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Automatic mesofluidic system combining dynamic gastrointestinal bioaccessibility with lab-on-valve based sorptive microextraction for risk exposure of organic emerging contaminants in filter feeding organisms

María Rosende[a], Ailette Prieto[b,c], Nestor Etxebarria[b,c], Gabriel Martorell[d] and Manuel Miró[a*]

a) FI-TRACE group, Department of Chemistry, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Spain, www.fitrace.es

b) Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), P.O. Box 644, 48080 Bilbao, (Basque Country), Spain

c) Research Centre for Experimental Marine Biology and Biotechnology (PIE), University of the Basque Country (UPV/EHU), Areatza z/g, 48620 Plentzia, (Basque Country), Spain

d) Serveis Cientificotècnics, University of the Balearic Islands, Carretera de Valldemossa, km 7.5, 07122 Palma de Mallorca, Spain

Abstract

An automatic mesofluidic system combining dynamic oral bioaccessibility with lab-on-valve (LOV)-based sorptive microextraction is herein proposed for the first time for exploring the kinetics of leaching of incurred rather than spiked organic emerging contaminants (*viz*, methyl paraben, butyl paraben, diclofenac and triclosan) from exposed mussels based on the Versantvoort's fed-state physiologically extraction test. Our method is capitalized on programmable flow analysis, in which the gastrointestinal extracts are obtained on-line by pumping a simulated biorelevant gastrointestinal fluid across a large-bore column (maintained at 37.0 ± 2.0 °C) loaded with 250 mg of freeze-dried and powdered mussel onto a PVDF filter membrane. The physiologically-relevant extracts are then cleaned-up and analytes preconcentrated onto a dedicated reversed-phase SPE (Oasis-PRIME-HLB) microcolumn, which is captured into the channels of an LOV mesofluidic platform. The aim behind is to obtain analyte-laden eluates with ACN/MeOH (90:10, v/v) in unsupervised mode for direct injection into LC-MS. The μSPE-LOV minicolumn (≤ 25 mg) is automatically disposed of and renewed in every individual fraction on account of the strong retention of (phospho)lipids by the copolymeric sorbent. The proposed dynamic bioaccessibility test features a significant shortening of the extraction time against the batch methods (28 min vs 240 min) while avoiding overestimation of potentially bioavailable fractions. The trueness of the on-line gastrointestinal extraction method was confirmed using mass balance validation following ultrasonic-assisted solid–liquid extraction of the original mussel sample and the residual (non-bioaccessible) emerging contaminant fraction.

[*] Corresponding author. E-mail: manuel.miro@uib.es. Phone: +34-971173426 (Prof. Manuel Miró)

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The acceptance that chronic human diseases are likely to result from the combination of exposures to several chemical and physical stressors along with human genetics has launched a new multidisciplinary research field that is called exposomics. The (bio)analytical challenge of the exposomics workflow is the need of exploring all of the potential exposures of an individual throughout time in a holistic format, including those related to the environment, diet, behaviour, and endogenous processes.[1,2]

Food is a major source of human exposure to xenobiotics, but it is well recognized that the impact of contaminated food on human health cannot merely be assessed by measuring the total concentration of food-borne contaminants[3,4], because only a fraction of those contaminants is able to reach the systemic circulation up to the target organs or tissues.[5] Hereto, the bioaccessibility concept that embraces the mobilizable pools of chemical species/contaminants from the solid food matrix into the human gastrointestinal tract (GI), that is, those that are potentially available for absorption across a physiological membrane, is crucial throughout exposome studies.

The recent increase of the use of *in-vitro* GI bioaccessibility tests for assessment of human exposure to organic contaminants is due to the rapid information they could provide against the *in-vivo* gastrointestinal counterparts, which are time and cost consuming, and are currently restricted by animal ethical issues. In fact, *in-vitro* bioaccessibility methods with mock digestive fluids meet REACH requirements with respect to the avoidance of *in-vivo* ecotoxicological tests and serve as a proxy of human GI digestion processes.[6] Over the past two decades a large number of *in-vitro* oral digestion methods dedicated to bioaccessibility studies of legacy organic contaminants have been reported[7-10] but unfortunately, its applicability to emerging organic pollutants (EOC)[11-13] in food samples has been barely reported.[14-17] So far, only the bioaccessibility of perfluorinated compounds,[14,16] flame-retardants,[16] personal care products,[16] and bisphenol A[15] has been studied in animal and plant-based solid foodstuff. To the best of our knowledge, researchers have merely used batchwise biomimetic digestion protocols, in which the human GI tract is simulated using synthetic digestive fluids, *namely*, saliva, bile, gastric phase and duodenal and colon fluids. These protocols are generally composed of two consecutive extraction steps[18]: (i) the gastric extraction that mimics the physiological conditions in the stomach incorporating gastric fluid and saliva to the food sample, and (ii) the GI extraction that simulate physiological conditions of the small intestine by adding the bile and the duodenal body fluids to gastric phase.

Two reported limitations of gastrointestinal bioaccessibility tests performed in batchwise mode are[19]: (i) the absence of a continuous removal of desorbed compounds from the surface of the solid matrix as soon as they are released, as an implicit assumption in the bioaccessibility concept as signaled by Brack *et al.*[20] and (ii) the lack of real-time information of the extraction

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3 rates of bioaccessible species. To tackle this shortcoming, recent trends focused on the development of novel i*n-vitro* dynamic/kinetic bioaccessibility assays that use fresh portions of gastrointestinal fluids for extraction of target components from the solid sample as contained in a flow-through chamber for simulation of the physiological processes throughout the gastrointestinal tract.[21-27] It should be, however, noted that all of the on-line/dynamic GI extraction systems reported in the literature refer to the bioaccessibility of trace elements and nutrients. To the best of our knowledge, automatic flow-through physiologically based extraction methods have not been applied so far to the determination of organic contaminants in solid matrices. In contrast to the analytical workflow for quantification of metal bioaccessibility in GI extracts, which are usually analysed directly by atomic spectrometry, the analytical method for organic contaminants in foodstuff does necessitate further extract pre-treatment steps. These normally include analyte preconcentration and/or matrix clean-up in a batchwise format using organic solvents and/or sorptive materials[17] because of the high concentration of lipids and proteins contained in the GI food extracts that may interfere with the ensuing chromatographic and/or mass spectrometric analysis.

The sequential injection/lab-on-valve (SI-LOV) platform is an enabling (meso)fluidic technology for miniaturization of (bio)chemical assays and on-line automatic sample treatment.[28-30] The Bead Injection (BI) concept[31,32] integrated in the SI-LOV format enables micro-solid phase extraction (μSPE) protocols with on-line sorbent packing and withdrawal of solid entities from the LOV in every single assay, thus overcoming analyte carryover effects and the irreversible uptake of interfering effects of on-line SPE counterparts using permanent/reusable sorbent cartridges.[31]

In this work, an automatic mesofluidic device combining dynamic GI bioaccessibility with SI-BI-LOV microextraction is herein presented for the first time to evaluate the leaching kinetics across the GI tract of four organic contaminants of emerging concern including pharmaceutical, and personal care products, *namely*, methyl 4-hydroxybenzoate (MeP), butyl 4-hydroxybenzoate (BuP), triclosan (TCS) and diclofenac (DCF), the latter included in the first Watch List on potential water pollutants of the European Commission, in incurred mussel tissues based on the Versantvoort's fed-state physiologically extraction method. A modified reversed-phase sorbent (Oasis-PRIME HLB) that is specially designed for the handling of biochemical samples with high concentration of (phospho)lipids is exploited for the unsupervised LOV-BI analyte preconcentration and clean-up of the *in-situ* generated dynamic GI extracts aimed at direct (at-line) injection of processed eluates into LC-MS.

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EXPERIMENTAL

Chemicals, sorbents, and reagents for the oral bioaccessibility methods

Methanol (MeOH), acetonitrile (ACN) and formic acid (85%) for solid-phase extraction (SPE) and chromatographic analysis were of LC/MS grade and purchased from Fisher Scientific (Loughborough, UK). Ultra-pure water was obtained from a Milli-Q water purification system (<0.05 S/cm, Milli-Q model 185, Millipore, Bedford, MA, USA). Standards of MeP, BuP, TCS and DCF, and methyl 4-hydroxybenzoate-ring-^{13}C6 (MeP-^{13}C6), butyl 4-hydroxybenzoate-ring-^{13}C6 (BuP-^{13}C6) and diclofenac-(acetophenyl ring-^{13}C6) sodium salt hemi(nonahydrate) (DCF-^{13}C6) used as surrogate internal standards were obtained from Sigma Aldrich (St. Louis, MO, USA) while triclosan-d3 (TCS-d3) was purchased from LGC standards (Barcelona, Spain). Stock solutions of all of the emerging contaminants and their isotopically labelled analogs (10 mg L^{-1}) were prepared in methanol and stored at -20ºC.

Two different types of commercially available reversed-phase copolymers with hydrophilic-lipophilic balance, namely, Oasis HLB and Oasis PRIME HLB (Waters, Mildford, USA, average 30 µm) and one in-house synthetized Ag-nanoparticle embedded copolymer beads[33] on account of potential strong Lewis acid-base interactions between Ag and electron-rich aromatic compounds have been tested for the clean-up and uptake of the four emerging contaminants from the mussel-laden human digestive extracts.

The chemical composition of the physiologically based extractants including enzymes, organic species and salts is based on Versantvoort's test[5] (see Table S1). All mock digestive fluids were prepared the day prior to their use. Each biomimetic fluid (viz., saliva, gastric and duodenal fluids and bile) results from the combination of an inorganic solution, an organic solution and a given number of enzymes. Once the inorganic and organic solutions and the GI enzymes are combined, each fluid is placed under magnetic agitation at 100 rpm for at least 3 hours. Then, the pH of every biofluid is monitored to match the pH values recommended by Versantvoort *et al.*[5] If necessary, pH of every solution is adjusted by dropwise addition of 1 mol L^{-1} NaOH or concentrated HCl. On the day of the on-line GI extraction, all fluids are placed at 37°C for at least one hour for activation pending use. In our work, a composite GI extractant was prepared by mixing 10 mL of saliva, 20 mL of gastric fluid, 20 mL of duodenal fluid, 10 mL of bile and 3.3 mL of 1.0 mol L^{-1} $NaHCO_3$, the last one to adjust the final pH to 6.5 (further information is provided in Results and Discussion).

Samples and exposure experiments

Mussels, *Mytilus galloprovincialis* (5–6 cm shell length) were collected in Gorliz (Bay of Biscay, Spain). The filter-feeding organisms were acclimated for three weeks at the Aquatic Facility from the Plentzia Marine Station (PIE), University of Basque Country, under a flow-

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3 through system using real seawater from PIE. The facility was equipped with two
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5 polypropylene tanks (control and dosing) containing aerated and each two days renewed 40 L-
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7 seawater with 75 mussels per tank. The experiments were performed under controlled
8 temperature (18 °C) and light (14:10 h light: dark cycles) for 8 days. The organisms were
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10 exposed every two days to a concentration level of 30 ng mL^{-1} each MeP, BuP, DCF and TCS.
11 A control tank (mussels with only seawater) was maintained under virtually identical conditions
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13 throughout the 8-day experiment. In the course of the exposure testing no mussel mortality was
14 observed. The exposed and control mussel tissues were pooled, minced and freeze-dried
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16 (Cryodos-50 laboratory freeze-dryer, Telstar Instrument, Sant Cugat del Valles, Barcelona,
17 Spain) and stored at −20°C until further processing.
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21 **Analytical instrumentation**
22 A diagrammatic description of the mesofluidic SI-LOV setup for assessment of dynamic
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24 bioaccessible GI fractions of MeP, BuP, DCF and TCS in exposed mussels is shown in Fig. 1.
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26 The flow manifold is composed of (i) a standalone 3000-step bidirectional micro-syringe pump
27 (SP; CAVRO XP3000, Tecan group, Männedorf, Switzerland) equipped with a 5 mL-gastight
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29 glass syringe (Hamilton, Switzerland) for automatic fluid handling, (ii) a three-way rotary valve
30 at the syringe head that allows communication with either the mesofluidic system or the carrier
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32 (Milli-Q water) reservoir, (iii) an eight-port selection valve (Vici Valco, Schenkon, Switzerland)
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34 operating as actuator and (iv) an LOV conduit platform mounted on top of the selection valve
35 that acts as a valve stator. The LOV mesobore assembly fabricated from chemically resistant
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37 polyetherimide (ULTEM) contains a central port that enables sequential communication with
38 either of eight peripheral LOV channels/ports of 1.2 mm ID and 14 mm length each, as
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40 illustrated in Fig. 1, by turning of the valve's rotor. The actuator is equipped with a grooved
41 polytetrafluoroethylene (PTFE) disk that serves as a Communication Channel (CC in Fig. 1)
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43 between the central port and the external channels. The central port of the LOV is connected to
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45 a holding coil (HC) made of 300 cm ×1.5 mm ID PTFE tubing (Supelco, Bellefonte, USA). The
46 microchannel in port 5 (see Fig. 1) serves as a microcolumn position for the renewable sorptive
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48 material. To contain the sorbent beads within the cavity of the LOV conduit module and prevent
49 them from escaping, the outlet of the column (port 5) was equipped with a 20 µm-polyethylene
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51 frit operating as a filter (Supelco). The suspension of reversed-phase beads was prepared by
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53 suspending of ca. 500 mg of sorbent material in 1 mL of 90:10 (ACN: MeOH) and was
54 contained in a 1.0 mL glass syringe (Hamilton) that was mounted vertically on port 2 of the
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56 LOV (see Fig. 1). The eluent (ACN:MeOH (90:10)) and rinsing solvent (5% (v/v) MeOH)
57 reservoirs were attached to the peripheral ports 6 and 4, respectively, whereas port 3 was
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59 employed for air aspiration with the purpose of preventing mixing across liquid zones in the HC
60 and fostering on-line dispersion of the sorbent (in port 1) pending aspiration into the LOV.

Three additional ports (7, 8 and 1) of the LOV served to attach the GI extractant reservoir, a large bore column extractor, and a sample/extract homogenizer that is composed of a 5-mL polypropylene pipette tip, respectively. The flow setup is also composed of an ancillary standalone 3000-step micro-syringe pump (CAVRO XP3000) furnished with 0.5 mL glass syringe (Hamilton) for the addition of minute volumes of internal standards (MeP-^{13}C6, BuP-^{13}C6, DCF-^{13}C6 and TCS-d3) to the GI extract contained in the sample homogenizer flask in port 1. The idea behind is to offset potential analyte breakthrough across the µSPE-LOV column.

Cocosoft 4.7 software package written in Python[34] was utilized as a freeware for automatic control of the motion and the flow rate of the syringe pumps as well as the selection of various channels/ports of the LOV mesofluidic platform.

The dedicated flow-through large bore column (LBC) with an inner volume of ca. 2.5 mL (height: 22 mm and diameter: 47 mm) was described elsewhere.[21] In short, the small aspect-ratio extraction unit is composed of (i) two threaded polypropylene filter holders ended with fitting for standard ¼-28 female to male luer connection, (ii) a polyvinylidiene difluoride (PVDF) membrane filter (5.0 µm pore size and 47 mm diameter, Merck Millipore) and (iii) two PTFE gaskets. The two filter holders contain eighteen rectangular apertures (1 mm width and 5 mm long) for minimization of pressure drop effects while permitting the free flow of the GI fluid. A weighed freeze-dried exposed mussel sample (250 mg) was placed over the membrane filter and the overall components of the container were securely clamped prior to connection to the LOV platform. A heating and magnetic stirring device (set at 300 rpm to ensure a stable sample-GIT fluid dispersed phase) and coupled to a digital thermoregulator (VELP Scientifica, Usmate Velate Monza e della Brianza, Italia) was used for adjusting the temperature of the GI fluid and the mussel-housing LBC to 37.0 ± 2.0 °C.

Reversed-phase LC systems coupled to either an Agilent 6430 triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA) or a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific, Walthman, MA) operating with a heated electrospray interface (HESI) in negative electrospray ionization were employed for the identification and quantification of MeP, BuP, TCS and DCF, of their isotopically labelled analogs and of potential degradation/hydrolytic products. Experimental details are given in SI.

Analytical procedure for on-line and batchwise GI bioaccessibility assays

The operational sequence of the dynamic biomimetic method for GI extraction of MeP, BuP, TCS and DCF from exposed mussels using the automatic LOV-BI-µSPE system prior to LC-MS/MS analysis is composed of five sequential steps: (i) bead packing into the LOV and sorbent conditioning (26.2 ± 1.9 mg Oasis PRIME), (ii) dynamic physiologically-based extraction of EOCs from the seafood using a given volume (1 or 3 mL) of body fluid (iii)

extract loading and clean-up by BI-LOV to remove unwanted matrix components; (iv) elution of analytes in 90:10 (v/v) ACN:MeOH; and (v) in-line renewal of the beads by automatic disposal to waste and rinsing of the flow system to avoid cross-contamination. The entire procedure is repeated 15 times in a fully automatic mode to get insight into the leaching kinetics under GI simulated conditions of the four EOC in food commodities.

Detailed descriptions of the (i) automatic dynamic procedure, (ii) batchwise test, and (iii) analytical procotol for determination of residual fractions and total content of EOCs are available as Supporting Information.

RESULTS AND DISCUSSION

Selection of the sorbent material for on-line disposable LOV-BI-µSPE

Preliminary experiments were undertaken in a batchwise SPE mode using the Visiprep™ SPE Vacuum Manifold (Supelco) to evaluate the extraction performance of two commercially available sorptive materials namely, co-polymeric Oasis HLB, and Oasis PRIME HLB, and an in-house synthetized Ag-nanoparticle (NP) embedded copolymer sorbent[33] for clean-up of GI extracts and analyte uptake. To this end, 3 mL of the Versantvoort's GI extract of the control mussels obtained by the batchwise protocol described in SI were spiked with 100 ng mL^{-1} each of MeP, BuP, DCF and TCS and loaded through 30 mg of sorbent. The retained analytes were eluted quantitatively in 1.0 mL eluent following manufacturers' recommendations, that is, 100% MeOH and 90:10 (v:v) ACN:MeOH for Oasis HLB and Oasis PRIME-HLB, respectively, and 100% ACN for the Ag-NP copolymer beads as indicated by Navarro-Pascual-Ahuir *et al.*[33] Prior to LC-MS/MS analysis, 30 ng of isotopologues (MeP-^{13}C6, BuP-^{13}C6, DCF-^{13}C6 and TCS-d3) were added to all of the eluates to minimize potential ion suppression effects.

The extraction performance, which is calculated on the basis of the retained and eluted amount of each EOC, is illustrated in Fig. 2. The four EOCs are proven to be quantitatively retained and eluted using marketed SPE sorbents, with recoveries >70%. On the contrary, the lab-made AgNP embedded copolymer beads rendered recoveries down to 50% for MeP, BuP and TCS and down to 10% for DCF. The lack of chemical stability of the sorbent occasioned by the progressive oxidation of the silver might account for the experimental findings. Experimental results demonstrated that Oasis PRIME-HLB features superior extraction performance in mussel laden GI fluids for the suite of EOCs as compared to Oasis HLB (see Fig. 2) with relative recoveries spanning from 80-105% against 75-82%, respectively. In fact, the proprietary Oasis PRIME sorbent was launched for SPE protocols in biological samples and food commodities for the efficient removal of phospholipids and fats,[35] resulting in cleaner extracts for direct injection into LC-MS systems without risks of short column lifetimes or MS source

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fouling.[36] It is important to note that the average content of phospholipids in mussels, which are major lipid components of biological membranes, ranges from 3.6 to 6.4 g kg^{-1}.[37]

The main asset of the Oasis PRIME-HLB material, that is, the strong uptake of (phospho)lipids, becomes a clear disadvantage for its potential reuse in on-line μSPE procedures, as is the case herein with the evaluation of the kinetics of the EOC bioaccessibility from exposed mussels for which several on-line GI extracts are to be processed automatically by μSPE prior to LC-MS/MS analysis. Using 90:10 (v:v) ACN:MeOH according to manufacturer's specifications, (phospho)lipids and fats are irreversibly retained onto the copolymeric hydrophobic/hydrophilic sorbent while analytes are quantitatively retrieved. This makes the reutilization of the bead material impracticable unless additional washing steps are added to the analytical workflow. To tackle this shortcoming, the LOV-μSPE minicolumn (≤ 25 mg) is automatically disposed of in this work via BI procedures and renewed in every analysis of EOC-laden GI extracts. The morphology of the Oasis PRIME HLB was evaluated by scanning electron microscopic (see Fig S1). The almost perfectly spherical and uniform in size bead distribution make this sorbent a suitable candidate for facile on-line handling of suspensions in the LOV format.[28] Our experimental results corroborate the fact that the automatic loading and packing of the LOV sorbent microcolumn is highly repeatable with RSD <8%.

Optimization of the LOV-μSPE-BI method for on-line handling of GI extracts

Because one-at-a-time univariate procedures would not necessarily afford optimum experimental conditions, a two-level full factorial design was selected to assess those experimental factors with the greatest effect on the extraction efficiency (eluted to loaded mass ratio) of EOCs in GI fluids under LOV-BI-μSPE conditions (see SI). In this work, the volume of GI extract, the loading flow rate across LOV-μSPE column and the matrix (mussel control) concentration in the GI extract were selected as the main quantitative factors. The volume of the GI extracts spanned from 1.0 to 5.0 mL as a compromise between enrichment factor and sample throughput. The flow rate of the GI extracts was investigated within the range of 0.5 to 1.5 mL min^{-1} on the basis of preliminary experiments that revealed the absence of pressure drop in the LOV-μSPE system. Matrix (mussel) concentration in the GI fluid ranged from 6.8 to 13.5 mg L^{-1} and is obtained by effecting the batchwise Versantvoort's test using three distinct sample amounts, *viz.*, 0.25, 0.375 and 0.5 g. The same amount of analytes (namely, 500 ng) was loaded across the LOV microcolumn regardless of the sample volume (1.0-5.0 mL) for investigation of potential breakthrough effects and thus the concentration levels of the target species were affixed to 500, 167 and 100 μg L^{-1} for GI extract volumes of 1.0 mL, 3.0 mL and 5.0 mL, respectively. The extraction efficiencies of the overall EOCs as determined in every of the

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3 thirteen individual runs by LC-MS/MS using external calibration with isotopologues are listed
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5 in Table S1.
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Evaluation of the effects of the main factors and the second-order interactions on the extraction efficiency (analytical response) for the four targets was conducted by ANOVA using the desirability function (see SI). The standardized factor effects of the desirability function are illustrated via Pareto charts (see Fig. S2), in which the standardized effects of the main factors and interactions thereof are arranged in a descending order. Pareto's bar lengths are equivalent to the calculated Student's *t* values. The cross vertical line (in our case, 2.88), signals the *t*-critical value at a 0.05 significance level for four degrees of freedom. The positive (light grey) bars denote those scenarios where the extraction efficiency improves by increasing a given factor from the lowest to the highest coded level in the experimental domain, while negative (navy blue) bars represent the opposite case. The matrix (mussel) concentration is the only variable which is deemed statistically significant at the 0.05 significance level according to the Pareto chart (Fig. S2). The lower the mussel concentration loaded onto the LOV-µSPE column the better was the extraction efficiency, thereby indicating the negative influence of the dissolved matter on the sorptive uptake of the EOCs. It should be also born in mind that the concentration of (mussel) matrix dissolved in the GI extracts is variable throughout the dynamic bioaccessibility assay. To alleviate these effects, isotopically labeled internal standards of the suite of EOCs were added to every GI extract fraction before on-line handling by µSPE using a flow rate (non-statistically significant variable) of 1.5 mL min^{-1}. As to the on-line GI extract volumes, the first ten fractions were fixed to 1.0 mL for proper resolution of the temporal profiles of bioaccessible EOCs and then increased to 3.0 mL until a total volume of GI fluid of 28 mL.

Validation of the first-order model was effected by application of a lack of fit test[38] to determine whether the full factorial design is adequate to describe the observed data or a second-order model should be selected instead. This test is performed by comparing variability between observations at replicate settings of the factors, in our case, at the central point level, against the variability of the model predicted errors. The first-order model appears to be appropriate to describe the analytical system at the 95% confidence level (*p*-value = 0.157) with no need of further optimization by surface response methodology.

Configuration of the mesofluidic system

A thorough investigation of the experimental conditions for on-line dynamic GI extraction using mock up digestive fluids and LBC setups has been reported elsewhere.[21] Hereto, the flow rate of the digestive fluid, the filter type, the pore dimensions, and the extraction temperature were fixed to 1.0 mL min^{-1}, PVDF material, 5.0 µm, and 37ºC, respectively for a sample amount of 250 mg.

The Versantvoort's method[5] is composed of two sequential extraction steps: (i) gastric digestion and (ii) intestinal digestion, using four different body fluid surrogates, namely, saliva and gastric fluid for the stomach compartment and duodenal fluid and bile for the small intestine compartment. Aiming to simplify the analytical procedure the role of the two sequential steps for bioaccessibility assessment of the target EOCs in mussels was evaluated. To this end, the *in-vitro* batchwise Versantvoort's test was realized by two slightly different procedures. In the first procedure, the standard batchwise Versantvoort's method was used without modification (see Experimental section). In the modified procedure, the four digestive fluids were first mixed and applied to the mussel in a single extraction step which lasted 4 h as is the case of the conventional Versantvoort's method. A good agreement is found for the bioaccessible concentrations of MeP, TCS and DCF in both protocols (see Fig. S3). The *t*-test of comparison of means revealed the inexistence of significant differences between the reported and the modified batchwise Versantvoort's method at the 0.05 significance level for the overall EOC ($t_{exp(MeP)}$ = 2.82; $F_{exp(DCF)}$ = 2.72; $F_{exp(TCS)}$ = 1.31 against $t_{crit}(0.05, 2)$ = 4.30). This is probably a consequence of the ineffective extraction of the EOC in the stomach phase because that target organic compounds are mostly associated to the lipid phase, which is digested throughout the intestinal phase due to the action of lipase and bile salts.[14] A single-step biomimetic digestion protocol, with a composite GI extractant containing saliva, gastric fluid, duodenal fluid and bile was thus selected for the ensuing studies. It should be noted that the total content of BuP of the exposed mussels is 23, 86 and 95 times inferior to that of MeP, TCS and DCF, respectively, thus rendering a GI bioaccessible concentration of BuP below the detection limit (LOD) of LC-MS (LOD_{BuP}=2.3 μg L^{-1}, LOD_{MeP}=2.5 μg L^{-1}, LOD_{TCS}=1.8 μg L^{-1}, LOD_{DCF}=1.2 μg L^{-1}, all calculated based on the $3S_{y/x}$ criterion). BP is either insignificantly bioacummulated by the mussels or degraded in the time course of the exposition experiment, yet only a hydrolytic product of TCS in the GI extracts could be inferred by LC-Orbitrap-MS based suspect screening studies (see SI).

Evaluation of the on-line dynamic GI extraction and comparison with the batchwise counterpart

The temporal profiles of the GI bioaccessible pools of MeP, DCF and TCS in exposed mussels as obtained by combining dynamic GI extraction with LOV-BI-μSPE are illustrated by the graphical plot of the extracted concentrations (given as μg kg^{-1} or μg $(kg{\cdot}min)^{-1}$) against the cumulative biomimetic extractant volume or extraction time (see Fig. S4). The composite GI fluid is programmed to flow through the real sample contained in the LBC for number of cycles of leaching and LOV-μSPE until the readily bioaccessible EOCs are quantitatively leached out of the mussel. A total number of sixteen subfractions (28 mL of GI fluid) was needed to reach

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3 background LC-MS readouts inasmuch as the increase of contaminant leached in three
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5 subsequent subfractions (9 mL) was below 10% of the cumulative extracted amount.
6 The extraction profiles of target EOC (four replicates) from the exposed mussels were adjusted
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8 ($p > 0.05$) to a first order kinetic equation[39] (equation 1) as follows:
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$$Q = A\left(1 - e^{-k_A t}\right) \qquad (1)$$
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15 where Q is the total GI bioaccessible content of a given EOC (μg kg^{-1}) measured at average time
16 *t*, A is the readily GI bioaccessible (labile) pool, and k_A (min^{-1}) the associated rate constant. The
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18 parameters of the mathematical model are summarized in Table 1. The lack of fit test (p=0,99)
19 suggests that slowly GI bioaccessible pools are not released under dynamic conditions. The
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21 predicted (A) and the experimental dynamic bioaccessible concentrations (obtained by
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23 summation of leached concentrations in every subfraction) were compared with the batchwise
24 physiologically based test (see Table 1). The batchwise test afforded larger concentrations of
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26 bioaccessible EOC with 1.5, 2.9, and 1.9-fold increase of MeP, TCS, and DCF, respectively.
27 Similar findings were reported for batchwise bioaccessibility testing using strong reagents in
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29 soil materials containing readily and poorly mobilizable pools of trace elements.[40,41] The
30 dissimilarity of the underlying fundamental principles of batch against dynamic extraction
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32 modes might account for the differing extractabilities. The continuous renewal of the
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34 biomimetic fluids in dynamic methods might not offset the shorter extraction times (< 30 min in
35 our case) compared with the batchwise counterpart (4 h). Further, physical disaggregation of the
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37 solid sample is accelerated by end-over-end agitation of the batch method, which does not
38 properly mimic physiological extraction conditions. The mathematical model described above
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40 indicates that 72, 20 and 60% of the total content of MeP, TCS and DCF, respectively, is readily
41 bioaccessible (see A values in Table 1), as determined by on-line leaching methods. On the
42
43 contrary, batchwise bioaccessibilities of 104, 50 and 105% of MeP, TCS and DCF, respectively,
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45 in the exposed mussels suggest that both readily and slowly labile fractions of EOC are released
46 from the solid sample by conventional extraction tests. Therefore, the potential bioavailability
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48 of EOCs, and human exposomics of contaminated mussel tissues are most likely overestimated
49 by the conventional Versantvoort's method.
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53 **Validation of on-line gastrointestinal bioaccessibility combined with LOV-BI sorptive**
54 **microextraction**
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56 The lack of bias (trueness) of the proposed fluidic approach combining dynamic gastrointestinal
57 bioaccessibility with lab-on-valve sorptive microextraction was assessed by mass balance
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59 validation as applied to individual target EOCs. To this end, the sum of the GI bioaccessible
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concentrations (determined by external calibration with isotopologues within the dynamic linear range of 2.5-300 µg L^{-1} each EOC) and the residual (immobilized) fraction (determined by UASLE) was statistically compared against total EOC concentration determined by enzyme-assisted UASLE. Because of the heterogeneity of the food commodity, outliers were identified and discarded by resorting to the median/MAD (median absolute difference) statistical test.[42] Relative recoveries spanned from 89 to 132% (Table 2). The *t*-test of comparison of means[43] for every individual target indicated that there were no statistically significant differences at the 0.05 level for any EOC. The combination of LOV-BI with the addition of isotopologues is thus proven suitable for elimination of potential additive and multiplicative matrix interferences from the dynamic GI extracts, making the use of the standard addition method unnecessary.

CONCLUSIONS

A hyphenated mesofluidic LOV system incorporating on-line dynamic bioaccessibility testing of EOCs in incurred seafoodstuff, and automatic processing of the extracts by on-line disposable µSPE has been for the first time assembled, optimized and validated for fast and reliable human risk assessment of contaminated mussels. Biorelevant body fluids and GI extracts containing salts, fats and enzyme suspensions were automatically handled and processed through the conduits of the flow manifold and LOV platform by pressure-driven flow without clogging of the system. Monitoring of the temporal extraction profiles permitted shortening of the GI bioaccessibility test from 4 hours to merely 28 min while offering insight into the leaching rates of organic pollutant-laden foodstuff entering the duodenal compartment. Our fluidic concept also offers a more realistic model for estimation of readily available pools of organic contaminants in foodstuff by the action of GI enzymes and bile components than that of standard (single-equilibrium) batch methods that tend to overestimate bioaccessibility on account of the unfeasibility of discrimination between readily and slowly leachable organic compounds.

Further work is underway in our group for exploring new applications of flow-through biomimetic/GI extraction combined with on-line LOV-BI sorptive microextraction for automatic analysis of real fish commodities and untargeted identification and quantification of oral bioaccessible pools of emerging contaminants at trace level concentrations in incurred environmental solid matrices.

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Supporting information: Detailed description of (i) LC-MS/MS methods for identification and quantification of EOCs and degradation products in incurred mussels; (ii) automatic dynamic extraction method with on-line LOV-μSPE processing of the gastrointestinal extracts; (iii) batch physiologically-based extraction test; (iv) analytical method for analysis of residues and raw samples aimed at mass balance validation, and (v) multivariate design for the investigation of the critical variables influencing the extraction efficiency of the LOV-μSPE system for the target emerging contaminants.

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Figure Caption

Figure 1: Schematic illustration of the automatic mesofluidic system combining on-line gastrointestinal bioaccessibility with LOV-Bead Injection sorptive microextraction for assessing the extraction rates of MeP, BuP, TCS and DCF in exposed mussels under simulated physiologically based conditions. HC, holding coil; CC, communication channel; LOV, Lab-On-Valve; LBC, Large-bore column; C, carrier; IS, internal standard; GI; gastrointestinal; LC-MS/MS: Liquid chromatography tandem mass spectrometry.

Figure 2: Batchwise evaluation of the extraction performance Oasis-HLB, Oasis-PRIME HLB and Ag-nanoparticle embedded copolymer beads for the uptake of diclofenac, triclosan, methyl paraben and butyl paraben from spiked gastrointestinal extracts of unexposed mussel tissues. Errors bars stand for standard deviation (n = 3).

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Figure 1

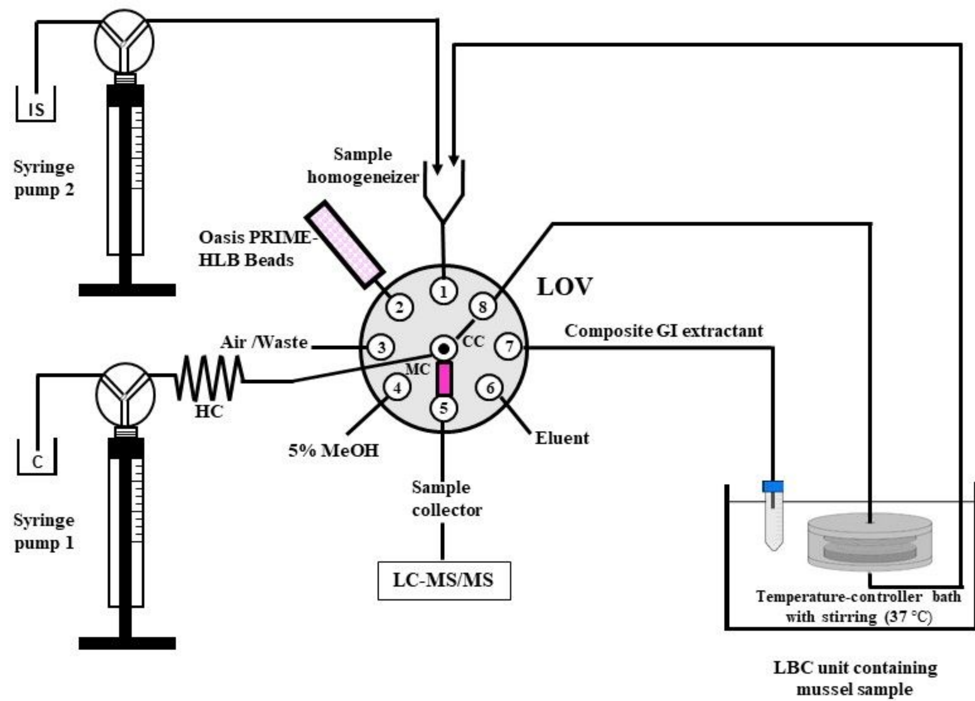

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Figure 2

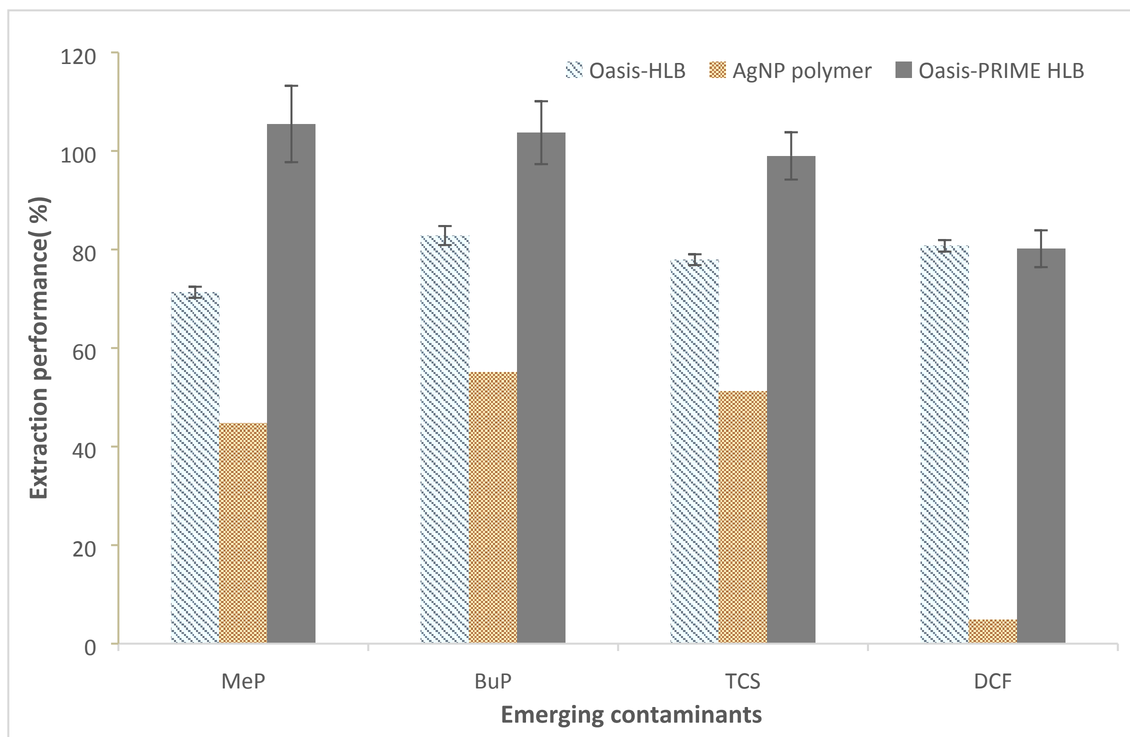

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Table 1: Model parameters of the first order kinetic equation for assessment of the readily GI bioaccessible concentrations of MeP, TCS and DCF in exposed mussels.

Model parameter	MeP	DCF	TCS
A (µg/kg mussel)	262 ± 10	956 ± 40	294 ± 10
k_A (min^{-1})	0.082 ± 0.006	0.084 ± 0.007	0.066 ± 0.004
p lack of fit (>0.05)	0.99	0.99	0.99
Predicted readily bioaccessible EOC (µg EOC/kg mussel)	262 ± 10	956 ± 40	294 ± 10
Bioaccessible EOC (µg EOC/kg mussel) determined by the proposed dynamic system (28 min)	243 ±24	882 ± 64	252 ±23
Bioaccessible EOC (µg EOC/kg mussel) determined by the conventional batchwise Versantvoort's method (240 min)	375 ±29	1672 ± 21	725 ± 106

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Table 2. Gastrointestinal bioaccessible concentrations of MeP, TCS and DCF from exposed mussels and mass balance validation of the on-line oral bioaccessibility test hyphenated to LOV-BI-µSPE

EOC	GI bioaccessibility (µg /kg^{-1}) (% referred to total amount)*	Residue (µg kg^{-1})[a]	Total (µg kg^{-1})[b]	Total UASLE (µg kg^{-1})	Recovery (%)	t_{exp}
MeP	243 ± 24 (67)	114 ± 19	357 ± 36	362 ± 21	99±14	0.24[c]
TCS	252 ± 23 (17)	1029 ± 138	1282 ± 140	1447 ± 101	89±15	1.92[c]
DCF	882 ± 64 (55)	1303 ± 362	2185 ± 356	1639 ± 46	132± 18	2.86[d]

* GI bioaccessibility of BuP is < 2.3 µg /kg of mussel

Results are expressed as mean of four replicates ± standard deviation.

[a] Concentration of non-bioaccessible EOC determined by UASLE (see SI)

[b] Sum of the GI bioaccessible and the residual EOC concentration

[c] t_{crit}= 2.45 (d.f. = 6)

[d] t_{crit}= 4.30 (d.f. = 2). Application of Welch's t-test (unequal variances)

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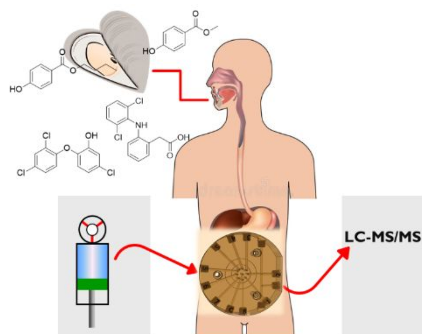
TOC graphic



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