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Solid-phase extraction method for stable isotope analysis of pesticides from large volume environmental water samples

Clara Torrentó^{a,†}, Rani Bakkour^b, Gaétan Glauser^c, Aileen Melsbach^d, Violaine Ponsin^a, Thomas B. Hofstetter^b, Martin Elsner^{d,e}, Daniel Hunkeler^a

^aCentre for Hydrogeology and Geothermics (CHYN), University of Neuchâtel, 2000 Neuchâtel, Switzerland
^bEawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland
^cNeuchâtel Platform of Analytical Chemistry (NPAC), University of Neuchâtel, 2000 Neuchâtel, Switzerland
^dHelmholtz Zentrum München, Institute of Groundwater Ecology, 85764 Neuherberg, Germany
^eTechnical University of Munich, Chair of Analytical Chemistry and Water Chemistry, 81377 Munich, Germany

Abstract

Compound-specific isotope analysis (CSIA) is a valuable tool for assessing the fate of organic pollutants in the environment. However, the requirement of sufficient analyte mass for precise isotope ratio mass spectrometry combined with prevailing low environmental concentrations currently limits comprehensive applications to many micropollutants. Here, we evaluate the upscaling of solid-phase extraction (SPE) approaches for routine CSIA of herbicides. To cover a wide range of polarity, a SPE method with two sorbents (a hydrophobic hypercrosslinked sorbent and a hydrophilic sorbent) was developed. Extraction conditions, including the nature and volume of the elution solvent, the amount of sorbent and the solution pH, were optimized. Extractions of up to 10 L of agricultural drainage water (corresponding to up to 200,000-fold pre-concentration) were successfully performed for precise and sensitive carbon and nitrogen CSIA of the target herbicides atrazine, acetochlor, metolachlor and chloridazon, and metabolites desethylatrazine, desphenylchloridazon and 2,6-dichlorobenzamide in the sub- $\mu\text{g L}^{-1}$ -range. $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios were measured by gas chromatography-isotope ratio mass spectrometry (GC/IRMS), except for desphenylchloridazon, for which liquid chromatography (LC/IRMS) and derivatization-GC/IRMS were used, respectively. The method validated in this study is an important step towards analyzing isotope ratios of pesticide mixtures in aquatic systems and holds great potential for multi-element CSIA applications to trace pesticide degradation in complex environments.

Keywords: Compound-specific isotope analysis, triazine herbicides, chloroacetanilide herbicides, chloridazon, desphenylchloridazon, 2,6-dichlorobenzamide.

Introduction

Due to abundant agricultural use, pesticides are frequently found in soil and groundwater.¹⁻⁵ Pesticide leaching is a problem for groundwater quality and a threat to human health if contaminated groundwater is used as drinking water. To mitigate existing pollution, it is critical to identify sources and to assess the fate of the

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3 numerous pesticides present in the environment. Existing methods for elucidating pesticide degradation
4 include, for example, monitoring of parent compound disappearance, detection of transformation products, and
5 evidence of intrinsic transformation potential by molecular biology tools.⁶ Nevertheless, measuring the
6 concentration of the parent compounds does not allow distinguishing transformation from other processes such
7 as dilution or sorption, especially in the vadose zone, where concentrations tend to fluctuate strongly under
8 transient and varying hydrological conditions. Furthermore, for the metabolites of many pesticides,
9 transformation reactions are not well known. Degradation processes may therefore not become evident from
10 analysis of the concentration dynamics.

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Compound-specific isotope analysis (CSIA) is a powerful tool to track and quantify pollutant degradation in
environmental systems.⁷⁻¹⁰ Molecules with light isotopes in the reactive position are degraded at different rates
than molecules containing heavy isotopes. Consequently, temporal and spatial shifts in isotope ratios are
indicative of degradation and enable tracking degradation processes. In addition, monitoring the changes of
isotope signatures of two (or more) elements is recommended to obtain a more reliable assessment of
degradation and to derive the extent and relative contribution of different reaction mechanisms.^{9,11,12}

Although CSIA is in widespread use to study the fate of many contaminants^{11,13-15}, applications are still emerging
for pesticides.¹⁶ Various analytical methods have been recently developed.¹⁷⁻²⁹ Nonetheless, application of CSIA
of pesticides to field studies remain scarce^{24,30-34} because of two major challenges. First, isotope effect-free
extraction and pre-concentration methods are required to allow CSIA at low environmentally relevant
concentrations by gas chromatography-isotope ratio mass spectrometry (GC/IRMS) or – in the case of
compounds like desphenylchloridazon for which GC/IRMS-based carbon isotope analysis does not work – by
liquid chromatography-IRMS (LC/IRMS).³⁵ To accomplish accurate (i.e. true and precise)³⁶ CSIA in specific
compounds, the required analyte amount is much higher than for concentration analysis (up to 5 nmol of C and
10 nmol of N on-column may be necessary).³⁷ In environmental water samples, most pesticides and their
metabolites are present in trace concentrations (ng L⁻¹ to µg L⁻¹ range), which are relevant for regulatory
purposes; the EU Directive 98/83/EC establishes maximum permissible concentrations of 0.1 µg L⁻¹ for individual
pesticides and relevant degradation products and 0.5 µg L⁻¹ for total pesticides. A more than 100,000-fold pre-
concentration (i.e. 10-100 mg L⁻¹) may therefore be required for CSIA. Hence, there is a need for an efficient
preparative procedure from large volumes of environmental water samples that can provide the required large
amounts of analyte without compromising accurate CSIA. Second, GC/IRMS and LC/IRMS analyses require
baseline separation of the target compounds from interfering substances and complex mixtures.^{9,16,38} Indeed,
during sample pre-concentration, matrix components are enriched together with the target compounds,
potentially compromising the chromatographic resolution of the latter. Furthermore, environmental samples
usually contain mixtures of pollutants and thus, to minimize workload, pre-concentration methods should be

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3 suitable for extracting various compounds at the same time, covering a broad polarity range but ensuring good
4 chromatographic resolution. View Article Online
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7 A method of choice for the extraction and pre-concentration of pesticides from large volumes of aqueous
8 solution is solid-phase extraction (SPE). The sorbent is selected depending on the characteristics of the analytes
9 to be retained and on the complexity of the sample matrix. A great variety of sorbents with a broad range of
10 properties are commercially available. Excellent reviews regarding sorbents mostly used for micropollutants³⁹⁻
11 ⁴², as well as several examples of pesticide extractions from large-volume water samples^{43,44} have been
12 published. Schreglmann et al.³⁰ validated a SPE-CSIA method for atrazine and desethylatrazine extraction from
13 up to 10 L of tap water spiked at concentrations from 0.5 to 50 µg L⁻¹ by using a hydrophilic divinylbenzene
14 sorbent. However, no extraction and pre-concentration methods applied to large-volume samples for accurate
15 CSIA of mixtures of herbicides covering a broad polarity range at nanomolar concentrations in environmental
16 waters are currently available.

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18 The main objective of the present study is therefore to develop and validate a SPE-CSIA method for determining
19 carbon and nitrogen isotope ratios of pesticides and metabolites covering a wide range of polarities that
20 commonly occur together in groundwater at nanomolar concentrations. The mixture selected for this work
21 (atrazine -ATR-, desethylatrazine -DEA-, desisopropylatrazine -DIA-, acetochlor -ACETO-, metolachlor -METO-,
22 chloridazon -CLZ-, desphenylchloridazon -DPC-, methyl-desphenylchloridazon -M-DPC-, and 2,6-
23 dichlorobenzamide -BAM-) displays a broad range of polarity (octanol-water partition coefficient, log K_{OW},
24 ranging from -0.4 to 3.1) (Table 1). To this end, we (i) optimized SPE conditions for the mixture of the target
25 compounds; (ii) scaled the selected SPE method to large-volume samples; (iii) validated instrumental methods
26 for carbon and nitrogen CSIA of the selected compounds; and (iv) validated the entire SPE-CSIA procedure for C
27 and N CSIA of the target compounds in agricultural drainage water samples.

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Experimental section

Optimization of the SPE method at small scale

We first compared the performance of different SPE sorbents for extracting the target compounds from small-volume samples of distilled water. The sorbents were selected based on previously published studies as follows. For ATR, DEA and DIA extraction from environmental water samples, polystyrene-divinylbenzene (PS-DVB)-based sorbents with hydrophilic character such as Oasis HLB and Strata-X have been mainly used^{45-47,48}, but also silica-based⁴⁹, graphitized carbon-based⁴⁹ and hypercrosslinked polystyrene-divinylbenzene (HC-PS-DVB) sorbents.⁵¹ CLZ has been successfully extracted with Oasis HLB^{49,50} and HC-PS-DVB sorbents.^{51,52} Schatz (2012)⁵³ found that only the HC-PS-DVB sorbents Bakerbond SDB-1 and LiChrolut EN were able to retain DPC. Nevertheless, other authors have used Oasis HLB for extracting DPC and M-DPC, although recoveries have not

been reported.^{5,54} Successful extraction of the chloroacetanilides ACETO and METO has been reported using the PS-DVB sorbents Oasis HLB and Strata X^{45,47,49,51}, as well as C18 bonded silica.⁵⁵ The reported approaches for extracting BAM have mainly used Oasis HLB^{56,57} and other DVB phases.⁵⁸ Based on this body of literature, and considering the wide range of polarity of the selected pesticides, the following sorbents were tested in this study: one graphitized carbon-based sorbent (Supelclean ENVI-Carb), one styrene-divinylbenzene (ST-DVB) sorbent (Strata-SDB-L), two PS-DVB sorbents with hydrophilic character (Oasis HLB and Septra ZT, which is the bulk phase of Strata X) and two hydrophobic HC-PS-DVB sorbents with ultra-high surface area (Bakerbond SDB-1 and LiChrolut EN). Details about the properties of the tested SPE sorbents can be found in Table S1 (Supplementary information).

The performance of the method was evaluated in terms of extraction efficiency from a mixture of the target compounds under different conditions, based on previous studies: concentration levels (from 1 to 25 $\mu\text{g L}^{-1}$), water volumes (from 20 to 500 mL), pH values (3 and unmodified pH), sorbent mass (0.2 to 1 g), and elution solvents (ethyl acetate and methanol). Atrazine- d_5 , alachlor- d_{13} and chloridazon- d_5 were added to the samples at 1.25, 5.0 and 5.0 $\mu\text{g L}^{-1}$, respectively, as surrogate standards. The internal standard terbutylazine was added to the final extracts at 50 $\mu\text{g L}^{-1}$. Samples were then analyzed by ultra-high pressure liquid chromatography quadrupole time of flight mass spectrometry, as explained below. Recoveries were determined by comparing the peak areas obtained in the spiked samples with those obtained in standard solutions at equivalent concentrations. The overall recovery of each pesticide was calculated as the mean recovery of the spiked samples extracted on different days using the same method and the same equipment. Repeatability was expressed as relative standard deviation (RSD).

Optimization of the large-volume water extraction procedure

The SPE approach was further evaluated to rule out SPE-induced isotope fractionation from large-volume samples. First, efficiency of the SPE approach was tested with 5 L and 10 L tap water spiked with 0.1 $\mu\text{g L}^{-1}$ of the target compounds. Second, the method performance was evaluated with spiked environmental aqueous samples to identify potential interferences from matrix compounds during chromatographic separation. Samples from the drainage water of lysimeters filled with arable soils were used.⁵⁹ Sorbent performance was evaluated in 10 L filtered (0.7- μm glass fibre filters) drainage water samples spiked with 0.1 to 50 $\mu\text{g L}^{-1}$ of the target compounds. Finally, the integrity of the isotope values after large-volume SPE was assessed. Validation tests consisted of ten liter samples spiked with standards of the target compounds with known isotope signatures (at 0.5 to 50 $\mu\text{g L}^{-1}$), for which SPE was performed following the optimized method.

Analytical methods

A detailed description of the analytical methods is available in the Supplementary information. Briefly, concentrations of the target compounds in the SPE eluates were determined by ultra-high pressure liquid chromatography quadrupole time of flight mass spectrometry (UHPLC-QTOF-MS), using the qualifier and quantifier ions listed in Table S2 (Supplementary information). Among the target compounds, DPC is the most polar one and is therefore not directly amenable to gas chromatographic separation. For CSIA, for that reason, the following strategy was chosen. Whereas the other compounds were analyzed by GC/IRMS without prior modification, LC/IRMS was used for carbon isotope analysis of DPC, and GC/IRMS after derivatization was used for nitrogen isotope analysis of DPC, respectively.³⁵ Carbon and nitrogen CSIA of ATR, ACETO, METO, DEA and BAM in ethyl acetate (EtAc) was performed by GC/IRMS according to modified methods.^{19,23,30,60} Carbon CSIA of DPC in water was performed by LC/IRMS as explained elsewhere.³⁵ For measuring N isotope signatures of DPC, derivatization with trimethylsilyldiazomethane was performed prior to GC/IRMS analysis.³⁵ The CSIA methods were validated following quality assurance recommendations from the US-EPA.⁷ The trueness of the isotope measurements was expressed as the deviation of isotope signatures measured by GC/IRMS and LC/IRMS from reference isotope ratios of the calibrated in-house standards of known carbon and nitrogen isotope ratios, which were previously determined by Elemental Analyzer (EA)/IRMS based on two-point normalization using the international organic reference materials USG 40 (L-glutamic acid), USG 41 (L-glutamic acid) and IAEA 600 (caffeine), provided by the International Atomic Agency (Vienna, Austria).³⁵ Isotope ratios of the in-house standards are listed in Table S3 (Supplementary information). Carbon and nitrogen isotope values are reported in per mil (‰) using the delta notation relative to the international standards Vienna PeeDee Belemnite (V-PDB) and air, respectively:

$$\delta \text{ (in \%)} = \left(\frac{R}{R_{std}} - 1 \right) \quad (1)$$

where R and R_{std} are the isotope ratios of the sample and the standard, respectively. All reported isotope ratios are expressed as arithmetic means of replicate measurements with 1 standard deviation ($\pm\sigma$) in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values as a measure for instrumental precision.

Limits of precise isotope analysis, amount dependency, and reproducibility

The GC/IRMS and LC/IRMS instrumental limit of precise isotope analysis ($\text{Limit}_{\text{instrument}}$) was determined for each target compound according to the moving mean method^{61,62}, using standard solutions of known isotope composition and uncertainty intervals of $\pm 0.5\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 1\text{‰}$ for $\delta^{15}\text{N}$. The linearity range (i.e. the range between the smallest and the largest concentration for which the standard deviation of the mean isotope ratio value is within the projected uncertainty intervals) was determined for each compound for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. The reproducibility and long-term stability of the GC/IRMS and LC/IRMS systems were established for different concentrations within the linear range over a period of time ranging between 1 and 3 months.

The limit of precise isotope analysis of the whole SPE-CSIA method ($Limit_{method}$) (i.e. the minimum concentration in water needed to reach the $Limit_{instrument}$), for both $\delta^{13}C$ and $\delta^{15}N$, was estimated for each target compound according to Eq. (2).

$$Limit_{SPE-CSIA\ method} = \frac{Limit_{instrument}}{Conc.\ factor} \times \frac{1}{Recov.} \quad (2)$$

where *Recov.* and *Conc. factor* are the extraction recoveries and pre-concentration factors achieved with the optimized large-volume SPE method, respectively. The validation tests with spiked drainage water samples were also used for experimental validation of the estimated $Limit_{method}$.

Results and Discussion

Optimization of the extraction methods

Sorbent screening. An initial screening study was performed to select the most promising sorbents in terms of extraction efficiency for the mixture of selected herbicides and metabolites. For all the selected sorbents, 0.2-g bulk phase was used, except in the case of the sorbents with the lowest specific surface area (ENVI-Carb and Strata SDB-L), for which 0.5-g of sorbent was utilized. Extraction efficiencies for each sorbent were evaluated in replicate trials from a mixture of the target compounds at the same mass load (0.25 μg of each compound, except DPC, for which 0.5 μg was added) and sample volume (20 mL) and using the same eluent solvent (EtAc) and the same eluent volume (3 mL).

Results of this sorbent screening are shown in Table 1. The two sorbents with the lowest surface area (ENVI-Carb and Strata SDB-L) were not able to retain neither CLZ nor DPC. M-DPC was not retained by ENVI-Carb, whereas partial recovery (54%) was achieved with Strata SDB-L. Recoveries for the rest of the target compounds were lower than 70%, except for the chloroacetanilides with ENVI-Carb (84-94%). Regarding the hydrophilic (i.e. with presence of polar moieties in their structures, and thus polar functionalities) PS-DVB sorbents, better performance was achieved with Septra ZT than with Oasis HLB. Except for DPC (less than 10% recovery with both sorbents), recoveries for all compounds were satisfactory with Septra ZT, ranging from 89 to 106%, with RSD values lower than 18% in all cases. Complete recovery of DPC (90-99%) was only achieved with the two hydrophobic hypercrosslinked HC-PS-DVB sorbents with ultra-high surface area (SDB-1 and LiChrolut EN), in accordance with the results of Schatz.⁵³ M-DPC was partially retained by SDB-1 (77%) and not tested with LiChrolut EN. For the rest of the analytes, high recoveries were also achieved using the HC-PS-DVB sorbents, ranging between 84 and 105% (RSDs up to 16%) with SDB-1 and between 83 and 103% (RSDs up to 16%) with LiChrolut EN.

Optimization at small scale (20-500 mL). Given the results of the sorbent screening, Septra ZT, LiChrolut EN and SDB-1 were selected for further optimization of the extraction method. Extraction efficiency from a mixture of

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the target compounds at different concentration levels (from 1 to 25 $\mu\text{g L}^{-1}$) and for different water volumes (from 20 to 500 mL) was investigated by varying the following key parameters, one at a time: pH of water sample, and type and volume of elution solvent. Similar results in terms of extraction efficiency of Septra ZT and SDB-1 were obtained with the three tested elution procedures: 3 mL EtAc, 3 mL MeOH and elution with a sequence of the two solvents (3 mL EtAc followed by 3 mL MeOH) (Fig. 1). Elution with 3 mL EtAc was finally selected due to higher recovery for DPC. Mass load effect was assessed for 0.2 g-cartridges of Septra ZT. Increasing the mass by a factor 25 (from loading 0.02 μg to 0.5 μg) did not cause important changes in the extraction efficiency, except for M-DPC, BAM and ACETO (Fig. 2).

With the hydrophobic HC-PS-DVB sorbents, two strategies for increasing DPC recovery were tested: modifying the pH of the sample and increasing sorbent mass.⁵³ First, triplicate extractions using 0.2 g-cartridges of SDB-1 and LiChrolut EN were performed with 20 mL of distilled water containing 25 $\mu\text{g L}^{-1}$ DPC with unmodified pH and with pH adjusted to 3 with HCl. For both sorbents, higher DPC recovery has been reported at pH 3 than at pH 7.⁵³ In the present experiments, however, no significant changes were observed for SDB-1 (113 \pm 5 and 109 \pm 8, respectively) and DPC recovery using LiChrolut EN was only marginally enhanced at pH 3 (104 \pm 3 vs. 92 \pm 3 with unmodified pH). Similar results were also obtained for the rest of the metabolites (Fig. S1, Supplementary information). Second, to determine if an increase in sorbent mass would result in an increase in DPC recovery, two SDB-1 sorbent masses (0.2 and 1 g) were tested for extracting samples spiked with 0.005 to 0.5 μg DPC. The mass of 0.2 g resulted in complete recoveries of DPC for mass load of 0.25 and 0.5 g. For DPC mass load lower than 0.25 μg , however, 1 g of sorbent was required (90-100% recovery), rather than 0.2 g (40-60 % recovery) (Fig. S2, Supplementary information).

Finally, the breakthrough effect related to sample volume was assessed. The hydrophilic sorbent Septra ZT and the hydrophobic hypercrosslinked sorbents with ultra-high surface area SDB-1 and LiChrolut EN were tested. For developing an efficient method that allows extracting all the analytes in one run in order to decrease workout, time and costs, layered cartridges containing one hydrophilic and one hydrophobic hypercrosslinked sorbent were also tested. Distilled water samples (20, 100 and 500 mL) spiked at 0.5 μg of each analyte (1 μg for DPC) were loaded onto the cartridges. For the commercial cartridges, elution was performed with 3 mL EtAc, whereas 6 mL EtAc were used for the homemade-layered cartridges due to the doubled sorbent mass. With Septra ZT, all metabolites except BAM showed breakthrough (Table 2), most pronounced for M-DPC and DPC. Regarding the parent compounds, the breakthrough effect was also significant for ATR and METO. The HC-PS-DVB sorbents with ultra-high surface area (SDB-1 and LiChrolut EN) reduced, as expected, breakthrough volume for the most polar compounds, but, surprisingly, performed poorly for the chloroacetanilides. In contrast, using the layered column with Septra ZT and SDB-1, breakthrough volumes higher than 100 mL were obtained for all analytes, except for DPC (between 20 and 100 mL). Therefore, the combination of the hydrophilic sorbent Septra

ZT and the hydrophobic hypercrosslinked sorbent SDB-1 provided the best performance to deal with the broad range of polarity of the selected analytes.

Scale-up to large volumes. Sorbent mass and breakthrough effects were assessed for cartridges containing Septra ZT and SDB-1 alone and combined. Tap water samples (5 and 10 L) spiked with $0.1 \mu\text{g L}^{-1}$ of each analyte were loaded onto cartridges containing different sorbent masses (5 and 10 g of single sorbents and 8 g each for the combined approach) (Table 3). For 5 L samples, cartridges with 5 g of Septra ZT, as expected, failed to recover DPC. Furthermore, recoveries for the rest of the compounds were low for M-DPC, ACETO and METO (19-36%) and acceptable for DIA, DEA, BAM, CLZ and ATR, but in all cases with high RSDs (up to 49%). Increasing the sorbent mass to 10 g resulted in excellent recoveries (92-108%) and RSDs (4-19%) for all the compounds, except DPC. Using 10 g of SDB-1, DPC was more strongly retained (46%) and similar results as those with Septra ZT were achieved for the rest of the compounds, except in the case of DIA, DEA and BAM, for which recoveries were slightly lower (70-80%). The best results were obtained with the combination of 8 g of each sorbent in a layered cartridge. Excellent recoveries (90-109%, RSD <17%) were achieved for all the compounds, even for DPC (76±17%). The breakthrough effect was assessed increasing the sample volume to 10 L for the three cartridges (10 g Septra ZT, 10 g SDB-1 and 8 g each) (Table 3). No compound displayed important breakthrough, except DPC, for which recovery slightly decreased (from 76% to 53%).

Finally, for the layered cartridges containing 8 g of SDB-1 and 8 g of Septra ZT, matrix spikes were extracted to assess the influence of matrix components of real samples. To this end, known amounts of the target analytes (0.1 to 5 μg) were added to 10 L filtered (0.7- μm glass fibre filters) samples of agricultural drainage water⁵⁹ (Table 3). At $0.1 \mu\text{g L}^{-1}$, similar recoveries were obtained for drainage water and tap water, except for DPC (24±30%) and DIA (35±14%). Increasing the load by a factor of 50 (from 0.1 μg to 5 μg) did not result in changes in the extraction efficiency, except for a significant improvement for DIA. Loading 50 μg led to excellent retention of all compounds, except DPC, for which similar results were obtained independently of the mass load (approx. 25%).

The optimal SPE procedure for large-volume samples is detailed in the Supplementary information (section 3).

CSIA methods

We determined the trueness, precision, reproducibility and amount-dependency of the CSIA methods for each target compound using EtAc solutions of standard of known isotope composition determined by EA/IRMS. Results are shown in the Supplementary information (Table S4, Fig. S3). Briefly, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the ATR, DEA, ACETO, METO and BAM working standards measured by GC/IRMS were reproducible over the whole period of analysis. Good precision (expressed as $\pm\sigma$) of ± 0.3 - 0.5‰ ($n=52$ - 94) for $\delta^{13}\text{C}$ and ± 0.1 - 0.8‰ for $\delta^{15}\text{N}$ analysis were achieved ($n=71$ - 84 for ATR, DEA, ACETO and METO, and $n= 11$ for BAM). For ATR, ACETO and METO

measurements, the trueness was within $\leq \pm 0.5\text{‰}$ for $\delta^{13}\text{C}$ and $\leq \pm 0.9\text{‰}$ for $\delta^{15}\text{N}$ and was thus within typical uncertainties of $\pm 0.5\text{‰}$ and $\pm 1\text{‰}$, respectively. DEA, on contrast, showed very reproducible offsets of $+2.0 \pm 0.3\text{‰}$ for $\delta^{13}\text{C}$ and $+1.1 \pm 0.1\text{‰}$ for $\delta^{15}\text{N}$ analysis compared to the reference EA/IRMS values. BAM isotope analysis also showed a reproducible offset for $\delta^{13}\text{C}$ ($-1.6 \pm 0.5\text{‰}$), whereas consistent $\delta^{15}\text{N}$ values were obtained ($\Delta\delta^{15}\text{N} = +0.3 \pm 0.8\text{‰}$). The occurrence of these offsets in GC/IRMS usually result from incomplete combustion of the target analytes. Since these deviations are reproducible, true analysis can be nevertheless be achieved by bracketing samples with external compound-specific standards and subsequent offset correction. The LC/IRMS method for $\delta^{13}\text{C}$ analysis of DPC showed excellent reproducibility over the whole period of analysis ($\pm 0.4\text{‰}$, $n=74$), but with an offset of $+3.2\text{‰}$ compared to the reference EA/IRMS value. This offset is likely related to incomplete wet oxidation of DPC.³⁵ Offset correction is therefore also required for true analyses. Regarding the derivatization-GC/IRMS method for determining $\delta^{15}\text{N}$ values of DPC, good precision was achieved ($\pm 0.4\text{‰}$, $n=12$), with an offset of -1.6‰ compared to the reference EA/IRMS value.³⁵ For direct injection of M-DPC, good precision was also achieved ($\pm 0.4\text{‰}$, $n=46$), with the offset of -1.6‰ compared to the reference EA/IRMS value.

The performance of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements was also assessed as a function of injected concentration (Fig. S4 and Fig. S5, Supplementary information) and limits of precise isotope analysis ($\text{Limits}_{\text{instrument}}$) were then derived according to the moving mean procedure.⁶¹ For $\delta^{13}\text{C}$ analysis by GC/IRMS for ATR, ACETO, METO and DEA, instrumental limits of 0.15 to 0.20 mM (corresponding to 1.2-2.8 nmol C) were obtained. For BAM, a higher $\text{Limit}_{\text{instrument}}$ of 1.0 mM (7.1 nmol C) was obtained. Similarly, $\text{Limits}_{\text{instrument}}$ for $\delta^{15}\text{N}$ ranged between 0.10 and 0.25 mM (0.7-6.3 nmol N) for ATR, ACETO, METO and DEA, whereas for BAM the limit amounted to 0.50 mM (2.5 nmol N). No amount dependency of the precision was observed between 0.15 and 4.1 mM for $\delta^{13}\text{C}$ and from 0.10 to 7.5 mM for $\delta^{15}\text{N}$ for ATR, ACETO, METO and DEA. The linearity ranges for BAM were 1.0-4.1 mM and 0.5-4.0 mM, respectively. The determined $\text{Limit}_{\text{instrument}}$ for LC/IRMS analysis of DPC was 0.14 mM (27.5 nmol C for an injection volume of 50 μL), and the linearity range was 27.5-133 nmol C. For $\delta^{15}\text{N}$ of DPC, $\text{Limit}_{\text{instrument}}$ was 0.69 mM (corresponding to 2.1 nmol N), and the linearity range was 0.69-6.9 mM.³⁵ Obtained values are summarized in the Supplementary information (Table S5).

Validation of the SPE-CSIA procedure for the determination of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the target compounds in water samples

For evaluating the effect of the SPE extraction procedure on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, standards of the target compounds of known isotope ratios were spiked into 10 L agricultural drainage water samples to give concentrations in the range of 0.5-50 $\mu\text{g L}^{-1}$. Extraction was performed using the cartridges containing 8 g of SDB-1 and 8 g of Septra ZT. Results are shown in Fig. 3 and Fig. 4 and listed in the Supplementary information (Table S6). In most cases, both trueness and precision were comparable between SPE extracts and freshly prepared standards. The SPE-CSIA method therefore induced negligible isotope fractionation, which was within

the uncertainty of analysis. The deviation from the EA/IRMS values of the $\delta^{13}\text{C}$ values for DEA and DPC and $\delta^{15}\text{N}$ values for DPC was almost identical to the offset from the reference value measured without SPE, indicating that this deviation did not originate from the enrichment procedure. It is worth noting that precise $\delta^{13}\text{C}$ values for DPC were obtained despite relatively low extraction efficiencies. In general, for GC/IRMS measurements of the spiked samples, proper chromatographic separation of the target compounds was achieved. Contrary to GC/IRMS, LC/IRMS measurements are more susceptible to interferences that could compromise the accuracy of isotope analysis produced by the concomitant enrichment of organic matrix. The chromatographic resolution was lower and thus there was a higher probability that analytes overlap with matrix compounds. The agricultural drainage water used in this work comes from lysimeters filled with 2.5 m arable soils, containing up to 2% organic matter, mostly in the first 70 cm.⁵⁹ Dissolved organic carbon (DOC) content in the water samples, measured by organic-matter combustion, ranged between 1.1 and 2.4 mg C L⁻¹. Although no attempts were made to characterize organic compounds of the drainage water, a high content of fulvic acids is expected.⁶³ A previous study using the same hydrophobic HC-PS-DVB sorbent with ultra-high surface area (Bakerbond SDB-1 cartridges, with a surface area of 1060 m²g⁻¹) demonstrated that fulvic acids are not retained at neutral pH and thus no co-retention/co-elution with the target pesticides occurred.⁵² Similarly, in our study, the DOC did not interfere with CSIA analysis. Precise $\delta^{13}\text{C}$ -DPC values were obtained even at concentrations below the instrumental limit of 27.5 nmol C on-column. Examples of resulting GC/IRMS and LC/IRMS chromatograms for both standards and spiked drainage water samples are shown in the Supplementary information (Fig. S6).

SPE-CSIA method limits for precise isotope analyses in water samples

The minimum concentrations of the target analytes in water samples necessary for precise and true isotope analysis (Limits_{method}) were calculated following Eq. (2). For calculations, extraction recoveries of 25% for DPC and 95% for the other compounds (Table 3) were applied. A reconstitution volume of 80 μL was assumed, except for LC/IRMS analysis, for which 150 μL was used. The obtained Limit_{method} for ATR, ACETO, METO and DEA ranged from 0.3 to 0.5 $\mu\text{g L}^{-1}$ for $\delta^{13}\text{C}$ and from 0.2 to 0.4 $\mu\text{g L}^{-1}$ for $\delta^{15}\text{N}$ values (Table S5, Supplementary information). Higher limits were obtained for BAM (1.6 and 0.8 $\mu\text{g L}^{-1}$, respectively) and DPC (1.2 and 3.2 $\mu\text{g L}^{-1}$, respectively). Consistent with these calculations, in the validation tests, accurate $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (i.e. deviation from bracketing standards and precision within typical uncertainties of $\pm 0.5\%$ and $\pm 1\%$, respectively) were obtained for ATR, ACETO and METO at concentrations as low as 0.5 $\mu\text{g L}^{-1}$. For DEA, the SPE-CSIA method was also validated for water samples at concentrations as low as 0.5 $\mu\text{g L}^{-1}$ for $\delta^{13}\text{C}$, whereas higher concentrations (2.5 $\mu\text{g L}^{-1}$) were required for accurate measurements of $\delta^{15}\text{N}$. True results (meaning that the deviation from bracketing standards was within $\pm 1.0\%$) were obtained for $\delta^{15}\text{N}$ of BAM, although the method was only tested with environmental water samples at concentrations much above the Limit_{method} (4 to 10 $\mu\text{g L}^{-1}$). For $\delta^{13}\text{C}$, however, deviation from bracketing standards (up to +2.2%) was higher than the typical uncertainty of $\pm 0.5\%$.

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Nevertheless, the two samples investigated at 0.5 and 1 $\mu\text{g L}^{-1}$, at concentrations below Limit_{method}, resulted still in true results (deviation from bracketing standards within $\pm 0.7\%$).

The accuracy of $\delta^{15}\text{N}$ analysis of DPC was also validated at concentrations as low as 1.0 $\mu\text{g L}^{-1}$. For $\delta^{13}\text{C}$ -DPC for few spiked samples at concentrations close to the calculated method limit, the accuracy of LC/IRMS measurements was compromised since lower signal (m/z 44) sizes than expected were obtained and/or the concomitant enrichment of organic matrix led to interferences. Nevertheless, as stated above, when amplitudes were higher than those corresponding to the Limit_{instrument}, accurate $\delta^{13}\text{C}$ -DPC values were achieved.

Conclusions

With this study, we validate a SPE-CSIA method for analyzing carbon and nitrogen isotope ratios of mixtures of herbicides and metabolites that commonly occur together in environmental water samples. Isotope ratios at analyte concentrations in the sub- $\mu\text{g L}^{-1}$ -range can be measured accurately with substantial pre-concentration of up to 200,000-fold. For the optimized SPE method, a hydrophobic hypercrosslinked sorbent with ultra-high surface area (SDB-1) and a polymeric sorbent chemically modified introducing polar functional groups (Septra ZT) were used to obtain an appropriate balance between selectivity and the ability to retain as many analytes as possible, covering a broad polarity range. SPE followed by CSIA enables carbon and nitrogen isotope measurements of a mixture of herbicides and metabolites with a wide range of polarity (K_{ow} from -0.4 to 3.1) in agricultural drainage water. We developed the SPE-CSIA method for compounds that frequently occur in groundwater. For common unconsolidated sand and/or gravel aquifer, the DOC concentration in groundwater tends to decrease rapidly as function of depth to the water table⁶⁴, thus limiting matrix effect. Our lysimeter study⁵⁹ with a rather shallow layer (1.5 m) between the organic carbon-rich top soil and the water sampling point represents a rather unfavorable case. Thus, we expect that our method is widely applicable for groundwater. For water samples containing higher DOC contents than in our study (e.g. river water, groundwater from aquifers with organic deposits, waste water treatment plant influent or effluent), additional clean-up steps may be required to reduce the effect of the organic matrix and thus obtain reliable isotope measurements. Further extracts clean-up can for example be performed with preparative HPLC, a technique previously used for various pesticides.^{30,65,66} Selective clean-up by molecularly-imprinted polymers has also recently been proposed for CSIA of enriched organic micropollutants.⁶⁷ The described SPE-CSIA method can also be extended to other elements, such as hydrogen, given that analytical methods have recently been validated for $^2\text{H}/^1\text{H}$ analysis of some pesticides.^{18,68,69} Nevertheless, a higher limit for precise isotope analysis is expected for hydrogen than for carbon, since larger amounts of hydrogen usually need to be injected.³⁷

The method proposed in this study is an important step towards analyzing isotope ratios of pesticide mixtures in aquatic systems. Isotope fractionation during pesticide degradation has been proven at lab scale¹⁶, demonstrating the potential of CSIA for tracing transformation of these contaminants in the environment. Our

study demonstrates that, despite the analytical challenges, multi-element CSIA of wide-polarity mixtures of pesticides at environmentally relevant concentrations is viable. This method will thus enable the application of CSIA to field sites for tracking pesticide sources and transformation processes.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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ELECTRONIC SUPPLEMENTARY INFORMATION

Further details on chemicals and reagents, analytical methods, optimization of the extraction methods, CSIA methods and validation of the SPE-CSIA procedure.

AUTHOR INFORMATION

Corresponding Author

* Phone: +34 934 033 773; e-mail: clara.torrento@gmail.com

Present Addresses

† Grup MAiMA, Departament de Mineralogia, Petrologia i Geologia Aplicada, Facultat de Ciències de la Terra, Universitat de Barcelona (UB), C/ Martí i Franquès s/n, 08028, Barcelona, Spain.

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Table 1. Log K_{ow} values of the target compounds, sorbent characteristics and average extraction recoveries (%) for sorbent screening using the six selected sorbents. Sorbent specific surface area increases from left column to right column. The average extraction recoveries were obtained from replicated experiments (n= 6 or 11, except for SDB-L, with n= 2). 20-mL of distilled water spiked to 12.5 $\mu\text{g L}^{-1}$ of each pesticide (25 $\mu\text{g L}^{-1}$ for DPC) were extracted. The relative standard deviations (RSD %) are shown in parenthesis. na= for some of the tests, the metabolites M-DPC, DIA and DEA were not added because the standards were not available when the tests were performed.

		ENVI-Carb	Strata SDB-L	Sepra ZT	Oasis HLB	Bakerbond SDB-1	LiChrolut EN
Type of sorbent phase		graphitized carbon-based	styrene-divinylbenzene (ST-DVB)	polystyrene-divinylbenzene (PS-DVB), hydrophilic		hypercrosslinked polystyrene-divinylbenzene (HC-PS-DVB)	
Sorbent area (i.e. amount \times specific surface area)		60 m ² (0.5 g, 120 m ² g ⁻¹)	250 m ² (0.5 g, 500 m ² g ⁻¹)	152-166 m ² (0.2 g, 760-830 m ² g ⁻¹)		183-240 m ² (0.2 g, 915-1200 m ² g ⁻¹)	
	log K_{ow}	n=6	n=2	n=6	n=6	n=11	n=11
DPC	-0.40	0	0	9 (43)	5 (26)	99 (16)	90 (26)
M-DPC	-0.30	0	54 (18)	90 (6)	57 (3)	77 (14)	na
DIA	1.15	na	na	106 (7)	na	103 (10)	103 (8)
DEA	1.51	na	na	103 (11)	na	99 (9)	98 (15)
BAM	0.77	67 (11)	52 (28)	90 (13)	73 (5)	85 (16)	93 (8)
CLZ	1.14	0	0	89 (5)	76 (3)	84 (9)	83 (6)
ATR	2.61	47 (55)	16 (28)	95 (17)	18 (7)	95 (7)	94 (10)
ACETO	3.03	84 (6)	42 (33)	95 (12)	69 (6)	100 (8)	98 (14)
METO	3.13	94 (9)	57 (18)	102 (14)	73 (5)	105 (7)	85 (16)

Table 2. Breakthrough effect. Mean recoveries (%) and relative standard deviation (RSDs) are shown, obtained on loading different sample volumes of distilled water spiked with 0.5 µg with each analyte (1 µg for DPC) on 0.2 g-cartridges of Septra ZT, SDB-1 or LiChrolut EN, and on layered columns containing 0.2 g Septra ZT and 0.2 g SDB-1. Tests were performed in duplicate (n=2), except for 100 mL with LiChrolut EN, for which only one test was done. na= for some of the tests, M-DPC was not added because the standard was not available when the tests were performed.

		Septra ZT			Bakerbond SDB-1		LiChrolut EN		Septra ZT + SDB-1	
		20 mL (n=2)	100 mL (n=2)	500 mL (n=2)	20 mL (n=2)	100 mL (n=2)	20 mL (n=2)	100 mL (n=1)	20 mL (n=2)	100 mL (n=2)
	log K _{OW}	Recovery [%] (RSD)			Recovery [%] (RSD)		Recovery [%] (RSD)		Recovery [%] (RSD)	
DPC	-0.40	19 (10)	0	0	110 (3)	110 (5)	111 (2)	102	91 (1)	62 (18)
M-DPC	-0.30	65 (3)	56 (8)	20 (8)	65 (3)	na	na	na	93 (5)	80 (12)
DIA	1.15	111 (4)	69 (7)	77 (6)	92 (13)	101 (12)	100 (3)	110	85 (3)	82 (3)
DEA	1.51	106 (4)	70 (7)	72 (16)	96 (6)	94 (16)	92 (6)	85	98 (1)	96 (14)
BAM	0.77	82 (5)	92 (9)	87 (9)	87 (1)	80 (4)	100 (4)	90	100 (1)	96 (5)
CLZ	1.14	91 (2)	90 (10)	89 (12)	75 (2)	76 (4)	81 (7)	85	94 (1)	88 (15)
ATR	2.61	100 (8)	60 (12)	65 (17)	90 (1)	92 (1)	96 (2)	81	89 (7)	94 (5)
ACETO	3.03	61 (13)	44 (6)	36 (9)	38 (15)	18 (3)	99 (9)	34	89 (8)	101 (8)
METO	3.13	83 (9)	51 (6)	45 (6)	56 (3)	39 (6)	82 (14)	81	83 (3)	107 (5)

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Table 3. Scale-up of SPE procedure to large sample volumes. Mean recoveries (%) and RSDs (in parenthesis) obtained on loading 5 or 10 L of tap or agricultural drainage water spiked with 0.5 to 500 µg of each analyte on cartridges containing 5 or 10 g of Septra ZT, 5 or 10 g of SDB-1 or two layers of 8 g each sorbent. Replicated tests were performed (n=2 to 9), except for 10 L drainage water at 5 µg L⁻¹ with the layered cartridge, for which unfortunately only one sample was available. na= for some of the tests, M-DPC was not added because the standard was not available when the tests were performed.

Extraction material	Septra ZT (5 g)	Septra ZT (10 g)		Bakerbond SDB-1 (10 g)		Septra ZT (8 g) + SDB-1 (8 g)					
Water source	tap water	tap water		tap water		tap water		drainage water			
Volume	5 L	5 L	10 L	5 L	10 L	5 L	10 L	10 L	10 L	10 L	10 L
Conc. (µg L ⁻¹)	0.1 (n=9)	0.1 (n=7)	0.1 (n=2)	0.1 (n=2)	0.1 (n=2)	0.1 (n=2)	0.1 (n=2)	0.1 (n=2)	1.0 (n=2)	5.0 (n=1)	50 (n=2)
	Recovery [%] (RSD)										
DPC	0	3 (126)	0	46 (28)	33 (20)	76 (17)	53 (12)	24 (30)	46 (15)	23	24 (25)
M-DPC	35 (10)	92 (19)	95 (25)	100 (10)	92 (2)	90 (3)	100 (4)	na	na	na	na
DIA	96 (15)	96 (13)	89 (4)	68 (7)	79 (3)	104 (9)	97 (10)	35 (14)	30 (13)	28	108 (2)
DEA	81 (37)	99 (11)	104 (6)	71 (8)	93 (21)	92 (9)	109 (16)	105 (7)	98 (17)	97	108 (3)
BAM	81 (49)	107 (9)	100 (5)	83 (9)	89 (5)	107 (14)	90 (22)	111 (3)	104 (10)	114	90 (12)
CLZ	101 (28)	102 (13)	113 (3)	101 (2)	97 (7)	91 (10)	97 (19)	92 (11)	74 (29)	80	109 (6)
ATR	103 (3)	108 (5)	112 (5)	94 (4)	101 (5)	109 (16)	93 (21)	95 (10)	100 (10)	109	113 (4)
ACETO	19 (20)	96 (12)	101 (14)	96 (9)	91 (25)	95 (8)	75 (14)	110 (4)	110 (17)	113	104 (4)
METO	36 (14)	95 (4)	92 (1)	87 (8)	94 (12)	100 (17)	97 (17)	99 (14)	112 (5)	113	110 (1)

Figure captions

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Figure 1. Elution procedure optimization. Extraction efficiencies (mean recoveries, %) for the three different elution procedures using Septra ZT and Bakerbond SDB-1 0.2g-cartridges for 20-mL distilled water samples spiked to 12.5 $\mu\text{g L}^{-1}$ each analyte (25 $\mu\text{g L}^{-1}$ for DPC). Error bars show RSDs (n=3). Herbicides are shown in the upper panel and metabolites, in the lower panel.

Figure 2. Mass load effect. Extraction efficiencies (mean recoveries, %) of Septra ZT 0.2g-cartridges for 20-mL distilled water samples spiked with 0.02 to 0.5 μg each analyte. Cartridges were eluted with 3 mL EtAc. Error bars show RSDs (n=3).

Figure 3. Validation of the SPE-CSIA procedure for the determination of carbon isotope ratios of the target compounds in 10-L drainage water samples (blue circles) spiked with 0.5 to 5 $\mu\text{g L}^{-1}$ for ATR, ACETO and METO, 0.5 to 10 $\mu\text{g L}^{-1}$ for DEA and BAM, and 0.5 to 50 $\mu\text{g L}^{-1}$ for DPC. Results of the amount-dependence tests (red circles) and EA/IRMS analysis of the standards (green lines) are also shown. The error bars indicate the standard deviations of quadruplicate measurements. The dashed lines represent the interval of $\delta^{13}\text{C}$ measured by EA/IRMS $\pm 0.5\%$. The molecular structure of the target analytes is also shown. For DPC, amplitude and not analyte concentration is shown because different injection volumes (10 to 100 μL) were used and thus there is not a straightforward correlation between injected C mass and DPC concentration.

Figure 4. Validation of the SPE-CSIA procedure for the determination of nitrogen isotope ratios of the target compounds in 10-L drainage water samples (blue circles) spiked with 0.5 to 5 $\mu\text{g L}^{-1}$ for ATR, ACETO and METO, 1.0 to 10 $\mu\text{g L}^{-1}$ for DEA and BAM, and 1.0 to 20 $\mu\text{g L}^{-1}$ for DPC. Results of the amount-dependence tests (red circles) and EA/IRMS analysis of the standards (green lines) are also shown. The error bars indicate the standard deviations of quadruplicate measurements (triplicate for DPC). The dashed lines represent the interval of $\delta^{15}\text{N}$ measured by EA/IRMS $\pm 1.0\%$. The molecular structure of the target analytes is also shown.

Figure 1

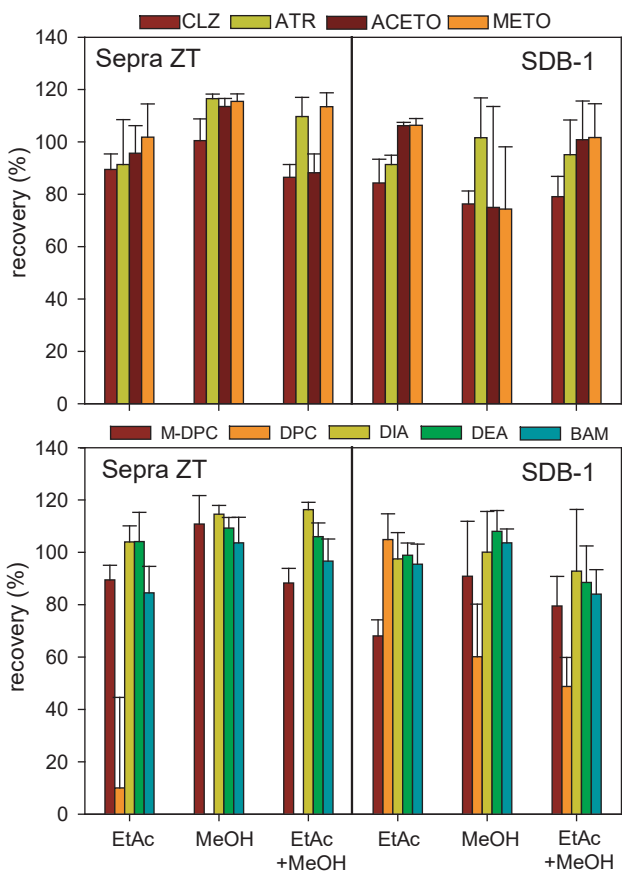


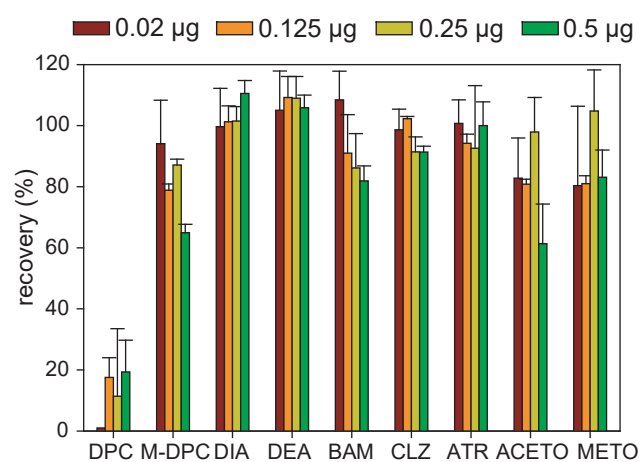
Figure 2

Figure 3

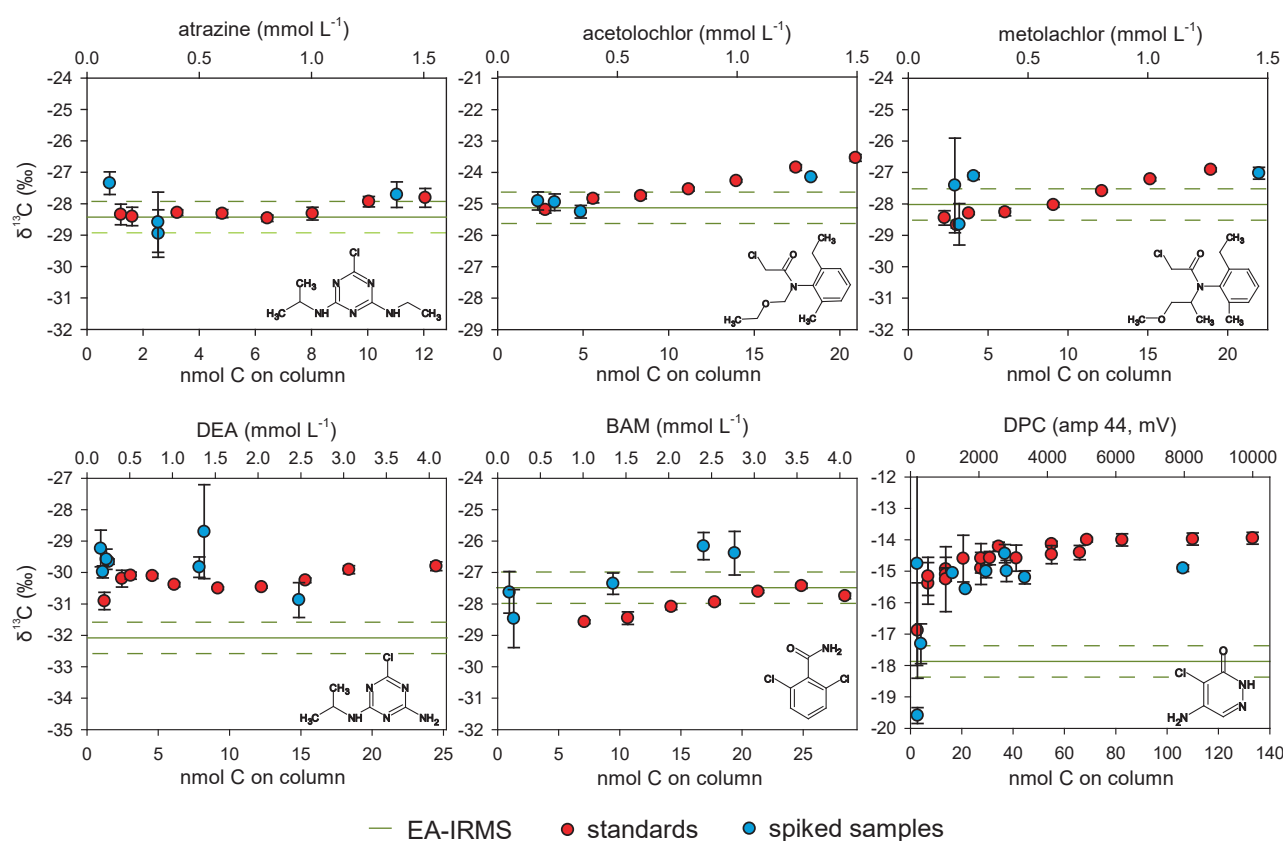
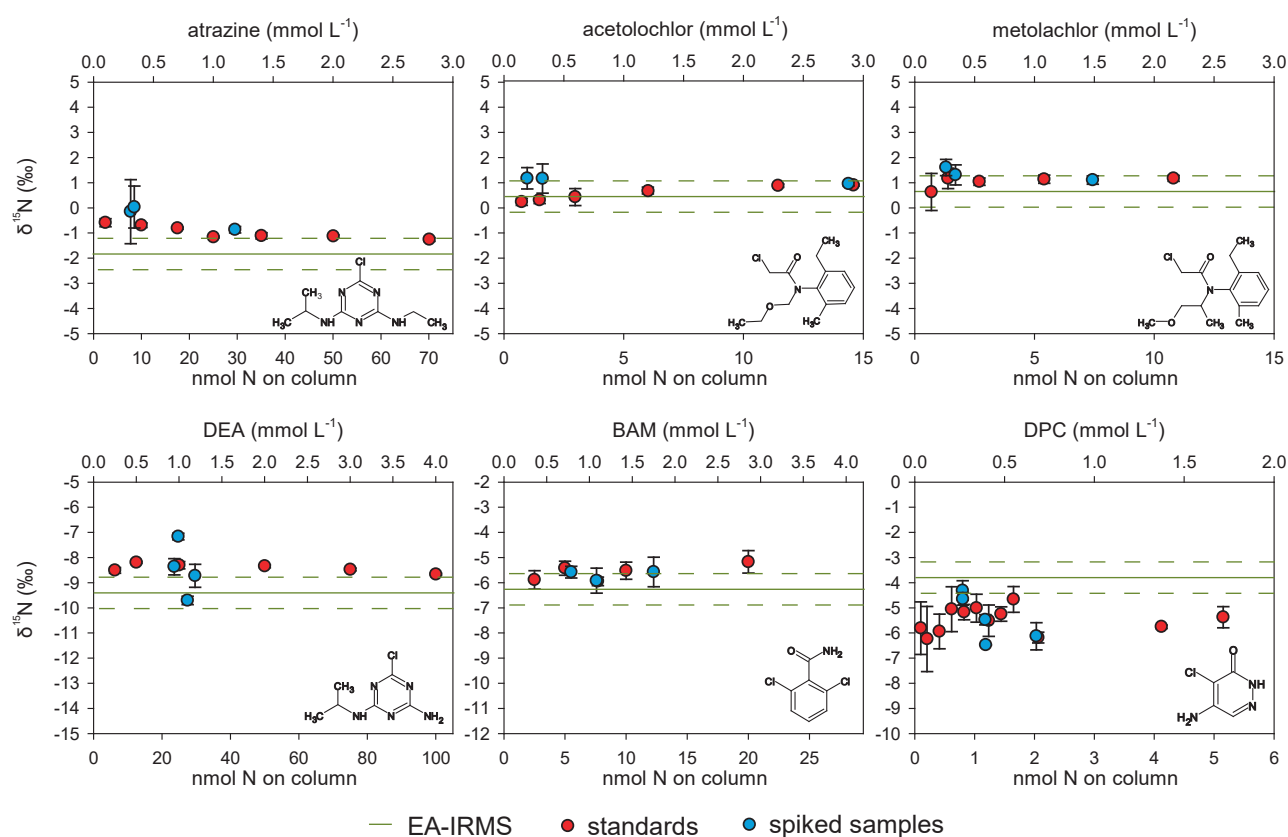
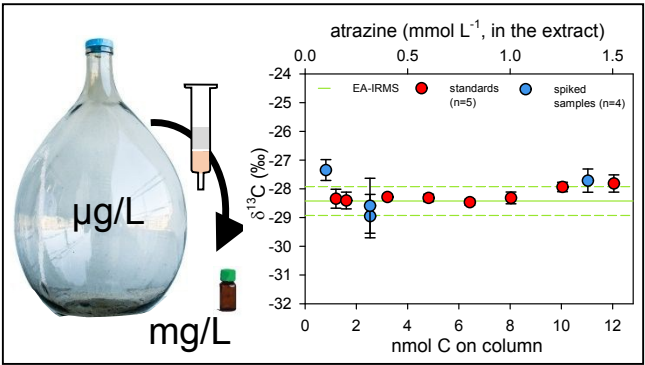


Figure 4



Graphical abstract

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We validated a SPE-CSIA method that allows analyzing isotope ratios of mixtures of pesticides that commonly occur together in environmental water samples