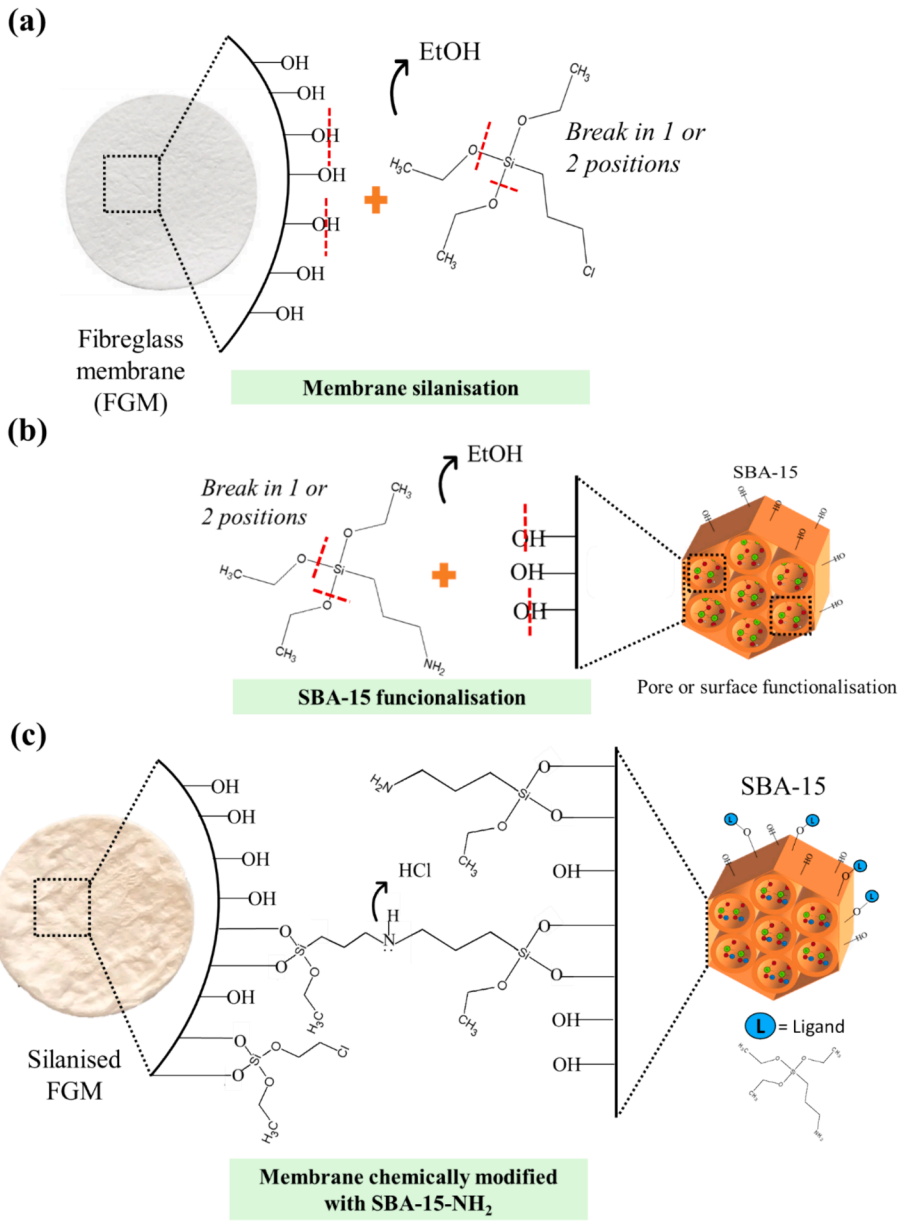


Fig. 1. Schematic representation of the synthesis of the functionalised membrane (FGM-SBA-15-NH_2) (a) membrane silanisation, (b) SBA-15 silica functionalisation with amino (NH_2) groups and (c) membrane chemically modified with SBA-15-NH_2.

et al. [34]. Briefly, 19.4 g of pluronic 123 (P123) were dissolved in 576 mL of HCl (2 M) and 144 mL of Milli-Q H_2O. The dissolution was stirred at 400 rpm and 35 °C. Afterwards, 40.8 g of TEOS was added dropwise and left to stir for 20 h. After that time, the stirring was stopped, the temperature was raised to 80 °C and left for 24 h (ageing process). After, it was filtered and washed with distilled water to remove the surfactant (P123). The material was then air-dried, transferred to a porcelain dish and calcined (8.5 h ramped up to 500 °C and then held for 12 h at 500 °C).

Bare SBA-15 silica was functionalised with NH_2 groups according to previous work [35]. First, 4 g of SBA-15 was dried on the vacuum line (150 °C using a sand bath, 24 h). Then, the dried SBA-15 was mixed with 40 mL of dry toluene and 4 mL of 3-aminopropyl-triethoxysilane (in a 1:1 ratio *w/v*). The mixture was stirred in a silicon bath at 300 rpm at 80 °C for 24 h under a nitrogen atmosphere. The SBA-15-NH_2 material was then filtered and washed with 40 mL of dry toluene, 40 mL of EtOH, and 40 mL of ethyl ether and left to dry overnight under vacuum. The mesostructured silica were characterised with different techniques according to previous work of our research group [35].

2.3.3. Chemical modification of FGM with SBA-15-NH_2

First, 0.5 g of SBA-15-NH_2 and a FGM were dried for 2 h in a vacuum line using a sand bath at 60 °C. After this time, 25 mL of dry toluene were added, and the membrane and the solid were stirred at 50 rpm for 30 min. Later, the temperature was raised to 80 °C and the stirring at 250 rpm. The reaction was carried out in an inert atmosphere for 48 h. Then, the solvent was recovered together with the leftover solid (SBA-15-NH_2), which was dried and stored for other uses. The membrane was washed twice with 25 mL of dry toluene and 25 mL of acetone. Afterwards, the modified membrane, denoted as FGM-SBA-15-NH_2 (Fig. 2a) was dried and then placed in a holder as shown in Fig. 2b. A silicone seal was used to prevent leakage and the holder was closed.

2.4. Characterisation of the materials

The FGM and FGM-SBA-15-NH_2 were characterised using nitrogen

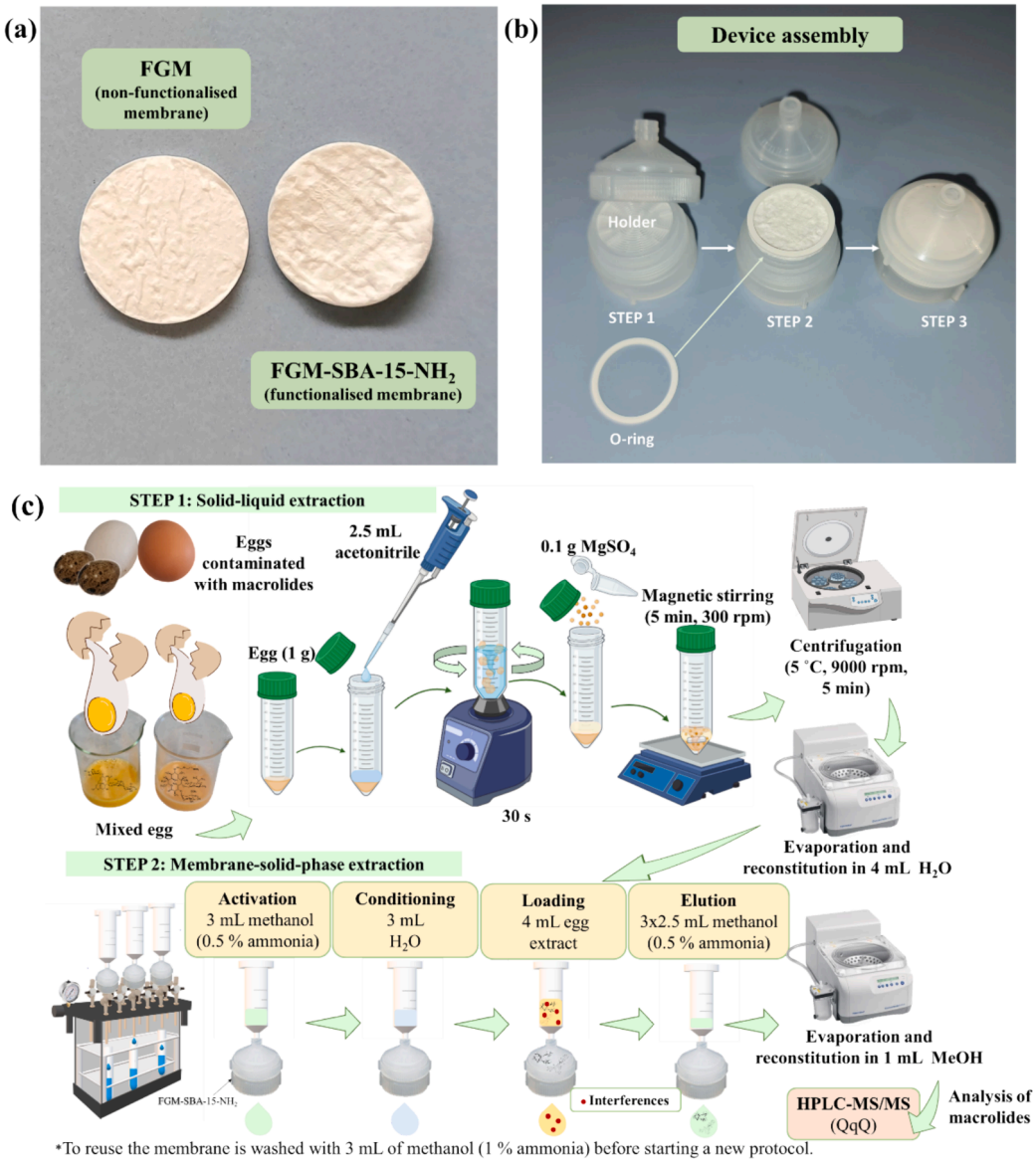


Fig. 2. (a) Fibreglass membrane before (FGM) and after functionalisation with SBA-15-NH_2 (FGM-SBA-15-NH_2), (b) assembling of the membrane in the holder and (c) schematic representation of the optimised sample preparation protocol with the functionalised membrane (FGM-SBA-15-NH_2).

gas adsorption–desorption isotherms, elemental analysis (EA), attenuated total reflection Fourier transform infrared spectroscopy (ATR-FT-IR), scanning electron microscopy (SEM) and thermogravimetric analysis (TGA). Nitrogen gas adsorption–desorption isotherms were carried out using a Micromeritics ASAP 2020 analyser. Prior to analysis, the materials were dried in a vacuum line overnight and then degassed at 90 °C under vacuum for 10 h in the degassing port of the porosimeter. Nitrogen adsorption–desorption data were recorded at a liquid nitrogen temperature of −196 °C. A Flash 2000 Thermo Fisher Scientific Inc. analyser was used to perform EA of nitrogen (% N) to estimate the functionalisation degree of NH_2 groups attached to FGM-SBA-15-NH_2. ATR-FT-IR spectra were carried out using a PerkinElmer Spectrum Two FT-IR spectrophotometer (PerkinElmer, Waltham, Massachusetts, USA) in the region 4000–450 cm^{-1} to identify the main functional groups before and after functionalisation process. The surface morphology was observed using an EM-30AX Plus COXEM from JASCO (COXEM, Korea) for SEM. Prior to the analysis, the samples were coated with Au using a SPT-20 sputter coater. The membranes were placed in a metal stub using a sticky carbon disc, and they were coated with 50 nm of gold (300 s, 50 mA). The samples were then observed at an accelerated voltage of 20 kV and a magnification between 70 and 100,000 times. TGA was investigated to know the thermal stability of FGM and FGM-SBA-15-NH_2. TGA was carried out using a Discovery SDT650 equipment (TA instruments, USA) with a heating rate of 5 °C min^{-1} and a flow rate of 100 mL min^{-1} ranging from 25 to 800 °C under N_2 atmosphere.

2.5. *Optimisation of the membrane-SPE protocol and experimental design*

The FGM-SBA-15-NH_2 placed in the holder as indicated in section 2.3.3 was used to optimise the membrane-SPE protocol. After several preliminary trials with standard solutions, at various concentrations and testing different loading and elution solvents, a response surface methodology (RSM) was applied. RSM was used to achieve the best recovery efficiency in egg samples spiked with the five macrolides at 150 ng/g. Box-Behnken Design (BBD) was applied to express the effect of three independent variables (i.e., the loading volume in the range between 1 and 6 mL, the elution volume in the range between 1 and 8 mL and the ammonia content (%) in the elution solvent in the range between 0 and 1 %) and three-level experiment (−1, 0 and +1) to predict the best conditions involved in membrane-SPE protocol with the FGM-SBA-15-

NH_2. The design consisted of seventeen experiments with five replicates at the central point, and the data were processed using Statgraphics Centurion XVI (version 16.1).

2.6. Sample preparation with the optimised protocol

Eggs were cracked open and beaten with a spatula followed by the SLE step based on previous studies with slight modifications [6,20]. Then, 1 g of egg was weighed in a Falcon® tube. Immediately, 2.5 mL of ACN was added and the mixture was vortexed for 30 s. Next, 0.1 g of $MgSO_4$ was placed on top of the stirred mixture. The new mixture was stirred on a magnetic plate for 5 min at 300 rpm. Finally, it was centrifuged at 9000 rpm, for 5 min and at 5 ℃ in a Digicen 21 R centrifuge from Ortoalresa (Madrid, Spain). The supernatant was collected and evaporated to dryness in an Eppendorf® Concentrator Plus from Eppendorf SE (Hamburg, Germany). The evaporated sample was reconstituted in 4 mL of Milli-Q H_2O and filtered with a nylon filter before the next step.

To carry out the membrane-SPE protocol with the FGM-SBA-15-NH_2, the holder containing the membrane was coupled with a Scharlab ExtraVac® vacuum manifold and the extraction process was started. First, the membrane was activated with 3 mL of MeOH with ammonia solution (0.5 %, *v/v*) and next it was conditioned with 3 mL of Milli-Q H_2O. Then the reconstituted sample was loaded onto the FGM-SBA-15-NH_2 and finally the analytes were then eluted with 3 × 2.5 mL of MeOH with ammonia solution (0.5 %, *v/v*). The extract was evaporated to dryness and reconstituted in 1 mL of MeOH before being injected into HPLC-MS/MS. To reuse the membrane, it was washed with 3 mL of MeOH with ammonia solution (1 %, *v/v*). The optimized protocol is shown in Fig. 2c.

2.7. Reproducibility and reusability of the FGM-SBA-15-NH_2

The evaluation of the reproducibility of the FGM-SBA-15-NH_2 was carried out in two steps, with standard solutions and later with spiked samples of egg. For this purpose, six batches of the FGM-SBA-15-NH_2 were prepared on different days and months following the conditions set out in Section 2.3. The synthesised FGM-SBA-15-NH_2 were named with an M and numbered from 1 to 6 according to the order of their synthesis. All membranes were stored in closed Falcon® tubes in the dark. The membranes were evaluated as sorbents by introducing them into a holder as described in Section 2.3.3.

First, the six batches were compared with standard solutions using the preliminary conditions. That is, the membranes were activated with 3 mL MeOH with ammonia solution (1 %, *v/v*), then the membrane was conditioned with water (2 × 3 mL), and then the standard solution (100 ng/mL) containing the five macrolides was loaded onto the membrane. Then the analytes were eluted with MeOH (3 mL) and MeOH with ammonia solution (1 %, *v/v*, 2 × 3 mL) and injected into HPLC-MS/MS. To calculate the recovery percentage, the process was carried out by loading 3 mL of standard solution (100 ng/mL), and in parallel loading 3 mL of water (in the latter case the eluate was doped with the appropriate amount of the target analytes after the SPE process). Subsequently, to confirm that reproducibility was maintained by applying an egg sample, two membranes were selected (M2 and M3). The protocol applied using egg samples spiked at 150 ng/g was the final one obtained through optimisation with design of experiments and explained in Section 2.6. Each membrane was tested three consecutive times (n = 3) to obtain the standard deviation and RSD (%) of each experiment.

On the other hand, to demonstrate the reusability of the membrane, batch 2 (M2) of FGM-SBA-15-NH_2 was used to perform most of the optimisation and validation studies. For this, the final protocol described in Section 2.6 was applied to about 70 extracted of egg samples doped at a concentration of 150 ng/g with the five target antibiotics, 15 μL of the 10 mg/L solution in ACN of the 5 analytes together in 1 g of egg. These tests were performed on different days, it took 2 weeks to obtain the results shown. Each day, the batch 2 (M2) of FGM-SBA-15-NH_2 was reused between 3 and 10 times in succession using the final protocol. Between days, the membrane was air-dried overnight to reduce dispersion. The recovery values obtained from these two weeks were averaged.

2.8. HPLC-MS/MS analysis

A Varian 1200L triple quadrupole coupled to a Varian Prostar HPLC (Varian Ibérica, Spain) was used for analysis. The chromatographic separation was carried out with an HPLC with the following modules: an autosampler equipped with a 100 μL loop (ProStar 410), two solvent deliver modules (ProStar 210/215) and a thermostatted compartment for the column. The column used was a reverse phase column C18 Kromaphase 100 column (150 mm × 2.0 mm, 3.5 μm particle size) with a C18 Kromaphase guard column (10 mm × 4.0 mm I.D., 5 μm particle size) at 30 °C acquired from Scharlab (Barcelona, Spain). The injection volume was 10 μL. Polar mobile phases with a gradient were used. The mobile phases were ACN (solvent A) and Milli-Q H_2O (solvent B), both with 0.1 % of FA. The separation was performed with a flow rate of 0.25 mL min^{-1} combining solvent A and solvent B as follows: 90 % B eluent decreased linearly to 12 % in 13 min. Then B recovered to 90 % in 2 min, and these conditions are maintained for 3 min. The total run-time was 18 min. The graphical representation of the gradient is shown in Fig. S1d (Supplementary material).

The data acquisition system in the mass spectrometry detector was performed with a Workstation version 6.8 system and using an electrospray ionisation (ESI) source operating in positive mode. The conditions were as follows: N_2 was used as drying gas (350 °C, 22 psi) and nebuliser gas (58 psi). Argon was set at 1.90 mTorr as collision gas, and detector voltage was set at 1480 V. The capillary voltage was 5000 V, and the shield was 600 V. Mass peak width Q1 was 2.0, mass peak width Q3 was 2.5 and scan width in a multiple reaction monitoring (MRM) was 3.5 s. Macrolide antibiotics were monitored at a cone voltage of 60 V. Table S2 lists the mass spectrometer parameters for the five analytes studied.

2.9. Instrumental and method validation

Standard solutions were analysed in the HPLC-MS/MS to evaluate the chromatographic method. Linearity, repeatability, within-laboratory reproducibility, detection and quantification limits (LOD and LOQ) were evaluated for instrumental validation. The linearity was determined by using six standard concentration levels in a range between 1 and 500 ng/mL for ERY, JOS and ROX or 5–500 ng/mL for SPI and TYL. Repeatability and within-laboratory reproducibility were evaluated at three levels of concentration 1 ng/mL, 150 ng/mL and 500 ng/mL. To assess repeatability a standard working solution for each concentration level was injected six times in one day (n = 6) in the HPLC-MS/MS. The within-laboratory reproducibility was evaluated by injecting three times a standard solution of each level evaluated during three days (n = 9). The sensitivity of the instrument was assessed by determining LOD and LOQ as three and ten times the signal-to-noise (S/N) ratio, respectively, corresponding to the lowest concentration standard working solution analysed (1 ng/mL).

The method validation was performed following the criteria described in several guides such as the Commission Implementing Regulation (EU) 2021/808 [21] and SANTE guidelines [36], this last guide used for the calculation of the matrix effect (ME). The method was validated in terms of selectivity/specificity, ME, linearity, method detection (MDL) and quantification (MQL) limits, decision limit (CCα), accuracy (trueness in terms of recoveries), intra- and inter- day precision. The selectivity/specificity of the proposed analytical method was evaluated by checking the presence of peaks with a S/N ratio of three at the retention time of each analyte (tolerance ±0.1 min). For this purpose, 20 egg extracts were injected, and the chromatogram was checked

for the absence of peaks at two of the transitions. ME was calculated to assess the effect of other components of the egg matrix on the analytes [7]. The following formula was used, ME = ((slope matrix-matched calibration/slope solvent calibration) − 1) × 100 [36]. Linearity was evaluated through matrix-matched calibration curves prepared on different days using the coefficient of determination (R^2). To obtain the R^2, the curves were constructed by plotting the peak area of each macrolide antibiotic versus analyte concentration and were fitted by linear regression analysis. Matrix-matched calibration curves were prepared by spiking the egg extract (after SLE and membrane-SPE protocol) with an appropriate aliquot of a standard solution containing the five macrolides to achieve the desired concentration level. The range was between 1 and 500 ng/mL for ERY, JOS and ROX and between 5 and 500 ng/mL for SPI and TYL. The curves were plotted with six points within the described ranges. MDL and MQL of the complete method (SLE, membrane-SPE protocol and HPLC-MS/MS) were calculated for each analyte as three and ten times the S/N ratio for the chromatographic response. The lowest concentration level of the matrix-matched calibration curve was used for this purpose. The CCα was calculated based on the legislation [21]. CCα is considered the limit in which it must be concluded with a probability of error α (5 %) that a sample is non-compliant (positive). For analytes with a fixed MRL, such as ERY (150 ng/g) and TYL (200 ng/g), the CCα (α error 5 %) parameter was determined by analysing twenty blank samples spiked to the MRL level and using the standard deviation of MRL in the formula CCα = MRL+1.64 SD_{MRL}. For analytes without a fixed MRL (SPI, JOS and ROX), CCα was calculated, using the formula CCα = 1st spiking level + 1.64 SD1st spiking level. The detection capability for screening (CCβ) was not calculated because in the legislation [21] is not necessary for confirmation methods. Accuracy (trueness) was evaluated as recovery percentage (%). Recovery values were calculated by comparing the areas of the spiked samples (on the egg sample) with the areas of the simulated samples (spiked samples at the end of the sample preparation procedure). For this experiment six different samples (n = 6) were assessed by spiking them at three concentration levels (low, medium and high). The medium level was selected according to the MRL, i.e. ERY (150 ng/g) and TYL (200 ng/g). For analytes without MRL, the MRL value for ERY 150 ng/g was selected. The low level was set at 5 ng/g and the high level at 500 ng/g, approximately 3 times the MRL, ensure that good recoveries are obtained at both concentrations since there is no legislation for most analytes studies. For the doping process, for example at high level (500 ng/g), 1 g of sample was taken, and 50 μL of a 10 mg/L solution in ACN containing all 5 analytes together was added. Then, the final protocol was then carried out. For the simulated sample, the same aliquot of 10 mg/L solution was added to the collected elution and the doped extract was evaporated. Method precision expressed as relative standard deviation (RSD%) was evaluated in terms of repeatability (intra-day precision) and within-laboratory reproducibility (inter-day precision). The levels selected were the same as those selected in accuracy. Intra-day precision was evaluated through six replicates on one day (n = 6) at the three levels, and inter-day precision by analysing three replicate samples on three different days (n = 9) at each validation level.

3. Results and discussion

3.1. Characterisation of the synthesised materials

Table S3 shows the textural characteristics obtained for the FGM-SBA-15-NH_2 synthesised in this work, in comparison with FGM and mesostructured silica. Surface area (S_{BET}), pore volume and pore size were calculated through the analysis of nitrogen gas adsorption–desorption isotherms. S_{BET} was calculated by Brunauer–Emmett–Teller method, total pore volume was measured at relative $P/P_0 = 0.97$, and pore size distribution was estimated according to the Barret–Joyner–Halenda (BJH) method in the desorption branch. The pore size distribution of the FGM-SBA-15-NH_2 compared to FGM is shown in Fig. S2. As shown in Table S3, the pore size of the FGM was not measurable, as can be seen in Fig. S2a. In contrast to FGM-SBA-15-NH_2 exhibited a narrow pore size distribution, attributed to the modification of FGM with the SBA-15-NH_2 (Table S3, Fig. S2b, Supplementary material) which, as shown in Table S3, has a high S_{BET} (363 m^2 g^{-1}) and pore size (47 Å), in line with previous work [35]. Fig. S2c and d show the nitrogen adsorption–desorption isotherms of the unfunctionalised (FGM) and functionalised membrane (FGM-SBA-15-NH_2). The FGM-SBA-15-NH_2 (Fig. S2d) showed a type IV isotherm according to IUPAC classification with a narrow H_1-type hysteresis cycle representative of mesoporous materials such as SBA-15 with uniform cylindrical pores. In contrast, the FGM showed a type III or V isotherm with no point B, these isotherms are uncommon, and can appear in porous sorbents or materials like polyethylene whereas the interactions between sorbent-sorbate are weak. The hysteresis cycle can be assigned as H_3-type, characterised by the absence of limiting adsorption at high P/P_0 ratios. This is observed in aggregates, and in this case, it is due to the overlapping fibres of the membrane (Fig. S2c).

EA revealed the degree of functionalisation (L_0) of the FGM-SBA-15-NH_2. For this, the degree of functionalisation of NH_2 was estimated with respect to the %N. FGM-SBA-15-NH_2 showed a binding of 0.17 g of SBA-15-NH_2 per g of FGM (Table S3) confirming the modification of the FGM with the SBA-15-NH_2 (1.7 mmol ligand/g).

ATR-FT-IR spectra are shown in Fig. 3a and b. A broadband between 1003 and 1053 cm^{-1} assigned to the siloxane groups (Si–O–Si) can be observed in the FGM and FGM-SBA-15-NH_2. The band around 950 cm^{-1} corresponds to the Si–OH vibration, the band at 800 cm^{-1} corresponds to symmetric vibrations of the Si–O bond of the siloxane groups and band 459 cm^{-1} corresponds to the torsion vibration of the Si–O–Si bond. The C–N stretching vibration was observed as weak bands at 1193 cm^{-1} in the FGM-SBA-15-NH_2 demonstrating the functionalisation with NH_2 groups.

Fig. 3c and d illustrate the SEM images of the membrane changes after the chemical modification process. In Fig. 3c, FGM shows an asymmetric structure with intertwined fibres. In contrast, the FGM-SBA-15-NH_2 (functionalised) (Fig. 3d) maintains the same structure but with quasi-spherical particles attached to the glass fibres, corresponding to SBA-15-NH_2, demonstrating the successful functionalisation of the membrane with the functionalised mesostructured silica.

Fig. S3 shows the TGA of the non-functionalised membrane (FGM) and the functionalised membrane (FGM-SBA-15-NH_2). As can be seen, the FGM is fully thermally stable in the range of 25–800 °C with a slight loss of weight of around 0.9 % (Fig. S3a), this loss may be due to physisorbed water in the membrane fibres. On the other hand, the FGM-SBA-15-NH_2 is completely stable up to 100 °C and a small total weight loss of about 3 % is observed after heating the sample until 800 °C due to the loss of the organic ligand tethered to the silica fibres (Fig. S3b). This demonstrates its functionalisation and is in accordance with the EA results.

3.2. Optimisation of HPLC-MS/MS conditions

To achieve an adequate separation of the five macrolide antibiotics, different gradients were tested using ACN and Milli-Q H_2O, both with FA (0.1 %, *v/v*) as mobile phases. Standard solutions in MeOH were injected into the HPLC-MS/MS system. These phases were selected as they are the most commonly used phases for separation in the analysis of macrolide antibiotics [4]. The first gradient, gradient 1 (Fig. S1a), started in 90 % Milli-Q H_2O with FA (0.1 %, *v/v*, mobile phase B) and was changed to 100 % ACN with FA (0.1 %, *v/v*, mobile phase A) in 15 min. These conditions were maintained for 5 min and then in 3 min it returned to the initial phase with 90 % B and these conditions were maintained for 2 min. A flow rate of 0.2 mL/min. The problem with these conditions was that SPI presented a chromatogram with a tail at the peak. All other analytes were narrow, well-defined peaks. Two new gradients were then tested (Fig. S1b and c), and the flow was slightly increased to 0.25 mL/

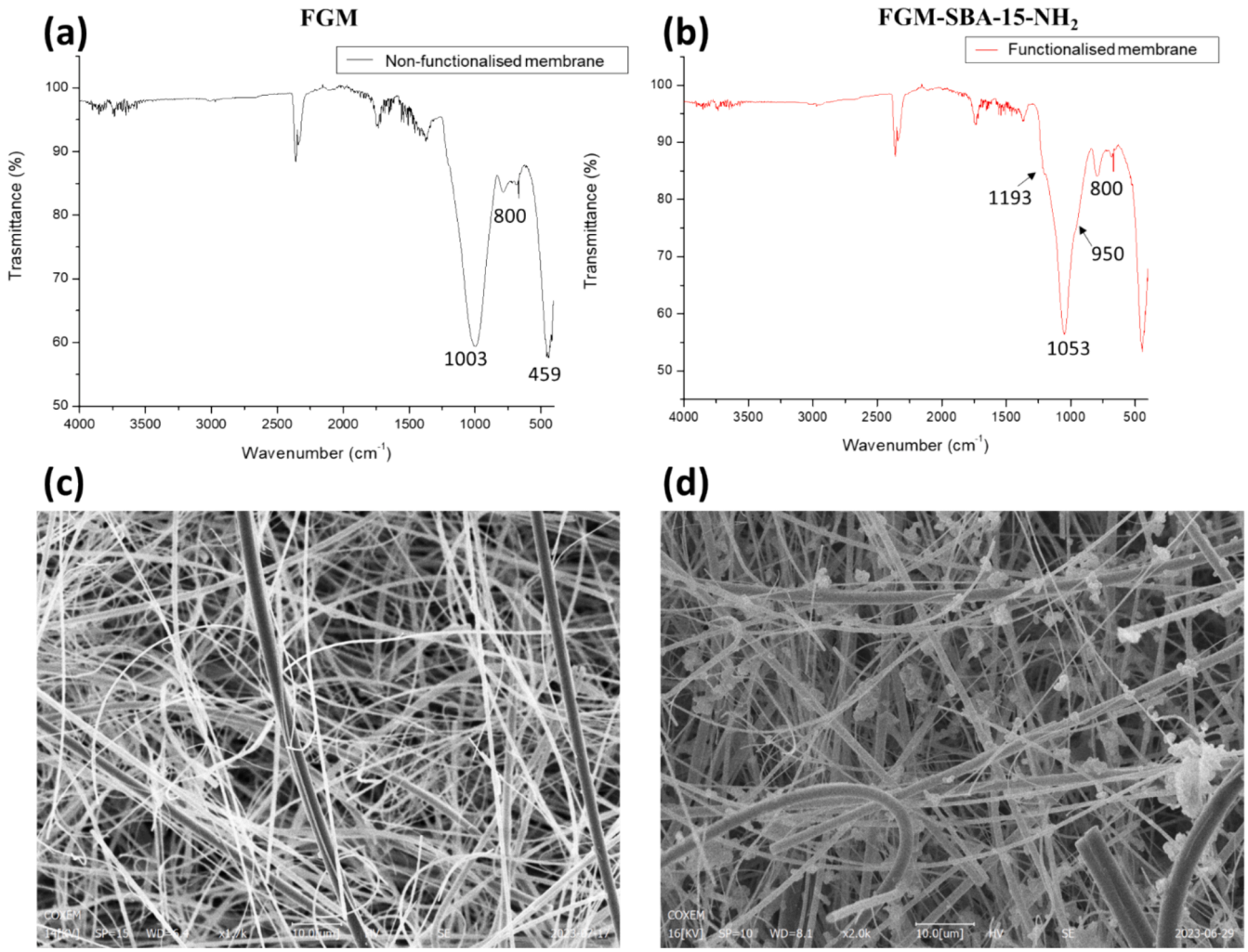


Fig. 3. (a) ATR-FT-IR spectra of non-functionalised membrane (FGM) and (b) functionalised membrane (FGM-SBA-15-NH_2). SEM micrographs of (c) non-functionalised membrane (1,700×, FGM) and (d) functionalised membrane (2,000×, FGM-SBA-15-NH_2).

min. Gradient 2 (Fig. S1b) started in 50 %-A:50 %-B (*v/v*), maintaining these conditions for 10 min, then the conditions changed to 100 % ACN with FA (0.1 %, *v/v*, mobile phase A) in 5 min and a further 5 min the gradient returned to initial conditions. The total run-time was 20 min. In this gradient, the five compounds came out before 5 min, but the SPI and the ERY coeluted in 2.1 min and peaks were very wide. On the other hand, gradient 3 (Fig. S1c) was tested but separation between ERY, JOS, ROX and TYL was not possible, and coeluted, as these analytes are more soluble in organic solvents than in water. Finally, regarding the results obtained with the three tested gradients, a gradient similar to gradient 1 was tested, but without reaching 100 % of the mobile phase A. So gradient 4 (Fig. S1d) was tested starting with 10 % of A that was increased to 88 %. Then it was returned to the initial conditions in 2 min (10 % A and 90 % B). These conditions were maintained for another 3 min. An attempt was made to reduce the final 3 min to shorten the total time of each run, but it was not possible since a splitting of SPI was observed. Therefore, gradient 4 was chosen as the best one, with a total time of 18 min. With this gradient, the retention time of the analytes was 8.0 min for SPI, 9.0 min for ERY, 9.2 min for TYL, 10.1 min for ROX and 10.6 min for JOS. Fig. S4 shows the separation of the five analytes with gradient 4 (Fig. S1d) in the quantification ion and the mass spectra of each analyte.

Mass parameters such as Dwell time at 0.1 s and 0.25 s were tested in the mentioned gradients. Dwell time at 0.25 s showed narrower peaks and was therefore chosen in the final conditions. Finally, it was also tried to inject with the final conditions in other media such as water or ACN. In the case of water, the signal of the analytes was lower than with MeOH and with ACN, the signals were like when injected in MeOH. For this reason, MeOH was selected as the optimum injection medium.

3.3. Optimisation of sample preparation protocol

3.3.1. Preliminary membrane-SPE assays with FGM-SBA-15-NH_2

First, preliminary tests were carried out to evaluate the performance of the prepared membrane in terms of recovery retention. For this purpose, a previous work was followed as reference [33]. For this reason, a peristaltic pump and Tygon® tubing were used as connectors to pass the solvent through the membrane. Standard solutions were prepared in different media (ACN, Milli-Q H_2O, Milli-Q H_2O with FA (0.1 %, *v/v*) at a concentration of 250 ng/mL. The FGM-SBA-15-NH_2 was conditioned with the same loading solvent (3 mL), and the analytes were eluted with 3 mL of MeOH. Between assays, the membrane was washed with 3 mL of MeOH. The results obtained from this experiment are shown in Table S4 (Supplementary material). The recovery percentages were extremely low in the case of loading in ACN and Milli-Q H_2O with FA (0.1 %, *v/v*). With Milli-Q H_2O, the results were slightly better with recoveries between 41 and 68 % for all five analytes.

To speed up the membrane-SPE process the FGM-SBA-15-NH_2 was placed in the holder as explained in the section 2.3.3 and then coupled to the Scharlab ExtraVac® vacuum manifold. In this test, the concentration

of the standard solutions was reduced to 100 ng/mL, and the elution volume was increased to 3 × 3 mL of MeOH. Two new loading solvents (Milli-Q H_2O/ACN (90/10, *v/v*) and Milli-Q H_2O/EtOH (90/10, *v/v*)) were also included to improve the results obtained. The results of this experiment are reported in Table S5 (Supplementary material). The results showed that ACN is not a good loading solvent either alone or when used in small proportions together with water (Milli-Q H_2O /ACN (90/10, *v/v*)). Milli-Q H_2O with FA (0.1 %, *v/v*) showed good recoveries for JOS, ROX and TYL, but poor recoveries for SPI and ERY. The same was true for the Milli-Q H_2O/EtOH (90/10, *v/v*) mixture. In the case of Milli-Q H_2O, it showed the best recovery percentages than in the previous test. The retention of the target analytes with this solvent may be due to Van der Waals forces and H-bonds. Furthermore, it seems that the increased elution volume and the lower macrolide content improved the recovery rates, which were between 51 % and 89 % for all five analytes. For this reason, water was selected as the loading solvent by favoring the interactions described above. Finally, different elution media (3 × 3 mL MeOH; 3 mL MeOH + 3 mL MeOH with HAc (1 %, *v/v*) + 3 mL MeOH; 3 mL MeOH + 2 × 3 mL ACN; 3 mL MeOH + 2 × 3 mL MeOH with ammonia solution (1 %, *v/v*)) were tested and compared after loading the standard solutions (100 ng/mL) in water to verify which medium is best able to break the bonds generated between the membrane and the analytes. Table S6 (Supplementary material) showed acceptable values for all analytes (76–124 % for all analytes) and proven elution solvents, except for SPI, which only showed good percentages (102 %) in the case of the elution medium containing ammonia (3 mL MeOH + 2 × 3 mL MeOH with ammonia solution (1 %, *v/v*)). It seems that the small percentage of ammonium allowed to break the bonds formed between the SPI and the membrane. Therefore, MeOH was selected to be evaluated along with the addition of ammonia in the BBD design.

3.3.2. Evaluation of the SLE extraction protocol

Before performing the membrane-SPE process, the egg sample must be extracted by SLE due to the viscosity of the egg sample. Based on the bibliography consulted, ACN was the most suitable solvent used as it favours the precipitation of proteins that may interfere with the process [4]. Therefore, the SLE was based on previous studies with some modifications [6,20]. For this purpose, 1 g of egg was weighed, and 2.5 mL of ACN was added. The mixture was vortexed (30 s) and 0.1 g of $MgSO_4$ was added to remove water and interferences. After magnetic stirring and centrifugation, the slightly cloudy and yellowish coloured extract was filtered and directly injected into HPLC-MS/MS. To evaluate the recovery, two samples were doped at the beginning and one at the end of the SLE protocol at the same concentration of 150 ng/g (15 μL of the 10 mg/L solution in ACN of the 5 analytes together). The recovery percentages ranged from 91 to 116 % for the five analytes, confirming that there are no analyte losses during the SLE process. Considering the preliminary results for the membrane-SPE, after SLE an evaporation and reconstitution step in water was necessary to favour the interaction of the analytes with the membrane as concluded in Section 3.3.1.

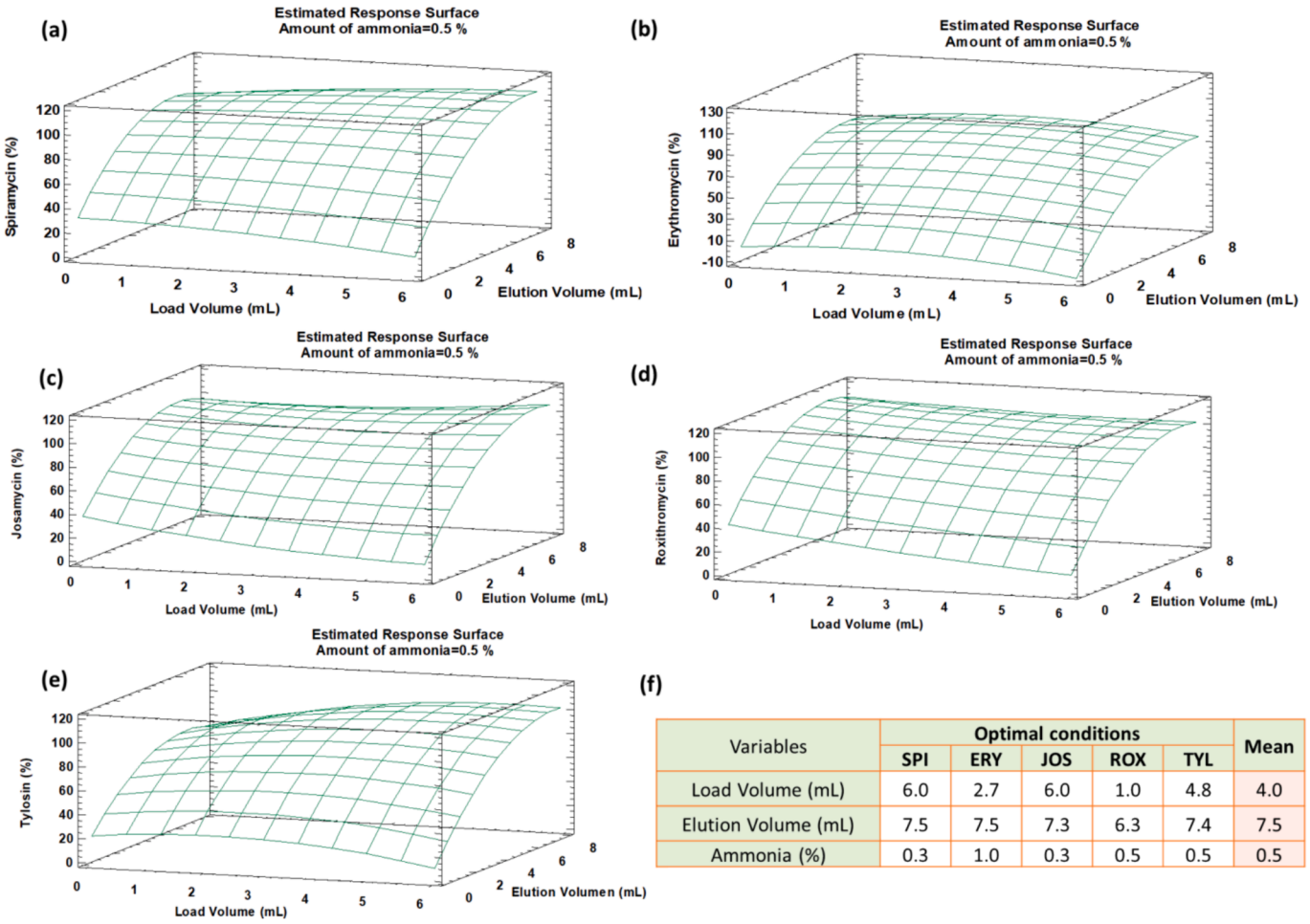
3.3.3. Selection of optimal membrane-SPE conditions using BBD

Considering the conditions in the preliminary studies explained in Section 3.3.1, a design of experiments was used to finalise the optimisation of the membrane-SPE protocol. For this, a RSM was used, specifically BBD, to find out how volume affects the loading and elution stages of the membrane-SPE protocol with FGM-SBA-15-NH_2. For this reason, it was optimised as an independent factor the loading and elution volumes and the ammonia content in the elution. In the preliminary assays, water was set as the loading solvent and MeOH and MeOH with ammonia solution (1 %, *v/v*) as the elution solvent. For this reason, it was decided to study the ammonia content (%) in the elution phase as a third variable. The dependent factor was the recovery (%). The results of the 3-variable, 3-level design with five central points of experiments are shown in Table 1. Experiments were conducted randomly to reduce systematic error. As the design was applied on doped egg samples, after the SLE step, the sample had to be evaporated during the ACN extraction and reconstituted in the appropriate amount of Milli-Q H_2O, according to the design experiment. The data obtained in Table 1 were used to obtain the analysis of variance (ANOVA) report and the second-order polynomial regression equations. The equations for each analyte are shown in Table S7 (Supplementary material). By solving these equations, the response (Y) i.e. the percentage recovery of each macrolide, can be estimated (A=Loading Volume, B=Elution Volume, and C=Amount of ammonia). The results of the ANOVA analysis shown in Table S7 (Supplementary material) showed significance ($p < 0.05$) for variables B and BB and no significance for the rest of the variables. On the other hand, the R^2 and $R^2_{adj.}$ values were 0.91 and 0.80 for SPI, 0.97 and 0.93 for ERY, 0.96 and 0.91 for JOS, 0.94 and 0.85 for ROX and 0.95 and 0.89 for TYL, respectively. These results showed a high degree of fit and small experimental error, indicating that the model can adequately predict the % recovery of macrolide antibiotics. On the other hand, to determine the influence of the interaction effect between the loading (A) and elution volume (B) (the third variable was set to 0.5) three-dimensional response surface plots (Fig. 4) were constructed using Statgraphics Centurion XVI (version 16.1). Fig. 4(a–e)

Table 1
Box-Behnken design and response obtained for the extraction of 5 macrolides antibiotics in egg at 150 ng/g.

Run	Experimental variables						Response (% Recovery ± SD)*				
	A	B	C	A: Load Volume (mL)	B: Elution Volume (mL)	C: Ammount of ammonia (%)	Spiramycin	Erytromycin	Josamycin	Roxitromycin	Tylosin
1	0	−1	1	3	1	1	41 ± 7	29 ± 6	39 ± 8	43 ± 5	41 ± 11
2	0	0	0	3	4	0.1	84 ± 1	76 ± 11	82 ± 12	88 ± 12	89 ± 14
3	1	1	0	6	8	0.1	110 ± 2	76 ± 12	107 ± 8	105 ± 6	98 ± 9
4	0	0	0	3	4	0.1	84 ± 9	76 ± 16	82 ± 10	88 ± 9	83 ± 17
5	−1	1	0	1	8	0.1	100 ± 1	86 ± 12	99 ± 9	111 ± 3	85 ± 12
6	−1	0	−1	1	4	0	59 ± 14	68 ± 11	73 ± 16	75 ± 15	61 ± 4
7	0	0	0	3	4	0.1	84 ± 6	76 ± 10	82 ± 8	88 ± 6	83 ± 11
8	−1	−1	0	1	1	0.1	34 ± 2	34 ± 8	41 ± 6	46 ± 7	40 ± 0
9	0	1	−1	3	8	0	88 ± 12	85 ± 19	88 ± 14	94 ± 11	88 ± 17
10	0	−1	−1	3	1	0	45 ± 2	48 ± 5	41 ± 3	48 ± 3	48 ± 1
11	0	1	1	3	8	1	84 ± 4	98 ± 18	85 ± 1	88 ± 1	93 ± 16
12	−1	0	1	1	4	1	91 ± 14	84 ± 16	92 ± 14	101 ± 14	80 ± 14
13	1	0	1	6	4	1	78 ± 5	71 ± 7	84 ± 14	79 ± 11	79 ± 12
14	0	0	0	3	4	0.1	84 ± 12	76 ± 9	82 ± 2	88 ± 10	83 ± 9
15	0	0	0	3	4	0.1	84 ± 18	76 ± 15	82 ± 12	88 ± 15	83 ± 13
16	1	−1	0	6	1	0.1	21 ± 3	17 ± 0	24 ± 2	25 ± 4	22 ± 0
17	1	0	−1	6	4	0	91 ± 2	68 ± 16	87 ± 5	91 ± 2	83 ± 9

* n = 3.



(f)

Variables	Optimal conditions					Mean
	SPI	ERY	JOS	ROX	TYL	
Load Volume (mL)	6.0	2.7	6.0	1.0	4.8	4.0
Elution Volume (mL)	7.5	7.5	7.3	6.3	7.4	7.5
Ammonia (%)	0.3	1.0	0.3	0.5	0.5	0.5

Fig. 4. Response surfaces plots for (a) spiramicyn (SPI) (b) erythromycin (ERY), (c) josamycin (JOS) (d) roxithromycin (ROX), (e) tylosin (TYL) and (f) optimal conditions for SLE-membrane-SPE.

shows that for small elution volumes, the recovery rates drop below 50 % for all five analytes. Observing the graph of main effects (Fig. S5, Supplementary material) we can confirm this fact, that is, volumes of around 7 mL are needed to be able to break the interactions between the membrane and the analytes (Van der Waals forces and H-bonds). In the case of the ERY (Fig. 4b) and ROX (Fig. 4d) it appears that the best extraction percentages are obtained with relatively small loading volumes (Fig. S5b and d, Supplementary material), around 3 and 1 mL, respectively, and large elution volumes, approximately 7 mL. But this does not seem to be a problem compared to the rest of the macrolides, if loading volumes of about 4 mL are taken, recovery values for ERY of about 80 % and for ROX of about 90 % would be obtained as shown in Fig. S5b and d (see Supplementary material).

Based on the results of this analysis, the optimum conditions were shown in Fig. 4f. As for some analytes there were discrepancies in the loading volume, so it was decided to take the average volume as the optimum. Values were loaded with 4 mL of water, elution 3 × 2.5 mL of MeOH with ammonia solution (0.5 %, *v/v*). Under these conditions, a new experiment (n = 3) was carried out with egg samples doped with five macrolides at 150 ng/g. In addition, these conditions were tested on a FGM to check the efficiency of the process. The results obtained are shown in Fig. 5. Recovery percentages of 107 ± 15 % were found for SPI, 87 ± 11 % for Ery, 108 ± 4 % for JOS, 117 ± 1 % for ROX and 100 ± 10

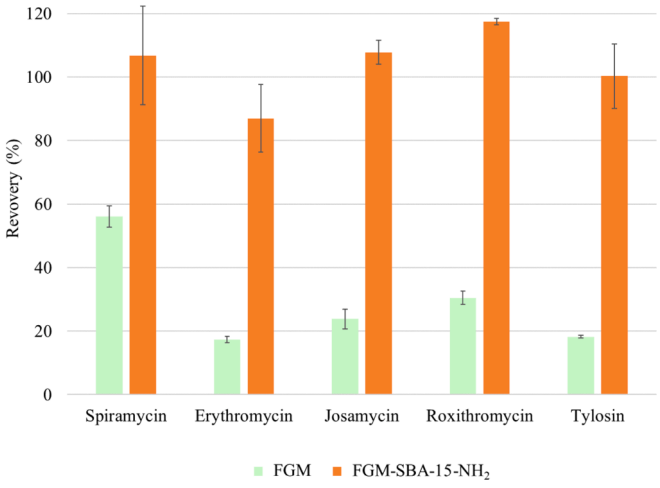


Fig. 5. Recoveries (%) obtained with the optimised protocol using non-functionalised (FGM) and functionalised (FGM-SBA-15-NH_2) as membrane-based sorbents for the purification of egg samples doped with the five macrolides at 150 ng/g.

% for TYL in the case of the FGM-SBA-15-NH_2 and recovery percentages of 56 ± 3 % were found for SPI, 17 ± 1 % for ERY, 24 ± 43 % for Jos, 30 ± 2 % for ROX and 18 ± 1 % for TYL in the case of the FGM. These results suggested that the response surface model correctly reflects the expected optimization, and FGM-SBA-15-NH_2 is shown to respond correctly as predicted compared to FGM.

3.4. Instrumental and method validation

Different standard solutions at three concentration levels (1, 150 and 500 ng/mL) were injected into the HPLC-MS/MS to evaluate the performance of the chromatographic method. Results are shown in Table S8 (Supplementary material). Linearity was evaluated showing $R^2 \geq 0.995$. Repeatability and within-laboratory reproducibility showed acceptable values of % RSD, between 3 and 16 % for the five analytes studied. Low LOD and LOQ were obtained: 1.0 and 2.6 ng/mL for SPI, 0.5 and 1.6 ng/mL for ERY, 0.4 and 1.2 ng/mL for JOS, 0.4 and 1.4 ng/mL for ROX, 0.3 and 1.1 ng/mL for TYL, respectively.

For selectivity, 20 blank samples were analysed with the optimised protocol to verify the absence of intrusive peaks in the retention times of the five macrolides. For this purpose, the uncontaminated sample H-Egg 9 was taken. The results showed clean chromatograms, as shown in Fig. S6 (Supplementary material), with no peaks in the retention times of the analytes compared to a sample doped to 150 ng/g. Fig. S6 (Supplementary material) shows the ions used for quantification. The selectivity was also checked in the rest of the daughter ions, and no peaks were found in the retention times of the analytes.

Table 2 compiles the results obtained from the full validation of the proposed methodology. Good linear regression for five macrolide antibiotics was reached (Table 2), obtaining $R^2 \geq 0.993$. The ME was verified in the five analytes by comparing the slopes of the matrix-matched calibration curve and solvent calibration curve and using the formula explained in Section 2.9. The ME is considered significant if it is ± 20 %. Table 2 shows that there is no ME for ERY, JOS, ROX and TYL, but there is a significant positive ME (63 %) for the SPI analyte. This means that solvent calibration lines can be used for all analytes except SPI. MDL and MQL were calculated using the lowest point of the matrix-matched calibration curve. MQL ranged between 2.1 and 1.1 ng/g and MDL between 0.3 and 0.6 ng/g for the five analytes studied. These values correspond to the same values in ng/mL considering the sample treatment (extract injected in HPLC-MS/MS after SLE and membrane-SPE). The values obtained in this validation demonstrate that the proposed methodology is very sensitive. Demonstrating MQL around 100 lower than the MRLs established for ERY and TYL. On the other hand, CCα was calculated with the formula described in Section 2.9. The relevant data are shown in Table 2. The CCα of the authorised substances as macrolides will be higher, but as close as possible, to the MRL. Table 2 shows how the ERY (187.7 ng/g) and TYL 208.4 (ng/g) values are slightly higher than the MRL. For the rest of the compounds without legislation, the values were lower than the medium validation limit (150 ng/g) and higher than the low one (5 ng/g).

The accuracy and precision of the proposed methodology were evaluated at three concentrations (5 ng/g, 150 ng/g (except for TYL), 200 ng/g) and 500 ng/g). The Commission Implementing Regulation (EU) 2021/808 [21] has set acceptable recovery values between 50 and 120 % for mass fractions ≤ 1 ng/g, 70–120 % for mass fractions > 1–10 ng/gand 80–120 % for mass fractions ≥ 10 ng/g. In this sense, the proposed method complies with the minimum ranges of accuracy (trueness) proposed by the legislation [21] since in the range > 1–10 ng/g (5 ng/g), the recovery percentages were between 87 and 100 % for the five analytes and in values of validation higher than ≥ 10 ng/g (150 ng/g, 200 ng/g and 500 ng/g) the recovery percentages were 85–98 % for the five analytes, as shown in Table 2. On the other hand, both inter-day and intra-day RSD percentages were below 20 %. The legislation recommends that for mass fractions <10 ng/g, the RSD should be less than 30 %. For the low level of intra-day and inter-day precision, the % RSD was for SPI 14 % and 9 %, for ERY 13 % and 16 %, for JOS 14 % and 13 %, for ROX 14 and 16 % and for TYL 14 % and 12 %, respectively. For

Table 2
Analytical performance of developed methodology.

Analyte	Level (ng/g)	Accuracy (recovery ± SD, %)	Intra-day precision (RSD, %) n = 6, 1 day	Inter-day precision (RSD, %) n = 9, 3 days	Linear range (ng/g)	Matrix matched calibration (R^2)	CCα[a] (ng/g)	MRL[b] (ng/g)	MDL[c] (ng/g)*	MQL[d] (ng/g)*	ME[e] (%)
SPI[f]	5	92 ± 10	14	9	5–500	$2.4 \times 10^5x + 1.8 \times 10^6$	9.4	N.L[k]	0.6	2.1	63
	150	94 ± 9	10	13		(0.998)					
	500	94 ± 11	15	12							
ERY[g]	5	100 ± 12	13	16	1–500	$3.4 \times 10^5x + 1.3 \times 10^6$	187.7	150	0.4	1.2	4
	150	85 ± 10	13	14		(1.000)					
	500	85 ± 13	13	11							
JOS[h]	5	98 ± 11	14	13	1–500	$3.1 \times 10^5x + 1.6 \times 10^6$	10.1	N.L[k]	0.3	1.1	5
	150	91 ± 11	12	13		(0.999)					
	500	98 ± 5	15	6							
ROX[i]	5	98 ± 8	14	16	1–500	$2.7 \times 10^5x + 3.3 \times 10^6$	9.3	N.L[k]	0.3	1.1	6
	150	92 ± 10	11	14		(0.993)					
	500	86 ± 13	15	9							
TYL[j]	5	87 ± 9	14	12	5–500	$1.7 \times 10^5x + 2.9 \times 10^6$	208.4	200	0.5	1.6	−2
	200	90 ± 8	11	13		(0.994)					
	500	94 ± 7	7	10							

Abbreviations: [a]CCα: Decision limit for confirmation. [b]MRL: Maximum residue limit. [c]MDL: Method detection limit. [d]MQL: Method quantification limit. [e]ME: Matrix effect. [f]SPI: Spiramycin. [g]ERY: Erythromycin. [h]JOS: Josamycin. [i]ROX: Roxithromycin. [j]TYL: Tylosin. [k]N.L.: Not legislated. * Expressed in ng/mL in the extract before HPLC-MS/MS are the same, considering all the steps performed in the sample treatment.

mass fractions between >10 ng/g–1000 ng/g, it recommends RSD below 22 %. In the proposed methodology, the medium and high levels did not exceed 15 % for intra-day precision and 16 % for inter-day precision. Therefore, it can be concluded that the method complies with the parameters of precision and accuracy established by the legislation [21].

3.5. Reproducibility and reusability studies of the FGM-SBA-15-NH_2

The results obtained after assessing the reproducibility of FGM-SBA-15-NH_2 are shown in Table S9 (Supplementary material). First, the 6 batches of membranes were tested with standard solutions at 100 ng/mL, applying preliminary conditions described in Section 2.7. The results show good recovery percentages among the 6 batches evaluated, obtaining RSD values between 4 and 16 % for the 5 analytes. The mean of the recovery percentages of the batches was 80 ± 13 % for SPI, 95 ± 7 % for ERY, 92 ± 4 % for JOS, 86 ± 6 % for ROX and 92 ± 5 % for TYL. On the other hand, the selected M2 and M3 FGM-SBA-15-NH_2 were applied to egg samples doped with the five macrolides at 150 ng/g, showing mean recovery percentages and standard deviations similar to those obtained with the previous test (98 ± 2 % for SPI, 85 ± 10 % for ERY, 96 ± 1 % for JOS, 96 ± 5 % for ROX and 90 ± 10 % for TYL). If the RSD values, between 1 and 12 % (Table S9), are compared with those shown in Table 2, they are similar, considering the number of assays, for inter-day (10–13 % for the 5 analytes) and intra-day (13–14 % for the 5 analytes) precision at medium level. These studies demonstrated the good reproducibility of the membrane preparation process.

Alternatively, the optimised protocol described in the Section 2.6 was applied around 70 times to egg samples doped with 150 ng/g of the five macrolides. For this, FGM-SBA-15-NH_2 batch M2 was selected. The results showed recoveries of 86 ± 13 % for SPI, 77 ± 14 % for ERY, 89 ± 14 % for JOS, 91 ± 12 % for ROX and 83 ± 12 % for TYL. The RSD values of this experiment, between 15 and 18 %, slightly higher than those explained above, but still valid values according to the validation guidelines, confirming the high reusability of the membrane. So, the values demonstrate that the membrane can be reused for at least 70 cycles and up to 10 times a day without affecting the extraction performance. It should be added that due to the nature of the membrane, made of silica, and the strong covalent union between the membrane and the modified silica, its stability is very high both thermally, mechanically and chemically. Therefore, by properly washing the membrane with the previously indicated solvent, the cycles can even exceed 70 cycles.

3.6. Application of the methodology in real egg samples

Ten different samples of chicken eggs and four quail eggs, obtained from smallholdings, and local supermarkets, were analysed. Each sample was analysed in triplicate with the SLE-membrane-SPE protocol and injected three times in the HPLC-MS/MS. None of the analysed macrolide antibiotic analytes was found at a concentration level higher than their CCα in the analysed egg samples. Only two samples tested positive for the analytes studied. To confirm the presence of these analytes, the retention time in the three replicates was confirmed by comparing with the retention time of a doped sample and the ionic ratios were checked, and both samples complied with the requirements established in the law [21]. The positive samples (Table S10, Supplementary material) were H-EGG-1 which was positive for SPI but with concentrations between MDL and MQL, and Q-EGG-1 which was positive for two macrolide antibiotics, JOS and ROX. In this case, the concentrations were also between MDL and MQL. The other samples studied were negative for the five analytes. Fig. 6 shows the chromatograms of the positive samples compared to a doped sample, demonstrating the applicability of the

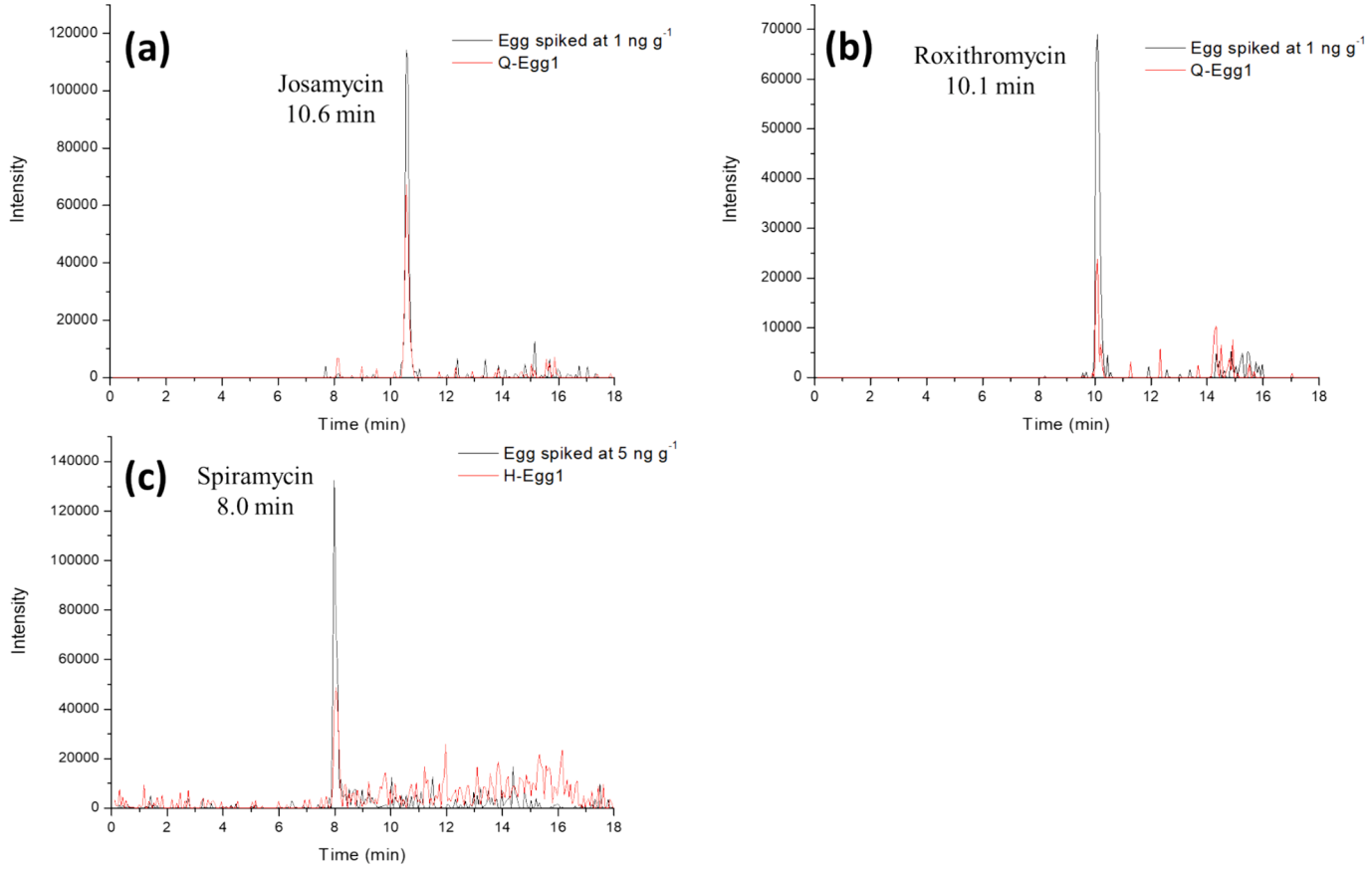


Fig. 6. Extracted ion chromatograms of samples contaminated with (a) tylosin (m/z 917.4 > 174.0), (b) roxithromycin (m/z 837.9 > 158.0) and (c) spiramycin (m/z 422.6 > 174.1) compared to a sample doped to 1 and 5 ng/g.

proposed method in samples contaminated by macrolide antibiotics.

3.7. Comparison with other methods

The method described in this study, based on the application of a novel membrane-based sorbent (FGM-SBA-15-NH_2), and MDLs, MQLs and recovery rates shown in this work were compared with those of other methods proposed for the analysis of macrolide antibiotics in egg samples. Information on the limited number of methods developed and published for the analysis of macrolides in egg samples is presented in Table S11 (Supplementary material). In general, all methodologies for egg samples use an extraction with large quantities of ACN (between 8 and 20 mL) [6,8,9,19,20,24] unlike the proposed method which only uses 2.5 mL of ACN in the SLE step. Generally, the methodologies include the SLE stage with ACN followed in some cases by salts such as Na_2EDTA, Na_2SO_4, NaCl or $MgSO_4$ to promote precipitation, remove sample water and interferences. On the other hand, SLE can generally be accompanied by SPE. The SPE technique is generally applied with commercial cartridges such as OASIS® HLB and OASIS® MCX. Generally some of the methods [8,9,19,24] using these cartridges have shown slightly higher limits than the proposed method. Also the OASIS® HLB cartridge has shown slightly lower recoveries for some analytes such as ERY (75 %) [19]. In other works recovery percentages have not been shown [24] or have been validated at low concentration levels [20]. Therefore, the proposed methodology allows the extraction and purification of samples without affecting the recovery from 5 ng/g up to a maximum concentration of 500 ng/g. Therefore, this methodology allows the quantification at high and low concentrations of the five analytes, being for the non-legislated analytes (SPI, JOS and ROX) a powerful and sensitive methodology that allows the determination of the analytes at low concentrations thanks to the excellent MQLs (between 1.1–2.1 ng/g) shown in Table S11. Also, techniques such as MSPD with C18 and florisil or modified QuEChERS have been compared to SLE followed by SPE but they have shown poor recoveries for the extraction of macrolide antibiotics [19]. The new proposed sorbent (FGM-SBA-15-NH_2) present some advantages compared with the other methodologies based on SLE-SPE described in Table S11, such as it can be reused for numerous cycles (up to 70 uses, without loss of binding capacity) and the small volumes of solvents required, avoiding the generation of waste, which is in accordance with the principles of green analytical chemistry. If we take into account the environmental impact and evaluate the greenness of the proposed methodology with the AGREE metric tool [37], we can demonstrate the slight improvement compared to similar methodologies presented in the Table S11 for the analysis of macrolides in egg samples. Our method achieved a score of 0.41 (Fig. S7a, Supplementary material), which is the highest among those compared. Other methods received scores of 0.29 (Fig. S7b, Supplementary material) [9] and 0.35 (Fig. S7c, Supplementary material) [24]. The main reason for this difference is how much sample, sorbent, and liquid we use during preparation. Our method uses less solvent in the SLE stage (2.5 mL *vs* 10–12 mL [9,24]). This is different from other methods, which use much more solvent in SPE, specially in conditioning stage (3 mL *vs* 25–30 mL [9,24]), possibly because they use a lot more sorbent (between 225–500 mg), unlike our thin FGM-SBA-15-NH_2 sorbent. Also, one of the methods we compared with had worse results because it used hexane, while the others only used MeOH, ACN, and water. So, overall, our method is a bit more environmentally friendly than the others for analysing macrolides in eggs, even though the exact score we achieve is similar to the average score (0.4) of other methods used for tetracycline antibiotics [38].

4. Conclusion

In this work, a new membrane-based sorbent chemically modified with SBA-15 functionalised with NH_2 groups (FGM-SBA-15-NH_2) was developed and designed for its application as SPE sorbent for use in the extraction and purification of five macrolide antibiotics (SPI, ERY, ROX, JOS and TYL) from egg samples. The morphological studies of the FGM-SBA-15-NH_2 by FTIR and SEM showed the success of the synthesis procedure. The proposed method, consisting of a SLE step followed by membrane-SPE and HPLC-MS/MS analysis, was optimised with a BBD design of experiments and successfully validated, showing good linearity, low limits, acceptable CCα, precision and accuracy. Furthermore, the method provided to be reproducible when testing different batches of the synthesized FGM-SBA-15-NH_2, and the membrane was reusable a large number of times. Finally, the applicability of the developed method was demonstrated on different hen and quail egg samples. Notably, the FGM-SBA-15-NH_2 sorbent's reusability establishes a sustainability edge over current macrolide analysis methods in eggs, marking a significant advancement in analytical techniques for improved environmental and economic impact.

CRediT authorship contribution statement

Lorena González-Gómez: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Sonia Morante-Zarcero:** Validation, Supervision, Resources, Methodology. **Damián Pérez-Quintanilla:** Supervision, Software, Resources, Investigation, Conceptualization. **Gema Paniagua González:** Investigation, Resources, Writing – review & editing. **Rosa M. Garcinuño:** Writing – review & editing, Supervision, Funding acquisition. **Pilar Fernández Hernando:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.111232.

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