Non-targeted analysis of lipidic extracts by high-resolution mass spectrometry to characterise the chemical exposome: comparison of four clean-up strategies applied to egg

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Abstract

Biota samples are used to monitor chemical stressors and their impact on the ecosystem and to describe dietary chemical exposure. These complex matrices require an extraction step followed by clean-up to avoid damaging sensitive analytical instruments based on chromatography coupled to mass spectrometry. While interest for non-targeted analysis (NTA) is increasing, there is no versatile or generic sample preparation for a wide range of contaminants suitable for a diversity of biotic matrices. Among the contaminants' variety, persistent contaminants are mostly hydrophobic (mid- to non-polar) and bio-magnify through the lipidic fraction. During their extraction, lipids are generally co-extracted, which may cause matrix effect during the analysis such as hindering the acquired signal. The aim of this study was to evaluate the efficacy of four clean-up methods to selectively remove lipids from extracts prior to NTA. We evaluated (i) gel permeation chromatography (GPC), (ii) Captiva EMR-lipid cartridge (EMR), (iii) sulphuric acid degradation (H_2SO_4) and (iv) polydimethyl siloxane (PDMS) for their efficiency to remove lipids from hen egg extracts. Gas and liquid chromatography coupled with high-resolution mass spectrometry fitted with either electron ionisation or electrospray ionisation sources operating in positive and negative modes were used to determine the performances of the clean-up methods. A set of 102 chemicals with a wide range of physico-chemical properties that covers the chemical space of mid- to non-polar contaminants, was used to assess and compare recoveries and matrix effects. Matrix effects, that could hinder the mass spectrometer signal, were lower for extracts cleaned-up with H_2SO_4 than for the ones cleaned-up with PDMS, EMR and GPC. The recoveries were satisfactory for both GPC and EMR while those determined for PDMS and H_2SO_4 were low due to poor partitioning and degradation/dissociation of the compounds, respectively. The choice of the clean-up methods, among those assessed, should be a compromise that takes into account the matrix under consideration, the levels and the physico-chemical properties of the contaminants.

Keywords

Non-targeted analysis; Clean-up; Lipid removal; High-resolution mass spectrometry; Recoveries; Matrix effects.

1. Introduction

Over the past 30 years, more than 150,000 chemicals have been registered for commercial use in Europe, the United States and Canada [1]. The Chemicals Abstracts Service has assigned over 204 million registry numbers as of June 2023, i.e. 64 million more than in September 2018 and 164 million more than in 2002 [2]. These chemicals' hazard and fate are less well characterised than the legacy contaminants. A portion of them may be released into the environment in various ways and may enter food chains with potential health issues for wildlife and humans [3,4]. Also, the transformation products, which may in certain cases be more persistent or toxic than the parent compound [5], broaden the spectrum of physico-chemical properties of the compounds to be characterised.

Hazardous and persistent chemicals need to be monitored to manage the risks they pose and ensure healthy ecosystems. Their analysis in biotic samples generally involves sample preparation, combining extraction and purification followed by data acquisition [6]. Chromatography coupled with mass spectrometry makes it possible to separate, identify and quantify contaminants. Non-targeted strategies, including suspect screening, are designed to describe samples' comprehensive fingerprints. As opposed to targeted analysis, non-targeted analysis (NTA), based on full scan mode high-resolution mass spectrometry (HRMS), is characterised by its non-selective data acquisition for thousands of chemicals over a wide mass range. A major drawback of full scan mode is its lower sensitivity compared to acquisition modes used for targeted analysis such as multiple reaction monitoring [7]. Furthermore, when performing mass spectrometry analysis on biotic samples, the significant presence of matrix interferents can cause ion suppression or enhancement, hindering the signal acquisition [8,9].

The development and implementation of sample preparation for contaminants analysis in biota by NTA is a challenge due to their wide physico-chemical properties (octanol-water partition coefficient K_{ow}, molecular mass, stability) and the complexity of these matrices (essentially lipids, proteins, water). Nevertheless, due to the lack of generic sample preparation methods for NTA, most studies available in the literature used sample preparation inspired from targeted methods to extract (solvent

extraction, QuEChERS) and clean-up (GPC, SPE, multilayer silica) the samples [10]. In NTA workflows, mid- to non-polar persistent contaminants are generally extracted from biotic environmental and food samples with solvent using pressurised liquid extraction [11–14], ultrasonication [15,16] or maceration [17,18]. These extractions involve the use of non-polar solvent or a mixture of non-polar and slightly polar solvents resulting in the co-extraction of matrix components such as lipids (triglycerides, phospholipids etc.) which could cause analytical problems such as poor chromatographic separation or alteration of the signal acquired. To preserve most of the contaminants in the extract while ensuring compatibility with analytical instruments based on chromatography coupled with HRMS, selective lipid clean-up methods should be used after biota extractions. Recently Dubocq et al. [19] compared several extractions and clean-up methods for fish tissue, a fatty matrix, and concluded that ultrasonication extraction followed by deactivated silica clean-up presented the best recoveries and repeatability for mid- to non-polar contaminants (pesticides, flame retardants). Since deactivated silica clean-up involves hydrophobic interactions, it is selective for both lipids but also hydrophobic contaminants that can be removed from the extract. In this study, four less selective clean-ups methods for which the efficiency of isolating lipids and other matrix components from contaminants in lipid extracts were assessed. These methods, as detailed below, were specifically selected for their potential to remove lipids, via their ability to take advantage of the physico-chemical properties of lipids such as their molecular size and reactive chemical group.

Most classes of lipids consist of linked fatty acids with 18 carbon atoms in their hydrocarbon chains, although this number could theoretically range between 4 and 26 carbon atoms [20]. These lipids, which may be larger than the chemical contaminants, can thus be separated using gel permeation chromatography (GPC), a separation technique based on size exclusion. GPC was used as early as 1972 to isolate pesticides and PCBs from fish lipids [21]. Since then, GPC has been used for both targeted analysis [22] and NTA [13] on complex fatty extracts from biotic samples by collecting cleaned-up extracts after the elution of the largest lipids amount and other large matrix components. However,

when using this method, chemicals similar in molecular size to lipids are lost in the process, such as contaminants with long carbon chain (e.g., phthalates, chlorinated paraffins, PFAS).

Faced with this challenge associated to the absence of a satisfactory protocol, and to respond to this demand, the analytical industry took into account the problems posed by lipid extracts and proposed an easy-to-implement solution. In 2017, Agilent commercialised Enhanced Matrix Removal (EMR)-Lipid, a phase, initially used as a dispersive phase, embedded into a cartridge that combines size exclusion and hydrophobic interactions to selectively remove lipids from extracts. To improve the efficiency of lipid removal, it has been recommended to load an extract containing at least 10% water [23,24]. The lipid selectivity of this cartridge appears promising for cleaning-up lipidic extracts with recoveries between 60% and 120% and minor matrix effects (8% of mean signal enhancement) [25,26].

As fatty acids are mostly bound to glycerol or phosphoric acid in the form of esters they can be hydrolysed with sulphuric acid and water [27,28]. The literature reports examples of comparing this strategy with GPC after the cleaning step, concluding that acidification had a negative effect on several compounds such as bromoindole and halogenated methyl bipyrroles [29]. It is assumed that only non-dissociated compounds under acidic conditions, acid-resistant compounds (POPs and other persistent chemicals) and degradation products generated during the process could be detected after such destructive clean-up [30].

Other strategies such as the use of polydimethylsiloxane (PDMS) as a sampler, used to extract contaminants from adipose tissues in order to avoid cleaning steps, have also been reported. Indeed, contaminants are extracted from tissues by passive diffusion towards the PDMS. PDMS has been used as passive sampler in several geometries: solid phase microextraction coating [31], microtube [32] and thin-film [33,34]. The kinetics equilibrium in diverse mammalian tissues was extensively reviewed [35] and the more lipid-rich a tissue is, the faster is time to equilibrium with lipid-rich tissues and pure lipid reaching steady state within 24 h [36]. As the partition coefficient between PDMS and lipid varies by less than one to two orders of magnitude for chemicals covering 10 orders of magnitude in

hydrophobicity [37,38] , partition with PDMS is in principle amenable to NTA because all chemicals are extracted with similar efficacy.

In order to comprehensively address the problem linked to the presence of lipids in extracts dedicated to the analysis of non-polar contaminants, this study aimed to compare the performances of these four techniques by adapting the published methods (GPC, EMR, H_2SO_4 and PDMS). Hen eggs were used as a model matrix due to their high lipid content but also the diversity of lipid classes present [39]. A set of 102 chemicals exhibiting a broad range of physico-chemical properties were selected to evaluate recoveries and matrix effects. Cleaned-up extracts were analysed by GC-EI(+)-HRMS and LC-ESI(±)-HRMS. Linearity, limit of detection (LOD) and combination of the analytical methods were evaluated prior the methods performances assessment.

2. Materials and methods

2.1. Chemicals and materials

Toluene, acetone, cyclohexane, ethyl acetate (LV-GC SuperTrace grade) and dichloromethane (Dioxins, Pesti-S, Furans, PCB's Analysis grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Water and acetonitrile LC-MS grade were obtained from VWR (Radnor, PA, USA) and Riedel-de-Haën (Seelze, Germany), respectively. Concentrated sulphuric acid (98%) was provided by Panreac (Barcelona, Spain). Anhydrous sodium sulphate was purchased from Merck (Emsure® grade, Darmstadt, Germany). Captiva EMR-lipid cartridges (6 mL, 600 mg) were obtained from Agilent Technologies (Santa Clara, CA, USA). PDMS sheets (SSP-M823, Special Silicone Products, Ballston, USA) with thickness of 1 mm and a density of 1.17 g cm^{-3} were provided by Shieldings Solutions (Great Notley, Great Britain).

The performance of the methods was assessed using a spiking solution constituted of 102 compounds at concentration range from 0.05 to 1 $ng.\mu L^{-1}$ depending on the concentration of the individual reference standards (Table B.1). In order to simplify the discussion, we will refer to compound

concentrations as the dilution of the spiking solution hereafter. Extensive details such as acronyms, molecular formula, InChiKeys, compound class and supplier are also provided in Table B.1. $^{13}C_{10}$-anti-Dechlorane Plus (anti-DP), used as external standard for the fraction analysed by GC-EI(+)-HRMS, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). $^{13}C_{12}$–Tetrabromobisphenol A (TBBPA) and $^{2}H_{18}$-β-hexabromocyclododecane (HBCDD), used as external standard for the fraction analysed by LC-ESI(±)-HRMS, were obtained from Wellington Laboratories (Guelph, Ontario, Canada).

To minimize procedural contamination, several precautions were taken. All glassware was heated at 400 °C for 4 h before use. Teflon caps and magnetic bar were rinsed with dichloromethane before use. Handling of samples and extracts was carried out in an overpressure room as much as possible.

2.2. Model lipid extract solution

Eggs from caged hens were purchased at a local store (Nantes, France) in November 2021. Whole eggs were pooled and freeze-dried resulting in a weight loss of 75.4%. The fat content of the pool was determined gravimetrically to be 10.4% (*l.w./w.w.*) following a method detailed by Bichon et al. [40]. Microwave assisted extraction (Anton Paar, Graz, Austria) was used to extract the lipids from the freeze dried samples for subsequent assessment of the clean-up methods. For each glass tube, approximately 1 ± 0.2 g freeze dried sample was extracted with 20 mL toluene/acetone mixture (70:30, *v/v*). Within 2 min, the temperature reached 130 °C and was maintained for 20 min. Agitation was done by a magnetic bar, at 600 rpm during the entire extraction. Centrifugation was applied to separate the solid and the liquid phases (1000 *g*, 10 min, 20 °C). Liquid extracts, containing lipids, were collected, pooled and evaporated to obtain 9 g of dried lipid extract. The nine grams of lipids were suspended in a cyclohexane/ethyl acetate mixture (50:50, *v/v*) at 200 $mg_{lipid}.g_{extract}^{-1}$.

2.3. Clean-up methods

The clean-up methods were selected for their efficiency to remove lipids. The workflow depicted in Figure 1 was developed to evaluate their performances to preserve contaminants in the extract while remove the matrix interferents. Considering that the GPC device capacity was limited to 200 mg of

lipids, we selected this sample amount for all clean-up methods in order to avoid biases in the comparison interpretations.

2.3.1. Size exclusion using gel permeation chromatography (GPC)

This clean-up procedure was adapted and modified from Abdel Malak et al. [41]. Aliquots of 1 g of the lipidic extract at 200 $mg_{lipid}.g_{extract}^{-1}$ were dried under a gentle stream of nitrogen and suspended in 500 µL of cyclohexane/ethyl acetate mixture (50:50, *v/v*) and fractionated through a GPC column (58 cm × 24.4 mm) packed with Bio-Beads SX-3 (Bio-Rad, Philadelphia, PA, USA) using cyclohexane/ethyl acetate mixture (50:50, *v/v*) as mobile phase at a flow rate of 5 mL min^{-1} during 70 min.

A preliminary experiment aimed at optimising the collection of fractions to remove the lipids was carried out. To do this, the extract was fractioned into 28 eluates collected every minute between 5 and 33 min, then dried under a gentle stream of nitrogen and weighted. Gravimetric determination of dried residues showed that lipids were eluted within the first 21 min of analysis. Therefore, the fraction between 22 min and 70 min (end of elution) was collected in a round-bottom flask. The collected extracts were concentrated and transferred to new glass tubes.

2.3.2. Size exclusion and hydrophobic interaction using Captiva EMR-lipid (EMR) cartridges

This clean-up procedure was adapted and modified from Zhao et al. [26]. Aliquots of 1 g of the extract containing 200 $mg_{lipid}.g_{extract}^{-1}$ were dried under a gentle stream of nitrogen, suspended in 2 mL acetonitrile/ethyl acetate mixture (75:25, *v/v*) and vortexed. The extracts were warmed up to 40 °C for 1 h to accelerate contaminants partitioning between the lipids and the solvent phases. After centrifugation under cooled temperature to make easier the phase separation and to minimize the lipid amount solubilize into the solvent layer (700 *g*, 10 min, 0 °C), the solvent phase was separated. This procedure was repeated once and solvent phases were combined. Water (1 mL) was added to the solvent phase. Captiva EMR-lipid cartridges were rinsed and conditioned with 4 mL acetonitrile/ethyl acetate/water (20:60:20, *v/v/v*). The solvent phases were loaded onto the cartridges and eluates collected by gravity without adding any further eluent. Eluates were concentrated until

193 approximatively 10 µL under at gentle stream of nitrogen and suspended with 2 mL toluene.
194 Anhydrous sodium sulphate was added to eliminate water traces. The eluates were centrifuged (700
195 *g*, 2 min, 20 °C) and the organic layer was transferred to new glass tube.

196 *2.3.3. Ester hydrolysis using sulphuric acid (H_2SO_4)*

197 This clean-up procedure was adapted and modified from Cariou et al. [11]. Aliquots of 1 g of the extract
198 solution at 200 $mg_{lipid}.g_{extract}^{-1}$ were dried under a gentle stream of nitrogen and suspended in 12 mL
199 cyclohexane. The extracts were partitioned with 2 mL concentrated sulphuric acid. After centrifugation
200 (700 *g*, 10 min, 20 °C), the organic layer was separated. This procedure was repeated four times at
201 more drastic conditions as the ester hydrolysis is a slow chemical reaction (60 °C, 6 h, frequent vortex-
202 mixing). The organic layer was neutralised three times with 2 mL water to obtain pH = 7 in the aqueous
203 layer. The organic layer was then dried with anhydrous sodium sulphate, centrifuged (700 *g*, 2 min, 20
204 °C) and transferred to new glass tube.

205 *2.3.4. Differential partitioning using polydimethylsiloxane (PDMS)*

206 This clean-up procedure was adapted and modified from Baumer et al. [35]. While the authors had
207 directly extracted lipid-rich tissue with PDMS, here we used the method to partition from the already
208 enriched lipids. The advantage is that a pure lipid phase reaches equilibrium faster than tissue with
209 lower lipid content. For egg yolk, it would be possible to extract directly but, for applicability to lean
210 tissue and comparison with the other three methods, we applied PDMS to the lipidic extracts. PDMS
211 disks were cleaned by Soxhlet extraction using ethyl acetate for 24 h and stored at room temperature
212 in amber bottles containing ethyl acetate. The PDMS disks were air dried during 2 h and the initial
213 masses were determined prior to the experiment.

214 Aliquots of 1 g of the extract at 200 $mg_{lipid}.g_{extract}^{-1}$ were dried under a gentle stream of nitrogen. Solvent
215 cleaned PDMS disks (125 mg, 12 mm × 1 mm) were embedded in the viscous lipid phase and
216 dynamically exposed to the dried extracts on an orbital shaker during 24 h at 40 °C. The PDMS disks
217 were cleaned with lint-free paper wipes and water, then weighted to determine the coextracted matrix

component. Then PDMS disks were extracted twice with 2.5 mL ethyl acetate during 2 h and the two fractions were combined into new glass tube.

2.4. Analysis and processing

2.4.1. Cleaned-up extract conditioning

Each cleaned-up extract was reconstituted in 40 µL toluene with 5 ng $^{13}C_{12}$-TBBPA and 10 ng $^{13}C_{10}$-anti-DP for GC-HRMS analysis (GC fraction). A 20 µL aliquot of the GC fraction was further dried under a gentle stream of nitrogen and reconstituted in 20 µL acetonitrile with 4 ng $^{2}H_{18}$-β-HBCDD for LC-HRMS analysis (LC fraction).

2.4.2. GC-EI-HRMS analysis

The GC fractions were analysed in a single sequence according to the method developed by Simonnet-Laprade et al. [42] with minor modifications. Briefly, a Trace 1310 gas chromatograph coupled to an Orbitrap Q Exactive GC mass spectrometer (Thermo Scientific, San José, CA, USA) was used. Injection (1 µL) was performed at 300 °C in the splitless mode onto a DB-5MS column (30 m × 0.25 mm, 0.25 µm, Agilent, Palo Alto, CA, USA). The carrier gas was helium at a flow rate of 1 mL min^{-1}. The temperature gradient started at 100 °C (held for 2 min) and raised at 10 °C min^{-1} to a final temperature of 325 °C (held for 10 min). Electron ionization source was operated at an electron energy set at 70 eV and data were acquired in full-scan mode over the range of 120-800 *m/z* at a resolving power of 120,000 at *m/z* 200 (acquisition window = 5-32 min). The automatic gain control target was set at 5.10^5 and the maximum injection time at 200 ms.

2.4.3. LC-ESI(±)-HRMS analysis

The LC fractions were analysed in two sequences according to the method developed by Cariou et al. [11] with minor modifications. Briefly, an Ultimate 3000 UHPLC pumping system coupled to an Orbitrap Q Exactive mass spectrometer was used. Five µL were loaded on a reverse phase C_{18}-like column (Hypersil Gold, 100 × 2.1 mm, 1.9 µm fitted with a Hypersil Gold guard column, 10 × 2.1 mm, 1.9 µm,

242 Thermo Fisher Scientific) that was kept at 45 °C. The mobile phase consisted of water (A) and
243 acetonitrile/water (99:1, *v/v*, B) each containing 10 mM ammonium acetate with a flow rate set at 0.4
244 $mL.min^{-1}$. The gradient started with 20% B (held for 2 min), ramped linearly at $2.5\%.min^{-1}$ to 40% B then
245 at $2\%.min^{-1}$ to 100% B (held for 6 min) before returning to 20% B at $40\%.min^{-1}$ for equilibration (held
246 for 4 min). The total run time was 52 min. Data were recorded in both negative and positive modes in
247 the same run with heated ESI source parameters as follows: sheath gas flow = 50 arbitrary units (AU),
248 auxiliary gas flow = 5 AU, auxiliary gas temperature = 150 °C, capillary temperature = 350 °C, spray
249 voltage = 2.5 kV, s-lens radio frequency = 50 AU. HRMS system was operated in full scan mode over
250 the range 120-1000 *m/z* at a resolving power of 70,000 at *m/z* 200 (acquisition window = 0-48 min).
251 The automatic gain control target was set at 5.10^5 and the maximum injection time at 250 ms.

252 *2.4.4. QA/QC*

253 Procedural blanks were prepared in triplicates at the same time and using the same methods in order
254 to assess only the clean-up procedural contamination. Water was used as blank for the PDMS
255 procedure.

256 To assess the mass spectrometer detector response during the sequence, a pooled QC sample
257 constituted with aliquots of each final extract was prepared. For GC-HRMS, the pooled QC sample was
258 injected every 10 randomised samples. For LC-HRMS, the pooled QC sample was injected five times
259 before the samples (column conditioning) and then every 10 randomised samples.

260 *2.4.5. Data processing*

261 Peak integration was performed using Skyline software [43,44] for both GC- and LC-HRMS data. For
262 each compound, 3 ions were searched in GC-HRMS data (1 quantifier and 2 qualifiers) and 2 ions were
263 searched in LC-HRMS data (1 quantifier and 1 qualifier) (Table B.1).

264 In order to correct the detector response variation during the sequences and considering that mimetic
265 internal standards were not available for all the compounds, peak areas were corrected by the pooled

QC sample using the "batch correction" module implemented into Workflow4Metabolomics [45,46]. Loess pool regression method was used for both analytical methods [47].

2.5. Methods performance assessment

The performance of the methods was assessed using the spiking solution containing diverse set of 102 mid to non-polar compounds including pesticides and their transformation products, polycyclic aromatic hydrocarbons (PAHs), phthalates, POPs and flame retardants (Table B.1), at concentrations in the range of 6.25-125 $ng.g^{-1}$ lipids (0.625 to 12.5 $ng.g^{-1}$ w.w.). These spike levels are higher than those reported in food analysis, but are in the same concentration ranges of those reported in the literature for environmental eggs like sea turtle eggs [48], terrestrial bird eggs [28,49] and seabird eggs [50,51]. The selected compounds covered a wide range of physico-chemical properties: molecular weight (122.6-706.1 Da), water solubility expressed as log K_{ow} (-0.9 to 10.6), molecule size expressed as collisional cross section (CCS) value (119.4 to 267.4 Å), halogenation degree and detectability in GC-EI and/or LC-ESI(+) and/or LC-ESI(-). We assumed that the previously mentioned extraction method is ideal for extracting hydrophobic compounds from the sample. However, we opted for mid-to non-polar compounds to encompass a slightly wider range of the chemical space.

Before assessing the performance of the clean-up methods, instrumental sensitivity and linearity, expressed by the limit of detection (LOD) and the coefficient of determination (R^2) respectively, were checked with an external calibration curve at dilution of *1/40*, *1/20*, *1/10*, *1/5*, *1/2.86*, *1/2*, *1/1.6*, *1/1.33* and *1/1* of the spiking solution concentration (Table B.1). The LOD was determined as the lowest concentration which allows to detect chromatographic peak defined with at least 5 consecutives scans, considering the slow acquisition rate of HRMS in full-scan mode, and *S/N* higher than 3. Spiked compounds were included to the performance assessment if their LOD was greater than *1/5* dilution. The linearity was accepted if R^2 was greater than 0.9 with at least 5 calibration concentrations. This R^2 value is less stringent in NTA than the acceptation criterion commonly used in targeted analysis

(R^2>0.98 or 0.99) because the signal variation in NTA is not normalised with fully mimetic internal standards.

Absolute recovery and matrix effect were selected criteria for the method performances assessment. Calculation was achieved for the compounds of the spiking solution detected in the pooled QC sample for which LOD and linearity were accepted. If a compound was detected by several techniques (GC-EI and/or by LC-ESI(+) and/or LC-ESI(-)), all values were considered and checked for consistency. Recoveries of compounds detected by several techniques appeared globally consistent. Recovery and matrix effect were calculated with equations (1) and (2), respectively. Equation (2) corrects for the endogenous levels.

$$\text{Recovery (\%)} = \left(\frac{\text{Area}_{\text{comp. spiked be. ext.}}}{\text{Area}_{\text{comp. spiked af. ext.}}}\right) \times 100 \quad (1)$$

$$\text{Matrix effect (\%)} = \left(\frac{\text{Area}_{\text{comp. spiked af. ext.}} - \text{Area}_{\text{comp. non-nspiked sample}}}{\text{Area}_{\text{standard}}} - 1\right) \times 100 \quad (2)$$

With “comp” the considered compound, “be. ext.” and “af. ext.” meaning before and after extraction, respectively.

For the recovery assessment, an aliquot (15 g) of the lipidic extract solution at 200 $mg_{lipid}.g_{extract}^{-1}$ was spiked with 375 µL of the spiking solution (Table B.1), resulting in 25 µL spiking solution per g of lipidic extract solution. This solution was vortex-mixed during 5 min and stored at -20 °C to equilibration for a night. For the matrix effects, extracts were spiked with 25 µL of the spiking solution just prior to the reconstitution in toluene. Non-spiked extracts were prepared with the same clean-up methods to check endogenous contamination.

3. Results and discussion

3.1. Detection and instrumental performances

312 The selected compounds of the spiking solution (n = 102) cover a wide range of physico-chemical
313 properties as indicated above. This was confirmed by comparing the chemical space covered by these
314 102 compounds with those covered by compounds listed in the CECscreen database with molecular
315 weight below 1000 $g.mol^{-1}$ [52] (Figure 2A).

316 The complementarity between selected separation and ionisation techniques is illustrated Figure 2B.
317 In total, 97 out of the 102 compounds were detected with at least one technique in the spiking solution.
318 Naphthalene, acenaphthylene and acenaphthene were not detected, likely due to losses during
319 evaporation process (volatile compounds) or inappropriate GC parameters. Malathion dicarboxylic
320 acid and 6-chloronicotinic acid, two pesticides transformation products, were not detected, likely due
321 to inappropriate ionisation parameters.

322 GC-EI-HRMS led to the detection of 64 compounds including 39 that were also detected by LC-ESI(±)-
323 HRMS. LC-ESI(±)-HRMS led to the detection of 73 compounds, 35 for the positive polarity only, 31 for
324 the negative polarity only and 7 for both polarities. Most of the phthalate compounds could be
325 detected both by GC-EI and LC-ESI(+) and their LODs were quite similar (Table B.1). All pesticides and
326 pesticide transformation products could be detected by LC-ESI(±) with low LODs except deltamethrin,
327 4,4'-DDE, β-HCH and HCB that could be detected only by GC-EI. Flame retardants, PAHs and POPs could
328 only be detected by GC-EI, except α-HBCDD, TBBPA and 2,4-dibromophenol, while hydroxy-PBDE have
329 only been detected by LC-ESI(-). Globally, phenolic compounds were more sensitive to ESI(-) due to the
330 hydrogen lability. Although expected, these observations confirmed that the combination of GC-EI and
331 LC-ESI(±) allowed covering a broad range of compounds for the suspect screening or NTA. A majority
332 of compounds was detected at the highest dilution factor with at least one of the analysis techniques.
333 Only a few compounds, including PAHs (fluorene, fluoranthene, pyrene and dibenzo (a,e) pyrene),
334 phthalates (DMT, DAP, DiNcH, TBR-DEHP), hydroxyl-BDEs (OH-BDE-28, OH-BDE-85 and OH-BDE-137),
335 BPF, deltamethrin, mecoprop, chlormequat, DETP, triclosan sulphate, triclosan glucuronide and FPrPA,
336 presented LODs greater than the highest dilution factor used for all analytical conditions.

In the present study, suspect screening was applied to assess the performances of the four tested clean-up methods for a wide range of contaminants by direct comparison of the peak areas between samples and only one calibration dilution. For this purpose, linearity was accepted if R^2 for the external calibration curve was greater than 0.9. In that respect, linearity appeared acceptable for a majority of compounds with at least one of the analysis techniques (Table B.1). DMT, chlormequat, FPrPA, 2,4-D and DMDTP were the only compounds with R^2 below 0.9 whatever the analysis techniques.

Since signal area was batch corrected using the pool QC samples, only the compounds detected in all the pool QC samples were used for assessing the clean-up methods performances. Consequently, DiNA was not considered in GC-EI-HRMS, BPF and p-toluensulfonamide were not considered in LC-ESI(-)-HRMS and benzophenone-3, DAP, DPhP and fenhexamid were not considered in LC-ESI(+)-HRMS.

3.2. Clean-up methods performances assessment

Eggs from caged hens were a relevant model matrix due to their high lipid content associated with a wide diversity of lipid classes [39]. In addition, low contamination levels were described for this type of egg, which helps prevent any potential bias involving spiked compounds that may arise from endogenous contamination [53,54]. Before applying equations (1) and (2) to calculate recoveries and matrix effects, respectively, the endogenous contamination level in non-spiked extracts was checked to minimise bias in the calculations of recovery.

3.2.1. Co-extracted matrix after clean-up

Extract appearance could present an overview of the clean-up performance based on the matrix elimination. While extracts cleaned-up by H_2SO_4 were colourless and transparent, extracts cleaned-up by PDMS, GPC and EMR were coloured with a gradient from yellowish to orange, respectively. However, when these extracts were reconstituted in acetonitrile before LC-ESI(±)-HRMS analysis, the extracts were totally solubilised in one liquid phase, suggesting that triglycerides and phospholipids, the main lipophilic lipid classes in poultry eggs, were removed [39,55].

The PDMS disks gained about 0.6 mg after the partitioning. This suggests that 0.3% of the initial lipid mass was taken up into the PDMS disks. This weight gain represents 0.48 ± 0.1% of the PDMS disk mass, which is in the range of that recorded by for PDMS exposed to pork adipose tissue [35] and dugong blubber [34]. The LLE step prior the EMR-lipid cartridge extracted 32 mg of lipids and 96.4% of these lipids were removed by the EMR-lipid cartridge resulting of a 99.6% overall lipid removal. These values were in accordance and even better than those reported in the literature. Pedersen et al. [24] reported that 97.2% of the matrix was removed from QuECHERS extracts of marine mammal bubbler cleaned-up with one Captiva EMR lipid cartridge. Zhao et al. [26] also determined that 85% of the matrix was removed from olive oil extract obtained with a similar LLE prior the EMR-lipid cartridge loading. Muz et al. [25] reported an average of 98.2% of lipids were removed from a salmon lipid extract using an LLE process involving acetonitrile. We estimated that 13.5 mg (27%) of salmon lipids were extracted in this LLE condition. We hypothesized that if more lipids are extracted during an LLE involving a more non-polar solvent mixture, more hydrophobic contaminants are also extracted.

Weights of residual matrix components were not determined for H_2SO_4, and GPC. The preliminary experiments carried out to determine the GPC fraction collection indicated that no measurable mass remained after 22 min (Figure A.1). However, a slight deposit was visible after drying.

3.2.2. Matrix effect assessment

Matrix effect results from unremoved matrix components that may cause signal suppression or enhancement in mass spectrometry [56–58]. If signal alteration can be characterised and corrected with the addition of mimetic labelled standards with targeted analysis, such matrix effect may impede compound detection with NTA. NTA interpretation is particularly vulnerable to matrix effect as a few standards, not necessarily mimetic of the matrix effect, are added to the sample. Matrix effects disturbing compounds detected by several techniques appeared independent and variable from one technique to another. Indeed, the presence of interferences and their competition for charges at a

target compound retention time greatly depend on the chromatographic separation and the ionisation mode.

For GC-EI-HRMS, the ratio of the spiked compounds presenting signal suppression or enhancement between ± 20% was 60% with H_2SO_4 clean-up, while this ratio decreased to 20%, 14% and 6% with PDMS, EMR and GPC, respectively (Figure 3A, Table B.2). This observation was in line with the matrix components detected among the chromatograms for the 4 tested clean-ups methods since signal intensity was lower for H_2SO_4 modality (Figure 4A). For instance, unknown matrix components were detected in the extracts from the four clean-up procedures at 7.85 min (*m/z* = 145.0648), 12.47 min (*m/z* = 186.1039), 13.08 min (*m/z* = 236.1772), 21.24 min (*m/z* = 316.2394). These signals could be related to hydrocarbon compounds with unsaturation between 4 and 6 and might be representative of remaining unsaturated fatty acids. However, H_2SO_4 modality exhibited prominent chromatographic hump between 19 and 25 min, mostly resulting from signals corresponding to $[C_xH_y]^{+\bullet}$ ions. Although the origin of these ions could not be determined, they did not affect the detection of co-eluted spiked compounds more than other spiked compounds.

For LC-ESI(±)-HRMS, the ratio of the spiked compounds presented a signal suppression or enhancement between ± 20% was 50% with H_2SO_4 clean-up (Figure 3B, Table B.3). For GPC and PDMS this ratio was around 33% of spiked compounds (75% of common compounds among them) whereas for EMR this ratio dropped to 25%. EMR, GPC and PDMS total ion chromatograms (TICs) showed intense interferences, mostly from hydrophilic matrix components, while H_2SO_4 TIC profile was similar to the *1/1.6* spiking solution dilution (Figure 4B and 4C). These observations confirmed that H_2SO_4 procedure remove more matrix components than the EMR, GPC and PDMS.

Matrix effects resulting from EMR were compared to those reported by Pourchet et al. [59] for breast milk samples as the same instruments were used for both studies. The authors observed no significant matrix effect for LC-ESI(±)-HRMS whereas significant signal alterations were observed for GC-EI(+)-HRMS. Results from both studies were in the same range for only a few common spiked contaminants

(in both studies about 20% and -25% for metolachlor and β-HCH, respectively, by GC-EI-HRMS analysis, and about 0% for fenhexamid and fipronil by LC-ESI(±)-HRMS). Matrix effects calculated were not within the same range for all the other common contaminants in both studies. This statement might indicate that the matrix removal with EMR procedure depends on the matrix composition such as lipids, free fatty acids or cholesterol. In addition, chromatographic conditions might be involved as spiked contaminants and matrix components' retentions were different. Since GPC matrix removal depends on the fraction collected, the lipid composition and the mobile phase composition, direct comparison is at risk. Last, to the best of our knowledge, there are no studies focusing on matrix effects using H_2SO_4 and PDMS to compare with.

3.2.3. Recoveries

Matrix component removal during the sample preparation is important for NTA to minimise matrix effects. Furthermore, the maximisation of the compounds' preservation in the extract during this process is also important to acquire a comprehensive fingerprint of the samples. Recoveries calculated with equation (1) were independent of the matrix effect since the latter applies equally to extracts spiked before and after clean-up.

With both GC- and LC- based techniques, recoveries could be calculated for only 36 detected compounds after H_2SO_4 clean-up procedure (Figure 5, Tables B.2 and B.3 for detailed results). Acidic conditions likely degraded many compounds sensitive to low pH, such as most pesticides and phthalates. In addition, hydrophilic compounds with log $K_{ow} \leq 3$ or dissociated species under acidic or neutral conditions (*e.g.* simazine, 3-phenoxybenzoic acid, mecoprop, fipronil, triclosan, prochloraz) were probably back-extracted into the aqueous phase and thus removed. Because phthalates are acid-sensitive, their presence was mostly due to a procedural contamination occurring after the neutralisation step. However, flame retardants, which are hydrophobic (log $K_{ow} > 3$), were resistant to acidic conditions as their recoveries were 50% in average. The use of the H_2SO_4 procedure is limited to

434 the analysis of contaminants that are acid-resistant, hydrophobic and not prone to dissociation, which
435 prevents a comprehensive screening.

436 PDMS clean-up led to the detection of 69 spiked compounds. Most of them (n = 63) exhibited
437 recoveries lower than 20%, in accordance with the estimated partition coefficient between lipid and
438 PDMS $K_{lipid/PDMS}$ at the equilibrium (Text A.1). The uptake did not seem to be related to molecular weight
439 or log K_{ow} (Figure A.3), which is also consistent with the partitioning theory. PDMS was expected to
440 partition a maximum fraction in PDMS f_{PDMS} of 6% for $K_{lipid/PDMS}$ of 10 and 2% for for $K_{lipid/PDMS}$ of 30,
441 which are the typical range of literature data [60] (calculation is detailed in Text A.1). The applicability
442 to partitioning contaminants with PDMS in the tested condition appeared to be confined to the highly
443 concentrated chemicals when applying NTA. To overcome this issue, experimental conditions could be
444 modified to increase the PDMS uptake quantity. According to Smedes et al. [61], increasing
445 *extract/PDMS* mass ratio increases the equilibrium concentration in the PDMS but also increases the
446 time for the equilibrium to be attained *i.e.* enhances the PDMS exposition time.

447 Size exclusion influenced both EMR and GPC to remove lipids characterised by long alkyl chains.
448 Additionally, EMR involved hydrophobic interactions to retain compounds [26]. EMR and GPC led to
449 the detection of 88 and 86 spiked compounds, respectively. Compounds characterised by the highest
450 CCS values presented lower recoveries than the other ones with GPC, in line with the theory [62]
451 (Figure A.4). Due to the size of these compounds, their elution might overlay with lipids. Due to their
452 sizes, the elution of these compounds likely overlapped with the discarded fraction containing lipids.
453 Conversely, regarding the EMR protocol, the recoveries do not seem to be correlated with the CCS
454 values or log K_{ow} (Figure A.5), although the retaining phenomena involves these physico-chemical
455 properties. Although these two methods presented similar average recoveries, the repeatability,
456 expressed in relative standard deviation (RSD), appeared better with EMR than with GPC (Table B.2
457 and B.3).

458 The recoveries after EMR reported in the literature [25,26] are higher than those presented here and
459 could be explained by the combination of two experimental considerations. The first consideration
460 might be that the solvent strength of the acetonitrile/ethyl acetate (75:25, *v/v*) mixture was not strong
461 enough for partitioning the spiked compounds between the lipids and the solvent. Indeed, in the two
462 studies mentioned above, the spike was performed directly in a unique solvent phase to be loaded on
463 the SPE cartridge and containing the lipid, so that no losses could occur prior to that loading step. The
464 second consideration might be that no vacuum was applied to collect the solvent from the cartridge.
465 In order to avoid cross-contamination that could occur at the port of the vacuum manifold, in-house
466 made cartridge rack was used with which it was not possible to apply vacuum. The solvent volume
467 remaining into the cartridge was evaluated to 1 mL. Further optimisation of these two parameters
468 could increase the recoveries of the EMR procedure.

469

470 **4. Conclusion**

471 Effective sample preparation is necessary to take advantage of the recent advances in chromatography
472 and high-resolution mass spectrometry which can detect thousands of chemicals in a single analysis.
473 NTA requires non-selective sample preparation to isolate compounds from matrix components such
474 as lipids. Since it is difficult to isolate compounds with a wide range of physico-chemical properties, as
475 present in samples yet, we suggested a clean-up that selectively remove matrix components from lipid
476 extracts.

477 The procedure involving H_2SO_4 demonstrated matrix removal efficiency and reduced the procedural
478 contamination. On the other hand, only acid-resistant compounds, such as POPs, could be detected
479 following such rather destructive clean-up procedure impeding the acquisition of a comprehensive
480 fingerprint. In this clean-up method, the lipid mass can be increased to a few grams, allowing a
481 concentration that may facilitate the detection of less concentrated acid-resistant compounds.

Nevertheless, concentrated H_2SO_4 is hazardous for the operator and the applied procedure remains labour-intensive (5 days).

PDMS absorbed the compounds of interest while a small amount of lipids was taken up into it, demonstrating the effectiveness of matrix removal. Under the conditions tested, the partition ratio of compounds enriched in PDMS was low, so many compounds were not identified. However, increasing the *extract/PDMS* mass ratio could increase the amount of analyte partitioned to reach instrumental LOD. This method required relatively low volumes of solvent per samples (5 mL without the PDMS cleaning solvent). The matrix removal mainly depends on the operator's care to remove components adsorbed to the surface of the PDMS material. Advantages of PDMS are its ease of operation and affordability, requiring minimal technical equipment and allowing high sample throughput.

Since both the Captiva EMR-lipid cartridge and GPC involved size exclusion, their matrix removal and recovery were similar and satisfactory for most of the spiked compounds. EMR recoveries can be higher by eluting solvent that remains adsorbed on the phase, although there is a potential risk of eluting matrix components as well. The LLE step preceding loading onto EMR lipid could also be optimised to partition a larger quantity of compounds but, again, attention must be paid to the co-extracted matrix components. Conversely, the fraction collection could start later to enhance the matrix components removal, but the largest compounds of interest might be excluded. The first major difference between EMR and GPC was repeatability that was better using EMR. The second major difference between both procedures was the solvent consumption. It was reduced using EMR (about 10 mL) whereas over 350 mL per sample were used with GPC clean-up.

If EMR and GPC seem suitable for NTA, attention needs to be paid to the lipid composition (lipid class, fatty acid chain length, unsaturation), to extend these procedures to fatty matrices other than hen eggs. Using EMR, Zhao et al. [26] showed that the matrix removal may vary depending to the type of natural oil (olive, corn, soybean and canola). GPC collection start may vary depending to the matrix components such as lipid composition as well.

With the knowledge gained in this study on the performance and limitations of several sample preparation strategies, appropriate approaches could be selected to perform non-targeted analysis of contaminants in fatty matrices, in order to better describe the chemical universe to which wildlife and human are exposed.

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Associated content

Supporting material. The following files are available free of charge.

5. References

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Figures

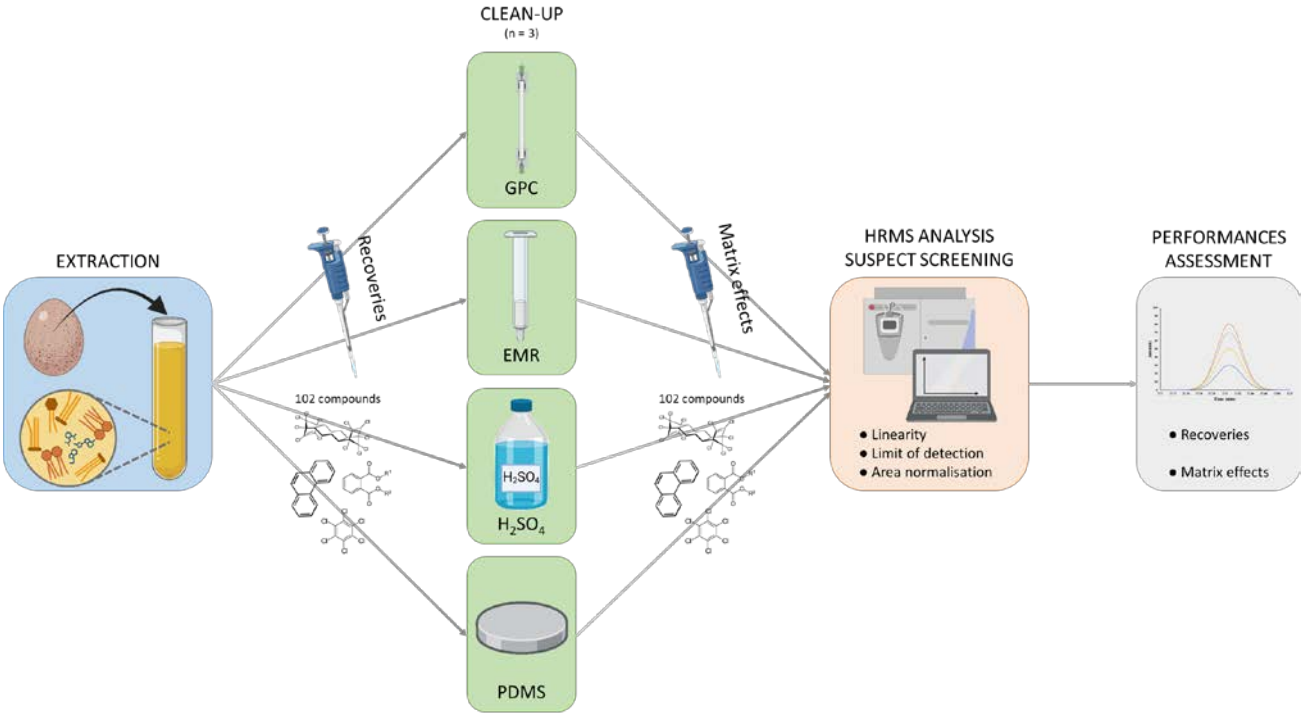
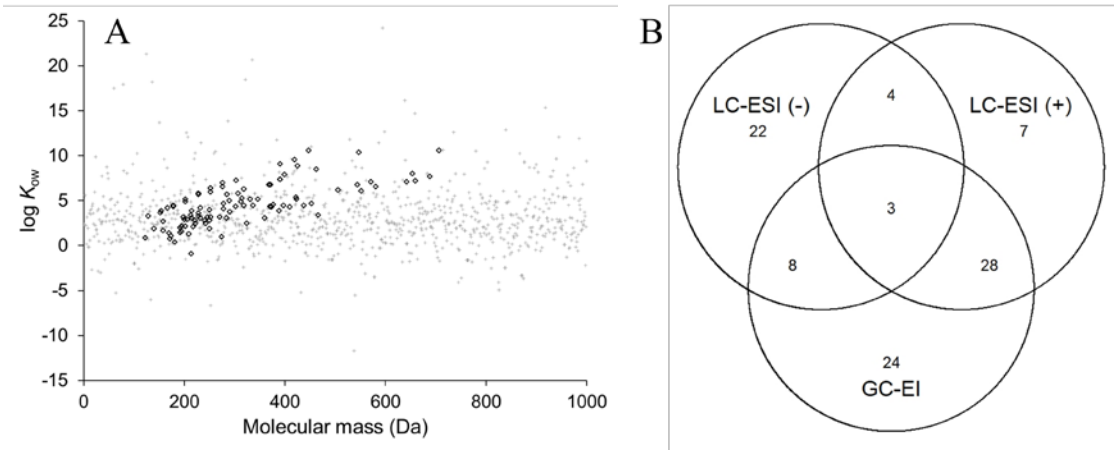


Figure 1: Detailed analytical workflow used to assess the clean-up performances. GPC: size exclusion using gel permeation chromatography method; EMR: size exclusion and hydrophobic interaction using Captiva EMR-Lipid cartridges; H_2SO_4: ester hydrolysis using sulphuric acid; PDMS: differential partitioning using polydimethylsiloxane.



742

743 *Figure 2: Chemical space covered by the 102 compounds of the spiking solution (black diamonds) within*

744 *the compounds with a molecular mass under 1000 g mol^{-1} from the CECscreen database (grey crosses,*

745 *n = 69,704 compounds) (A) and Venn diagram of the 98 detected compounds by GC-EI and LC-ESI(±)*

746 *(B).*

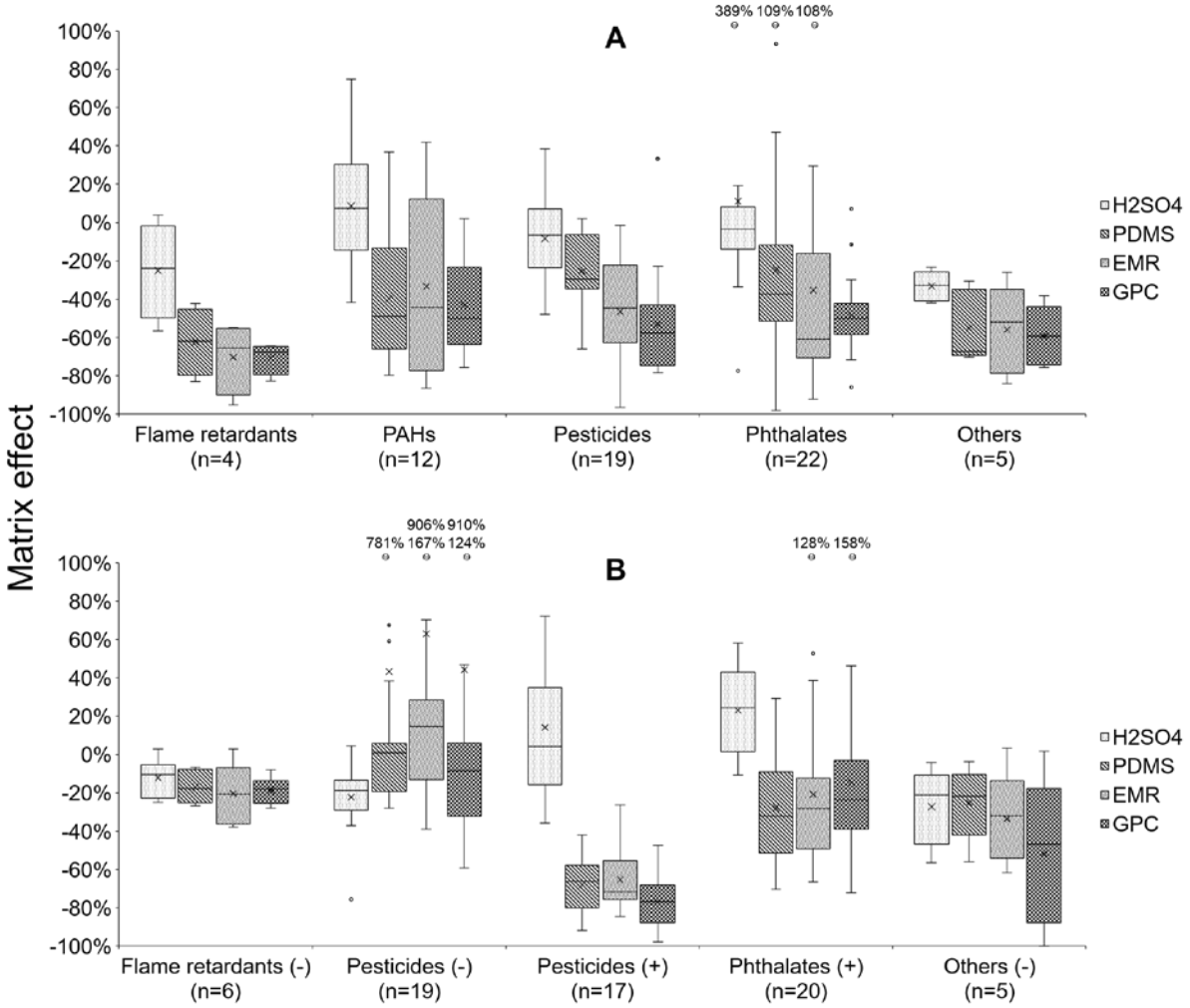


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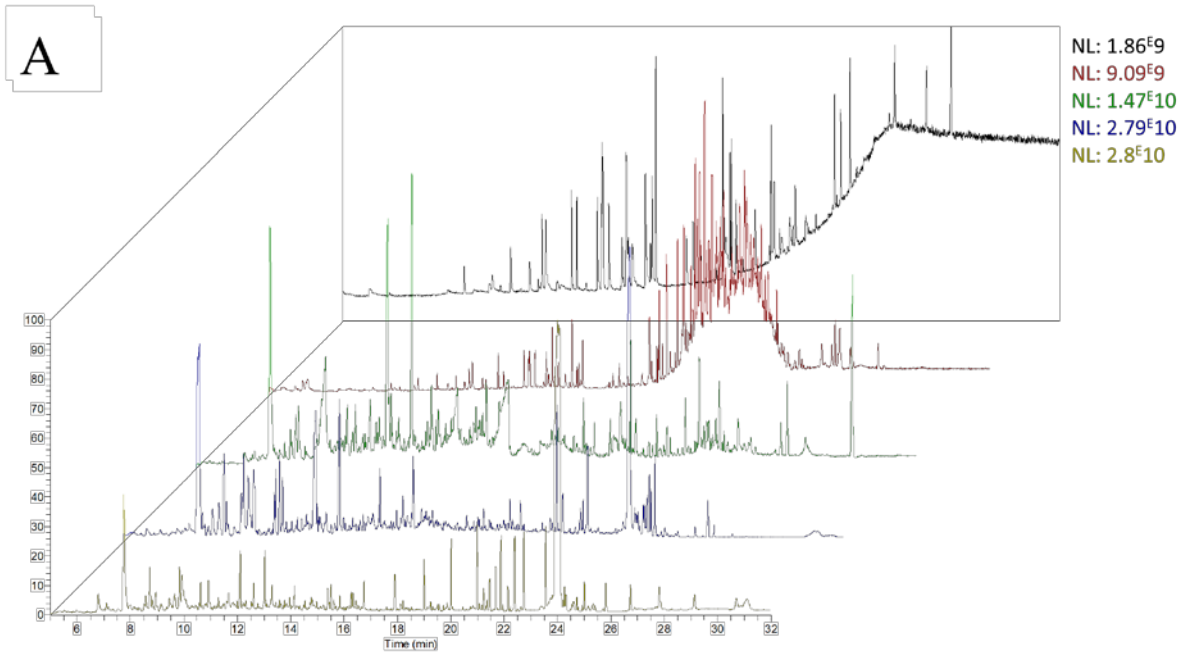
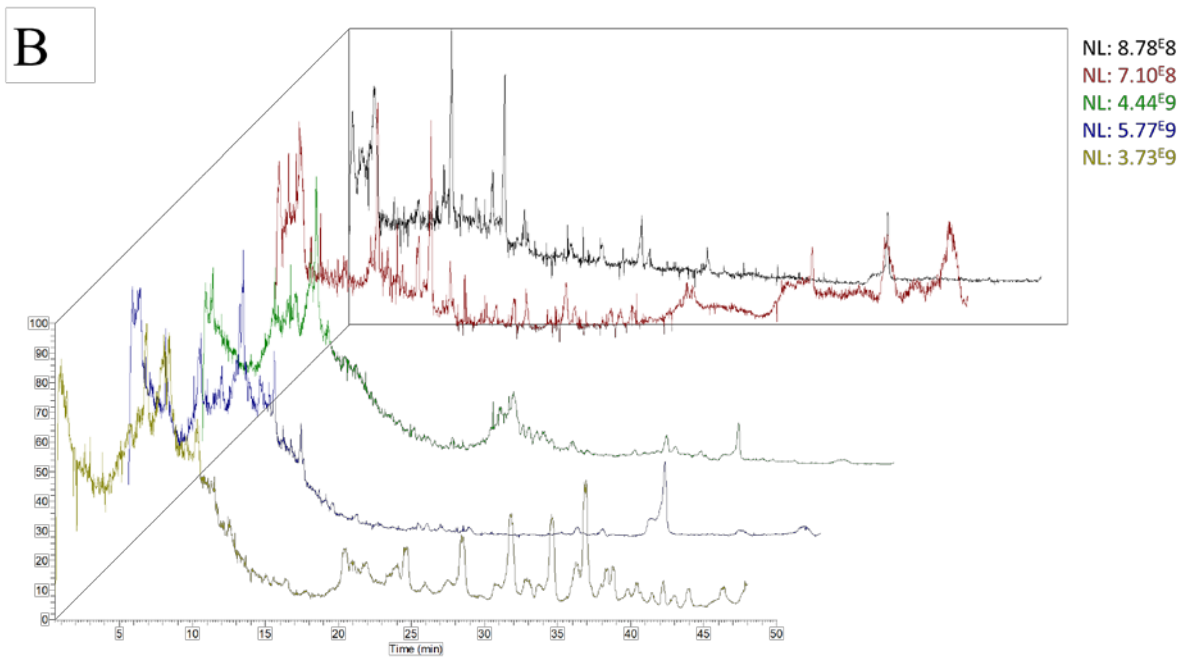
Figure 3: Matrix effects observed for the analysis of the spiked compounds by GC-EI-HRMS (A) and LC-ESI(±)-HRMS (B) with the four clean-up methods (n = 3). Compounds are grouped according to their classes and ESI polarity. Whiskers: min and max values; boxes: interquartile range; crosses: mean values; lines: median values; dots: outliers (data that are 1.5 times larger than the 3rd quartile or 1.5 times smaller than the 1st quartile).





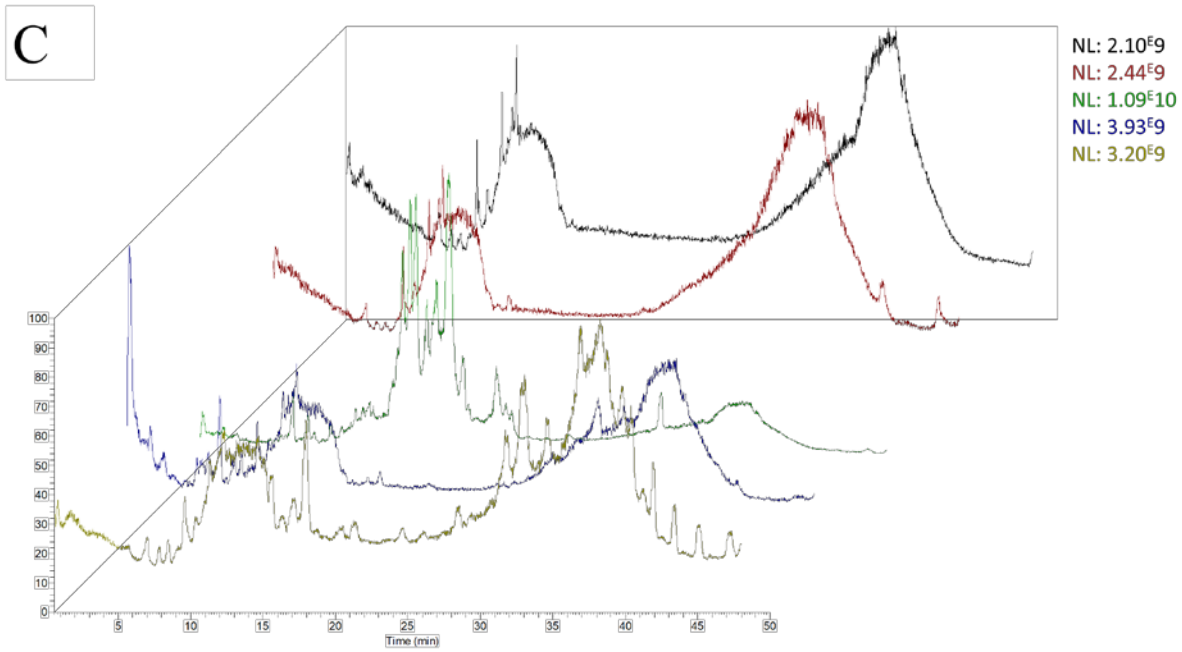
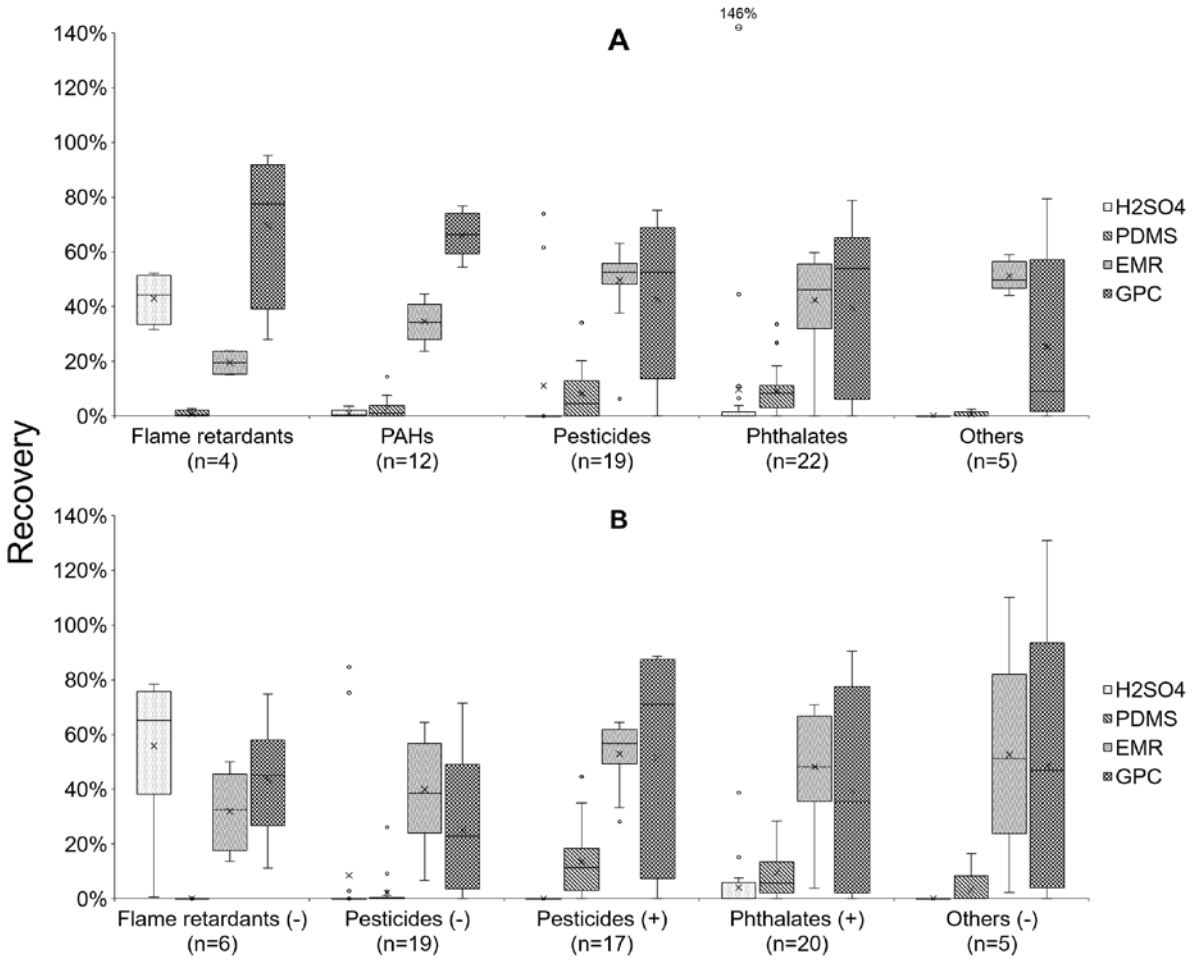


Figure 4: Representative TICs acquired by GC-EI-HRMS (A), LC-ESI(+)-HRMS (B) and LC-ESI(-)-HRMS (C) for the spiking solution at 1/1.6 dilution (black) and egg samples spiked after the H_2SO_4 (red), Captiva-EMR-lipid (green), GPC (blue) and PDMS (yellow) clean-up procedures. NL: normalised level.



759

760 *Figure 5: Absolute recoveries of spiked compounds analysed by GC-EI-HRMS (A) and LC-ESI(±)-HRMS*
761 *(B) with the four clean-up methods (n = 3). Compounds are grouped according to their class and ESI*
762 *polarity. Whiskers: min and max values; boxes: interquartile range; crosses: mean values; lines: median*
763 *values; dots: outliers (data that are 1.5 times larger than the 3rd quartile or 1.5 times smaller than the*
764 *1st quartile).*