**Supplementary material**

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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**Fig. S1.** Different gradients tested during the optimization of the chromatographic method and retention time of the analytes according to the gradient. (a) gradient 1, (b) gradient 2, (c) gradient 3 and (d) gradient 4 (definitive).



**Fig. S2**. Pore size distributions and nitrogen adsorption-desorption isotherms of (a, c) FGM (non-functionalised membrane) and (b, d) FGM-SBA-15-NH2 (functionalised membrane) respectively.



**Fig. S3.** Thermogravimetric analysis (TGA) of (a) non-functionalised membrane (FGM) and (b) functionalised membrane (FGM-SBA-15-NH2).



**Fig. S4.** (a) Extracted ion chromatogram of the five macrolide antibiotics at 150 ng mL-1 with the final chromatographic method and (b, c, d, e, f) mass spectra for each analyte.

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**Fig. S5.** Main effects plots for (a) spiramycin, (b) erythromycin, (c) josamycin, (d) roxithromycin and (e) tylosin

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**Fig. S6.** Extracted ion chromatogram for (a) spiramycin (*m/z* 422.6 > 174.1), (b) erythromycin (*m/z* 734.9 > 158.2), (c) tylosin (*m/z* 917.4 > 174.0), (d) roxithromycin (*m/z* 837.9 > 158.0) and (e) josamycin (*m/z* 828.9 > 174.1), of an uncontaminated sample (H-Egg9) compared to a sample doped to 150 ng g-1 for evaluation of the selectivity of the methodology.



**Fig. S7**. Evaluation of the greenest profile using the AGREE metric of (a) the proposed methodology based on membrane-SPE compared to (b and c) two similar methodologies that apply SPE with commercial cartridges [9,24].

**Table S1**

Description of the analysed egg samples

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Samples | Animal | Origin | Egg colour | Type of rearing |
| H-Egg-1 | Hen | Local market | Brown | Barn |
| H-Egg-2 | Hen | Smallholding | Brown | - |
| H-Egg-3 | Hen | Local market | Brown | Barn |
| H-Egg-4 | Hen | Smallholding | Brown | - |
| H-Egg-5 | Hen | Smallholding | Blue | - |
| H-Egg-6 | Hen | Smallholding | Brown | - |
| H-Egg-7 | Hen | Smallholding | Brown | - |
| H-Egg-8 | Hen | Local market | Brown | Cage |
| H-Egg-9 | Hen | Local market | Brown | Free range |
| H-Egg-10 | Hen | Local market | Brown | Cage |
| Q-Egg1 | Quail | Local market | Brown with spots | Free range |
| Q-Egg2 | Quail | Local market | Brown with spots | - |
| Q-Egg3 | Quail | Local market | Brown with spots | Free range |
| Q-Egg4 | Quail | Local market | Brown with spots | - |

* It means that it was not indicated on the label or that it is from a smallholding.

**Table S2**

Parameters of mass spectrometry analysis in positive ionization mode and retention time with gradient 4.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Analyte | Mw (g mol-1) | Precursor ions(Q1, m/z, [M+H]+) | Capillary (V) | MS2 Product Ions (Q3, m/z) | CE (V) | Dwell time (s) | Retention time (min) |
| Spiramycin  | 842.5 | 422.6 | 60 | 100.7 | 16 | 0.25 | 8.0 |
|  |  |  |  | 174.1\* | 20 | 0.25 |  |
| Erythromycin  | 733.9 | 734.9 | 60 | 115.4 | 24 | 0.25 | 9.0 |
|  |  |  |  | 158.2\* | 22 | 0.25 |  |
|  |  |  |  | 576.7 | 14 | 0.25 |  |
| Josamycin  | 827.5 | 828.9 | 60 | 108.9 | 36 | 0.25 | 10.6 |
|  |  |  |  | 174.1\* | 24 | 0.25 |  |
|  |  |  |  | 229.3 | 24 | 0.25 |  |
| Roxithromycin  | 836.5 | 837.9 | 60 | 116.1 | 22 | 0.25 | 10.1 |
|  |  |  |  | 158.0\* | 26 | 0.25 |  |
|  |  |  |  | 679.8 | 12 | 0.25 |  |
| Tylosin | 915.5 | 917.4 | 60 | 156.5 | 30 | 0.25 | 9.2 |
|  |  |  |  | 174.0\* | 30 | 0.25 |  |
|  |  |  |  | 773.0 | 22 | 0.25 |  |

\* Quantification ion.

**Table S3**

Textural properties and functionalization degree of FGM-SBA-15-NH2 compared with SBA-15.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Material | SBETa (m2 g-1) | Pore volumeb (cm3 g-1) | Pore sizec (Å)  | L0d (mmol ligand g-1) |  g SBA-15-NH2 g-1 FGM |
| FGM-SBA-15-NH2 | 28 | 0.045 | 45 | - | 0.17 |
| FGM | 2 | 0.003 | NMe | - | - |
| SBA-15-NH2 | 363  | 0.46 | 47 | 1.7 | - |
| SBA-15 | 999 | 1 | 56 | - | - |

a SBET: Specific surface area calculated by Brunauer-Emmett-Teller (BET) method. b Pore volume: Total pore volume was measured at relative P/P0=0.97. c Pore size: Pore diameter estimated by Barret-Joyner-Halenda (BJH) model applied in the desorption branch. d L0: Functionalization degree estimated by elemental analysis. e NM: Not mesurable.

**Table S4**

Preliminary studies to evaluate the loading solvent at 250 ng mL-1 using a peristaltic pump and tygon tubing for the dosing of solvents in the membrane-SPE protocol.

|  |  |
| --- | --- |
|  | Recovery (% ± SD)\*  |
| Load solvent (3 mL) | Spiramycin | Erythromycin | Josamycin | Roxithromycin | Tylosin |
| ACN | 16 ± 2 | 9 ± 13 | 8 ± 10 | 17 ± 2 | 9 ± 12 |
| Milli-Q H2O | 41 ± 16 | 50 ± 7 | 68 ± 15 | 59 ± 9 | 60 ± 9 |
| Milli-Q H2O with formic acid (0.1 %, *v/v*) | 18 ± 2 | 6 ± 6 | 34 ± 14 | 34 ± 22 | 34 ± 9 |

\* Elution with 3 mL of MeOH. n = 3

**Table S5**

Preliminary studies to evaluate the loading solvent at 100 ng mL-1 using a Scharlab ExtraVac® vacuum manifold for the dosing of solvents in the membrane-SPE protocol.

|  |  |
| --- | --- |
|  | Recovery (% ± SD)  |
| Load solvent (3 mL) | Spiramycin | Erythromycin | Josamycin | Roxithromycin | Tylosin |
| ACN | 44 ± 11  | 18 ± 7 | 21 ± 10 | 30 ± 12 | 19 ± 10 |
| Milli-Q H2O | 51 ± 6  | 76 ± 12 | 83 ± 8 | 87 ± 3 | 89 ± 12 |
| Milli-Q H2O with formic acid (0.1 %, *v/v*) | 39 ± 17  | 6 ± 1 | 94 ± 7  | 81 ± 5  | 98 ± 5 |
| Milli-Q H2O /ACN (90/10, *v*/*v*) | 24 ± 6 | 17 ± 1 | 47 ± 1 | 34 ± 1 | 28 ± 4 |
| Milli-Q H2O /EtOH (90/10, *v*/*v*) | 55 ± 1 | 40 ± 2 | 109 ± 1 | 87 ± 9 | 83 ± 7 |

\* Elution 3x3 mL of MeOH. n = 3

**Table S6**

Preliminary studies to evaluate the elution solvent at 100 ng mL-1 using a Scharlab ExtraVac® vacuum manifold for the dosing of solvents in the membrane-SPE protocol.

|  |  |
| --- | --- |
|  | Recovery (% ± SD)  |
| Elution Solvent  | Spiramycin | Erythromycin | Josamycin | Roxithromycin | Tylosin |
| 3x3 mL MeOH | 51 ± 6  | 76 ± 12 | 83 ± 8 | 87 ± 3 | 89 ± 12 |
| 3 mL MeOH + 3 mL MeOH with HAc (1%, *v/v*) + 3 mL MeOH | 6 ± 1  | 124 ± 8 | 113 ± 1 | 114 ± 13 | 118 ± 6 |
| 3 mL MeOH + 2 x 3 mL ACN | 16 ± 11  | 88 ± 7 | 82 ± 8 | 86 ± 11 | 100 ± 1 |
| 3 mL MeOH + 2 x 3 mL MeOH with ammonia solution (1 %, *v/v*) | 102 ± 13 | 99 ± 1 | 93 ± 5 | 89 ± 1 | 94 ± 3 |

\*Load in water (3 mL). n = 3

**Table S7**

ANOVA report and mathematical equations of response surface quadratic models of SLE-membrane-SPE protocol for macrolides antibiotics during Box-Behnken analysis.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Variablea | Spiramycin recovery (%) | Erythromycin recovery (%) | Josamycin recovery (%) | Roxithromycin recovery (%) | Tylosin recovery (%) |
| Regression coefficient | *p* -value | Regression coefficient | *p* -value | Regression coefficient | *p* -value | Regression coefficient | *p* -value | Regression coefficient | *p*-value |
| A | 1.5470 | 0.9964 | 4.4586 | 0.0640 | -6.3999 | 0.7698 | -4.2309 | 0.1565 | 5.7887 | 0.5363 |
| B | 23.1942 | 0.0003\* | 20.4863 | 0.0000\* | 22.9592 | 0.0000\* | 24.1015 | 0.0001\* | 18.9003 | 0.0000\* |
| C | 75.7875 | 0.9230 | -40.9711 | 0.3928 | 42.9485 | 0.7386 | 54.7368 | 0.8997 | 25.7430 | 0.5876 |
| AA | -0.3249 | 0.7309 | -1.0318 | 0.0724 | 0.5188 | 0.3998 | 0.2623 | 0.7403 | -1.1889 | 0.0866 |
| AB | 0.7008 | 0.2985 | 0.2234 | 0.5270 | 0.7489 | 0.1019 | 0.4539 | 0.4141 | 0.8805 | 0.0690 |
| AC | -4.8356 | 0.2463 | -0.6185 | 0.7722 | -1.8827 | 0.4651 | -3.9568 | 0.2574 | -1.3615 | 0.6047 |
| BB | -1.7739 | 0.0057\* | -1.5989 | 0.0003\* | -1.8822 | 0.0004\* | -1.8216 | 0.0019\* | -1.5783 | 0.0011\* |
| BC | -2.9976 | 0.3107 | 3.0925 | 0.0743 | -2.1197 | 0.2649 | -2.5127 | 0.3103 | 0.1669 | 0.9289 |
| CC | -44.5822 | 0.5397 | 33.0881 | 0.4033 | -25.0710 | 0.5875 | -30.4807 | 0.6150 | -18.7771 | 0.6920 |
| R2 | 0.9139 | 0.9684 | 0.9607 | 0.9357 | 0.9512 |
| R2 Adj. | 0.8033 | 0.9279 | 0.9102 | 0.8529 | 0.8884 |
| Equations | *y*= 4.99 + 1.54A + 23.19B + 75.78C – 0.32A2 + 0.70AB – 4.84AC – 1.77B2 – 2.99BC – 44.58C2 | *y*= 15.58 + 4.46A + 20.49B – 40.97C – 1.03A2 + 0.22AB – 0.62AC – 1.59B2 +3.09BC + 33.09C2 | *y*= 22.23 – 6.39A + 22.95B + 42.95C + 0.52A2 + 0.75AB – 1.88AC – 1.88B2 – 2.12BC – 25.07C2 | *y*= 22.64 - 4.23A + 24.10B + 54.74C + 0.26A2+ 0.45AB -3.96AC– 1.82B2 – 2.51BC –30.48C2 | *y*= 13.38 + 5.79A + 18.90B + 25.74C – 1.19A2 + 0.88AB – 1.36AC – 1.58B2 +0.167BC – 18.78C2 |

a A: Load Volume; B: Elution Volume; C: Amount of ammonia

*y*= represents the response (recovery)

\*significant *p* < 0.05.

**Table S8**

Instrumental validation parameters of the HPLC/MS-MS

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Parameters** | **Level** | **Spiramycin** | **Erythromycin** | **Josamycin** | **Roxithromycin** | **Tylosin** |
| Linear range (ng mL-1) |  | 5-500 | 1-500 | 1-500 | 1-500 | 5-500 |
| Linearity |  | 1.5·105x - 6.2·104 | 3.2·105x + 1.6·106 | 2.9·105x + 2.7·106 | 2.5·105x + 1.7·106 | 1.7·105x + 1.8·106 |
| R2 |  | 1.000 | 0.998 | 0.995 | 0.996 | 0.998 |
| LODa (ng mL-1) |  | 1.0 | 0.5 | 0.4 | 0.4 | 0.3 |
| LOQb (ng mL-1) |  | 2.6 | 1.6 | 1.2 | 1.4 | 1.1 |
| Repeatability, RSD%(Intra-day, n=6) | 1 ng mL-1 | 4 | 6 | 9 | 3 | 11 |
| 150 ng mL-1 | 7 | 5 | 4 | 4 | 4 |
| 500 ng mL-1 | 3 | 3 | 3 | 2 | 2 |
| Within-laboratory reproducibility, RSD%(Inter-day, n=9) | 1 ng mL-1 | 8 | 12 | 12 | 8 | 9 |
| 150 ng mL-1 | 6 | 10 | 14 | 12 | 8 |
| 500 ng mL-1 | 8 | 9 | 16 | 15 | 16 |

a Limit of detection. b Limit of quantification.

**Table S9**

Reproducibility studies of the FGM-SBA-15-NH2 using standard solutions at 100 ng mL-1 and egg samples doped at 150 ng g-1.

|  |  |
| --- | --- |
|   | Standard solutions at 100 ng mL-1 (Recovery, % ± SD), n = 3a |
| Batch | Spiramycin  | Erythromycin | Josamycin | Roxithromycin | Tylosin |
| M1 | 75 ± 6 | 104 ± 8 | 96 ± 1 | 94 ± 1 | 96 ± 2 |
| M2 | 102 ± 13 | 99 ± 1 | 93 ± 5 | 89 ± 1 | 94 ± 3 |
| M3 | 69 ± 6 | 92 ± 16 | 86 ± 16 | 80 ± 13 | 82 ± 16 |
| M4 | 87 ± 16 | 98 ± 11 | 95 ± 8 | 92 ± 6 | 96 ± 11 |
| M5 | 73 ± 13 | 83 ± 9 | 88 ± 16 | 81 ± 17 | 91± 11 |
| M6 | 71 ± 9 | 92 ± 9 | 92 ± 7 | 81 ± 5 | 90± 10 |
|  ± SD | 80 ± 13 | 95 ± 7 | 92 ± 4 | 86 ± 6 | 92 ± 5 |
| %RSD | 16 | 8 | 4 | 7 | 6 |
|  | Egg spiked at 150 ng g-1 (Recovery, % ± SD), n = 3b |
| Batch | Spiramycin  | Erythromycin | Josamycin | Roxithromycin | Tylosin |
| M2 | 96 ± 2 | 78 ± 8 | 95 ± 6 | 93 ± 5 | 83 ± 2 |
| M3 | 99 ± 3 | 92 ± 5 | 96 ± 6 | 99 ± 1 | 97 ± 3 |
|  ± SD | 98 ± 2 | 85 ± 10 | 96 ± 1 | 96 ± 5 | 90 ± 10 |
| %RSD | 2 | 12 | 1 | 5 | 11 |

aMembrane-SPE (Activation: 3 mL MeOH with 1 % ammonia solution; conditioned: 2 x 3 mL water; load: 3 mL of standard solution (100 ng mL-1) containing the 5 macrolides; elution: 3 mL MeOH and 2 x 3 mL MeOH (1 % ammonia solution))

aSLE-membrane-SPE protocol definitive for egg samples explained in section 2.6.

**Table S10**

Content of macrolide antibiotics in different egg samples obtained with SLE-membrane-SPE protocol validated.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Samples | Animal | Spiramycin (ng g-1) | Erythromycin(ng g-1) | Josamycin(ng g-1) | Roxithromycin(ng g-1) | Tylosin(ng g-1) |
| H-Egg-1 | Hen | MDL-MQL | ND | ND | ND | ND |
| H-Egg-2 | Hen | ND | ND | ND | ND | ND |
| H-Egg-3 | Hen | ND | ND | ND | ND | ND |
| H-Egg-4 | Hen | ND | ND | ND | ND | ND |
| H-Egg-5 | Hen | ND | ND | ND | ND | ND |
| H-Egg-6 | Hen | ND | ND | ND | ND | ND |
| H-Egg-7 | Hen | ND | ND | ND | ND | ND |
| H-Egg-8 | Hen | ND | ND | ND | ND | ND |
| H-Egg-9 | Hen | ND | ND | ND | ND | ND |
| H-Egg-10 | Hen | ND | ND | ND | ND | ND |
| Q-Egg1 | Quail | ND | ND | MDL-MQL | MDL-MQL | ND |
| Q-Egg2 | Quail | ND | ND | ND | ND | ND |
| Q-Egg3 | Quail | ND | ND | ND | ND | ND |
| Q-Egg4 | Quail | ND | ND | ND | ND | ND |

MDL-MQL: Between the limit of quantification and detection of the method

MDL/MQL for Spiramycin: 0.6/2.1 ng g-1

MDL/MQL for Josamycin and Roxitromycin: 0.3/1.1 ng g-1

ND: Not detected

**Table S11**

Comparison of proposed methodology with other approaches based on SLE or SLE-SPE for macrolide analysis in egg samples.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Analytes** | **SLE** | **SPE** | **Analytical technique** | **Recovery for macrolides (%)** | **MDL(μg kg−1)****MQL(μg kg−1)** | **[Ref.]** |
| SPI, ERY, TYL, TIL and oleandomycin | Egg (5 g), ACN (3 mL), vortex (15 s), ACN (9 mL), stirring (15 min) centrifugation (15 min), 2 g NaCl, hexane (10 mL), stirring (15 min) centrifugation (15 min), evaporation (only ACN) and reconstitution (7 mL) | OASIS® HLB Plus (225 mg) *Load:* extract (7 mL)*Elution:* MeOH (95 % *v/v)* (5 mL) | HPLC-MS/MS | 95-98 % | 0.1-0.9Not shown | [9] |
| ERY, TYL, CLA, polyether ionophores and lincomycin | Egg (2 g), ACN (4 mL x 2), vortex (15 s), stirring (30 min) centrifugation (5 min, 4 ºC) evaporation (only 250 µL) and reconstitution (1 mL) | - | HPLC-MS/MS |  79-168%  | 0.09-0.160.29-0.53 | [6] |
| ERY, TIL, TYL, JOS and 25 veterinary drugs  | Egg (1 g), ACN (10 mL), citrid acid 0.5 M (1 mL) and Na2EDTA 0.1 M (0.5 mL), vortex (1 min) | OASIS® HLB (200 mg) as clean up*Load*: extract (11.5 mL)*Elution*: Collect the extract only (11.5 mL)  | UHPLC-MS/MS |  75-97% (at 100 μg kg−1) | Not shown1.5-5.0  | [19] |
| 120 antibiotics including 18 macrolides | Egg (2 g), ACN:H2O (90:10*v/v)* (10 mL), UAE (10 min),centrifugation (10 min), evaporation to 1 mL remained, dilution andvortex (2 min), centrifugation (10min) | OASIS HLB® (500 mg)*Load:* extract (6 mL)*Elution:* FA:MeOH (5/95, *v/v*) (5 mL) and ethyl acetate (5 mL) | LC-MS/MS | Not shown | 0.5-3.01.5-10.0 | [24] |
| Gamithromycin | Egg (2 g), ACN (20 mL) andNa2SO4 (2 g), centrifugation (5 min) | OASIS® MCX (60 mg)*Load*: extract (5 mL) *Elution:* 5% ammoniated ACN (3 mL) | UHPLC-MS/MS | 96 % (at 10 μg kg−1) | 0.30–0.40 0.80 – 1.0  | [19] |
| TIL, JOS, Kitasamycin, AZI and other veterinary drugs | Egg (5 g), Na2EDTA (0.1 mM) (5 mL), 1% HAc in ACN (20 mL), vortex (1 min) and salts (Na2SO4, 4 g and NaCl, 1 g), vortex (1 min), centrifugation  | Silanized melamine sponge into a 2.5-mL syringe barrel*Load*: Extract (1 mL)*Elution*: MeOH-H2O solvents (50/50, *v/v*) (1 mL) | UPLC-MS/MS | 78-90 % (at 100 μg kg−1) | 0.2-20.5-5.9 | [8] |
| SPI, ERYJOS, ROX,TYL | Egg (1 g), ACN (2.5 mL) and MgSO4 (0.1 g), stirring (5 min), centrifugation, evaporation and reconstitution  | FGM-SBA-15-NH2*Load:* Extract (4 mL)*Elution:* MeOH (0.5 % ammonia) (3 x 2.5 mL) | LC-MS/MS | 85-92 % (at 150 μg kg−1) | 0.3-0.61.1-2.1 | This work |

*Abbreviations*: ACN: Acetonitrile; ACE, Acetylspyramycin; AZI: Azithromycin; CLA: Clarithromycin; FA: Formic acid; HAc: Acetic Acid; ERY: Erythromycin; MeOH: Methanol; MDL: method detection limit; MQL: method quantification limit; MSPD: Solid phase matrix dispersion; QuEChERS: Quick, Easy, Cheap, Effective, Rugged & Safe; ROX: Roxithromycin; SLE: Solid-liquid extraction; SPE: Solid-liquid extraction; SPI: Spyramycin; TIL: Tilmicosin; TYL: tylosin,