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Effect of extraction method on EPS from activated sludge: An HPSEC investigation

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Abstract

The extracellular polymeric substances (EPS) contained in activated sludge flocs resulting from two-sewage treatment plants were extracted according to eight methods referred to in the bibliography. Extracted EPS were characterized by their extraction yield, carbon concentration, their biochemical composition, their HPSEC chromatograms and, where possible, molecular weight (MW) distributions. With HPSEC chromatograms, the use of the mobile phase containing methanol allowed a hydrophobic mechanism for EPS, extracted partly by chemical methods, to be identified. An MW distribution (from 0.1 to 600 kDa) was established for EPS extracted by control and physical methods only, from calibration. Except for the resin and heating extraction methods, the EPS extracted from the two sludges displayed the same trend in their HPSEC fingerprints but not in their MW distribution. Results show that the extraction methods using chemical reagents strongly affected the HPSEC fingerprints of EPS, whereas, the physical methods influenced only MW distribution but not HPSEC fingerprints. The use of heat to extract EPS seems to induce hydrolysis of a part of EPS. The HPSEC fingerprint is a good indicator for the appreciation of the consequences of EPS extraction methods on the EPS extracted and the distribution of EPS with low MW in particular.

Keywords: Activated sludge; EPS; Extraction method; HPSEC chromatography

1. Introduction

Extracellular polymeric substances (EPS) represent a major component of activated sludge flocs, biofilms or microbial granules [1,2]. They play a major role in flocculation [3], settling and dewatering of activated sludge [4–6]. EPS and microbial cells inside the flocs are cross-linked forming a polymeric network, which with its pores and channels, is capable of adsorbing nutrients, minerals, pollutants and heavy metals [7–10]. EPS may be classified as either weakly (called "soluble") or strongly (called "bound") bound to the flocs [11].

EPS result from active bacterial secretion, cell lysis and molecules from effluents [12]. Frölund et al. [13] showed that EPS consist essentially of a variety of organic substances: carbohydrates [14] and proteins [15] as major constituents and humic substances [16], uronic acids and nucleic acids [17] in smaller quantities. The EPS composition depends on wastewater type and the operating conditions of the treatment plant [18].

There are many methods proposed for extracting EPS [2]. They can be extracted by centrifugation alone but most researchers treat the sludge by various physical and/or chemical means in order to increase the amounts of EPS released by flocs. Physical extractions include sonication and ultracentrifugation [15], cation exchange resin [13] and heating [19], whereas, common chemical extractions include use of alkaline reagents [13], EDTA [9] and aldehydic solutions [20,9]. Comte et al. [21,22] compared eight EPS extraction methods and analyzed their influence on the EPS composition as well as biosorption properties towards Pb and Cd. Their findings show that, where extraction was carried out by a chemical method, there was contamination of EPS by the chemical extractant. The EPS characterizations show qualitative and quantitative differences depending on the method used. Moreover, the biosorption properties of EPS for Pb and Cd could also be greatly affected by the extraction method used.

Until now mainly colorimetric methods have been used to characterize EPS, giving a total amount of polysaccharides or

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proteins for example [12]. Several authors [13,23–25] have studied the molecular weight (MW) distribution of the EPS, which according to Nielsen et al. [17], can be a useful tool for obtaining the EPS fingerprint.

Gel electrophoresis [11,26], low-pressure size exclusion chromatography (SEC) [1,27] and recently, high-pressure size exclusion chromatography (HPSEC) [13,25,28,29] have been used to determine the EPS MW. Higgins and Novak [11] evidenced a single "lectin-like" protein of about 15 kDa in activated sludge samples from different sources, SEC users have generally detected three peaks with MW ranging from 1 to 10^3 kDa for EPS and HPSEC users have revealed that EPS can be separated into a chromatographic profile composed of seven peaks ranging from 1 to $6.7.10^3$ kDa. Garnier et al. [29] investigated the molecular weight and nature of EPS from different activated sludges. Their results show that the molecular weight of proteins varied from small (10 kDa) to large (600 kDa), while all polysaccharides were smaller than 1 kDa.

The HPSEC method was successful in showing differences and similarities between EPS from two different sludge treatment plants [30], showing the degradation of EPS with sludge storage time [17] and the impact of extraction conditions on chromatographic fingerprints [31,30]. The aim of this study was to compare the impact of eight extraction procedures tested on activated sludge EPS, on the EPS fingerprint and eventually distribution size with HPSEC.

2. Materials and methods

2.1. EPS extraction

EPS extraction was carried out on two activated sludges obtained from the aeration tanks of two wastewater treatment plants (WWTP), called A and B, in order to obtain two EPS with different compositions. The WWTPs A and B presented different characteristics. The treatment capacity in inhabitant equivalent is 285,000 and 4000 for WWTP A and B, respectively. The organic loads are between 0.24–0.30 and 0.13–0.16 kg BOD_5 m^{-3} day^{-1} for WWTP A and B, respectively.

For all extraction protocols used, the sludges were concentrated by centrifugation at 4300 × *g* for 10 min, using an MR 23i (JOUAN) type centrifuge before extraction. The residues were recovered and re-suspended in ultra-pure water. EPS, for each sludge (A and B), were extracted using eight methods referenced in the literature [21].

One, a physical method, called the "control method", involved centrifugation (4000 × *g*) for 20 min, at 4 °C [2]. Three others were based on the use of chemical reagents: EDTA 2% (Prolabo, 99%) for 3 h at 4 °C [9], formaldehyde (Prolabo, 36.5%) for 1 h at 4 °C and then NaOH 1 M (98%, Prolabo) for 3 h at 4 °C [9], and glutaraldehyde (Aldrich, 25%) 10% for 12 h at 4 °C [20]. The other four were based on physical processes: sonication at 40 W for 2 min [8] using a sonopuls GM 70 (Bandelin) device, cation exchange resin (Dowex 50 × 8, Fluka) for 1 h at 4 °C [13], sonication at 40 W for 2 min associated to cation exchange resin (Dowex 50 × 8, Fluka) [32], and heating for 10 min at 80 °C (1 bar) [15]. The separation of EPS from treated sludges (control excepted) was carried out by two ultracentrifugations: the first at 20,000 × *g* for 20 min at 4 °C and the second at 10,000 × *g* for 15 min at 4 °C.

Finally, all extracted EPS were purified according to Liu and Fang [9]: 100 mL of EPS solution contained in a dialysis membrane (Cellu Sep, 3500D) were placed in 1000 mL of ultra-pure water for 24 h at 4 °C. The EPS were stored at −18 °C before use.

2.2. Main extracted EPS characteristics

The main characteristics of extracted EPS are summarized Table 1 [21]. EPS A and B were mainly composed of proteins, polysaccharides and humic substances for EPS extracted by control method and physical methods. The chemical extractions presented the advantage of the highest extraction yield but the composition of EPS was different from that of the EPS control and the EPS solutions were contaminated by extracting reagents.

The extent of contamination by molecules due to cell lysis during extraction is difficult to measure in undefined samples and measurement of specific enzymes (such as glucose-6-phosphate dehydrogenase) was not applicable for alkaline samples, samples treated by aldehyde or by heat, since high pH, heat, or alkylation are known to disrupt or greatly modify macromolecules such as enzymes and proteins [33]. Other authors have used the ratio of protein/polysaccharide or the nucleic acid content of the EPS extracted to estimate/give an idea of the extent of EPS solution contamination by molecules due to cell lysis during extraction [2]. Since the ratio of protein/polysaccharide was close to 2.3 [21] and the nucleic acid content was low (Table 1), it would appear that the EPS extracted in this study was not contaminated by a significant amount of intracellular materials [21].

2.3. HPSEC analysis of EPS

The separation of extracted EPS was carried out with a Merck Hitashi LA Chrom chromatograph equipped with an L7200 autosampler, an L7100 pump, an L7000 interface and a diode array UV detector (L7455). The HPSEC method was carried out with two columns: an Amersham Biosciences, Superdex peptide 10/300 GL and an Amersham Biosciences, Superdex 20010/300 GL.

All measurements were made using a mobile phase flow of 0.5 mL/min.

The detection was carried out at 25 °C with a diode array UV detector at several wavelengths (210, 260 or 280 nm). In the literature, UV detection at 280 nm is commonly used for EPS, but 260 nm [34] or 210 nm [25] wavelengths are also found. All samples were filtered with 0.20 μm filters (Millipore) prior to injection (40 μL).

Three polymer standards (α-poly(styrenesulfonic acid sodium salt) at 0.25, 1.4 and 4.3 kDa, FLUKA) were injected with a linear range between 0.1 and 7 kDa for the first column and five protein standards (Chymotrypsinogen 20.4 kDa, Albumin 67.4 kDa, Adolase 177 kDa, Ferritin 438 kDa and Thyroglobu-

Table 1
Extraction yields in % (EPS DW/TSS sludge) and biochemical composition of extracted EPS (in mg g^{-1} of EPS SS) [21]

Methods	Extraction yield %	Proteins	Polysaccharides	Humic substances	Uronic acids	Nucleic acids	%C (g^{-1} MS)
Control							
A	0.8	317	170	63	21	40	20
B	1.0	249	157	149	59	11	20
Chemical methods							
EDTA							
A	19.3	9	24	86	6	8	35
B	19.2	5	31	120	19	2	33
NaOH+							
A	39.0	107	53	83	85	9	10
Formaldehyde							
B	47.0	73	43	74	52	6	7
Glutar aldehyde							
A	25.1	129	ND	516	ND	81	ND
B	17.3	6	ND	363	ND	76	ND
Physical methods							
Sonication							
A	1.9	343	141	61	15	46	20
B	2.3	337	136	177	55	11	23
Resin							
A	2.5	301	132	107	47	24	14
B	3.1	322	126	129	54	16	17
Sonication + resin							
A	3.7	252	103	126	47	25	13
B	2.7	266	113	156	50	35	16
80 °C							
A	3.6	378	166	126	37	17	37
B	4.1	296	183	57	30	10	38

ND: not determined.

lin 703 kDa, Amersham Biosciences) were injected with a linear range between 100 and 600 kDa.

In our study, the mobile phase consisted of 9.0 mM NaCl, 0.9 mM Na_2HPO_4 [25]. An other mobile phase consisting of 5% (v/v) MeOH, 9.0 mM NaCl, 0.9 mM Na_2HPO_4 was also used in order to appreciate hydrophobic interactions between column material packing and EPS.

Frölund and Keiding [35] tested different pH values for a mobile phase and their results showed that optimum performance occurred at a pH of 7. A pH of 7 was used for all mobile phases in this study.

Görner et al. [25] compared different mobile phases and underlined that the change in ionic strength ($I = 0.26$–0.02 M) did not modify the peak retention times and the number of peaks determined for EPS. Thus, for these authors ionic exchange between EPS and column packing material did not occur. In consequence, in this study, such interactions have not been considered.

According to Frölund and Keiding [35], in order to obtain a pure size-exclusion separation, the sample compounds must elute according to their hydrodynamic volume. Hence interaction between sample components and column packing material, for example, ion adsorption, ion exclusion or hydrophobic adsorption, could occur. Such interactions influence EPS separation by steric exclusion chromatography. As a consequence, the absence of the interactions between column packing material and EPS compounds must be verified in order to calculate the MWs of EPS from a calibration curve. The use of a methanol mobile phase highlighted the presence of hydrophobic interactions between EPS and the column packing material used for the separation [25]. It was thus necessary to verify the presence of such interactions between EPS and column packing material. In order to identify or not the presence of a hydrophobic retention mechanism for EPS on the columns, the chromatograms performed with and without the methanol mobile phase were compared for both columns according to Görner et al. [25].

3. Results

3.1. Choice of working wavelength

Examples of HPSEC chromatograms recorded at a wavelengths 280, 260 and 210 nm are presented in Fig. 1. The results show that, for both columns, the chromatograms of EPS (A and B, whatever the extraction method used) at 280 and 260 nm were not different. Nevertheless, it can be seen that the absorbance of EPS is slightly higher at 260 nm than at 280 nm. At 210 nm, no absorbance was recorded for any of the EPS. Frölund and Keiding [35] studied EPS from activated sludges in HPSEC and found no additional information for 260 nm compared to 280 nm. Our

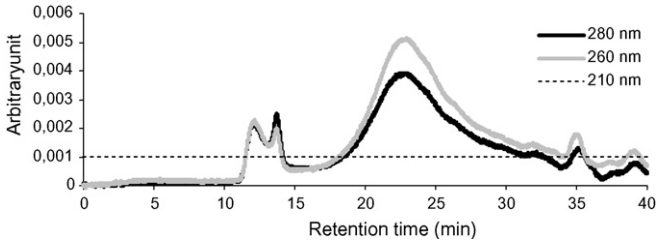

Fig. 1. Example of a chromatogram of EPS B extracted using the control method at 280, 260 and 210 nm wavelengths (column 0.1–7 kDa).

results were in accordance with the literature [25]. In this study, we choose to record the EPS chromatogram at a wavelength of 280 nm.

3.2. Mechanisms implicated for separation of EPS by HPSEC

Fig. 2 illustrates differences (Fig. 2a) and similarities (Fig. 2b) between chromatograms for both types of mobile phase (with and without methanol). For a same extraction method and for both EPS A and B, results obtained were the same for both columns. With the methanol mobile phase, the retention times of several peaks for chromatograms with EPS extracted by the method using formaldehyde + NaOH or glutaraldehyde were modified. The chromatograms of EPS extracted with control and physical methods do not show significant differences with or without methanol in the mobile phase (results not shown). According to Görner et al. [25], the use of the methanol mobile phase demonstrated the presence of hydrophobic retention mechanisms on the columns for EPS extracted by formaldehyde + NaOH and glutaraldehyde. The absence of hydrophobic retention mechanisms, which therefore does not allow apparent molecular weight as function of retention time to be calculated, with the EPS extracted by the other methods led to the conclusion that the peaks with low retention times corresponded to high MW molecules, whereas, the peaks with high retention times corresponded to low MW molecules.

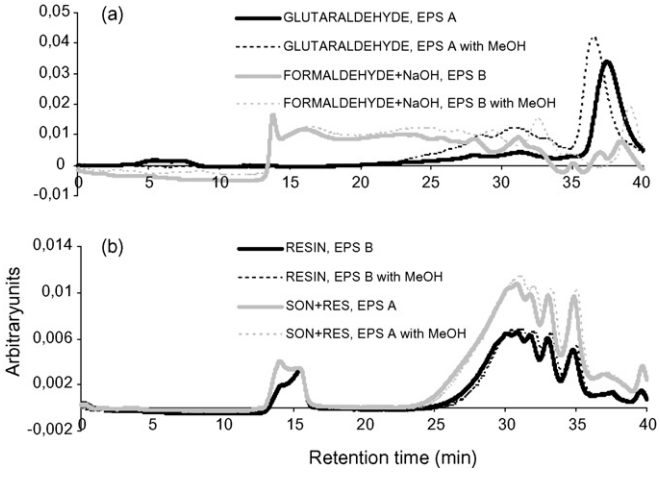

Fig. 2. HPSEC chromatogram of EPS without and with MeOH in the eluent phase: (a) column 0.1–7 kDa; (b) column 10–600 kDa.

However, the results of this study were different from those of the literature. Frölund and Keiding [35] and Görner et al. [25] demonstrated the presence of hydrophobic interactions between activated sludge EPS extracted by cationic resins (DOWEX) and the column used (Chrompack P 1000 GFC column 250 mm × 7.7 mm i.d. and Zorbax Bio Series column GF-250 9.4 mm i.d. × 25 cm). The composition of the packing material in the HPSEC column, the origin of the sludges used to extract EPS or the protocol with DOWEX resin used for extraction could explain such disparities between the results of this study and those of Frölund and Keiding [35] and Görner et al. [25].

3.3. Effect of the origin of EPS on HPSEC fingerprint

Chromatographic fingerprints of EPS A and B extracted by control and resin + sonication methods are presented Fig. 3 as examples. Note that the same results were obtained whatever the method used *i.e.* that EPS A and B had close chromatographic fingerprints for a same extraction method, whatever the method used. Afterwards, only the results of EPS A are presented as conclusions, from fingerprints, for EPS A and EPS B were similar.

Nevertheless, it can be seen that the distribution of MW for the EPS A and B seems to be different. Indeed, even if for EPS A or B, the retention time of each peak was the same, the area of the chromatographic peak could have been affected by the origin of EPS. Biochemical characterization (Table 1) revealed that EPS A contained more proteins and less humic substances than EPS B. Such differences in biochemical composition might explain the differences between the area of peaks for the EPS A and B chromatograms.

3.4. Influence of chemical extraction method on HPSEC fingerprints of EPS

The chromatograms of EPS A extracted by the control method and the chemical protocols are presented Fig. 4 for both columns used. Differences between chromatographic fingerprints of EPS extracted by the control and the other methods are noted. On Fig. 4a, EPS extracted with EDTA give a different fingerprint from those extracted by the control method as their chromatogram has a band with a retention time of 32 min. EPS extracted by formaldehyde + NaOH had a specificity in their fingerprint in that they had no molecules with a retention time inferior to 14 min. The EPS extracted with glutaraldehyde exhibited a band around 37 min but EPS extracted by the control method did not present a such band. On Fig. 4b, a difference between EPS fingerprints can be seen. Unlike the chromatogram of EPS extracted by the control method, the chromatogram of EPS extracted with EDTA has a peak eluted at 36 min, EPS extracted with formaldehyde in an extreme environment had no peak eluted between 10 and 20 min and EPS extracted with glutaraldehyde had not eluted at 30 min as well many molecules whose retention time was superior to 35 min. Note that the same observations were made for EPS B under the same extraction conditions.

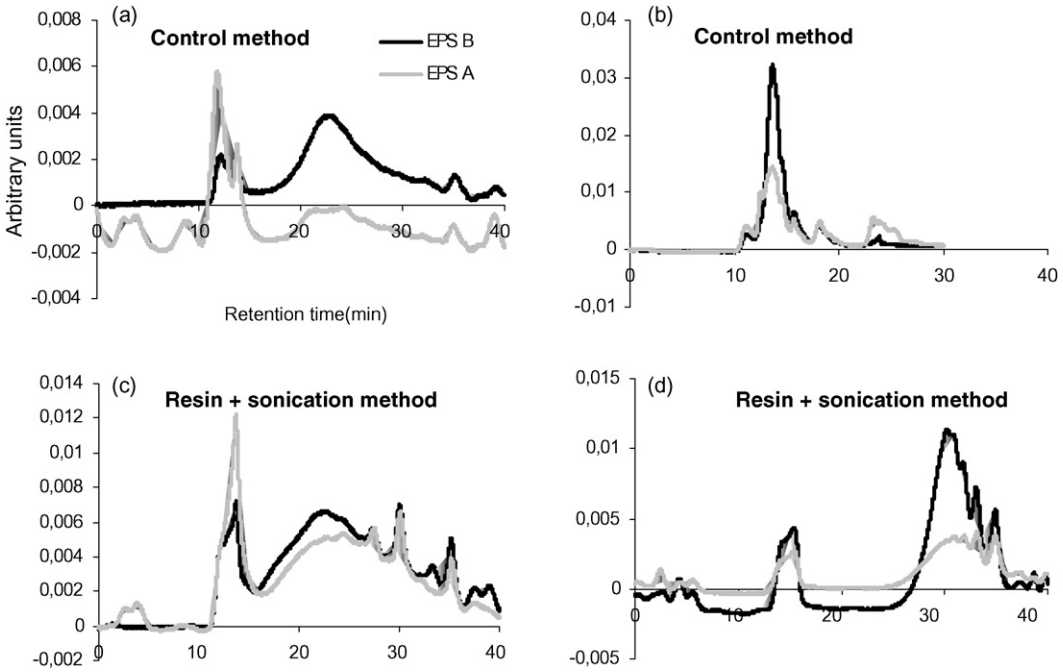

Fig. 3. Chromatogram of EPS A and B extracted from control and resin + sonication methods: (a and c) column 0.1–7 kDa; (b and d) column 10–600 kDa.

3.5. Influence of physical extraction method on HPSEC fingerprints of EPS

The HPSEC chromatograms obtained for EPS A with the control and the physical methods are presented on Fig. 5. The chromatographic fingerprints were very similar whatever the column used. For the exclusion column 0.1–7 kDa (Fig. 5a), the presence of four groups of EPS peaks can be seen: one group whose the retention time was less than 10 min, two peaks eluted at 12 and 14 min, another whose retention time was between 18 and 28 min and two peaks eluted at 35 and 39 min. As for the EPS extracted by resin, sonication + resin and heating methods, the peak eluted at 12 min shows a large decrease in area. At the same time as this peak disappears, for the heating method, a large increase in molecules whose the retention time was between 18 and 28 min can be seen.

For the 10–600 kDa separation column (Fig. 5b), several peaks or groups of peaks can be observed depending on the extraction method. With the column for separation of molecules with a high MW, two groups of EPS peaks, molecules with a retention time between 14 and 15 min and molecules with retention time between 30 and 40 min, can be seen. All of the HPSEC fingerprints obtained using physical extraction methods were similar to that of the control method. However, except for the control, the fingerprints of EPS extracted by physical methods showed molecules with a

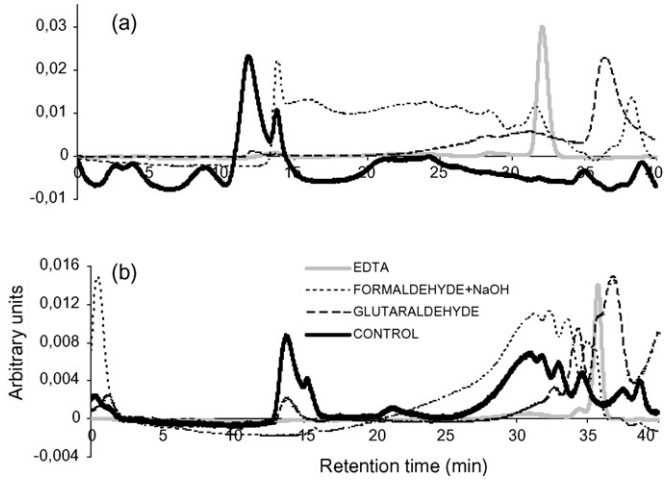

Fig. 4. Chromatogram of EPS A extracted by the control method and chemical methods: (a) EPS A (280 nm, column 0.1–7 kDa); (b) EPS A (280 nm, column 10–600 kDa).

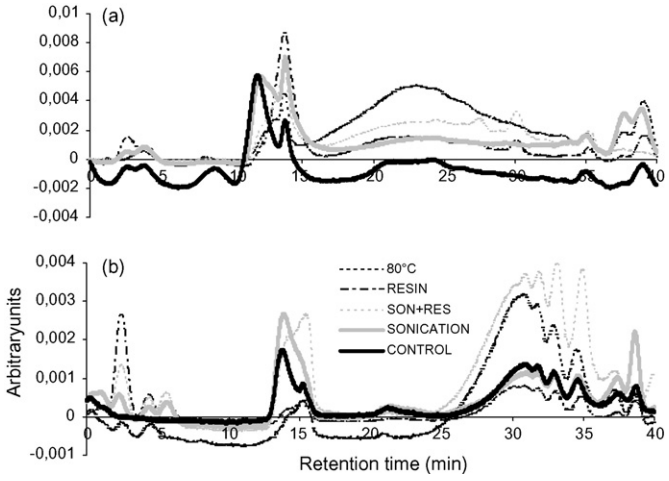

Fig. 5. Chromatogram of EPS A extracted by the control method and physical methods: (a) EPS A (280 nm, column 0.1–7 kDa); (b) EPS A (280 nm, column 10–600 kDa).

retention time inferior to 6 min, corresponding to molecules with high MW.

3.6. MW determination and distribution of EPS extracted by physical and control methods

Concerning the EPS extracted by the control and the physical methods, no mechanism of hydrophobic retention on the column packing material has been identified. It is therefore possible to associate the retention time with the MW of EPS (using the MW standard calibration curve). The MW distributions of EPS A and B extracted with the control and the physical methods are given in Table 2 for each column. Four groups of peaks for the 0.1–7 kDa column and two groups of peaks for the 10–600 kDa column have been distinguished.

The precedent results (Figs. 3 and 5) show that HPSEC fingerprints of the EPS A or B extracted with control or physical methods are very similar. The results (Table 2) demonstrate that the MW distribution can be different according to both (a) the control or physical methods and (b) for the EPS A or B. For the EPS with low MW, there was very little of the MW fraction between 0.16 and 0.3 kDa whatever the extraction method used and for both EPS A and B (except for EPS A extracted with sonication + resin). As for the control, sonication and sonication + resin extractions: for the EPS A, the greatest MW fraction represented was the 4.6–6 kDa fraction, whereas, for the EPS B, it was 2.7–0.7 kDa one. For both EPS A and B, extracted with resin and heating, the greatest fraction was 2.7–0.7 kDa. Concerning the presence of peaks with a retention time of less than 10 min, the MW attribution was not possible because it was out with the specificity of the 0.1–7 kDa column used. Table 2 (10–600 kDa column) shows very similar MW distributions for EPS with high MW depending on the extraction method and for EPS A or B, with a majority of MW between 16 and 190 kDa, except for EPS extracted by heating. Thus, the presence of molecules with low MW (0.1–7 kDa) underlines differences between EPS depending on their origin or extraction method used.

4. Discussion

The study of the chromatographic fingerprints and MW distribution of EPS has opened up a new approach to EPS characterization. In the present study, the use of two columns has allowed a better separation of EPS to be obtained. More peaks were separated than in previous studies as in the literature, only a maximum of seven MW peaks have been reported [35,13,25] due to the use of only one HPSEC column.

The results show that chromatographic fingerprints of EPS A and B were very similar for a same extraction method. Frölund et al. [30] studied EPS (extracted with two centrifugations of 15 min at 12,000 × *g*, 4 °C) from two different WWTPs: one working with carbonic compounds, the other working with nitrogen and phosphoric. Although EPS had different origins they did have very similar chemical compositions and HPSEC fingerprints. In our study and that of Frölund et al. [30], the HPSEC fingerprint of EPS was not characteristic of the origin of the sludges from which EPS were extracted, for the physical methods of extraction.

However, in our study, the HPSEC fingerprint of EPS did show differences according to the extraction method used. While HPSEC fingerprints of EPS extracted by physical methods were close to those of the control EPS (except for extraction by heating), the HPSEC fingerprints of EPS extracted by chemical methods were very different from those of the control EPS and specific to the method used. The chemical extraction meth-

Table 2
Molecular weight distribution of EPS A and B (column 0.1–7 kDa and column 10–600 kDa)

Fraction of EPS according to the extraction method used (%)	Retention time (t) in min or molecular weight (MW) in kDa				
	0.1–7 kDa column			10–600 kDa column	
	$12 \le t \le 14$, $4.6 \le MW \le 6$	$18 < t < 28$, $2.7 \le MW \le 0.7$	$35 \le t \le 39$, $0.16 \le MW \le 0.3$	$14 \le t \le 15$, $270 \le MW \le 275$	$30 \le t \le 40$, $16 \le MW \le 190$
Control					
A	47	34	18	27	73
B	11	80	6	18	82
Sonication					
A	79	12	10	39	61
B	43	55	2	49	51
Resin					
A	32	60	8	16	84
B	29	63	7	31	69
Sonication + resin					
A	64	17	19	17	83
B	34	54	11	39	61
80 °C					
A	10	88	2	8	90
B	11	89	–	11	89

ods greatly affected HPSEC chromatograms of the extracted EPS. The modification of the HPSEC chromatograms could be linked to the presence of extracting reagent in EPS solutions. Comte et al. [21] have demonstrated the contamination of EPS by chemical extraction reagents with an IR study. On the one hand, the reasons for these modifications could be a modification of EPS by the presence of chemical reagents in the solution and on the other hand, in the case of EPS extracted by chemical methods, another assumption could explain the difference in HPSEC chromatograms: a different selective extraction of EPS from flocs depending on the chemical method used. But due to lack of knowledge of the mechanism of EPS extraction by chemical extractants and the location of the EPS, it is not possible to estimate the contribution of each of the reasons assumed to explain the difference in HPSEC chromatograms.

The chromatograms obtained for EPS extracted with EDTA were different from those of the control EPS, this could show the presence of molecules from an EDTA/EPS reaction.

In the case of EPS extracted with formaldehyde + NaOH or glutaraldehyde, the chromatograms obtained with the methanol mobile phase demonstrate the presence of molecules with hydrophobic properties unlike the control EPS. Perhaps these methods can selectively extract EPS with hydrophobic properties. Another assumption could be that formaldehyde or glutaraldehyde have a strong affinity for amine functional groups and can react with them. The alkylation of amine functional groups of EPS could generate an increase in the EPS hydrophobicity. The presence of a hydrophobic retention mechanism in the columns does not allow retention time and MW to be related.

In the case of EPS extracted with the control or physical methods, the results show that the EPS retention time can be associated with MW as no hydrophobic retention mechanism was observed between the EPS and the columns with the methanol mobile phase. In this study, the calibration obtained (constituted of polymers and proteins standards) demonstrates that an increase in the retention time corresponded to a decrease in MW of molecules for the both columns used. According to Frölund and Keiding [35], the relationship between molecular mass and retention time (or elution volume) is valid only for the specific compound used in the calibration. But the EPS are a mixture of complex compounds (Table 1), which are difficult to calibrate. Görner et al. [25] used two calibrations to characterize EPS: one with polysaccharides and one with proteins. However, Jorand et al. [4] think that EPS are mainly composed of glycoproteins. Thus the use of proteins, polysaccharides or other molecules for the calibration is questionable. Thus the MW determined for EPS could be only apparent. As a result, in our study, when it was possible (no hydrophobic interactions between column packing material and EPS), the relationship between MW and retention time relating to the calibration was established with a commercial standard in order to give apparent MW of EPS extracted from A and B sludges.

The HPSEC fingerprints of EPS extracted with control or physical methods were very similar (except for the heating method). Some additional peaks at less than 6 min were observed on the fingerprints of EPS extracted with physical methods for 10–600 kDa (Fig. 5b). This can indicate that physical methods extract additional molecules with higher MW than control molecules. The flocs dispersion was better with physical methods than with the control method, as the extraction yields (Table 1) show. The "bound" EPS can also be released in greater quantities and we can assume that there is a greater diversity of EPS MW.

Concerning the MW distribution of EPS, even if the HPSEC fingerprints were very similar, they varied depending on the control or physical extraction used and the EPS A or B considered. For EPS extracted by control, sonication and sonication + resin, differences in the MW distribution of the EPS between A and B were noticed according to the slight difference in biochemical composition (Table 1). Moreover, the MW distribution could be linked to differences in the kind of proteins and polysaccharides present in EPS A or B. For EPS A or B extracted by heating at 80 °C, the increase in the peak with the retention time between 18 and 28 min (Fig 3a) indicates the presence of low MW molecules ($2.7 \leq MW \leq 0.7$ kDa). This could be due to hydrolysis of some EPS on heating. Karapanagiotis et al. [36] compared the MW distribution of EPS from activated digested sewage sludge extracted using five methods including two methods involving heating (boiling and steaming). They showed that the MW distribution of proteins contained in the EPS extracted by heating was more heterogeneous than the proteins contained in the EPS extracted by the other methods (resin DOWEX, sodium hydroxide extraction, phenol treatment) suggesting deterioration of molecular integrity of the sample.

The results of Liu et al. [23] for EPS from activated sludges (extraction at $20{,}000 \times g$ for 30 min) indicate that the biggest EPS fraction (30.8%) had a MW between 15 and 30 kDa. Zhou et al. [37] indicated that 48.3% of EPS had a MW superior to 25 kDa. Our results (Table 2) are in accordance with the literature. The biggest MW of EPS fractions represented were between 16 and 190 kDa for 10–600 kDa columns.

The biochemical composition of EPS extracted with the control and physical methods (Table 1) showed that EPS were mainly composed of proteins, polysaccharides and humic substances. Garnier et al. [29] showed that the MW of proteins from activated sludge EPS varied from 10 to 600 kDa, whereas, the MW of polysaccharides were inferior to 1 kDa. In this study, we can assume that low MW EPS separated with the 0.1–7 kDa column were polysaccharides and high MW EPS separated with 10–600 kDa column were proteins.

For humic substances, uronic acids and nucleic acids, no data concerning their identification in HPSEC is available in the literature. The extraction method has a crucial impact on the EPS composition and properties. Comte et al. [21] have demonstrated qualitative differences for EPS according to the extraction method used. The biosorption properties of EPS are affected by the extraction method used [22]. Others EPS properties such as their hydrophobicity and their surface charge have been studied. Liao et al. [5] have shown that it is the surface properties, hydrophobicity, surface charge and composition of EPS, of sludge, rather than the quantity of EPS, that govern bioflocculation. The MW of EPS may have an important role in sludge flocculation. According to Horan and Eccles [27], high MW EPS lead to round strong flocs while Higgins and Novak

[11] observed deflocculation after the enzymatic digestion of exocellular proteins. This study has shown the importance of the choice of extraction method on the HPSEC chromatogram and MW of EPS. It is thus important to know the influence of the EPS extraction method used in order to study EPS properties, all the more so when the size of EPS influences its own properties.

5. Conclusion

The HPSEC fingerprint is a good indicator of the 'identity' of EPS and consequently helps appreciate the consequences of the extraction method on the EPS extracted. Nevertheless, HPSEC highlights, very clearly, differences between EPS, on studying the distribution of EPS with low MW (<7 kDa in our study).

The use of two mobile phases (one with methanol, the other without) allowed hydrophobic retention mechanisms between EPS and the packing material of the column for EPS extracted by glutaraldehyde and formaldehyde + NaOH to be observed. For the other EPS extracted, a MW distribution was established with commercial standard molecules.

The results of this study show the influence of the extraction methods on the HPSEC fingerprints. Chemical extractions greatly affect the HPSEC fingerprints, whereas, physical extractions (sonication, resin + sonication, resin) do not modify them but influence the MW distribution of EPS. The MW distribution can demonstrate differences between EPS A and B extracted by control, sonication and sonication + resin. The fingerprints of EPS do not show differences between EPS A and B contrary to the MW distribution. The change in MW distribution between EPS A and B could be linked to the "slight" difference in EPS biochemical composition and/or the nature of the molecules (amino-acids, mono-saccharides ...) found in proteins/polysaccharides of EPS. With MW distribution, the possible hydrolysis of EPS molecules extracted by heating (at 80 °C) can be demonstrated. The MW distribution acts as an indicator that helps appreciate differences in the composition of EPS extracted with control and physical methods as low MW seems to be characteristic of polysaccharides and high MW of proteins.

The extraction method for EPS affects the HPSEC fingerprint of EPS and their MW distribution. It thus has an important role in the study of the EPS properties where the size of EPS plays an important role in the actual EPS property studied.

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