Method Article

Optimization of a new selective pressurized liquid extraction methodology for determining organic pollutants in wild boar livers

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A B S T R A C T

In this study, a new selective pressurised liquid extraction (SPLE) methodology was optimised for determining about 70 organic pollutants (OPs) including organochlorine (OCPs), organophosphate (OPPs) and pyrethroid (PYRs) pesticides, polychlorinated biphenyls (PCBs), polybromodiphenyl ethers (PBDEs), as well as, polycyclic aromatic hydrocarbons (PAHs) in wild boar liver samples considering the temperature, pressure and time of contact between the solvent and the matrix as influential variables. Clean-up of extracts was performed by solid-phase extraction (SPE) using EZ-POP cartridges. Detection of OPs was carried out by gas chromatography (GC) coupled to tandem mass spectrometry (QqQ-MS/MS). This new approach offers:

- A new non-time consuming SPLEx methodology for determining about 70 OPs in wild boar.
- Recoveries achieved ranged between 74 to 119 % with RSD less than 20 %.
- Detection and quantification limits in the low to mid pg/g range.

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A R T I C L E  I N F O

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It is well-known that living organisms are exposed to organic pollutants (OPs) release into the environment. These chemical inputs from different sources (industry, urban and agricultural areas) may create a vulnerable status especially to animals [1,2]. The great variety of OPs that could be responsible for this type of damage makes necessary the development of powerful analytical methodologies, which allow the identification of these substances.

**Reagents and standards**

A list of the target OPs and the labelled internal and surrogate standards including CAS and supplier is given in Table S1 and S2, respectively. In order to improve peak shape and reduce OP decomposition, the 3-ethoxy-1,2-propanediol (98 %), d-sorbitol (> 99 %) and l-gulonic acid γ-lactone (> 98 %) used as analyte protectants (APs) were purchased from Sigma Aldrich (Madrid, Spain). Individual stock standard solutions of APs were prepared in acetonitrile (50 g/L), acetonitrile:water (85:15, v/v, 5 g/L) and acetonitrile:water (80:20, v/v, 5 g/L), respectively. Mixes of 10 mg/L stock solutions of each family of OPs were prepared from the individual stock solutions standards in acetonitrile. From these solutions, standards ranging from 0.25 to 100 μg/L were prepared in APs and used to construct the calibration line. These solutions were stored in amber flasks at -18°C.

**SPLX optimisation**

Liver is a complex matrix that requires a sample preparation in order to improve the in-situ clean-up in sample procedure. The effect of the presence or the absence of additives (KOH (aqueous) (60 %, w/v) or KOH (MeOH) (35 %, w/v)) used in other analytic techniques such as matrix solid phase dispersion (MSPD) were tested. Several combinations of KOH (aq) (30-60 %, w/v) and KOH (MeOH) (10-35 %, w/v) were chosen for such purpose. With the use of KOH (60 %, w/v) fat elimination was observed. Therefore, different combinations of mL KOH (15, 10 and 7.5) as well as activated silica amounts (35, 30 and 25 g) were tested. The experimental runs were performed in 2.0 g liver samples spiked with OP concentration range at 0.25 ng/g and 0.50 ng/g. Response was evaluated in terms of the recoveries of the selected OPs. Determination by GC-QqQ-MS/MS were performed using a previous ones optimised by the present research team [2] (Table S3).

The optimal sample conditions were 7.5 mL KOH (60%, w/v), 35 g activated silica and 1.0 g of anhydrous sodium sulphate. To extract the maximum target analytes with minimum interferences, different SPLX parameters were optimized. The selected parameters were temperatures (100 °C, 137.5 °C and 175°C), static times (5 min, 10 min and 15 min) and pressures (100 ba, 125 ba and 150 ba) using acetonitrile as solvent by a Box and Benhken experimental design with three independent variables consisted of 15 random experimental runs including three replicates at the central point. The experimental design was generated and all analytical treatments were supported by the software Statgraphics Plus 5.1 version (Manugistics, Rockville, MD, USA). The results are shown in Fig. 1. The final working conditions were obtained at 100 °C, three extraction cycles (10 min) and 150 ba.

Dual-layer EZ-POP SPE cartridges were used after SPLX. The final acetonitrile extract (1.0 mL) was passed through the tandem of dual-layer EZ-POP SPE cartridges previously conditioned with 20 mL of acetone. Acetonitrile (40 mL) was used to elute the target analytes and the collected extract was again reduced until dryness at 30°C, re-dissolved in 100 μL of acetone containing 50 ng of the internal standards and the three APs for GC-QqQ-MS/MS.
Quality assurance/quality control (QA/QC)

The studied methods were in-house validated according to the criteria and recommendations of European guidelines for linearity, precision, trueness/accuracy, limits of detection and quantification (LODs and LOQs) and uncertainly values [3]. Internal linear calibration was used to quantify the targeted OPs in livers using the following internal standards: DDT-D₈ for OCPs, diazinon-D₁₀ for OPPs, PCB 30 and 195 for PCBs, trans-cypermethrin-D₆ for PYRs and PBDE 166 for PBDEs. Linear calibration curves fit reasonably (r² > 0.999) in a twelve-point calibration curve with a concentration scale of two or three orders of magnitude, depending on the compound (0.010 – 1.0 ng/g). The quality parameters of the optimised method are summarized in Table 2. Results obtained for the accuracy were in the range from 1.0 to 14 %.

For the validation of the analytical methodology, 29 liver samples from Ourense (Northwest of Spain) were analysed. The set of liver samples was processed each day together with: a reagent blank to test for contamination in the extraction process, a spiked blank and a spiked sample at an intermediate concentration (0.50 ng/g) to calculate the extraction efficiency. Surrogate standards (chlorpyrifos-D₁₀, α,γ-HCH-D₆; DDE-D₈; HCB-¹³C₆, Chrysene-D₁₂, PBDE 77, PCB 14, 65 and 166, trans-Permethrin-D₆) were also added to check the recovery rates in each extraction procedure.

Most of the target pollutants were detected in the selected liver samples with significant differences (p < 0.050) and the following mean level concentration order ΣPAHs > ΣOCPs > ΣNDLPCBs > ΣPYRs > ΣOPPs > ΣDLPCBs > ΣPBDEs. Fig. 2 shows the main contributors in each family of OPs. Fluoranthene and pyrene were the main PAHs found in liver. With regard to chlorinated pollutants, trans-Chlordane were the most abundant OCP followed by HCB, as well as, PCB 153, 138 and 180 for NDLPCBs. PCB 157 and PCB 126 were the most prevalent DLPCB congeners. Permethrin and chlorpyrifos were the detected PYRs and OPPs, respectively. To our knowledge no results were found about the levels of PYR and OPP pesticides in liver of wild terrestrial mammals. PBDEs were the OPs with the lowest contribution with PBDE 47, 100 and 99 as major congeners.

Additional information

To our knowledge, scarce literature about the concentration of OPs in wild boar liver is available due to the complexity of the selected biological sample [4–20]. For these reasons, it is required to
develop quick and simple techniques capable of efficiently detecting a wide range of contaminants. In this type of multiresidue methods the extraction process is perhaps the most critical step since it requires the development of special and suitable conditions to determine substances with different physico-chemical properties related to water solubility ($S_w$), octanol/water partition coefficient ($K_{ow}$) and organic carbon partition coefficient ($K_{oc}$). In recent years, OPs have been analysed in the liver of different wild animals (Table 1). Most of the studies focus on the determination of a single group of compounds. These researches use classical extractive techniques such as solid-liquid extraction (SLE) or soxhlet followed by clean-up steps using gel permeation chromatography (GPC) or solid-phase extraction (SPE with different absorbents (silica, alumina, florisil...) [4–6,8–17]. The main disadvantages of these techniques are the use of large amounts of solvent and the need for additional cleaning steps to avoid interferences, which involves possible loss of analytes and waste of time. Other alternatives are the use of high pressure extractive techniques such as accelerated solvent extraction (ASE) or also called pressurized liquid extraction (PLE) [7,18–22]. The combination of PLE with an in situ clean-up (in cell) of the extract is known as selective pressurized liquid extraction (SPLE). This technique avoids the need of subsequent cleanings and also improves the automation of the process. To the best

Table 1
Background of the analytical extraction methods for liver from wild mammals since 2001.

| Compounds | Specie     | Extraction | Clean Up           | References |
|-----------|------------|------------|--------------------|------------|
| OCPs, PCBs| Wild boar  | SLE        | Silica gel         | [4]        |
| OPPs      | Wild boar  | SLE        | SPE C18            | [5]        |
| OCPs, PCBs| Wild boar  | SLE        | Florisil           | [6]        |
| PCDDs/DFs, PCBs | Wild boar | ASE        | Acidic silica, florisil | [7] |
| PBDEs     | Wild boar  | Soxhlet    | Acidic silica gel  | [8]        |
| OCPs, PCBs| Wolf       | SLE        | Alumina            | [9]        |
| PAHs      | Otter      | Soxhlet    | GPC, silica gel    | [10]       |
| OCPs, PCBs| Lynx       | SLE        | Sulfuric acid      | [11]       |
| PCBs, OH-PCBs | Seal    | SLE        | Silica gel         | [12]       |
| PAHs      | Dolphin    | Soxhlet    | GPC                | [13]       |
| PBDEs     | Otter      | SLE        | GPC                | [14]       |
| PBDEs     | Otter      | SLE        | Florisil           | [15]       |
| PCDD/Fs, PCBs, PBDEs | Reindeer | SLE        | Multilayer column  | [16] |
| OCPs, PCBs| Mink       | SLE        | Florisil           | [17]       |
| PBDEs, PCBs| Sheep     | SPLE       | Acidic silica, sodium sulphate | [18] |
| PCBs      | Racoon dog | ASE        | GPC                | [19]       |

Fig. 2. Summary of the main OP contributors in each family.
Table 2
Mean recoveries (R) and relative standard deviations (RSD) at four spike levels (LOQs, 0.10, 0.25 and 0.50 ng/g), LOD (ng/g) and LOQ (ng/g) for each target compound are shown.

| OPs          | RT (min) | % R (RSD) | LODs | LOQs |
|--------------|----------|-----------|------|------|
| α-HCH-D_6    | 6.860    | 109 (10)  | -    | -    |
| α-HCH        | 6.912    | 89 (16)   | 0.010 | 0.050 |
| HCB-13C_6    | 7.043    | 94 (18)   | -    | -    |
| HCB          | 7.043    | 112 (16)  | 0.32 | 1.1  |
| PCB 14       | 7.079    | 108 (10)  | -    | -    |
| β-HCH        | 7.309    | 117 (9.1) | 0.020 | 0.19 |
| γ-HCH-D_6    | 7.344    | 93 (17)   | -    | -    |
| PCB 11       | 7.079    | 89 (9.0)  | 0.010 | 0.040 |
| Diazinon     | 7.544    | 94 (1.0)  | 0.11 | 0.38 |
| PCB 28       | 8.027    | 83 (7.2)  | 0.0010 | 0.0040 |
| Parathion Methyl | 8.654  | 106 (17)  | 0.10 | 0.33 |
| Heptachlor   | 8.765    | 106 (18)  | 0.010 | 0.040 |
| Aldrin       | 9.289    | 117 (4.0) | 0.010 | 0.040 |
| PCB 52       | 9.293    | 92 (5.0)  | 0.010 | 0.040 |
| PCB 65       | 9.293    | 105 (14)  | -    | -    |
| Chlorpyrifos-D_{10} | 9.387  | 88 (19)   | -    | -    |
| Fenthion     | 9.433    | 77 (5.0)  | 0.010 | 0.040 |
| Chlorpyrifos | 9.476    | 106 (15)  | 0.040 | 0.14 |
| Fluoranthene | 10.454   | 104 (11)  | 0.030 | 0.10 |
| trans-Chlordane | 10.880  | 95 (1.0)  | 0.010 | 0.040 |
| Pyrene       | 11.101   | 100 (10)  | 0.11 | 0.39 |
| cis-Chlordane | 11.267  | 93 (3.0)  | 0.010 | 0.040 |
| DDE-D_3      | 11.752   | 99 (17)   | -    | -    |
| PC 101       | 11.752   | 92 (2.3)  | 0.010 | 0.040 |
| PC 105       | 11.752   | 108 (9.0) | 0.010 | 0.040 |
| PC 77        | 11.753   | 102 (1.0) | 0.010 | 0.040 |
| PC 81        | 11.753   | 103 (11)  | 0.010 | 0.040 |
| o,p'-DDT     | 12.045   | 94 (4.0)  | 0.050 | 0.16 |
| Dieldrin     | 12.677   | 120 (8.0) | 0.010 | 0.040 |
| Endrin       | 12.677   | 119 (8.3) | 0.010 | 0.040 |
| PC 114       | 12.750   | 94 (14)   | 0.010 | 0.040 |
| PBDE 28      | 13.530   | 87 (11)   | 0.0010 | 0.0020 |
| p,p'-DDT     | 12.924   | 119 (12)  | 0.010 | 0.040 |
| o,p'-DDT     | 13.058   | 94 (18)   | 0.030 | 0.12 |
| PCB 138      | 13.364   | 89 (3.1)  | 0.010 | 0.040 |
| PBEB         | 13.628   | 115 (12)  | 0.010 | 0.040 |
| PCB123       | 13.979   | 92 (10)   | 0.010 | 0.040 |
| PCB 118      | 13.979   | 93 (11)   | 0.010 | 0.060 |
| p,p'-DDT     | 14.054   | 86 (18)   | 0.0080 | 0.020 |
| PCB 153      | 14.616   | 99 (1.0)  | 0.010 | 0.040 |
| PCB 166      | 14.616   | 108 (12)  | -    | -    |
| PCB 156      | 14.616   | 81 (16)   | 0.010 | 0.040 |
| PCB126       | 14.611   | 115 (16)  | 0.010 | 0.040 |
| Chrysene-D_{12} | 15.569  | 89 (12)   | -    | -    |
| Chrysene     | 15.553   | 99 (8.0)  | 0.010 | 0.060 |
| B[a]A        | 15.553   | 85 (5.4)  | 0.020 | 0.080 |
| PCB 180      | 15.942   | 106 (13)  | 0.010 | 0.040 |
| PCB 157      | 15.937   | 85 (18)   | 0.010 | 0.040 |
| PCB 167      | 15.937   | 117 (4.0) | 0.010 | 0.040 |
| PBDE 47      | 16.316   | 74 (9.2)  | 0.0020 | 0.0070 |
| PCB 169      | 16.764   | 102 (1.0) | 0.010 | 0.040 |
| PBDE 77      | 17.657   | 93 (11)   | -    | -    |
| PCB 189      | 18.037   | 90 (18)   | 0.010 | 0.040 |
| trans-Permethrin | 18.786 | 99 (5.0)  | 0.12 | 0.42 |
| cis-Permethrin | 19.026  | 99 (6.0)  | 0.12 | 0.42 |
| trans-Permethrin-D_6 | 19.087 | 97 (9.0)  | -    | -    |
| PBDE 99      | 19.100   | 99 (6.0)  | 0.0010 | 0.0040 |
| B[k]F        | 19.785   | 110 (13)  | 0.030 | 0.12 |
| B[b]F        | 19.785   | 100 (1.3) | 0.0030 | 0.010 |

(continued on next page)
of our knowledge, there is only a research in sheep liver where only PBDEs and PCBs were analysed by this technique [18]. Something new and promising is the inclusion of additives such as potassium hydroxide in SPLE to avoid the co-elution of unwanted matrix components allowing the extraction of about 70 OPs.

**Declaration of Competing Interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Supplementary materials**

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.mex.2021.101242.

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