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Studying the interaction between triazines and humic substances—A new approach using open tubular capillary eletrochromatography

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ABSTRACT

The strength of the interaction between a pesticide and the soil organic matter is a key parameter to assess the risk of it reaching to groundwater with potentially harmful effects to human health. In this work, a new approach that allows measuring such interactions in a few minutes using a purified fraction of the soil organic matter (humic substances) is detailed. The strength of sorption is assessed via the normalised difference of elution (retention factor, k') between the chemical of interest and a neutral marker transported via electroosmotic flow through an open tubular column supporting the immobilised humic substances (open tubular capillary electrochromatography). The immobilisation was achieved by incubating a capillary, pre-coated with a monolayer of humic acid, with an acidic solution of humic substances. This induces the formation of a supramolecular structure of humic substances as it occurs in soils. This aggregate can easily be removed using alkaline solutions, and a new structure assembled using other humic substances (HS) or different incubations conditions. The whole procedure takes 2 h. This approach has been tested using five triazines and three types of humic substances. The order of the strength of sorption of the triazines as expected from relevant literature and the relative standard deviation of k' was between 1 and 6%. Good repeatability was also observed after long period of wash, between re-coating and repeating of the full coating with a new capillary.

1. Introduction

Pesticides, fungicides and other phytosanitary products allow significantly to reduce the crop lost to pest [1]. They have been key products in the farming revolution [2]. However, numerous studies highlight the serious risks to health posed by such chemicals [3,4]. This especially concerns for the hydrophobic organic compounds (HOCs) because of their tendency to bio-accumulate [3]. The noxiousness of a spread chemical toward human life does not only depend on its toxicity but also, and more importantly, on its bioavailability. A pesticide that is strongly bound to the soil matrix will not leach to groundwater, independently of its toxicity, and will not pose a serious threat to human health.

Whereas it is well established that the soil organic matter (SOM) is the main sorbent of hydrophobic pesticides [5], their main sorption mechanisms are not fully understood. This is principally due to the complexity of the SOM [6], that is believed to be a supramolecular structure (SmS) made of relatively small molecules hold together via weak forces [7–10]. Despite recent breakthroughs, there is still no full agreement on their probable structure and composition. However, as with any supramolecular entities, the architecture of the SOM aggregate is as important as its chemical composition for sorption of pesticides [11].

To help in understanding the behaviour of SOM, numerous analytical approaches have been developed in recent years. They aim to measure the interactions between pesticides and purified fractions of the SOM (usually the humic acid (HA) and fulvic acid (FA) fractions but can also be other humic substances (HS) [12]) in a fast and accurate way. Those techniques include fluorescence quenching [13], ultracentrifugation, dialysis equilibrium [14], or affinity capillary electrophoresis [15] to name a few. However, all of those methods measure the interactions using HS in dissolved state, thus the HS can adopt structures that are different from the ones possible in solid state (formation of micelles for example). To work with HS in solid form, interactions are measured by batch experiments where the solid is in contact with an aqueous solution containing the chemical of interest in conditions where the precipitate remains stable (acidic pH) [16]. Whereas this approach is satisfactory, it requires a long incubation time (up to 24 h) as well as purification and separation steps to measure the concentration of free chemicals. Moreover the studies are limited to acidic medium with the only fractions that are less soluble. An interesting alternative has been proposed by André et al. [17,18]. HS were covalently linked to silica gel and used as a stationary phase in reversed phase

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Table 1
Elemental composition of the humic substances used in this work expressed as weight percent; O/C and H/C ratios are atomic percent.

Name	C	H	O	N	S	P	O/C	H/C
flu-HA[a]	65.79	5.51	37.79	0.71	3.16	<0.05	0.43	1.01
IHSS-HA[b]	58.13	3.68	34.08	4.14	0.44	0.24	0.44	0.76
IHSS-FA[b]	50.12	4.28	42.61	3.75	0.89	0.12	0.64	1.02

[a] Data obtained from [19].
[b] Data obtained from the IHSS (http://www.ihss.gatech.edu/elements.html).

HPLC. The retention factor (k') was used to measure the interactions between the chemical and the immobilised humic phase. This approach allows measuring the interactions in few minutes; however the immobilisation and packing are labour intensive and require equipments and know-how. This approach is not adapted to compare the interactions with different type of HS.

In this manuscript an alternative approach is explored where the formation of the humic supramolecular entity is induced in the inner wall of silica capillary that has been previously coated with a monolayer of humic acids. The interactions are then studied via open-tubular capillary electrochromatography (OT-CEC). For this explanatory work, three types of humic substances were tested and their interactions with five s-triazine measured. All of these are either phytosanitary products (atrazine, simazine and terbuthylazine) or main metabolites (atrazine desethyl and terbuthylazine desethyl).

2. Materials and methods

2.1. Chemicals

All chemicals were analytical reagent grade: acetic acid was from Fluka (Buchs, Switzerland), sodium hydroxide from Merck (Lisboa, Portugal), hexadimethrine bromide (polybrene) from Sigma Chemical Co (St Louis, MO, USA). Ultra pure water was obtained using a Milli-Q system (Milli-Q plus 185) from Millipore (Bedford, MA, USA). Atrazine, simazine, terbuthylazine, atrazine desethyl and terbuthylazine desethyl were from Riedel de Haen (Seelze, Germany). All triazines were dissolved at a concentration of 1000 ppm in 100% ACN. Each sample was prepared adding 15 μL of this stock solution, and 15 μL of acetone used as an EOF marker in 5 mL buffer. Samples were prepared daily.

Three samples of humic substances were used for this work, a humic acid from Fluka (flu-HA) and two samples from the international humic substances society, a soil standard humic acid (IHSS-HA, ref: 1S102H) and a soil standard fulvic acid (IHSS-FA, ref: 2S102F). The elemental composition of the humic substances used in this work can be found in Table 1 [19].

2.2. Capillary electrophoresis

All experiments were performed using a commercial instrument (Beckman P/ACE MDQ (Fullerton, CA, USA)) with a diode array detector. Running buffers were made of 40 mM acetic acid with pH adjusted to the required value with ammonium hydroxide (alkaline buffer) or 40 mM ammonium hydroxide adjusted to the required pH with acetic acid (acidic buffer). All buffers have a constant ionic strength of 40 mM. For the EOF measurements, separations were performed at 20 kV (direct and reverse mode) and 5% acetone in buffer was injected for 3 s at 3 kPa, and detected at 191 nm.

2.3. Monolayer coating of a silica capillary (flu-HA_{ML} coating)

A bare silica capillary (0.5 m length, 0.4 to detector, 50 μm ID, from Composite Metal Services (Worcester, UK)) was first conditioned by flushing at 138 kPa (20 psi) with 1 M NaOH for 30 min followed by Milli-Q water for 30 min. The coating was obtained by flushing sequentially 250 ppm polybrene in 50 mM Na_2CO_3 for 10 min, Milli-Q water for 10 min, 250 ppm flu-HA in 5 mM Na_2CO_3 for 10 min, 50 mM Na_2CO_3 for 10 min and Milli-Q water for 30 min.

2.4. Open tubular capillary electrochromatography

SmS of HS was self-assembled in the inner wall of the previously coated capillary by flushing an incubation buffer (IB) for 10 min and leaving it to incubate for 1 h. The capillary was then flushed with the running buffer for 30 min at 138 kPa. Before each run, the capillary was flushed with the running buffer for 3 min at 138 kPa. IBs were made dissolving 250 ppm of HS in 5 mM NaOH (final concentration) and adjusting the pH to the required value with acetic acid. The IBs were prepared just before the incubation step.

For the OT-CEC separation, the running buffer was 10 mM sodium hydroxide adjusted to pH 5.00 ± 0.05 with acetic acid. The samples were injected for 3 s at 3 kPa. The separation was performed in direct polarity for the HA coated capillaries and reverse polarity for the polybrene coated capillary at 20 kV. The capillary was thermostated at 25 °C. The triazine used as test analyte was detected at 223 ± 6 nm and the neutral marker at 260 ± 6 nm.

2.5. Peak analysis

Electrochromatographic peaks were analysed using commercial peak fitting software (PeakFit version 4, SPSS, Chicago, USA) following a procedure already described elsewhere [20]. However an exponentially modified function [21] was used instead of the Haarhoff–Van der Linde function to describe electrophoretic peaks [22,23].

3. Results and discussion

3.1. Immobilisation strategy

Whereas the exact composition and structure of HS remain unknown [6], a number of strong evidence point toward the formation of a SmS of relatively small molecules hold by weak forces [9,10,24]. This concept was the key to the immobilisation strategy used in this work. The capillary was primarily coated with a thin layer of commercial HS (flu-HA), and then incubated with an acidic (pH between 3 and 6) aqueous solution of humic substances under study, promoting the formation of an immobilised supramolecular structure of humic substances. The formation and stability of such a structure is believed to be due to the equilibrium between weak attractive and electrostatic repulsive forces. As the pH increases, the degree of deprotonation of existing carboxylic moieties within the HS structure increases, as well as the electrostatic repulsion. From the work of Brigante et al., best results are expected between pH 3 and 5 [25]. However, it should also be emphasised that numerous works highlight the importance of the type and concentration of the cation [26].

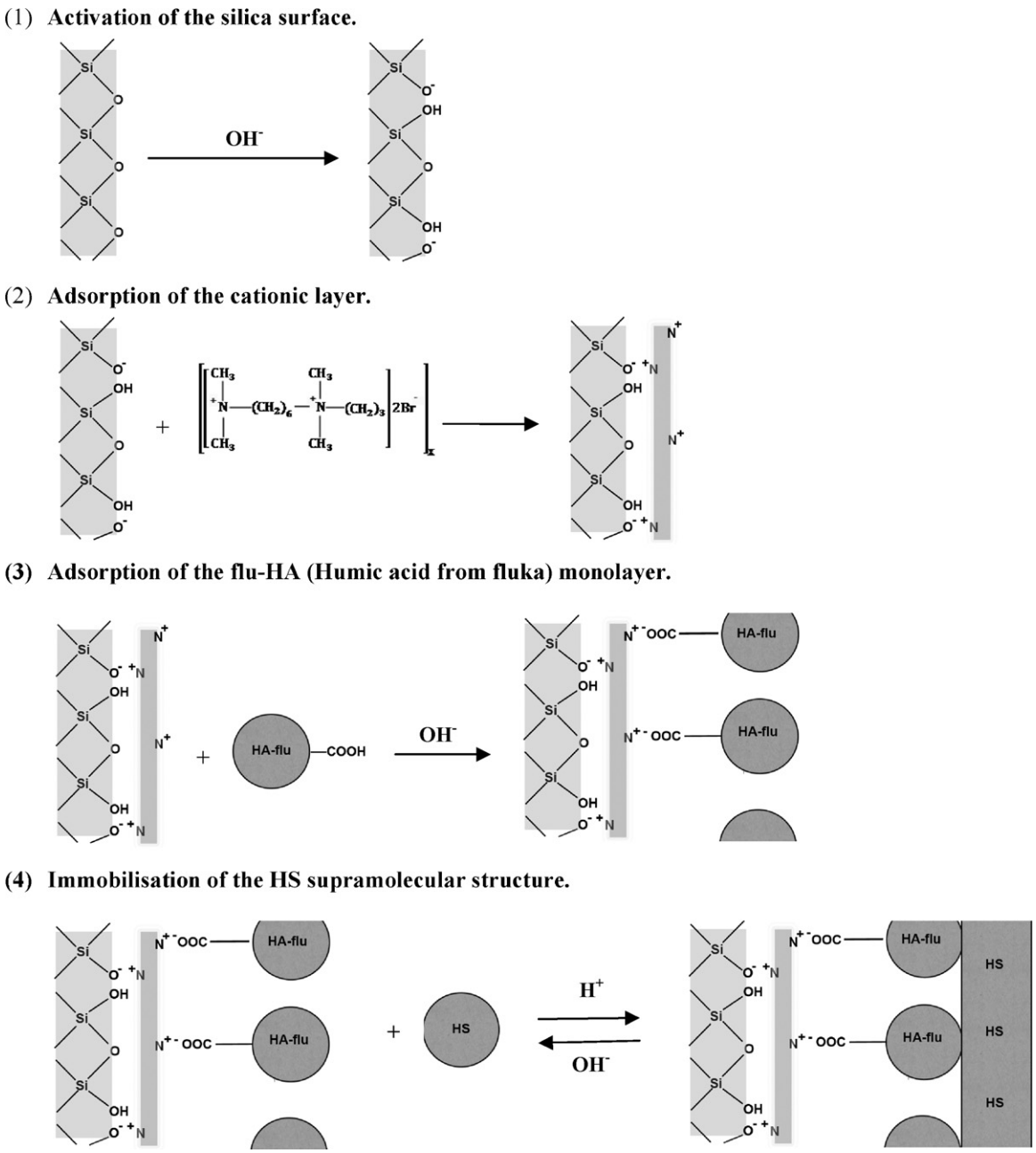


Fig. 1. Schematic of the coating approach.

A schematization of the approach is presented in Fig. 1. In brief:

(1) The surface is activated by flushing the capillary with an alkaline solution
(2) The surface is flushed with an aqueous solution polybrene (hexadimethrine bromide), a cationic polymer with a high affinity to silica surfaces, leading to a positively charged surface [27].
(3) A first layer of HS is immobilised in the surface. To do so, a commercial humic acid from Fluka (flu-HA) is dissolved in an alkaline solution (5 mM Na_2CO_3, pH 10.9), an environment favourable to the dissolution of humic aggregates, and thus adverse to the formation of supramolecular structures [25]. Dissolved humic substances, anionic entities, will naturally interact with the now cationic surface via electrostatic interactions to form a stable layer [28]. The capillary is then extensively washed with 0.1 M NaOH. This approach, often referred to as bilayer coating [29] or successive multiple ionic polymer layer (SMIL) [30] coating, has been chosen over covalent coupling because of its easiness. It is unknown, at this point, that the obtained layer is a monolayer or an aggregate of HS resistant to alkaline conditions (humin-like structure?). However, for simplicity, this layer will be referred to, in the subsequent discussion, as flu-HA monolayer (flu-HA_{ML})
(4) The monolayer is used as the starting layer to immobilise HS supramolecular structures. The HS of interest are dissolved in its incubation buffer and is flushed through the capillary. The capillary, filled with the IB, is left to incubate for 1 h before being rinsed with the running buffer. This approach is not as stable as a covalent coupling [31–33], however it allows easily to immobilise different types of HS in a few hours.

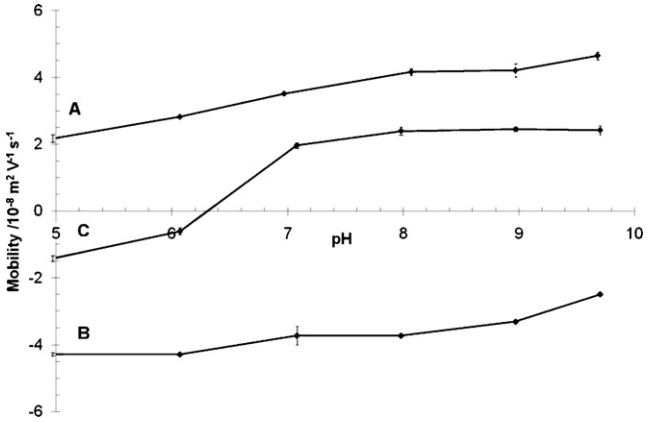


Fig. 2. Electroosmotic flow mobility as a function of pH measured in (A) a bare silica capillary, (B) a polybrene coated capillary and (C) Flu-HA_{ML} coated capillary. Separations were performed at 20 kV in direct or reverse polarity; acetone was used as a neutral marker and injected in 90% buffer for 3 s at 3.4 kPa. Alkaline buffers were made by adjusting 40 mM of acetic acid to the required pH with 0.1 M ammonium hydroxide, and acidic pH by adjusting 40 mM of ammonium hydroxide to the required pH with 0.1 M acetic acid. The error bars show the standard deviation measured with three replicates.

3.2. Characterisation of the flu-HA_{ML}

The flu-HA_{ML} coating was characterised by comparing the electroosmotic flow (EOF) at different pH using (A) a bare silica, (B) a polybrene coated and (C) a flu-HA_{ML} coated capillary. Results are displayed in Fig. 2. The EOF is related to the zeta potential of the surface [34], thus measuring it at different pH allows to compare the global charge of the different layers. With the bare silica capillary, the EOF mobility shows the expected dependence with the pH in the selected pH range [35]. After being coated with polybrene, a strong EOF towards the anode (anodic) is obtained, indicating that the silica surface is now positively charged at all pH. With the flu-HA_{ML} coated capillary, a low anodic flow is obtained below pH 6.3 and a cathodic flow at higher pH. The EOF velocity becomes constant over pH 7. The EOF of the coated capillary was observed to be relatively stable (RSD below 5% at all pH, $n = 5$). It is unclear if at low pH, the low anodic EOF is due to the presence of residual cationic sites in the polybrene layer, the presence of amine sites in the HA structure or both. The experiment, displayed in Fig. 2, was performed from the lowest (pH 4.97) to the highest (pH 9.7) pH and then repeated at the lowest pH. For the flu-HA_{ML} coated capillary, no significant differences were observed in the EOF mobility (μ_{EOF}) between the first and the last measurement at pH 4.97, $\mu_{EOF} = (-1.413 \pm 0.084) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ and $\mu_{EOF} = (-1.602 \pm 0.018) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$, respectively, showing the coating stability in the pH range 5–10.

3.3. Immobilisation and characterisation of the supramolecular structure of HS

The flu-HA_{ML} surface was used to induce the self-assembly of supramolecular structures using different type of humic substances. Three humic substances were tested, two humic acids (flu-HA and IHSS-HA) and one fulvic acid (IHSS-FA) (see Table 1). Before incubation with each IB, the capillary was flushed at 138 kPa with 50 mM Na_2CO_3 for 60 min. To characterise the SmS layer, 3 ppm of a triazine with 0.15% acetone in buffer were injected and separated in a pH 5.00 sodium acetate buffer. The key properties of the triazines used as test compounds are summarised in Table 2. In this table, K_{oc} is the soil organic partition coefficient, a measure of the strength of sorption of pesticides to soils [36]. Examples of electrochromatograms can be observed in Fig. 3, that show the separation of atrazine and acetone obtained with (A) a polybrene coated capillary, (B) a flu-HA_{ML} coated capillary, (C) a capillary incubated with flu-HA in a pH 4.00 IB (flu-HA_{SmSpH4}) and (D) a capillary incubated with flu-HA in a pH 3.00 IB (flu-HA_{SmSpH3}). As already discussed, both with the polybrene (A) and the flu-HA_{ML} coated capillary (B), an anodic EOF is obtained at the buffer pH (pH 5) and the separation was performed in reverse polarity (−20 kV). After incubation with flu-HA in acidic conditions (C and D), a cathodic EOF was obtained, and the separation was performed in direct polarity (+20 kV). The reversal of the EOF, measured after incubation ($\mu_{EOF} = (-1.413 \pm 0.084) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ with a flu-HA_{ML} coated capillary; $\mu_{EOF} = (3.63 \pm 0.12) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ with a capillary incubated with flu-HA in a pH 4.00 IB; $\mu_{EOF} = (3.56 \pm 0.06) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ with a capillary incubated with flu-HA in a pH 3.00 IB), is probably due to a higher coverage of the wall with humic substances, acidic species in the experimental conditions. This proves without ambiguity that an important amount of HS is immobilised after the incubation. However the EOF is again reversed if the capillary is washed with 5 mM of Na_2CO_3 showing that the formation of the SmS is reversible.

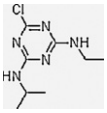
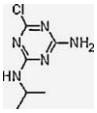
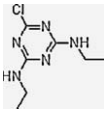
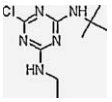
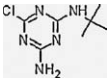
The interactions between atrazine and the surface can clearly be observed in this figure. Whereas with the polybrene and flu-HA_{ML} capillaries (Fig. 3A and B) similar migration times are measured for atrazine (1) and acetone (2), with flu-HA_{SmS} (Fig. 3C and D) an increase in the separation can be observed. Being the same buffer in all cases, the differences can confidently be assigned to a retention/partition mechanism rather than an electrophoretic process. This is further corroborated by the higher distortion in the peak of atrazine when atrazine and acetone are separated (asymmetry at 10%: Fig. 3A = 1.08 ± 0.01, Fig. 3B = 1.78 ± 0.11, Fig. 3C = 2.37 ± 0.53, Fig. 3D = 3.81 ± 0.58). This is due to slow mass transfer. In the absence of any residual electrophoretic transport, as in liquid chromatography, the strength of sorption can be assessed via the retention factor, k', defined as:

$$k' = \frac{t_{CEC} - t_{EOF}}{t_{EOF}} \quad (1)$$

where t_{CEC} and t_{EOF} are the time of the triazine and EOF peaks. It should be emphasised that this equation does only apply for fully neutral analytes as it is the case here (see Table 2) [37].

Values obtained with the five triazines using capillaries incubated with IB at different pH and with different HS (flu-HA, IHSS-HA and IHSS-FA) are resumed in Table 3. The electropherograms that have been used to calculate the k' can be found in the supplementary information. A good agreement is obtained in all conditions with the expected results, the trend of the order of k' are the same as the K_{oc} resumed in Table 2. The amino groups in position 2 and 4 are keys for the sorption properties of the chlorotriazines herbicides. Result shows that sorption properties of the chloretriazines are dependent on the complexity of the alkyl amine group in position 2 and 4. The lowest sorption is obtained with chemicals having at least one amino group (Atz-de and Tba-de) and the highest can be linked to the presence of a tert-butyl amine group (Tba). At identical incubation condition (pH 3.00), apart from Tba, higher k' was obtained using the humic acid from Fluka. The values were significantly lower with the fulvic acids. This lower binding capacity of FA was expected from relevant literature [38]. Whereas no significant differences were observed using an incubation buffer at pH 4.00 ± 0.05 or pH 5.00 ± 0.05, the k' values were roughly doubled using an incubation buffer at pH 3.00. This confirms the importance of the formation of the SmS for the sorption of pesticides. With the three IBs, similar EOF were obtained ($\mu_{EOF} = (3.59 \pm 0.11) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ with a capillary incubated with flu-HA in a pH 5.00 IB; $\mu_{EOF} = (3.63 \pm 0.12) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ with a capillary incubated with flu-HA in a pH 4.00 IB; $\mu_{EOF} = (3.56 \pm 0.06) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ with a capillary incubated with flu-HA in a pH 3.00 IB). This shows that the sorption of

Table 2
Structure and properties of the triazine used for this work.

Name		Solubility[a] (mg L^{-1})	pK_a[a]	K_{oc}[a] (mL g^{-1})	Structure
Atrazine	Atz	35	1.7	100	
Atrazine desethyl	Atz-de	3200		72	
Simazine	Sim	6.6	2	130	
Terbuthylazine	Tba	5	1.62	219	
Terbuthylazine desethyl	Tba-de	327.1		72.2	

[a] Data obtained from the Pesticide Properties Database (http://sitem.herts.ac.uk/aeru/footprint/index.htm).

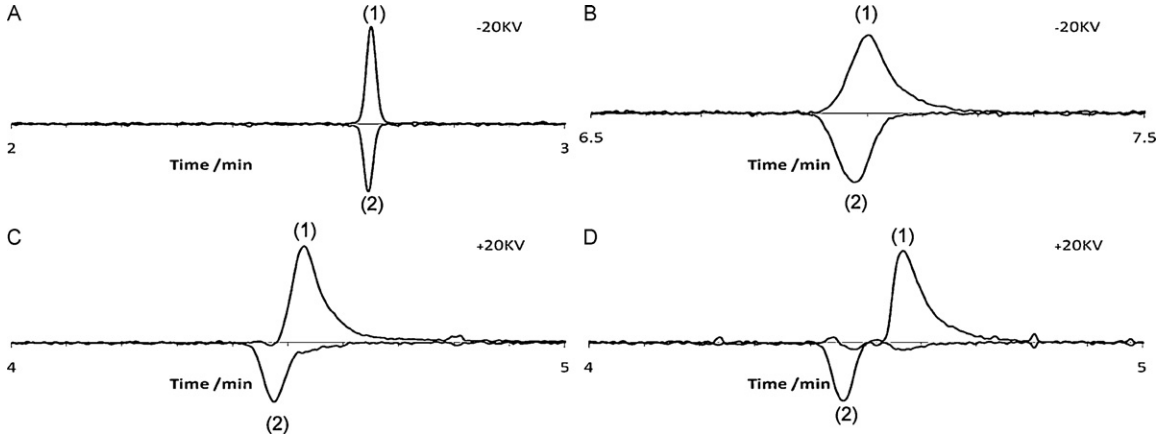


Fig. 3. Open tubular capillary electrochromatography separation in (A) a polybrene coated capillary, (B) a flu-HA_{ML} coated capillary, (C) a flu-HA_{SmSpH4} coated capillary and (D) a flu-HA_{SmSpH3} coated capillary. Experimental condition: capillary 0.5 m length (0.4 to detector), 0.5 μm internal diameter, applied voltage 20 kV (reverse polarity A and B, direct polarity C and D), buffer 10 mM ammonium acetate pH 5.00. Sample: 3 ppm atrazine, 0.15% acetone in buffer. The elution of (1) atrazine was recorded at 223 ± 6 nm and elution of (2) acetone at 260 ± 6 nm (reverse in this figure for clarity).

the tested triazines is mainly due to weak forces (hydrophobic, dipole–dipole, etc.) rather than electrostatic interactions. These results confirm the applicability of this approach to obtain a fast measurement of the strength of sorption of a triazine to a HS.

3.4. Repeatability

Atrazine was used to test the long-term stability and the repeatability of the coating. The standard deviation (SD) was measured using four successive runs with 3 min buffer wash in between. Between a series of runs, different processes were performed.

Table 3
Retention factor of the trazines measured with HS coated capillaries.

	$k'/10^{-2}$				
	flu-HA_{SmSpH5}[a]	flu-HA_{SmSpH4}[b]	flu-HA_{SmSpH3}[c]	IHSS-HA_{SmSpH3}[d]	IHSS-FA_{SmSpH3}[e]
Atz	1.0 ± 0.1	1.1 ± 0.2	2.3 ± 0.2	2.1 ± 0.2	0.9 ± 0.1
Atz-de	0.4 ± 0.1	0.4 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	0.2 ± 0.1
Sim	1.1 ± 0.4	0.9 ± 0.1	2.5 ± 0.4	1.5 ± 0.1	0.7 ± 0.1
Tba	5.0 ± 0.5	4.5 ± 0.6	7.5 ± 0.4	9.6 ± 0.6	4.6 ± 0.6
Tba-de	1.2 ± 0.3	0.8 ± 0.1	1.9 ± 0.2	1.4 ± 0.1	0.7 ± 0.1

[a] pH 5 IB with flu-HA.
[b] pH 4 IB with flu-HA.
[c] pH 3 IB with flu-HA.
[d] pH 3 IB with IHSS-HA.
[e] pH 3 IB with IHSS-FA.

Table 4
Repeatability measured using atrazine and a flu-HA_{SmSpH5} coated capillary.

Conditions	$k'/10^{-2}$	$\mu_{EOF}/10^{-8}$ ($m^2\,V^{-1}\,s^{-1}0$
(1)	2.2 ± 0.1	3.60 ± 0.04
(1)	1.9 ± 0.1	3.17 ± 0.02
(1)	2.0 ± 0.1	3.02 ± 0.01
(1)	2.0 ± 0.1	2.95 ± 0.02
(2)	2.4 ± 0.2	2.94 ± 0.01
(3)	2.1 ± 0.1	3.52 ± 0.07
(3)	1.9 ± 0.1	3.47 ± 0.06
(4)	2.3 ± 0.2	3.65 ± 0.08

(1) Between each series (four replicates) the capillary was washed 30 min with buffer. (2) Capillary left to stand for 48 h with buffer. (3) The SmS was washed and the capillary re-incubated. (4) The whole coating was done on new capillaries.

Results are displayed in Table 4, where (1) an half hour buffer wash was used between each series of experiments, (2) the capillary was left to stand for 48 h filled with buffer, (3) the SmS was washed with an alkaline solution (50 mM Na_2CO_3, 1 h) and the capillary was re-incubated with the IB and (4) the whole coating procedure was repeated with new capillaries. In all cases the SD is acceptable giving a relative standard deviation between 1 and 6%. Good repeatability was obtained with all experiments with an average value of $(2.2 \pm 0.2) \times 10^{-2}$ and an RSD of 8.7%.

4. Conclusions

In this work, supramolecular assemblies of humic substances were immobilised on a pre-coated silica capillary. This new coated capillary was used to measure the interactions between triazines and humic substances. This approach was demonstrated to be precise and repeatable. Moreover one measurement takes few minutes and the immobilisation and conditioning of a new supramolecular structure, 2 h. This approach has been validated using triazines that are interacting moderately with the soil organic matter (K_{oc} between 70 and 220 $mL\,g^{-1}$), however for species interacting more strongly, modifying the capillary dimensions should allow in obtaining similar results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.01.052.

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