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Binding of s-Triazines to Dissolved Humic Substances: Electrophoretic Approaches using Affinity Capillary Electrophoresis (ACE) and Micellar Electrokinetic Chromatography (MEKC)

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Dedicated to Prof. Dr. Werner Klein on the occasion of his 60th birthday

Abstract

Binding studies were conducted between s-triazines and soil and water extracted fulvic and humic acids (FA and HA) using capillary electrophoretic methods.

A first approach to estimate simultaneously the affinity of several s-triazines (hydroxyatrazine, ameline, atraton and ametryn) to dissolved humic substances (HS) was done with the affinity capillary electrophoresis (ACE) modus; the limits of the ACE method resulted in the measurement of the electrophoretic mobility of the pesticide-HS complexes. In a second approach, the partition of the s-triazines between the water and the dissolved humic substances was successfully described like in micellar electrokinetic chromatography (MEKC) using the humic substances as micellar phase. Similar to surfactants, humic acids (HA) behaved like ionic micelles in the aqueous running buffer at concentrations higher than a defined "*humic critical micellar concentration*" (HCMC). The low molecular weight acidic fulvic acids (FA) behaved the same but showing higher HCMC.

These results confirm the micellar properties of HS and the hydrophobic type of interaction of the s-triazines with hydrophobic sites of humic and fulvic ionic micelles.

Key words: capillary electrophoresis, humic substances, ionic micelles, s-triazines.

Introduction

Dissolved organic matter (DOM) contributes significantly to the behavior of anthropogenic chemicals in surface water and soil and sediment porewater. Humic substances (HS) can constitute up to 50% of DOM and are known to bind chemicals, governing the transport and bioavailability of xenobiotics and thus participating in the flux of pesticides and other contaminants to water reservoirs.

The measurement of the binding of organic contaminants such as pesticides to DOM is still a big challange. HS are usually chosen as model ligands in research related to this phenomenon. The main experimental problem in such research is the separation of the free from the bound pesticide fraction. In studies involving dissolved DOM, the low molecular weight DOM fraction often acts as a perturbation in the analytical system. Thus, in many binding studies the HS are handled in suspended form for good phase separation. Physical or chemical separation methods like centrifugation [1-3], filtration [4-5], solid phase extraction [6], supercritical fluid extraction [7], ultrafiltration [8, 9], dialysis [10, 11] or solvant extraction [12, 13] are the more often used techniques, even if they may interfere in the pesticide/DOM equilibrium and lead to over- or underestimation of the free pesticide form. The electrophoretic method presented here was considered a good alternative technique to study the binding of ionizable pesticides to DOM; the separation of the free pesticides from the organic ligands occurs in the capillary in aqueous buffers during the analysis without complemental fractionation procedures.

The electrophoretic behavior of both s-triazines and humic substances was studied by the authors recently with CE [14-17]. In this study we present the theoretical approach used in affinity capillary electrophoresis (ACE) and micellar elektrokinetic chromatography (MEKC) for the study of the binding of s-triazines to humic substances. These methods consider only the changes in electrophoretic mobilities of the triazines with complex formation upon addition of increasing amounts of humic substances to the separation buffer. A few possibilities in using these CE-techniques to determine distribution coefficients of several s-triazines analyzed simultaneously between humic and water phase will be presented. Variation in experimental conditions

(pH, ionic strength, temperature, compensation cations ...) and discussion on the type of binding occuring as a function of the structure of the organic matrices will be given elsewhere.

MATERIALS AND METHODS

1. Humic substances.

Scheyern-soil (Ap Horizon, 0-20 cm) was sampled from a cultivated *loamy brown soil* from the „Forschungsverbund Agrarökosystem München" (FAM) in Scheyern, Germany (sampling points 290/190 and 270/190). The extraction and purification procedures followed the guidelines of the International Humic Substances Society (IHSS). The characterization and electrophoretic behavour of these humic substances is given elsewhere [16, 17]. Standard soil (ref: IRI02H) and water humic substances (ref: IRI01H) [Suwannee River, US Geological survey Open File Report 87-557] were obtained from the International Humic Substances Society, Dr. Patrick MacCarthy, Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO, 80401. Commercial humic acids were obtained from Aldrich (Steinheim, Germany).

2. Capillary electrophoresis.

Instrumentation consisted of a Beckman P/ACE 2100 Series HPCE with Beckman System Gold Chromatography Software. The uncoated fused-silica CE column (75 μm id, 375 μm od, 50 cm length to the detector, total length 57 cm) was obtained from Beckman Instruments Inc. All measurements were conducted at temperature, 30° C; voltage, 20 kV; detector wavelength, 230 nm; 10 sec. hydrodynamic injection.

An acetate buffer (50 mM, pH 4.6) was chosen for its good baseline separation of the four s-triazines; it was composed of 0.05 M glacial acetic acid : 0.05 M sodium acetate : water, 1:1:2, v:v:v. The buffer inlet and outlet vials were limited at 480 μl volume.

3. Pesticides

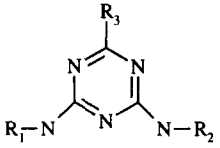
The hydroxy-alkylamino-s-triazines (Table 1), atraton and ametryn were purchased in greater than 99% purity grade from Dr. Ehrenstorfer GmbH, Augsburg, Germany or from Riedel de Haen (Pestanal grade), Munich, Germany. The hydroxy-arylamino-s-triazines ([H, Ar], [H, mAr], [iPr, Ar], and [iPr, mAr] - Table 1) were synthesized from the corresponding 2-chloro-4-arylamino-6-alkylamino-s-triazines by acidic hydrolysis in 1:1 acetonitrile/water at 80° C. The 2-chloro-4-arylamino-6-alkylamino-s-triazines were synthesized from cyanuric chloride as reasonable model compounds to study the fate of bound atrazine residues in soil and water systems; the hydroxy analogues have been found as their major abiotic degradation products.

THEORETICAL APPROACH

1. Affinity capillary electrophoresis (ACE)

Electrophoretic methods are routinely used in pharmacology to measure the binding affinity characteristics of proteins and ligands; both enzyme activities and affinities of drugs to their receptors are described by binding constants that are important parameters in the optimization of the therapeutic dose of a drug [18]. In the last four years ACE [19-22] has found application in the measurement of binding constants between proteins and, for example, sugars, amino acids, drugs and metals [23-27]. The binding of vancomycin to different proteins and peptides has been well described by ACE [24, 28, 29].

ACE were adapted from the gel permeation methods developed for protein binding studies by Hummel and Dreyer in the early sixties [30]; these early methods were already used to study the binding of atrazine to dissolved humic acids [31, 32]. The classical quantitative methods of ACE are based on the quantification of peak area and height and were reviewed recently by Kraak et al [33]: the Hummel Dreyer method, the vacancy peak method and the front analysis method.



pesticides and metabolites	usual or commercial names	R_1	R_2	R_3	short name *	order (Fig5a)
2-hydroxy-4-ethylamino-6-tertiarybuthylamino-1,3,5-triazine	hydroxyterbuthylazine	CH_2CH_3	$C(CH_3)_3$	OH	[Et, tBu]-ameline	1
2-hydroxy-4-isopropylamino-6-isopropylamino-1,3,5-triazine	hydroxypropazine	$CH(CH_3)_2$	$CH(CH_3)_2$	OH	[iPr, iPr]-ameline	5
2-hydroxy-4-ethylamino-6-isopropylamino-1,3,5-triazine	hydroxyatrazine	CH_2CH_3	$CH(CH_3)_2$	OH	[Et, iPr]-ameline	4
2-hydroxy-4-ethylamino-6-ethylamino-1,3,5-triazine	hydroxysimazine	CH_2CH_3	CH_2CH_3	OH	[Et, Et]-ameline	2
2-hydroxy-4-amino-6-tertiobuthylamino-1,3,5-triazine	hydroxydesethylterbuthylazine	H	$C(CH_3)_3$	OH	[H, tBu]-ameline	3
2-hydroxy-4-amino-6-isopropylamino-1,3,5-triazine	hydroxydesethylatrazine	H	$CH(CH_3)_2$	OH	[H, iPr]-ameline	6
2-hydroxy-4-amino-6-ethylamino-1,3,5-triazine	hydroxydesisopropylatrazine	H	CH_2CH_3	OH	[H, Et]-ameline	7
2-hydroxy-4-amino-6-amino-1,3,5-triazine	hydroxydiamino-triazine (ameline)	H	H	OH	[H, H]-ameline	8
2-hydroxy-4-isopropylamino-6-anilino-1,3,5-triazine	R234	$CH(CH_3)_2$	C_6H_5	OH	[iPr, Ar]-ameline	10
2-hydroxy-4-amino-6-anilino-1,3,5-triazine	R243	H	C_6H_5	OH	[H, Ar]-ameline	12
2-hydroxy-4-isopropylamino-6-(3´,4´-dimethoxyanilino)-1,3,5-triazine	R240	$CH(CH_3)_2$	$C_6H_3(OCH_3)_2$	OH	[iPr, mAr]-ameline	9
2-hydroxy-4-amino-6-(3´,4´-dimethoxyanilino)-1,3,5-triazine	R237	H	$C_6H_3(OCH_3)_2$	OH	[H, mAr]-ameline	11
2-methoxy-4-ethylamino-6-isopropylamino-1,3,5-triazine	atraton	CH_2CH_3	$CH(CH_3)_2$	OCH_3	-	-
2-thiomethyl-4-ethylamino-6-isopropylamino-1,3,5-triazine	ametryn	CH_2CH_3	$CH(CH_3)_2$	SCH_3	-	-

* short name as a function of different substitution of ameline

Table 1: s-triazines used for the binding studies with humic substances using the MEKC modus

The interactions between two molecules P (pesticide) and L (Ligand) to form a complex PL can be described by a association constant K_{ass} (assuming a monovalent interaction at equilibrium):

$$K_{ass} = \frac{[PL]}{[P].[L]} \qquad (1)$$

(where [P]: concentration (mol/l) of the free pesticide, [L]: concentration (mol/l) of ligand, [PL]: concentration (mol/l) of pesticide-ligand complex, with $[P]_{tot} = [P] + [PL]$; the dissociation constant K_d can be defined as $K_d = 1 / K_{ass}$)

The same theoretical approach is commonly used in enantiomeric separation using capillary electrophoresis (CE) with various chiral reagents. The addition to the CE buffer of the chiral reagents (e.g. a cyclodextrin) causes the formation of a complexe with each enantiomer of the analyte. The difference in the binding affinity of the (+) and (-) forms of the analyte provide different electrophoretic mobilities and separation of the two complexes [34, 35]. In affinity electrophoresis [19], as well as in chiral separations with cyclodextrins [36], the resulting effective electrophoretic mobility μ (directly proportional to the velocity) of the measured substance is a weighted product of the effective electrophoretic mobilities of all the free and bound forms of the analyte in the studied system.

$$\mu = \frac{[P]}{[P]_{tot}} . \mu_o + \frac{[PL]}{[P]_{tot}} . \mu_c \qquad (2)$$

(μ_o= electrophoretic mobility of the the free analyte for [L]=0, μ_c is the electrophoretic mobility of the formed complex)

The negatively charged wall of the capillary from the ionization of the silanol groups (the pI of fused silica is about 1.5) attracts positively-charged ions from the buffer, creating a electrical double layer. With application of the voltage across the capillary, the cations of the double layer migrate to the cathode creating a net flow of buffer solution to the negative electrode (electroosmotic flow - EOF). The retention time

does not reflect the effective electrophoretic mobilities μ of the analytes in the separation system which is independant of the EOF. The effective electrophoretic mobilities of the analytes can be calculated by subtracting the electroosmotic flow (μ_{eof}) from the measured electrophoretic mobilities (μ_{mes}) as an EOF-correction [37] and are used as absolute electrophoretic values for the calculation of the binding constants in this paper. The combination of (2) with (1) gives:

$$\mu = \frac{K_{ass} \cdot \mu_o + \mu_c \cdot [L]}{K_{ass} + [L]} \quad \text{or} \quad \mu = \frac{\mu_o + \mu_c \cdot K_d \cdot [L]}{1 + K_d \cdot [L]} \qquad (3)$$

The constant K_d (i.e. $1/K_{ass}$) can be determined by linear regression. μ_o and L are experimental values. Because μ_c is an unknown value, Shimura [20] proposed a graphical resolution where (3) can be written as:

$$\frac{1}{\mu - \mu_o} = \frac{1}{\mu_c - \mu_o} \cdot K_d \cdot \frac{1}{L} + \frac{1}{\mu_c - \mu_o} \qquad (4)$$

Graphical representation of the inverse of the difference between the actual mobility and the mobility of the free form, against the inversed ligand concentration gives a theoretical line ($y = a \cdot x + b$) and the estimation of the binding constant ($K_{ass} = b / a$, or $K_d = a / b$) can be made without knowing the mobility of the 1:1 complex (μ_c). Applications of these equations were done by the authors in the study of the interactions between s-triazines and monomolecular phenolic acids (phthalic acid, gallic acid, salicylic acid, p-hydroxybenzoic acid, vanillic acid and syringic acid) as structural models of humic substances [38]. The binding constants were correlated to the ionization degree of both s-triazines and ligands and the interactions were interpretated in terms of hydrogen bonding and of acid-base interactions of protonated s-triazines with the negatively charged carboxylic acids and fulvic acids. Additionally a relative higher extent of protonation of the s-triazines as predicted by the pH of the buffer and the pKa of the analytes was observed (induced increase in their apparent pKa due to their

stabilization as cations by negatively charges ligands - similar to clay surface interactions [39, 40]).

Because of the high molecular weight and the polydisperse structure of humic acids, the complexation does not occur at a 1:1 stoichiometry and the above mentioned equations are not verified. One can describe the interactions of HS with pesticides by the partitioning of the pesticides between the aqueous and the organic humic phase.

$$C_{ads} = K_p \cdot C_{eq} \qquad (5)$$

(where C_{ads} is the adsorbed pesticide amount defined as $C_{ads} = ([P_{tot}] - [P]) / L$ in mol/kg, where $[P_{tot}]$ is the initial pesticide concentration in g/l, [P] is the pesticide concentration in solution at equilibrium in g/l, L the ligand concentration of sorbent in kg/l)

Under these conditions one finds:

$$K_p = \frac{[P_{tot}] - [P]}{L.\,[P]} \qquad (6)$$

Using the same approximations as for equation (3) one finds:

$$K_p = \frac{1}{L} \cdot \frac{\mu - \mu_o}{\mu_c - \mu} \qquad (7)$$

However the mobility μ_c of the formed complexe between the HS and the pesticides is unknown. The graphical approach like with equation (4) does not give any linearity. In a first approximation, μ_c was choosed equal to zero as described in [38, 41] but the K_p values were overestimated as compared to $\mu_c \neq 0$. The corresponding graphical estimation (assuming $\mu_c = 0$) of K_p of four s-triazines (hydroxyatrazine, ameline, atraton and ametryn) is presented in Fig. 1 where ($\mu \cdot L$) is plotted against μ;

the slope is $-1/K_p$. The linearity of the plots is not verified over all the HS concentration range.

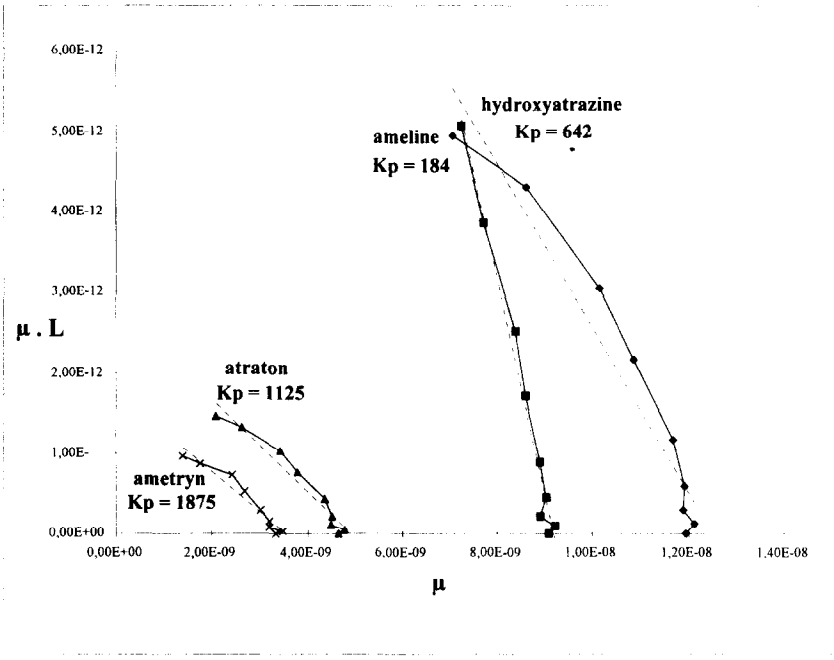


Fig. 1: Graphical ACE - estimation of K_p of four s-triazines with commercial Aldrich humic acids (assuming $\mu c = 0$) from the slope of the linearized experimental data.

The lack in informations about the mobilities of the complexes and the possible errors on the K_p values let the authors investigate a chromatographic modelisation as described in micellar electrokinetic chromatography (MEKC).

2. Micellar electrokinetic chromatography (MEKC)

This capillary electrophoresis technique also called micellar electrokinetic capillary chromatography (MECC) allows the resolution of uncharged molecules or charged molecules. The electrophoretic MEKC techniques takes advantage of the properties of surfactants to form ionic micelles in aqueous media at concentrations over

the critical micellar concentration (CMC). At lower concentrations the surfactant molecules are in a molecular disperse stage (possible associations in dimers, trimers or oligomers) and the CMC is a function of the structure of the surfactants [42]. The description of a separation uses the definition of a capacity factor k′ like in liquid chromatography:

$$k' = \frac{n_{mc}}{n_{aq}} \qquad (8)$$

(with n_{mc} and n_{aq} the amount of the analyte incorporated into the micelle and in the aqueous phase)

From (8), k′can be calculated from the retention times as well as from the effective electrophoretic mobilities:

$$k' = \frac{\mu - \mu_o}{\mu_{mc} - \mu} \qquad (9)$$

(μ and μ_o defined as in equation (2) and μ_{mc} the mobility of the micelle)

The relation between k′ and the distribution coefficient K_p (partition coefficient) of the analyte between the aqueous phase and the micellar phase is defined [43]:

$$k' = K_p \cdot \frac{V([S] - CMC)}{1 - V([S] - CMC)} \qquad (10)$$

(with [S] the total surfactant concentration in mol, V the molar volume of the surfactant in l/mol)

At low micellar concentrations a linear relation can be assumed:

$$k' \cong K_p \cdot V([S] - CMC) \qquad (11)$$

Using increasing concentration of SDS as a surfactant in the buffer, K_p of different solutes and the CMC of the surfactant can be determined graphically from equation (11) [42] by linear regression of [S] versus k.

RESULTS AND DISCUSSION

A similar approach as in MEKC was used in the present study: humic acids were added in increasing amount in a 50 mM acetate buffer at pH 4.6 (concentration in HS of the dilution serie: 10, 25, 50, 100, 200, 400, 700 mg/l). The pH of the buffer did not change in this HS concentration range. Only 2 mg humic acids were dissolved in 0.5 ml sodium acetate 0.1 M followed by 0.5 ml acetic acid 0.1 M and 1 ml water; this stock solution was diluted to fill two 500 µl buffer vials used for the separation.

Hydroxyatrazine, ameline, atraton and ametryn are baseline separated with an 50 mM acetate buffer at pH 4.6. The changes in retention times by addition of increasing amount of IHSS-Suwannee river humic acids are shown as an example in Fig. 2.

The capacity factors k´were calculated for each pesticide of each run according to equation (9) and modelised with equation (11). Under this hypothesis the average electrophoretic mobility (AEM) [17] of the humic acids with the same buffer was used for the mobility of the ionic micelles μ_{mc}.

	^{13}C NMR shifts (ppm) and relative abondance (%)					**density**
	1-60	**60-105**	**105-160**	**160-180**	**180-220**	**[kg/l]**
Scheyern HA	24,4 %	31,9 %	29,6 %	13,3 %	0,8 %	1.429
Scheyern FA	28,2 %	30,0 %	33,6 %	8,2 %	0 %	1.381
IHSS river HA *	17 %	18 %	42 %	16 %	7 %	1,501
IHSS river FA *	21,8 %	20,7 %	35,0%	16,9 %	4,9 %	1.454

Table 2: ^{13}C liquid NMR data of selected humic substances and their calculated densities (* from US Geological survey Open File Report 87-557)

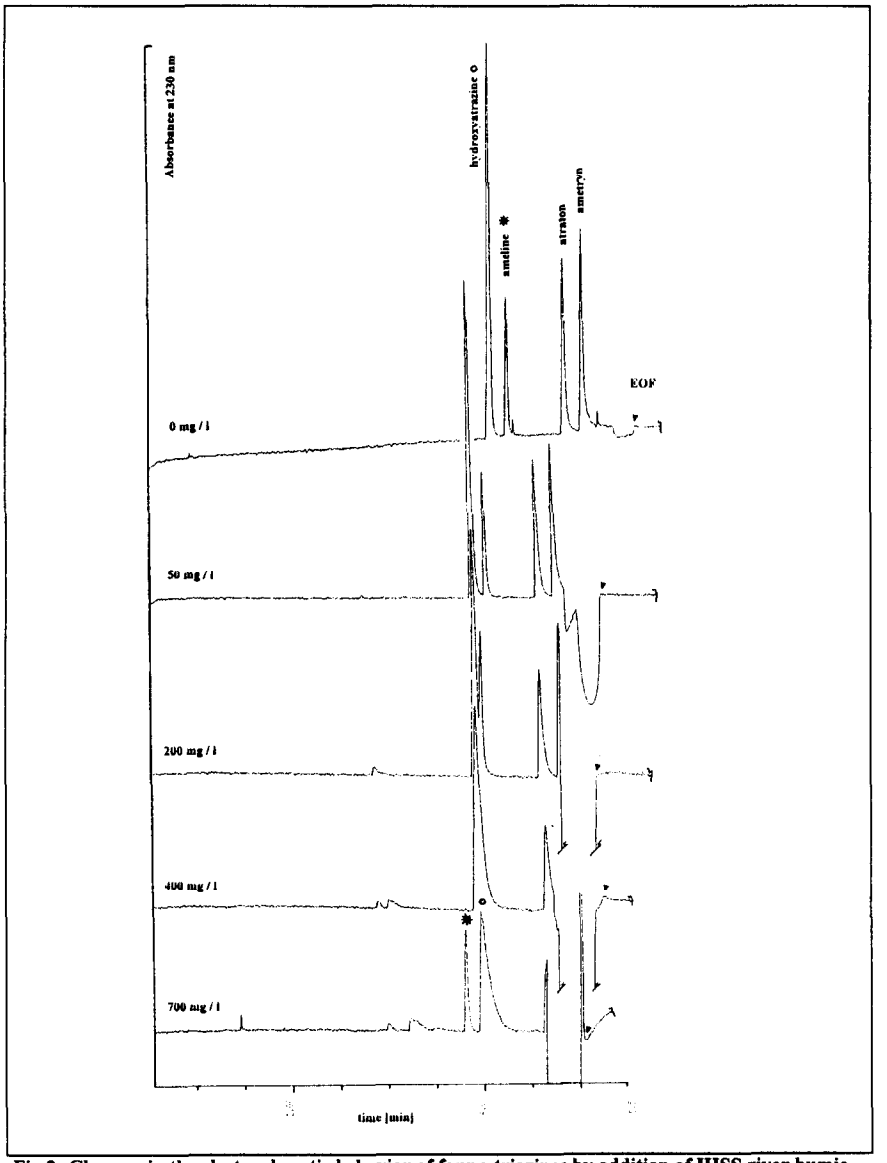


Fig.2: Changes in the electrophoretic behavior of four s-triazines by addition of IHSS river humic acids in the 50 mM acetate buffer (pH 4.6); one can see the change in migration order between hydroxyatrazine and ameline when increasing the HA concentration. Measurements are done in less than 6 min.

The molar volume necessary to calculate K_p from k´ is a known value for the surfactants used in MEKC; the molar volumes (l/mol) of humic material however, are not usual data. An fairly good estimation of the density (kg/l) of humic substances can be done using structural unit density values and functional group distribution estimated from liquide ^{13}C-NMR studies [US Geological survey Open File Report 87-557] (Table 2). No quantitative ^{13}C-NMR data were available for Aldrich HA and IHSS river HA; a density value of 1.550 was choosen for the calculation of the partition coefficients in accordance to their higher aromaticity and the higher density of HA as compared to FA (Table 2).

The graphical representation of the k´as a function of the HA concentrations (Fig. 3) showed good linearities ($r^2 > 0.97$) for all the studied HAS.

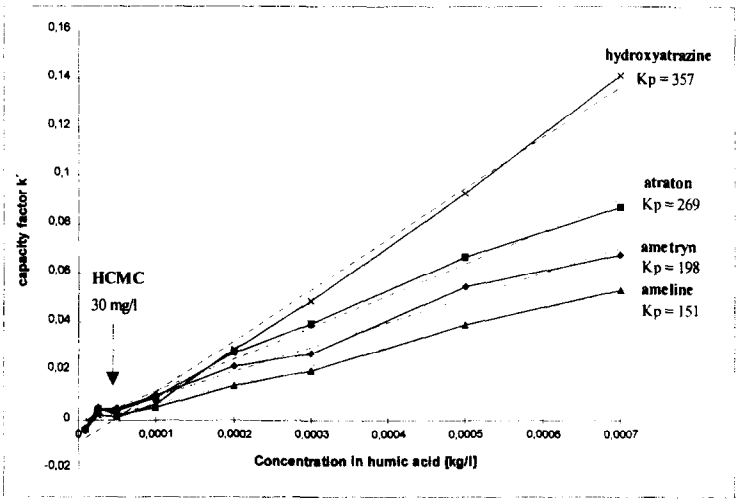


Fig. 3: Linearity of the capacity factor k´ of four s-triazines (5 ppm each) as a function of the concentration in Aldrich humic acids in the 50 mM acetate buffer (pH 4.6) (same data than Fig. 1)

This linearity implies that the partitioning coefficient remains constant in the studied HA concentration range and that the HA behave similarly to surfactants. They showed no micellar properties at low concentration (molecular disperse stage) and from Fig. 3 the minimal concentration in Aldrich humic acids can be determined from which

the HA behave like ionic micelles (around 30 mg/l); we defined it like in MEKC as „*Humic Critical Micellar Concentration*" (HCMC). This HCMC is dependant of the structure of the HA. The Scheyern fulvic acids behaved similarly with much higher HCMC values (around 30 mg/l); the IHSS river fulvic acids did not behave like micelles (no linearity) in these experimental conditions. Discussion of the influence of exchangeable cations, temperature, solution ionic strength or pH and humic substances chemical properties on the micellar properties of HA will be given elsewhere.

Humic acids have been described in the literature to have micellar properties having both hydrophilic and hydrophobic sites that are responsible for example for the enhancement of the solubility of organica in aqueous media or the lowering of the water surface tension [44-46]. These secondary structures of the HS are responsible for agregation phenomena between the humic molecules as a function of the solution condition (pH, salt concentration...) as well as for the binding anorganic or organic contaminants (entrapment in structural voids or hydrophobic domains). The pseudomicellar or membrane like properties of HS has been described in early studies when following the binding of PAHs to humic substances with fluorescence techniques [47, 48].

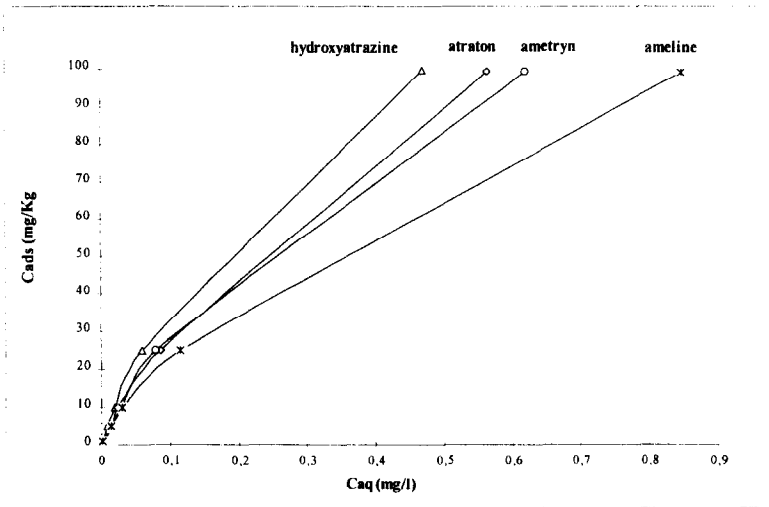
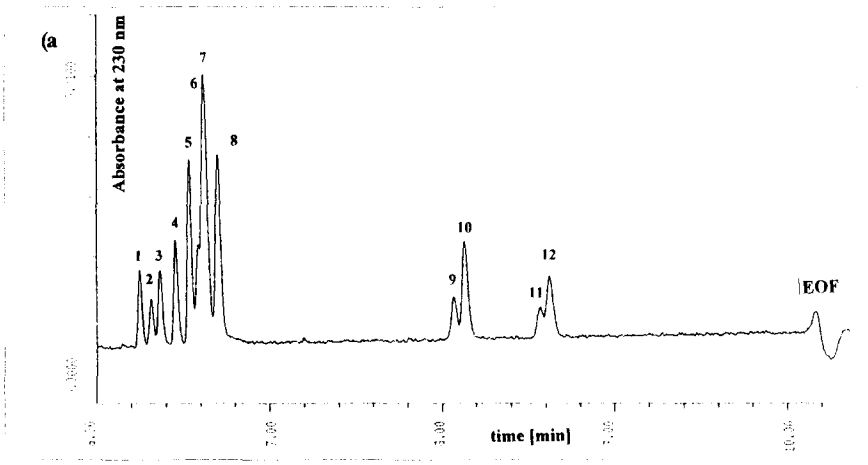


Fig. 4: Adsorption isotherme of the four s-triazines with the IHSS soil humic acid in a 50 mM acetate buffer (pH 4.6)

Using this method we investigated the changes in distribution coefficients as a function of the concentration of the injected sample (concentrations of 0.5, 1, 5, 10, 25, 100 ppm of hydroxyatrazine, ameline, atraton and ametryn in simultaneous runs). From the partition coefficient one can calculate the bound and the free amount of s-triazines for each measurement.

The adsorption isotherm obtained this way for the four pesticides with the IHSS soil humic acid are in Fig. 4. Humic acids show a saturation of the binding sites with increasing concentration in s-triazines (S curve).

Another example of application for this method is the simultaneous determination of the partition coefficient of 10 hydroxy-s-triazines with the Scheyern humic acids (structure of the s-triazines in Table 1). Twelve hydroxy-compounds could be separated with an 100 mM acetate buffer at pH 4.45 (Fig. 5a); the increase of the HS concentration in the buffer was followed by changes in electrophoretic mobilities of the triazines and the partition coefficients were calculated from equations (9) and (11). These values are given in Fig. 5b; hydroxy desethylatrazine did not give a good linear response ($r^2 < 0.9$) and hydroxydesisopropylatrazine was only detectable with difficulties in the s-triazine mixture: their K_p values were not determined



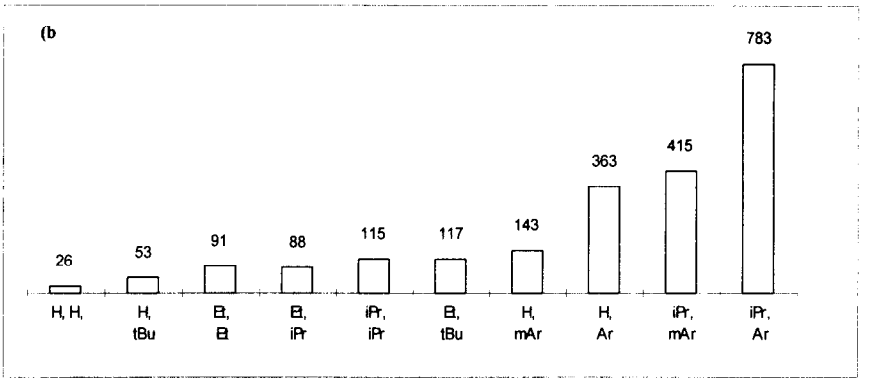


Fig.5: **(a) separation of the twelve hydroxy-s-triazines in an 100 mM acetate buffer (pH 4.45) (ellution order is given in Table 1).**
(b) K_p with Scheyern humic acids for ten of the hydroxy-s- triazines as a function of their triazine ring substitution.

The partitioning of the hydroxy-s-triazines between the water and the organic phase is shown as a function of the substitution of the triazinic ring; the higher the hydrophobicity of the compounds is, the higher is the distribution coefficient confirming the hypothesis of hydrophobic type of interactions. Changes in pH have a direct influence on the ionization properties of both hydroxy-compounds and humic substances changing their hydrophobicity (LogP as a function of pH) and the micellar properties of HS; these changes are under investigation.

CONCLUSION

Capillary electrophoresis measurements allowed the calculation of the partition coefficients of s-triazines between dissolved humic substances and the water phase. The experimental data were described the best like with MEKC technique confirming the micellar properties of humic substances. A humic critical micellar concentration (HCMC) was defined as the minimal concentration at which the humic substances associate to behave like ionic micelles. One advantage of this method is the rapid simultaneous measurement of the affinity of several ionizable pesticides to the HS ligands based on changes in migration times, thus allowing a direct comparison of results.

No changes in the pH, ionic concentration of the buffer, exchangeable cations (Cu^{++}, Fe^{+++}, Al^{+++}...) or temperature have been investigated in this paper. These parameters are essential, since they govern the steric organization of the HS and thus the possible interactions with pesticides or metals with hydrophobic or hydrophilic sites respectively. Studies are ongoing involving these experimental parameters and using different HS to give more indications on the type and localization of the binding of pesticides (structure / reactivity studies). This electrophoretic method is yet limited to ionizable pesticides but shows already good promesses in the study of the binding of xenobiotics (organics and metals) to natural DOM.

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