Microfluidic water-assisted trap focusing method for ultra-large volume injection in reversed-phase nano-liquid chromatography coupled to electron ionization tandem-mass spectrometry

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Abstract

Nano-LC gradient elution is the separation method of choice in many emerging LC-MS applications. This success is due to the synergistic effect of nano-flow rates and ionization efficiency. However, the advantages obtained from a higher solute concentration are limited by the small injection volumes typical of nano-LC. Solvent-based solute focusing of large sample volumes in a short trap column, operating in switching mode, can overcome this constraint. Herein this research study we present an efficient, column-switching method that relies on a custom-made T-union passive diffusion micromixer to assist water dilution and promote trap solute focusing. Five pesticides in different media were used as model compounds and the analyses were carried out with a triple quadrupole mass spectrometer equipped with a Liquid Electron Ionization (LEI) LC-MS interface in MRM mode and full scan. The system microfluidics were investigated using COMSOL modeling software. This method allows injecting 20 □L (or volumes greater than) of sample volume in an organic solvent onto a nano-LC column, speeding up the analysis time and, improving 400-fold the limits of quantitation for selected compounds. Reproducible results in terms of peak area, peak shape, and retention times were achieved, regardless the matrix composition. Matrix effects values in soil extracts spanned between 95.2% and 101.5%. Repeatability test on peak area variations were lower than 10%.

Key words
Ultra-high-volume injection; Nano-LC; Liquid electron ionization (LEI)-tandem mass spectrometry; Water-assisted trap focusing; Pesticides; Microfluidics.

1. Introduction

One of the key challenges in many analytical procedures is to lower limits of detection (LOD) and quantification (LOQ), enabling analyte detection at an ultra-trace level. Liquid chromatography-mass spectrometry (LC-MS) has become the preferred analytical technique for many such challenging applications thanks to selectivity, sensitivity, and wide range of LC-amenable substances of different polarity [1]. Despite the advantages of this technique, the presence of very low concentrated contaminants in environmental, forensic, food, and other applications requires to include efficient strategies to boost LC-MS sensitivity [2,3]. This increase can be achieved using different approaches such as instrument implementation [4], enrichment steps [5,6], or by increasing the volume of sample injected in the chromatographic column [7,8]. One of the essential parts of the LC-MS analytical workflow is sample pretreatment, especially when analyzing complex samples containing target analytes at low concentrations [9]. Offline or online solid-phase extraction (SPE) are widely used as preconcentration and pretreatment procedures, particularly for aqueous sample [10-12]. A typical SPE protocol is time-consuming, requires a sample size of a few milliliters, and adequate apparatus maintenance to achieve reliable results and avoid sample loss. Moreover, SPE procedures often require additional steps of solvent evaporation and residue reconstitution in the mobile phase, which represent possible error sources [13]. Furthermore, the idea of chemical processes and procedures more attentive to reducing environmental impact has become of increasing importance, and all laboratory practices should follow guidelines and principles of "green analytical chemistry" (GAC) to couple performance and sustainability [14-16].

A "green" and well-recognized alternative to solvent evaporation is the direct injection of a large volume (LVI) of sample. This strategy enhances sensitivity when a volume of sample dissolved in a non-eluting solvent, exceeding the 10% of the column void volume, is injected onto the column [17,18]. The maximum sample volume injectable is linked to column dimensions, analyte, mobile and stationary phase polarities, and working chromatography flow rates. The simplest way to perform LVI is to inject a large volume of sample dissolved in a non-eluting solvent to avoid peak broadening (single column system) [19]. For instance, in reversed-phase liquid chromatography (RP-LC), where

water is a non-eluting solvent, the volume of injected sample can span from 250 to 1800 μL, depending on column diameter [20,21]. Another way to realize LVI is called column-switching (coupled columns, CC-LVI). It involves an online SPE preconcentration step in a short pre-column after LVI. The pre-column is coupled with an analytical column via a switching valve that allows operating in back-flushing mode to elute the trapped analytes [22,23]. In literature, several existing LVI applications are reported, including the determination of pesticides, herbicides, and fungicides in vegetables and water [24-26]. In the last decades, miniaturization of LC instrumentation has led to the development of micro- and nano-LC, improving sensitivity by reducing sample dilution inside the column. However, the excellent mass sensitivity of nano-LC is diminished by the severe injection volume limitation. Several strategies have been adopted to focus LVI in a narrow band at the column head and exploit increased sensitivity and peak capacity in scaled-down LC columns. Wilson et al. [27] developed an automated instrumental method based on temperature-assisted on-column solute focusing (TASF), demonstrating the focusing of LVI of small molecules and peptides on the head of capillary columns of 0.1 mm of internal diameter (i.d.) in gradient mode. However, for non-polar compounds, most of the conventional LVI methods used in RP-LC cannot be applied. Target compounds should be dissolved in a hydrophilic solvent to be focused on the column head, leading to considerable sample loss due to very low water solubility, adsorption on sample vials, or injection lines. Màrta et al., developed a new method to improve LOD for the determination of steroid hormones in plasma samples by sample dilution in water/organic solvent at different concentrations using LVI in micro-LC [28].

Microfluidic devices (mixers and micromixers, depending on their internal volume) have attracted increasing attention in several fields of application thanks to the possibility to mix different fluids very rapidly. Mixers are predominantly used to enable lab-on-chip devices or to implement microfluidic technologies in the LC systems, ensuring efficient solvents mixing in gradient formation in HPLC/UHPLC pumps. Mixers can be classified into two main categories: active and passive. Active mixers require power input, whereas passive mixers —also called static mixers— rely on the design of microchannels to enhance molecular diffusion and chaotic advection for efficient mixing [29,30]. Recently, Ianovska et al. realized a small volume microfluidic chaotic mixer for two-dimensional liquid chromatography. Their device can properly mix two different mobile phases for two different stationary phases in a multidimensional separation technique, improving sample focusing and chromatographic performance [31].

Mixers are potentially usable in LVI applications, enabling proper dilution mixing between a highly aqueous mobile phase and a fixed volume of a strong eluting solvent where the analytes are dissolved. Zhong et al. developed a pre-column dilution LVI for the UHPLC-MS/MS separation of pesticides

in cabbages at trace level using an active mixer before the chromatographic column [32]. The analytes dissolved in a strong eluting solvent were diluted in water inside the mixer before chromatographic separation, thus focusing at the column head (2 mm i.d.).

In this study, we present and discuss a microfluidic water-assisted trap focusing (M-WATF) method to increase sensitivity in RP nano-LC using a liquid electron ionization (LEI) MS/MS system [33,34]. LEI represents an efficient approach for addressing the difficult conversion of a liquid-phase into a gas-phase for EI, using a nano-scale flowrate, generating library searchable spectra. Different interfacing systems based on EI have been also proposed by Amirav's and Mondello's groups [35-38]. M-WATF was applied to the determination and quantification of selected pesticides in soil samples. In the M-WATF method, a fixed volume of sample (20 μL) dissolved in a high percentage of a strong eluting solvent was injected and focused on a nano-trap column (0.3 mm i.d.) before elution and chromatographic separation. This volume represents a 400-fold increase in respect to the typical sample injection volume applied in nano-LC. A dual strategy was employed to achieve this goal: evaluation of the microfluidic dynamics before, inside, and after the micromixer, and the use of nano-LC columns of 0.075 mm i.d. to avoid sensitivity losses.

2. Experimental section

2.2 Standard and sample preparation

Clomazone, paclobutrazol, fludioxonil, metolachlor and pirimiphos methyl standards (purity grade >99%) were provided by Syngenta Ltd. (Bracknell, UK). Stock solutions were prepared gravimetrically in acetonitrile (ACN) (LC-MS grade, Merck, Rome, Italy) at 400 μg/mL. The working solutions were prepared volumetrically as combined suites at the concentration of 10 ng/mL in 100% ACN, 50:50 H_2O:ACN (v:v), and 100% H_2O (LC-MS grade, Merck, Rome, Italy). Soil samples were provided by Syngenta Ltd. and treated as follows: 1 g of soil was extracted with 2 mL of a mixture ACN:H_2O 50:50 (v:v) and shaken for 30 min using IKA Trayster digital shaker (VWR PBI International, Milan, Italy) at 60 rpm. The soil was re-extracted twice with 2 mL of a mixture ACN:H_2O 80:20 (v:v) for 30 min, then acidified at pH 3 with formic acid (Sigma Aldrich, Milan, Italy) and shaken again for 30 min. The supernatant (6 mL) was passed through a 0.22 μm disposable filter (UNIPREP, Whatman, Clifton, NJ) and centrifuged at 5000 rpm for 5 min before evaporation to 1 mL under a nitrogen flow (N_2 Generator, Claind, Milan, Italy). Ten extracts (1 mL each) were used for method validation. Five of them were fortified at the following concentrations: 2; 10; 50; 100, and 250 ng/mL for real sample calibration experiments. The other five extracts were fortified at 10 ng/mL for M-WATF performance evaluation and repeatability tests.

2.3 M-WATF nano-LC apparatus

Figure 1 a-b illustrates the M-WATF nano-LC system and its functioning in a) trap loading; b) trap elution. In Figure 1 a, an Agilent Infinity II pump (Agilent Technologies, CA, USA), working at 20 µL/min, provided a water flow rate for the dilution of the sample dissolved in a strong eluting solvent inside a custom-made T-union passive diffusion micromixer, T2 (VICI AG International, Schenkon, Switzerland). This aqueous flow rate was first directed to an unmodified stainless-steel tee-union, called T1 (internal volume: 0.57 µL, VICI AG International, Schenkon, Switzerland), where it was split into two streams of water: (A) at 18 µL/min and (B) at 2 µL/min. Stream A is directed to T2, while stream B passed through a six-port valve (V1) (Rheodyne, Milan, Italy) collecting the sample into the loop before reaching T2. V1 was equipped with a certified 20 µL sample loop (Rehodyne, Milan, Italy). A micro-liquid flow meter (Sensirion SLG-0075, Staefa, Switzerland) was placed between T1 and V1 to monitor stream B in real time. Stream A and the sample carried by stream B mixed inside T2, re-establishing the flow rate at 20 µL/min directed into a second valve (V2) (Rheodyne, Milan, Italy) equipped with a nano-trap column (Zorbax SB-C18, 0.3 mm x 5 mm, 3.5 µm i.d. particle size, Agilent Technologies, Palo Alto, CA, USA). This is a crucial point of the system because only if the mixing of the two streams is efficient, will the sample be transported and focused in the trap with a high percentage of water. A second pump, a 3000 Ultimate RSLC nano pump (Thermo Scientific, Milan, Italy), working at 400 nL/min was used to elute the trap column in back-flushing mode and for the chromatographic separation, as shown in Figure 1 b. The separation was carried out on an Agilent SB-C18 column (0.075 mm i.d. x 150 mm x 3.5 µm i.d. particle size). The mobile phase consisted of 3:97 (v:v) ACN:water (A) and ACN (B) in linear gradient elution, from 0% B to 100% B in 10 minutes. Details of the entire system are fully discussed in the Results and Discussion section.

2.4 LEI MS/MS instrumentation

An Agilent 7010B triple quadrupole mass detector (Agilent Technologies, CA, USA) was equipped with a Liquid Electron Ionization (LEI) interface. Further, details of the mechanism and performance of the interface can be retrieved elsewhere [33,34]. The ion source was kept at 280 °C during LEI analyses, and the vaporization micro-channel temperature was set at 400 °C. Data acquisitions were carried out in full scan and multiple reaction monitoring (MRM) modes. Physicochemical properties, full scan acquisition parameters, precursor and product ions, and optimized collision energy for each

analyte are listed in Table S-1 and in Table S-2 of the Supporting Information. The ion source pressure was 150 mTorr. Nitrogen (99.9 %, Air Liquide, Milan, Italy) was used as collision gas at a flow rate of 1.5 mL/min when operating in MRM. Helium was used as quenching gas in the collision cell at 4 mL/min flow rate. The filament current was set at 60 μA to prevent filament damages and increase lifespan.

3. Results and discussion

Narrow chromatographic peak bands typically require small injection volumes, especially when the sample is dissolved in a strong eluting solvent [39]. Commonly, in gradient mode, the injection volume with a strong eluting solvent in nano-LC columns (0.075 mm i.d.) must be lower than 50 nL at 400 nL/min flow rate. Therefore, many real-world applications are not possible due to the difficulty in reaching the required LODs without sample preconcentration step. Contrary, if a sample can be diluted in a non-eluting solvent, then it is possible to inject a larger volume into the column. This methodology is penalized by the extended time required (several minutes, depending on the volume, LC flow rate) to transfer the sample from the loop to the column head.

As mentioned in the Introduction, column-switching has been used to rapidly and efficiently transfer a large volume of sample into a suitable trap column before releasing the analytes in back-flushing mode [22]. This solution is successful if used with analytical columns after a proper sample dilution into a weak eluting solvent. However, at micro- and nano-flow rates, the water dilution necessary for trap focusing is less efficient or absent, impaired by the mostly laminar properties of the solvents involved in the dilution process. In RP nano-LC, a suitable alternative to mix a strong eluting solvent with water is needed to avoid sample handling and speed up analysis time.

In the M-WATF system, the microfluidic processes have been studied in detail. In particular, the behavior of two laminar flows has been investigated, one coming from the sample (in ACN) and the other from the pump (100% H_2O, see Experimental section), inside and after the T2 micromixer and the connection between T2 and V2. Two different designs for T2 were considered and evaluated. These studies permitted the investigation of the ideal combination of the microfluidic components to accomplish the efficient mixing of the two solvents, promoting analytes focusing on the trap column.

3.1 Evaluation of T2 passive diffusion micromixer

3.1.1 Governing balance equations

In order to study the velocity and concentration fields obtained after T2 for ACN in water, the numerical solution of the transport phenomena equations [40], which express the conservation of mass, momentum, and chemical species transport under the hypothesis of isothermal, incompressible Newtonian fluids and laminar mixing flow, is obtained by using COMSOL® (COMSOL Multiphysics, Brescia, Italy) modeling software.

The governing balance equations are the following:

$$\begin{aligned} &\left(\nabla \cdot \vec{u}\right) = 0 \\ &\rho\left(\frac{\partial \vec{u}}{\partial t} + \vec{u} \cdot \nabla \vec{u}\right) = -\nabla p + \mu \nabla^2 \vec{u} \\ &\frac{\partial c_A}{\partial t} + \left(\vec{u} \cdot \nabla\right) c_A = D_{AB} \nabla^2 c_A \quad with\ c_A = \rho x_A \end{aligned} \tag{1}$$

Where u is the velocity vector, c_A, x_A and D_{AB} are the concentration, mass fraction, and binary diffusivity of the species A for system A–B, respectively, while p, □ □and □ are the pressure, density, and dynamic viscosity of the fluids, respectively.

In Table 1 the values assumed by the main thermophysical properties of the liquids considered in this work are shown.

By applying appropriate boundary conditions to the geometrical configuration of interest a unique solution can be obtained numerically.

In a more general solution, the balance equations have been written in dimensionless form:

$$\begin{aligned} &\left(\nabla^* \cdot \vec{u}\right) = 0 \\ &\left(\frac{\partial \vec{u}}{\partial t^*} + \vec{u} \cdot \nabla \vec{u}\right) = \frac{}{Dt^*} = -\nabla p + \frac{}{\mathrm{Re}} \nabla^2 \vec{u} \\ &\frac{\partial c_A^*}{\partial t^*} + \left(\vec{u} \cdot \nabla\right) c_A = \frac{Dc_A^*}{Dt^*} = \frac{1}{\mathrm{Re}\, Sc} \nabla^{*2} c_A^* \end{aligned} \tag{2}$$

where a physical meaning can be assigned to the dimensionless parameters which appear in the equations. More in detail, the Reynolds number (Re) expresses the ratio between the inertia forces and the viscous forces. In this case, by considering the volumetric flow rate imposed at the inlet of the T2, Re at the exit of the micromixer assumes low values (Re=0.45-0.48), which means that in this case, viscous forces dominate on the inertia ones. In the meantime, the Schmidt number (Sc), defined as the ratio between momentum and mass diffusivity, assumes a value of 210 for ACN. Under the investigated conditions, a trade-off is obtained between the low values of Re and the high values

assumed by Sc for which it becomes mandatory to study numerically the minimum distance from the outlet of the T2 in correspondence of which a perfect mixing of the two liquids is expected. The numerical solution of Equations 2 can give a complete description of the variation of the mixing index as a function of the axial distance from T2 outlet towards V2.

The solutions for governing equations together with their boundary conditions were carried out by resorting to the commercial software COMSOL Multiphysics™ 5.2a., with a relative tolerance of 1e-06.

3.1.2 T2 passive diffusion micromixer volume and connection

At such low Re values, turbulent mixing does not occur, and diffusive species mixing plays an important role. Therefore, the choice of internal volume and exit capillary diameter connected to T2 was crucial for achieving a sufficient solvent mixing to focus analytes on the trap column [30]. Three prototypes were tested to evaluate the mixing features of T2 during the short travel from the exit of the union (0 mm) inside the first mm of the capillary. Because of the laminar nature of the flow, no further mixing is expected along the capillary. The first prototype was an unmodified stainless-steel T-union with 0.57 µL internal volume and 175 µm i.d. exit capillary; the second and the third ones were custom-made PEEK T-unions with internal volumes of 2.2 µL, and 175 µm i.d. and 20 µm i.d. exit capillaries, respectively. The comparison between the different prototypes was carried out by considering the average mass concentration of ACN exiting T2. In Figure 2, it can be observed that the unions with 0.57 and 2.2 µL internal volumes and 175 µm i.d. exit capillaries show similar results (12.3 and 12.1%, respectively) at 1 mm from T2 exit. It was noticed an increase of the contact area between water and ACN only when working with the union with 2.2 µL internal volume and 20 µm exiting capillary, enhancing the mixing effect by diffusion [41] and further decreasing the organic solvent percentage to 7%. Therefore, this custom-made union was selected to conduct the experiments. Diameters smaller than 20 µm were not used because they generate a very high backpressure.

3.1.3 M-WATF nano-LC system operation and method optimization

The sequence of system operation has been described in the Experimental Section 2.3 and illustrated in Figure 1. Before *trap loading*, the 20 µL loop is filled with the sample dissolved in ACN or various percentages of water-ACN. In *trap loading*, V1 is switched, and the sample is driven to T2 at 2 µL/min (stream B). Due to laminar regimes, this flow rate should be low enough to promote mixing

by diffusion in T2 with the 18 µL/min of 100% water (stream A) reaching the expected reduction of ACN concentration of 10% at the exit of T2. Two different T2 configurations were considered: the first one (red dots) has an internal volume of 0.57 µL and an exit capillary of 175 µm i.d.; the second one (blue dots) has an internal volume of 2.2 µL an exit capillary of 20 µm i.d (Figure 3). As it can be seen in the Figure, the red-dotted plot tends to reach the expected percentage (10%) as the distance from the mixer increases, but after 1 mm the percentage is still higher (>12%). Contrary, the blue-dotted line indicates the immediate dilution of the sample at the exit of T2.

Finally, streams A and B after mixing inside T2, carried the sample at 20 µL/min into the trap in a highly aqueous environment for an optimized sample focusing.

The total sample transfer time is approximately 10 min using a 20 µL loop. In *trap elution* V2 is switched, and the trap is back-flushed by the nano-LC pump. Then, the eluted analytes are delivered to the nano-LC column for chromatographic separation. The MRM profiles of the experiments are reported in Figure 4.The five-compound mixture (10 ng/mL) was dissolved in 50:50 H_2O:ACN (v:v) (b) and 100% ACN (c), and the results were compared with the same mixture dissolved in 100% H_2O (a). The profiles show similar peak shapes, and the average area values comparison (Table 2) spans from 77.6% and 102%. The area values differences within the five replicates spread between 2 and 8%. These results demonstrate a satisfying dilution in water and efficient trap focusing even in pure eluting solvent (100% ACN) with limited sample losses and excellent repeatability. It is relevant that the injected volume (20 µL) represents a 400-fold increase for a typical injection volume in nano-LC. It is also noticeable that the presence of a high concentration of ACN does not cause peak broadening or other modifications in the chromatographic profiles.

3.1.4 M-WATF nano-LC performance evaluation

The M-WATF performance was evaluated considering method LODs and LOQs, linearity, and matrix effects (ME). All measurements were conducted in triplicate. The results are reported in Table 3.

Linearity experiments were carried out using soil extracts, at the concentrations reported in the Experimental section, to calculate LODs and LOQs. All pesticides examined showed good linearity (R^2>0.995). High response for linearity is expected when using EI as the ionization mechanism, and this result is also confirmed in this case, thanks to the efficiency of the dilution step in T2 and the nano-LC column-switching approach. LODs and LOQs were calculated based on a signal-to-noise ratio 3:1, and 10:1, respectively considering the less intense MRM transitions. With a 20 µL injection, instrumental LODs spanned between 2 and 9 pg on-column, corresponding to 0.2-0.45 ng/g in soil

extracts. These results represent a significant sensitivity improvement due to the larger injection volume, with LODs and LOQs nearly 400-fold lower than those obtained with typical nano-LC injection volumes (~50 nL). Repeatability results indicate a stable response with a negligible influence of the matrix. The evaluation of MEs was conducted comparing the calibration curves of each pesticide in water and soil extracts. In ideal conditions, the total absence of MEs is observed when the two curves are overlapping. Figure S-1 shows the calibration curves obtained in 100% H_2O and soil extract for each selected compound. For this application, the overlapping ratio spans from 95.2% to 101.5% confirming the lack of MEs. These results are in agreement with other findings that indicate that LEI response is scarcely influenced by interfering coeluted compounds coming from the matrix compared to other LC-MS ionization techniques, in particular electrospray [29,30].

Studies of repeatability were carried out in post-extraction addition mode using soil matrix samples spiked with the five-pesticides mixture at 10 ng/mL. These experiments were aimed to evaluate the matrix impact on the system during routine analyses. It was essential to investigate the response of the system in real-world conditions to evaluate the impact of a complex matrix on the system and its effect on the analytes response. The soil extract was injected ten consecutive times into the system, and MRM profiles were recorded. The peak area values and RDS% are shown in Table S-3. As demonstrated, peak area variations were lower than 10%, which is considered a satisfactory result in real-world conditions.

4. Conclusions

A significant limitation of nano-LC is the very low injection volume, M-WATF is a new method to introduce an ultra-large volume of sample in a RP nano-LC-RP column. This large volume introduction leads to a noticeable sensitivity increase without affecting chromatographic resolution, using samples dissolved in solvent mixtures at varying percentages. A significant limitation of nano-LC is a very low injection volume. M-WATF contributes to widening the range of possible applications of nano-LC, especially when the concentrations of analytes are particularly low.

M-WAFT RP nano-LC enhances the results obtainable with the LEI-LC-MS, demonstrating negligible variations of signal response at different solvent percentages and absence of MEs, even in the presence of soil extracts as complex matrices. The design of M-WATF can be successfully utilized with any nano-LC-MS system, with other nano-scale analytical approaches whenever a significant increase in sensitivity is required.

Declaration of interest

The authors declare no conflicts of interest.

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Supplementary Material

Supplementary material related to this article can be found in SM_file.

Figure 1. M-WATF operation steps in sequence: a) trap loading; b) trap elution. A: 18 µL/min of 100% H2O; B: 2 µL/min of 100% H2O.

Figure 2. Average mass concentration of ACN obtained with different T2 prototypes in the first mm inside the exit capillary. Red trace: 0.57 µL - 175 µm i.d. exit capillary. Green trace: 2.2 µL-175 µm i.d..; Blue trace: 2.2 µL - 20 µm i.d.

Figure 3. Comparison of average mass concentration of ACN (%) obtained with different T2 configurations.

Figure 4. MRM profiles of a five-compound mix at 10 ng/mL dissolved in a) 100% H2O; b) 50:50 H2O:ACN (v:v); c) 100% ACN, respectively. 1) clomazone; 2) paclobutrazol; 3) fludioxonil; 4) s-metolachlor; 5) pirimiphos-methyl.

Table 1. Main properties of the working fluids.

	Water	***Acetonitrile***
Dynamic viscosity [mPa s] @ 25°C	0.89	0.347
Density [kg/m^3] @ 25°C	1000	786
Mass Diffusivity in water [m^2/s]	-	1.975 10^{-9}

Table 2. Comparison average of the peak areas values obtained from five-compound mix solutions in 100% H_2O:ACN(v:v), 50:50 H_2O:ACN (v:v), and 100% ACN.

Compound	***Area*** [a] ***At 100% H_2O***	***Area*** [a] ***at 50:50 H_2O:ACN (v:v)***	***Avg. area comparison (%)***	***Area*** [a] ***at 100% ACN***	***Avg. area comparison (%)***
1-clomazone	10107.0±123.5	8123.0±366.8	84.2	7844.9±171.8	77.6
2-paclobutrazol	6399.1±261.0	6001.3±120.5	94.7	5135.3±113.0	80.2
3-fludioxonil	12114.0±963.7	12562.0±909.5	102.3	10663.0±375.2	88.1
4-s-metolachlor	15051.1±717.4	12054.3±426.8	80.8	12511.7±503.1	83.0
5-pirimiphos-methyl	2282.7±96.7	1977.7±86.4	86.6	1987.2±84.5	90.8

[a] Average value of five replicates ± standard deviation

Table 3. Method validation data of the selected compounds in water and soil extracts.

Compound	***Method LODs (ng/mL)*** [(a)]	***Method LOQs (ng/mL)*** [(a)]	***Linearity range (ng/mL)***	***Equation (100% H_2O)***	***R^2***	***Equation (soil extracts)***	***R^2***	***Matrix effects evaluation*** [(b)]
clomazone	0.20	0.70	2-250	y=1044.4x-1277.4	0.9971	y=1060.4x-3627.9	0.9996	101.5 (+1.5%)
paclobutrazol	0.20	0.80	2-250	y=988.73x -5753.2	0.9955	y=958.46x-4821.6	0.9981	96.9 (-3.1%)
fludioxonil	0.10	0.25	2-250	y=1334.4x-4238.8	0.9988	y=1270.6x-3183.1	0.9998	95.2 (-4.8%)
s-metolachlor	0.25	0.60	2-250	y=1215.5x-3391.5	0.9983	y=1212.2x-1739.4	0.9938	99.7 (-0.3%)
pirimiphos-methyl	0.45	1.50	2-250	y=289.07x-333.69	0.9997	y=286.26x-219.89	0.9989	99.0 (-1.0%)

[(a)] LODs and LOQs were calculated in soil extracts

[(b)] soil extracts slope/water slope*100

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