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Influence of secondary interactions on high performance size exclusion chromatography. Application to the fractionation of landfill leachates

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Abstract. The leachates from municipal landfills are characterised by the complex chemical distribution and wide molecular weights range of their constituents. We have designed a high performance size exclusion chromatography method to take into account these two aspects. By using eluents of low ionic strength, electrostatic interactions between the eluted molecules and the negative sites of the stationary phase are enhanced. Thus, the compounds are eluted mostly according to their physico-chemical properties. Contrarily, using eluents of high ionic strength allows eliminating most of these electrostatic interactions which, in turn, permits to estimate the molecular weights, since steric exclusion is then the dominating mechanism of separation. A spectroscopic fingerprint may also be determined through multidetection (UV absorption, fluorescence and evaporative light scattering detection) coupled *on line* which allows to sort out the compounds into a few number of families.

Keywords. Landfill leachates – high performance size exclusion chromatography – secondary interactions – characterisation.

Introduction

Many authors undertook the chemical characterisation of the leachates generated by the municipal landfills. The review of the data of the literature (see [1]) shows that several separative and analytical techniques were primarily used *i.e.* the ultrafiltration, the liquid-liquid extraction, the permeation chromatography on Sephadex gels, the adsorption on Amberlite XAD resins, and the gas chromatography. This led us to use a different method: the high performance size exclusion chromatography (HPSEC).

We carried out a preliminarily study [1], which showed that leachates from domestic wastes contain molecules which could be filed in two main groups according to their molecular weights: one constituted of molecules of AMW (Apparent Molecular Weight) lower than 1000 Da and the other one with molecules of AMW higher than 10000 Da. Few products of intermediate molecular weights are present. Simultaneous monitoring of the samples by UV absorbance, fluorescence and evaporative light scattering (ELSD) reveals the diversity of the physico-chemical characteristics of the separated compounds. A thorough spectrometric study [2] enabled us to validate the choice of excitation/emission couples characteristic of the substances so-called humic-type and protein-type compounds used in the first study. We also showed that the chromatographic elution of the molecules is biased by secondary interactions. So, in the present article we propose to study the influence of these interactions on chromatographic separation.

The mechanism of steric exclusion is theoretically a purely entropic phenomenon, which warrants for the observed relation between the volume of elution and the hydrodynamic volume of the eluted molecule. One however notes experimental variations in retention times either due to adsorption (elution delayed) or exclusion (elution advanced). This is to say that interactions between the eluted molecules and the stationary phase modify the elution order of compounds with definite molecular weights. In aqueous solvents, very polar ionised groups are exposed at the water-soluble compounds surface and secondary enthalpic interactions are observed [3-5]. They are due to ion-exchange and ion-exclusion effects, hydrophobic interactions and hydrogen bonds. The solute-support interactions are of course undesirable for the study of the molecular weight distribution of the molecules but they can be made profitable for the optimisation of their separation. These interactions were previously described [4,6,7] and it was shown that the choice of the eluent is particularly critical in aqueous size exclusion chromatography. It is not trivial to eliminate such interactions but they can be, at least, strongly reduced by judiciously choosing the eluent properties (pH, ionic strength, addition of organic co-solvent...) [7]. Sodium nitrate is often preferred for the elution of humic substances in HPSEC. At a 20 mM concentration in the eluent, the elution of the fulvic acids on both polymeric- and silica- based columns is weakly disturbed by the ionic repulsion and the hydrophobic interactions [8]. Kato *et al*. [9] also recommend the use of this salt, at a 0.1 M concentration for the elution of

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hydrophilic polymers, and with addition of 20 % of organic co-solvent for the hydrophobic compounds.

To compare our results with those mentioned above, we first used $NaNO_3$ eluents. But with these eluents the use of the ELSD is not possible since $NaNO_3$ is not evaporated at the operating temperature of the detector and generates a significant background noise. Later, we used an eluent containing ammonium acetate, a salt which evaporates under the operating conditions of the ELSD.

Material and methods

The studied leachates come from a sanitary landfill of domestic wastes in exploitation since 1992. Their characteristics, sampling, storage and fractionation by ultrafiltration are described in our preceding publications [1,2].

Ultrafiltration leads to obtain four fractions noted F1 (compounds of AMW higher than 10000 Da), F2 (AMW ranging between 10000 and 3000 Da), F3 (AMW ranging between 3000 and 1000 Da) and F4 (AMW lower than 1000 Da), on which we carried out the steric exclusion chromatography. We used polymer-based columns, manufactured by Tosohaas, whose characteristics are given in table I.

We plotted the calibration curves from standard solutions of polyethylene oxides (PEO), and polyethylene glycols (PEG) from Sigma, of average molecular weight ranging between 400 and 10^6 Da. The eluent used is a 200 mM ammonium acetate solution. The blue-Dextran and sodium chloride determine the total- exclusion *Ve* and permeation *Vp* volumes. For the G3000 column (flow of eluent set to 0.5 ml/min) *Ve* is equal to 10.2 min and *Vp* to 18.4 min. For G6000-G5000 columns in series (flow of eluent set to 1 mL/min), *Ve* is equal to 12.5 min and Vp to 21.5 min. The solvents (water and methanol) used are of HPLC grade and the electrolytes (Carlo Erba) of analytical grade. The eluents are delivered by a Varian 9012 pump; the injected volume is set to 50 µl (Valco loop). Three detectors are used: an UV-visible detector (Varian 9050), a fluorimetric detector Lachrom L-7480 (Merck-Hitachi), and an evaporative light scattering detector (ELSD) Sedex 55 (Touzart and Matignon). The wavelengths are 254 nm for UV absorption, and for fluorescence 280/335 nm to monitor protein-type compounds and 345/475nm to monitor humic-type compounds [2].

Results and discussion

Study of the four fractions on a G3000 column

We analysed the chromatograms obtained with the following eluents: water/methanol (70/30), water/sodium nitrate and water/ammonium acetate. First let us note that, for the same detection conditions, the chromatographic profiles are identical whatever the salt used; one observes merely a slight alteration of the retention times which can be due to the influence of the nature of the salt on the matrix pore and leachate compound size.

A humic-type compounds family with an anionic character

Figure 1 displays, with the fluorimetric detection 345/475 nm (humic-type), the chromatograms of the F1 fraction obtained with the eluents water/methanol and water/sodium nitrate 20 and 200 mM. Under these detection and elution conditions, the chromatographic profiles of the three other fractions, F2, F3 and F4 are approximately identical to those presented in figure 1 and are, in addition, similar to the profiles obtained by UV detection (254 nm). For each eluent, retention times of the chromatographic peaks are also close for all of the fractions. These observations drive us to assume the presence of a unique family of humic-type compounds in the four fractions. We studied the effect of the salt concentration in the eluent on the chromatographic profile by recording the elution variation of the main peak. The elution of this peak is delayed as the salt concentration increases: its retention time is around 9.7 min in water/methanol (value close to *Ve*) and around 15 min at 200 mM $NaNO_3$ (the broad peak, common to the four fractions, is spread out from 14 to 21 min). One notes a significant variation of the retention time up to 50 mM; then, it tends to a plateau value. The compounds responsible for this chromatographic peak would exhibit an anionic character and undergo repulsion interactions at low ionic strength (water/methanol) on behalf of the negative sites of the matrix; they are thus excluded from the gel. The addition of an electrolyte in the eluent results in negative sites screening by the formation of an ionic layer, which reduces the effects of repulsion [10]. With this column, a minimal concentration of 50 mM in salt is necessary to suppress the repulsion.

Table I. Characteristics of the TSK-PW columns (from Tosohaas).

Columns TSK	*Particle size* (µm)	*Pore size* (Å)	*Fractionation ranges (Dalton)* PEG, PEO	Dextrans	Proteins
G3000 PW	10	200	< 50000	< 60000	$500\text{-}8.10^5$
G5000 PW	17	1000	$4000\text{-}10^6$	$5.10^4\text{-}7.10^6$	$< 10^7$
G6000 PW	17	>1000	$4.10^5\text{-}8.10^6$	$5.10^5\text{-}5.10^7$	$< 2.10^8$

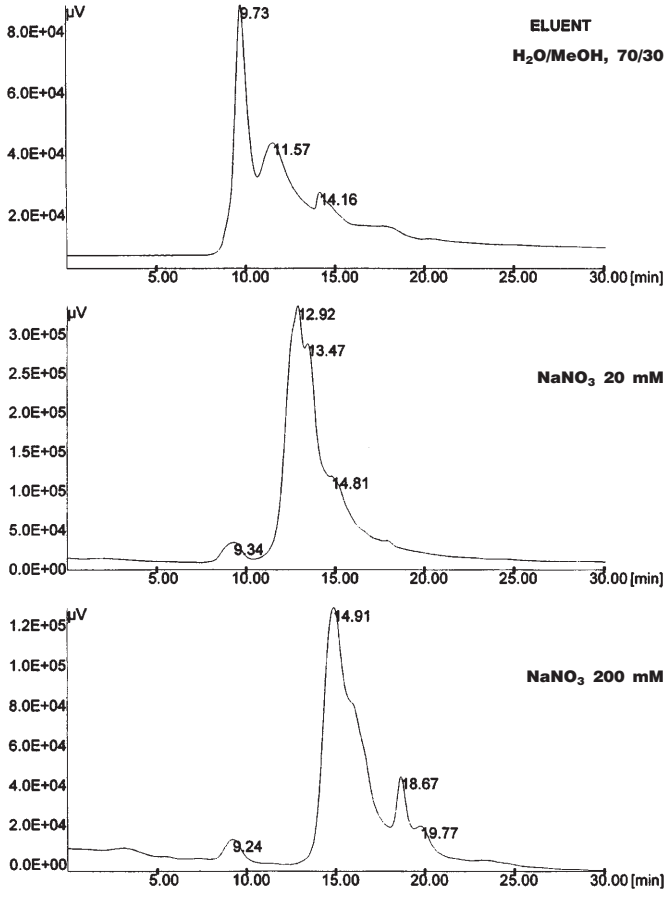

Figure 1. Chromatograms of the F1 fraction using the G3000 column: effect of elution conditions. Detection: fluorimetry at exc/em 345/475 nm.

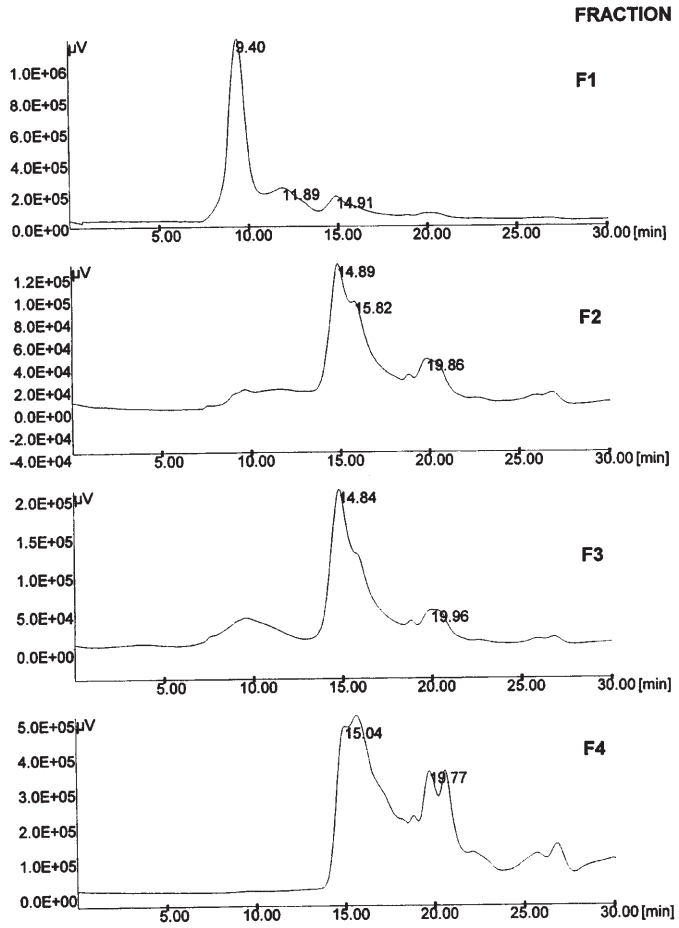

Figure 2. Chromatograms of the four fractions using the G3000 column. Eluent: $NaNO_3$ 200 mM. Detection: fluorimetry at 280/335 nm.

A protein-type family in the F1 fraction

With fluorimetric detection set to 345/475 nm, the F1 fraction differs from the three other fractions by the presence ca. 9.3 min of a chromatographic peak of low intensity (see Fig. 1); its retention time is independent on the eluent salt concentration. With fluorimetric 280/335 nm detection, this difference becomes drastic. Thus, by eluting with 200 mM $NaNO_3$ (Fig. 2), the four fractions exhibit similar chromatograms in the 14-22 min time domain, but the chromatogram of the F1 fraction is characterised by a very strong fluorescence peak at 9.4 min.

Hydrophobic compounds

By comparing the chromatogram (Fig. 1) obtained with the mobile phase water/methanol which removes, or at least reduces, the hydrophobic interactions and the chromatogram with water/sodium nitrate 20 mM, we do not observed adsorption phenomena.

On the other hand, few small chromatographic peaks appear at retention times higher than *Vp i.e.* 18.4 min with the eluent 200 mM $NaNO_3$. This may arise either from the retention of cationic compounds by the gel electronegative sites, or from the adsorption of hydrophobic compounds on the non-polar sites. The electrostatic retention is usually eliminated by salt addition, which results in an opposite effect on the hydrophobic interactions. We observed that the addition of 30 % methanol in the salt eluent decreases the retention time of these peaks, which tends to approach the *Vp* value; no other chromatographic peak appears.

This indicates that there are only weak hydrophobic adsorptions.

Anionic and cationic compounds without UV absorption (254 nm) nor fluorescence (280/335 nm-345/475 nm) signature

The comparison between the fluorimetric chromatograms and those revealed by ELSD detection is interesting (Fig. 3,

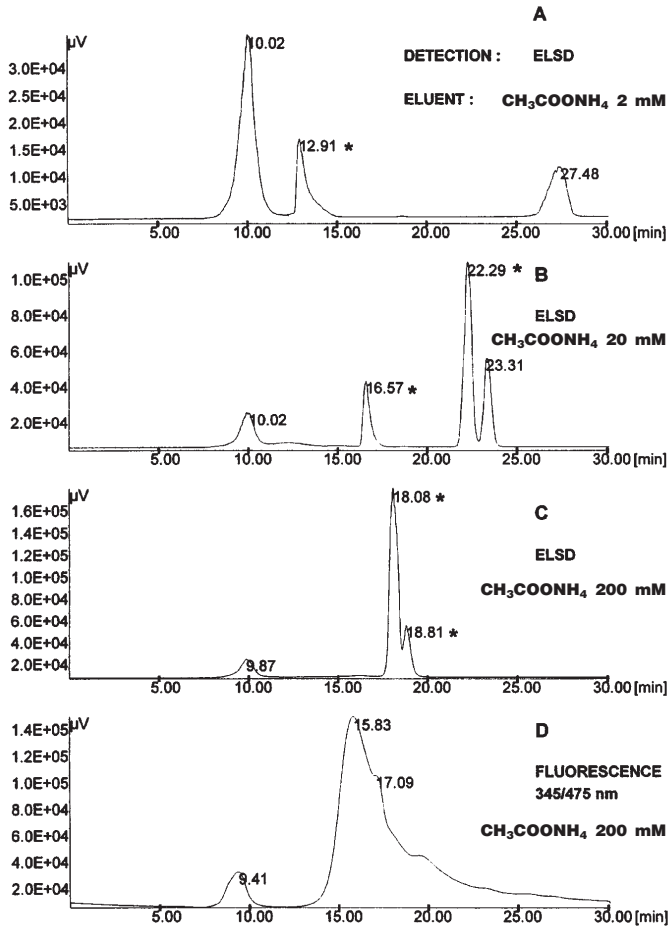

Figure 3. Chromatograms of the F1 fraction using the G3000 column: effect of elution conditions (salt concentration in the eluent) and of detection mode (ELSD and fluorimetry). The retention time of NaCl is labelled by an asterisk.

C and D). The prominent part of the fluorimetric massif (15-17 min) only yields a weak response in ELSD detection; on the contrary, two significant ELSD overlapping peaks are observed ca. 18-19 min in all the fractions whereas the fluorescence intensity remains weak. We made attempts to ascertain whether part of this signal could arise from the mineral fraction of the ultrafiltrates. This one primarily consists of sodium, chloride, carbonate and ammonium ions. The F4 fraction contains 80 % of them. The ions carbonate and ammonium are evaporated under the ELSD operating conditions but the sodium and chloride (≈ 150 mg/L in the F1-F3 fractions; 1.3 g/L in F4) ions are detected by this device in the form of two peaks. The comparison between the intensities of the chromatographic signals of the fractions and those of pure sodium chloride solutions at similar concentrations, shows that, for the fractions, other compounds that the ions mentioned above elute at the same retention time. On figure 3, the asterisked peaks correspond to elution times of NaCl. The retention of these peaks is dependent on the eluent salt concentration (Fig. 3, A, B, C). As this concentration decreases, the elution of the peak at 18.81 min is advanced whereas that of the second, at 18.08 min, is delayed so as not to appear on the chromatogram obtained with the 2 mM eluent. One may conclude that the two groups of substances observed with the ELSD are of anionic and cationic nature, respectively. Regarding the compounds chromatographic resolution, as long as the salt concentration is finely tuned, the low ionic strength eluents appear optimal to separate a maximum of compounds. Thus, for the F4 fraction one obtains many chromatographic peaks by using a 2 mM eluent (Fig. 4) by promoting the ionic, instead of steric, exclusion. To elute the compounds of cationic nature, the salt concentration must be increased up to 20 mM at least.

Estimation of molecular weights

Finally, with an eluent at 200 mM in salt, which eliminates most of the electrostatic interactions, whether attractive or repulsive, one can estimate the molecular weights, since steric exclusion is then the dominating mechanism of separation. However, because of the diversity of the compounds occurring in the leachates and, ignoring *a priori* their chemical nature or molecular sizes and forms, the choice of PEO and PEG as calibration standards is puzzling. Nevertheless, we used the calibration curve obtained with these standards under similar elution conditions (see Material and method), and the results were expressed in terms of Apparent Molecular Weight (AMW). Thus we estimate that the anionic humic-type compounds present in the four fraction have AMW between 300 and 1000 Da and that the anionic and cationic compounds without fluorescence nor UV absorbance, occurring in the four fraction, exhibit AMW < 200 Da. In the F1 fraction, the protein-type compounds eluted at the exclusion total volume exhibit AMW ≥ 10^6 Da; this drove us to further characterise this fraction by using other separation columns.

Study of the F1 fraction on the G6000 and G5000 columns in series

The columns G3000 and G6000 are more particularly fitted for the separation of compounds of AMW respectively lower and higher than 100000 Da. The G5000 column is intermediate between these two columns. The G6000-G5000 combination gave the best results in term of separation. Moreover, the G5000 and G6000 columns bear electronegative loads in less quantity than the G3000 column [11]; their ability to exchange cations is thus weaker. Indeed, by using a 2 mM ammonium acetate eluent, all the cations are eluted which is not the case with the G3000 column. At this salt concentration, the ELSD detection (Fig. 5) reveals:

– Cationic compounds at 37.4 min (in coelution with the Na^+ ions) and at 41.9 min. They are eluted at 21.6 min when the eluent is 200 mM. These compounds exhibit no fluorescent signal under the conditions used (Fig. 6).

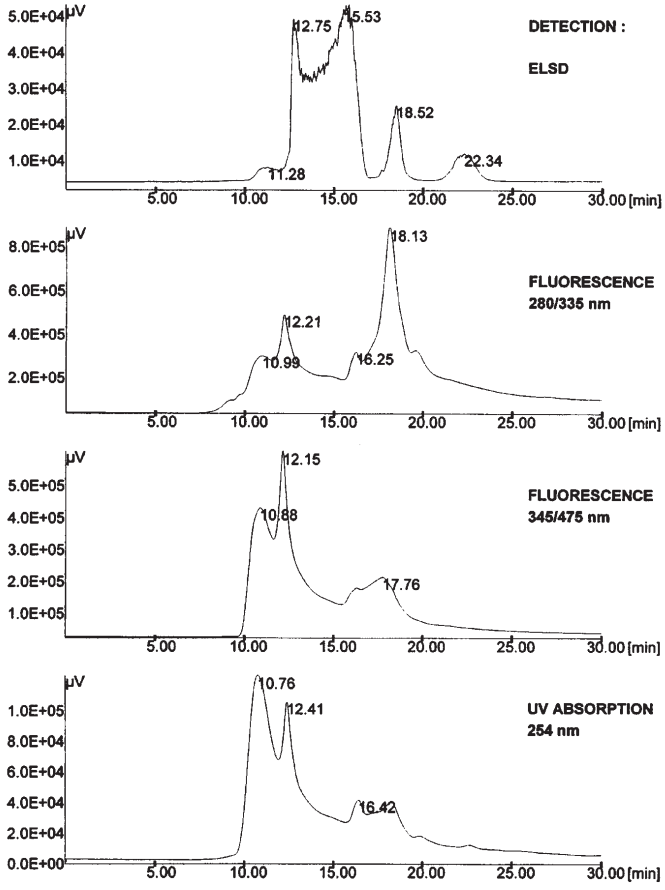

Figure 4. Chromatograms of the F4 fraction using the G3000 column: effect of the detection mode. Eluent: 2 mM $CH_3CO_2NH_4$.

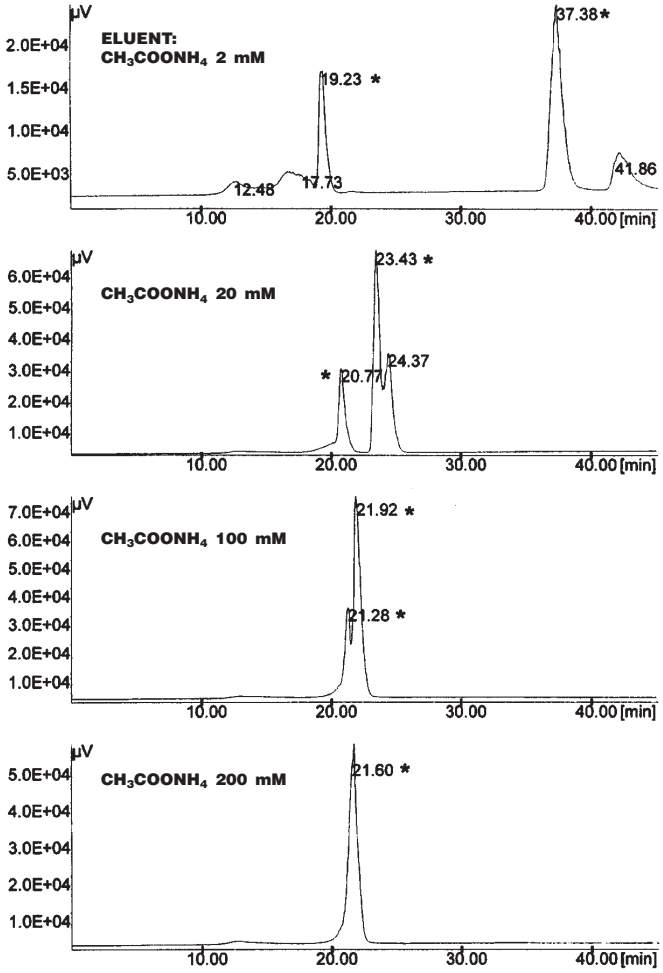

Figure 5. Chromatograms of the F1 fraction using the G6000 and G5000 columns in series: effect of the salt concentration in the eluent. Detection: ELSD. The retention time of NaCl is labelled by an asterisk.

– Anionic compounds at 19.2 min, which coelute with the Cl^- ions; they are characterised by a retention time which approaches that of the cationic compounds (21.6 min) when the salt concentration of eluent increases.

– Two broad but weak signals at 12.5 and 17.7 min, which are better observed with fluorimetric detection set to 280/335 nm (Fig. 6). Indeed, three main peaks (quoted 1 to 3) at 12.4, 15.5 and 18.17 min, respectively are observed, the peak 3 exhibiting a strong fluorescence at 345/475 nm. The evolution of retention times of these three peaks as a function of the salt concentration in the eluent is summarised in table II.

We observe that the peak #1 is little affected by the ionic strength of the eluent: its retention time remains close to 12.5 min whatever the salt concentration. On the other hand, the two other peaks exhibit an anionic character since their retention time increases with the salt concentration of eluent.

The F1 fraction thus consists of several groups of compounds, which AMW can be estimated by calibrating the series of columns under the same conditions of elution (*cf.* Materials and methods). We detect thus in the F1 fraction:

– A first protein-type group, which presents a wide range of AMW (broad chromatographic peak) centred on 1000000 Da;

Table II. Effect of the salt concentration in the eluent on the retention time (min) of the F1 fraction chromatographic peaks.

[Salt] in eluent	*2 mM*	*20 mM*	*100 mM*	*200 mM*
Peak # 1	12.43	12.58	12.71	12.69
Peak # 2	15.58	18.08	18.75	18.83
Peak # 3	18.17	20.34	20.85	21.02

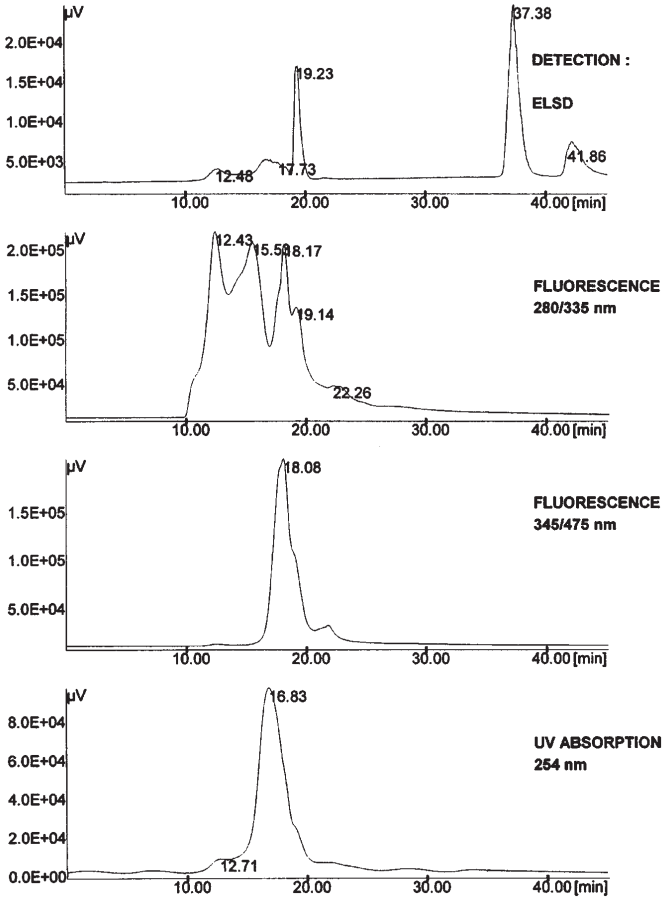

Figure 6. Chromatograms of the F1 fraction: effect of the detection mode. Columns: G6000 and G5000 in series. Eluent: 2 mM $CH_3CO_2NH_4$.

– A second group of protein-type compounds with anionic nature and a broad range of AMW centred on 10000 – 20000 Da;

– Humic and protein compounds with anionic character, of low AMW, (less than 1000 Da);

– A family made up of anionic compounds that elute with the chloride ions with AMW ≤ 200 Da;

– Two families of cationic compounds without spectroscopic signature under the conditions used, with weak AMW (< 200 Da).

Discussion

The presence in the F1 fraction of compounds with AMW lower than the threshold of the ultrafiltration membrane cut off (10000 Da) which have been already highlighted by using the G3000 column, is thus confirmed. One can hypothesise that these compounds are molecules trapped in structures of high molecular weights, but the membranes also carry charges that can cause the rejection of small molecules by electrostatic interactions, even though the pores are sufficiently large to let them go through [12]. These observations lead us to recommend cautiousness when ultrafiltration membranes are used to determine the molecular weight distribution of the leachates components.

UV absorbance and fluorescence, frequently used in chromatography detection are not sufficient to characterise the leachates organic matter. Some molecules, probably at high concentration, as seen from strong ELSD signals, do not generate significant UV or fluorimetric signals. This characteristic was also pointed out by Mejbri *et al.* [13] which reported variable Chemical Oxygen Demand/UV Absorption (254 nm) ratios: weak (< 100) for hydrophobic compounds and strong (1200) for compounds not retained on XAD resins. These observations reinforce those made by Crozes and *et al.* [14], who measured the UV absorption and the Total Organic Carbon (TOC) of chromatographic eluates of urban wastewater. Their results show that these values are not correlated. Some fractions exhibit significant UV absorbance along with a low TOC, characteristic of the unsaturated nature of the molecules; conversely, in other fractions, high TOC/UV Abs ratios are observed, suggesting the presence of saturated compounds.

Generally, it is tricky to compare results obtained by different techniques of fractionation. However, one finds some convergence between the present data and previous studies. As a matter of example, Mejbri *et al.* [13] points out that the substances retained on XAD4 resin exhibiting low UV absorbencies, are low molecular weights, hydrophilic compounds. Accordingly, the present work highlights anionic and cationic compounds, which lack spectral characteristics and whose molecular weights are below 200 Da. This author also notes than hydrophobic compounds retained on the XAD7 resin with strong UV absorbance, exhibit high molecular weights and constitute the major part of the leachate compounds. In comparison with the semi-quantitative information brought by ELSD detection (see Ref. [1]), it arises that the situation is reversed in the leachates we studied; hence, our conclusions are closer to those of Trebouet *et al.* [15] who observed a prominent fraction of AMW lower than 500 Da.

Conclusion

Whenever the secondary interactions are eliminated by salt addition in the eluent, the HPSEC allows to study the molecular weights distribution of the leachates compounds. The present study clearly indicates that they are dominated by low molecular weight molecules (AMW < 1000 Da) which are not optimally resolved by HPSEC.

Conversely, by making profitable the secondary interactions, one obtains a better chromatographic resolution since, in the leachates, many ionic or polar *species* interact with the negative sites of the stationary phase, inducing thereby

significant modifications of retention times. We therefore propose that a weak cation-exchange column must be used to perform an optimal separation method.

The eluted compounds were monitored after their UV and fluorimetric characteristics as well as by using an evaporative light scattering detector. The latter had allowed observing the presence of substances without spectroscopic signals under the conditions of the study. Nevertheless, UV and fluorimetric detections still remain appropriate to highlight the humic- and protein-types compounds. Clearly, it will be necessary to associate chromatographic separation with structural characterisation techniques, such as mass detection, to further progress in the elucidation of the leachate composition.

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