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Purification of oligouronides using hollow-fiber membrane functionalised with L-histidine

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Abstract

The pseudobioaffinity chromatography with L-histidines immobilized onto poly(ethylene-vinyl alcohol) hollow-fiber membranes was studied for a selective purification of anionic oligosaccharides. Oligoglucuronans with different degrees of polymerization and acetylation, prepared by an enzymatic degradation of bacterial polyglucuronic acid, were used as models. The adsorption and elution parameters were studied to optimise the selective adsorption. A better understanding of the physico-chemical phenomena governing this selectivity was attempted and a few hypotheses on the mechanism of selectivity are proposed.

Keywords: Anionic oligosaccharides; Interaction; Purification; Pseudobioaffinity; L-histidine

1. Introduction

The pseudobioaffinity chromatography using L-histidines immobilized onto poly(ethylene-vinyl alcohol) (L-His-PEVA) hollow-fiber membranes was developed, in the last decade, to separate and purify proteins at different scales [1–3]. Separation and study of oligosaccharides substituted or not is becoming a limiting step in studying their biological activity for pharmaceutical and other applications. In our previous reports [4,5], it was shown that oligomers of glucuronan, a β-(1,4) linked polyglucuronic acid partially acetylated on C2 and/or C3, were retained on L-histidines immobilized onto poly(ethylene-vinyl alcohol) (L-His-PEVA). Nevertheless, the eventual interactions of neutral or ionic substituted sugars with this support for their efficient purification, despite their importance, have not been focussed much. This absence of study was principally due to the lack of availability in large quantities of oligoglucuronans resulting from the insufficient production strategies. The oligoglucuronans and their derivatives have been previously described as bioactive molecules on animals [6] and as elicitors of defence responses in plants as pesticides and fertilizers agents [7]. To circumvent the lack of availability in large quantities, the screening of glucuronan cleavage enzymes was undertaken and a new fungal glucuronan lyase was identified and characterized [8]. This enzyme, by depolymerization of the polyglucuronan allowed the production of large amounts of unsaturated oligoglucuronans acetylated or not. In order to envisage new biological applications with these original anionic β-glucosyl oligosaccharides, it was necessary to produce pure glycuronide compounds. The purpose of this paper is to investigate an affinity chromatography with L-histidine ligand for the selective purification of large quantities of oligouronates obtained by enzymatic degradations of glucuronan.

2. Experimental

2.1. Glucuronan and oligoglucuronan production

The *Sinorhizobium meliloti* M5N1CS mutant strain (NCIMB 40472) was cultivated at 30 °C in a 20-l reactor (SGI, Toulouse, France) containing 15 l of *Rhizobium* complete (RC) medium

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[9] supplemented with sucrose 1% (w/v) to produce the glucuronan [10]. This anionic homopolymer is an (1 → 4)-β-D-polyglucuropyranosyluronic acids mainly *O*-acetylated at C-3 position (PGUac). The inoculum was a 1.5 l of RCS medium containing *S. meliloti* M5N1CS and incubated 20 h at 30 °C on a rotary shaker at 100 rpm. After 96 h of incubation, the broths were centrifuged at 33,900 × *g* for 40 min at 20 °C. Polysaccharides in supernatant were then precipitated using three volumes of isopropanol and collected by centrifugation at 33,900 × *g* for 20 min at 4 °C. In order to produce deacetylated glucuronan (PGU), a standard glucuronan was treated overnight by KOH (2 M, pH 12) at 50 °C and recovered by alcoholic precipitation.

All oligoglucuronates tested were acquired by enzymatic degradation of two glucuronan families: PGUac and PGU (a deacetylated glucuronan), using a glucuronan lyase from *Trichoderma* sp. GL2 [8]. Glucuronan solutions (3%, w/v) in 50 mM phosphate buffer at 20 °C were incubated with enzymatic preparation at appropriate dilution. After incubation, enzymatic β-elimination was stopped by dipping the reaction medium into 95 °C water bath during 5 min. The mixture of oligomers was then centrifugated at 15,000 × *g* for 20 min at 20 °C and the supernatant was recovered. Acetylated oligomers (ΔOGUac) from PGUac were deacetylated as described above to generate oligoglucuronates deacetylated fraction (ΔOGU). The average degree of polymerization (DP) and degree of *O*-acetylation (Dac) were estimated by ^{1}H NMR analysis (Brücker DPX 300 SB).

Unsaturated oligoglucuronate of DP 3 (ΔOGU_3) was size fractionated according to their size from OGU mix by low-pressure gel-permeation chromatography on a Biogel P6 fine Bio-Rad column (100 cm × 2.6 cm, Amersham Bioscience). The ΔOGU mixture was loaded (100–500 mg in 10 ml) and eluted with a 50 mM ammonium formate solution at a flow rate of 0.8 ml/min. Detection was done with a UV detector (UA-6 from ISCO) at 254 nm and a RI detector (Melz). Fractions (5 ml) were collected with a Foxy 200 (ISCO) collector. Those belonging to a same peak were pooled and freeze-dried. Purity of ΔOGU_3 was confirmed by mass spectrometry analysis as described previously [8].

2.2. Chromatographic procedure on L-histidine-PEVA hollow-fibers membranes

Pseudobioffinity chromatographic modules (surface: 110 cm^2 or 0.1 m^2) composed of L-histidines immobilized onto poly(ethylene-vinyl alcohol) hollow-fiber membranes were prepared as described in literature [3]. All chromatographic analysis was carried out at room temperature (20–22 °C) and at pH 4.8. Modules were equilibrated with corresponding buffers (50 mM, pH 4.8) at the flow rate of 0.4 ml/min in cross flow mode using two peristaltic pumps. ΔOGU samples (4 g/l) were injected on closed circuit during 2 h [4].

Membranes were then washed with the same buffer in all elution modes according to literature [11]. Finally, oligomers were eluted in back-flushing mode with increasing the ionic strength of buffer. Fractions of 2 ml collected were assayed by colorimetric method for their sugar content.

2.3. Oligosaccharides assay

ΔOGU were quantified by microscale colorimetric assay according to a microprocedure with metahydroxybiphenyl (mHBP) [12]. The colorimetric assays were achieved on microtitration plate using D-glucuronic acid as standard. A microplate spectrophotometer (Opsys MR, Dynex technologies, VA, USA) was employed for absorbance measurements.

2.4. ^{1}H nuclear magnetic resonance (^{1}H NMR) studies

^{1}H NMR analyses were carried out at 80 °C with a Bruker Avance 300 spectrometer of 300 MHz equipped with ^{13}C/^{1}H dual probe. The NMR experiments were recorded with a spectral width of 3000 Hz, an acquisition time of 1.36 s, a pulse width of 7 μs, a relaxation time of 1 s and a number of 256 scans. The HOD signal was presaturated by a presaturation sequence. All samples were previously dissolved in D_2O (99.9% D) and lyophilized to replace exchangeable protons with deuterium. The dried samples were then dissolved in D_2O at a 10–20 g/l concentration.

The average DP of deacetylated oligoglucuronans was estimated by comparaison between H-1 signal integration of β-Δ-(4,5)-glucuronic acid and all H-1 signals integrations (H-1Δ, H-1β, H-1α and H-1) as above described in literature [13]. The *O*-acetylesterification distribution and proportion for all populations were analysed by ^{1}H NMR spectroscopy with integration of the signals from downfield, upfield and acetyl regions according to Courtois et al. [14].

3. Results and discussion

Based on the rationale and hypotheses proposed for the purification and interaction of human immunoglobulin G by PEVA-histidine chromatography [3,15], the interactions of oligoglucuronans with L-histidines ligands immobilized onto PEVA hollow-fiber membrane were investigated. After cleavage of glucuronan by fungal glucuronan lyase, oligoglucuronans (ΔOGU_3, ΔOGUac and ΔOGU) were extracted or fractionated on Biogel P6 (ΔOGU_3), and analysed for their DP and acetylation content by ^{1}H NMR spectroscopy (Fig. 1) before further purification by pseudobioaffinity chromatography. The ΔOGUac fraction exclusively mono-acetylated on C-3 (the most representative) and C-2 possessed a Dac of 44%.

3.1. Influence of the buffer system on adsorption

Chromatographic parameters have been optimised to study the oligoglucuronans adsorption capacity of PEVA-histidine cartridge (110 cm^2) and to avoid the masking of binding sites on module by charge–charge interactions between buffer ions and L-histidines. Ammonium acetate/acetic acid (negative charge), Mes/ammonium acetate (neutral charge), phosphate (positive charge) and Tris/succinate (positive charge) buffers (50 mM) were applied at pH 4.8 in analogy to protein purification studies [15]. The pH choice was motivated by the necessity to gener-

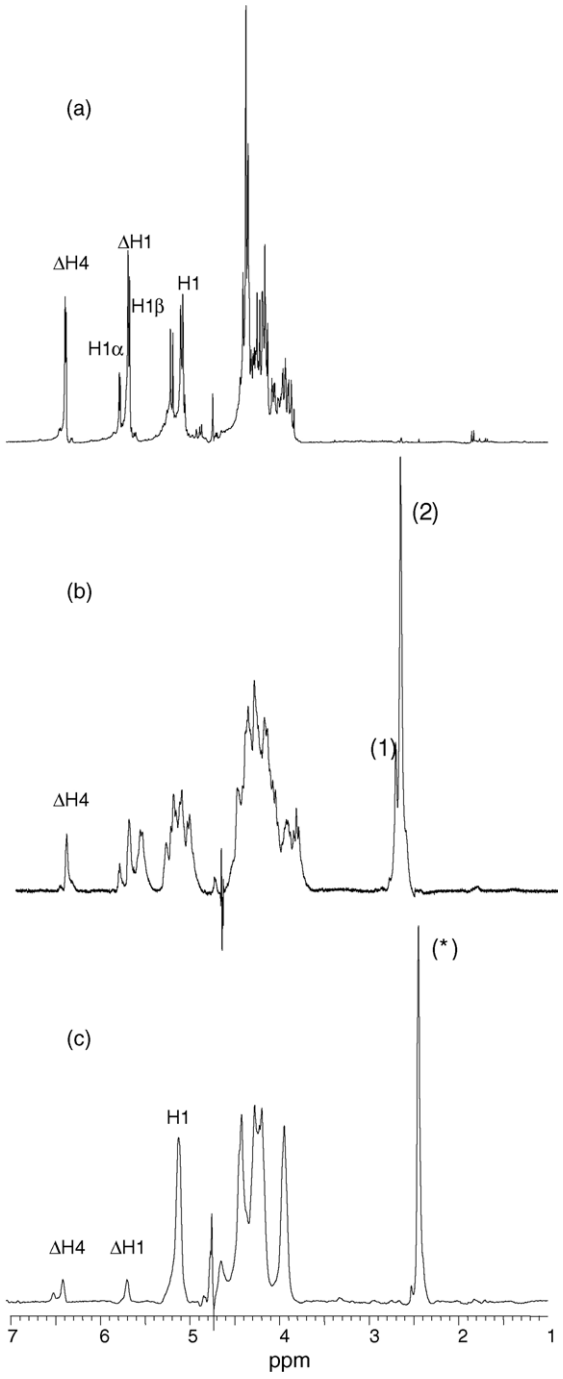

Fig. 1. ^{1}H NMR spectra of ΔOGU_3 (a), $\Delta OGUac$ (b) and ΔOGU (c). $\Delta H4$, H-4 of an unsaturated unit corresponding to the non reducing terminus unit of glucuronan; $\Delta H1$, H-1 of the unsaturated non-reducing terminus unit; H-1α and H-1β of the reducing terminus unit; H-1 of the non-terminal unit. (1) ^{1}H of the acetyl group at C-2 in the 2-*O*-acetylated residues; (2) ^{1}H of the acetyl group at C-3 in the 3-*O*-acetylated residues and (*) ^{1}H of the free acetyl group.

ate localised charges onto pseudobioaffinity system to engender specific ionic interactions between anionic ΔOGU and histidine ligands. It was previously noted that peptides and/or proteins were successfully purified on histidyl liganded support only at pH values at/or around their isoelectric point. Therefore, we have chosen an equilibrating adsorption pH of 4.8, due to the

Table 1
Influence of buffer composition on the adsorption of ΔOGU_{Ac}[a] onto L-histidine-PEVA cartridge

Buffer	Adsorption (%)	Capacity (μg/cm^2)
Acetate	28.1	40.9
Mes-acetate	27.5	40.0
Phosphate	11.0	16.0
Tris–succinate	10.0	14.5

Sixteen milligrams was injected on PEVA-histidine cartridge (110 cm^2) at pH 4.8 in the different buffer systems used at 50 mM.
[a] Unsaturated acetylated oligoglucuronan.

pK_a of polyglucuronic acid solutions: $4 \lesssim pK_a \lesssim 5$ [4,13]. Injections were done in cross flow mode with recirculation of both retentate and permeate to increase adsorption capacity by flow forcing on the surface and in the pore of hollow-fibers membrane. Oligouronates were eluted with increasing ionic strength of buffer.

As shown in Table 1, the specific $\Delta OGUac$ adsorption capacities of the histidyl-PEVA membrane vary from one buffer to another. As expected and in comparison to the reported data with protein retentions, the present observation indicated a high importance of the residual ion or small ionic loci present at pH 4.8, for oligouronates adsorption. In case of a zwitterionic buffer (Mes/ammonium acetate), the adsorption capacity of oligosaccharides was 40 μg/cm^2, whereas in the case of homogeneous charge buffers: positive (Tris–succinate) or negative (phosphate), the adsorption capacity was significantly low (16 and 14.5 μg/cm^2, respectively). In contrast, with acetate buffer (negative charge), the adsorption capacity (40.9 μg/cm^2) was similar to zwitterionic one. These results show an important role of the carboxyl substituents (polyanionic character) at the pH equal to pK_a of oligouronates since anionic charges induced by carboxylates (COO^-) was essential to oligosaccharide retention. So, we propose that electrostatic effect was predominantly depending to charge–charge interactions with positive charges of histidine imidazole rings as illustrated in Fig. 2. Nevertheless, the induced charge effects, like induced dipoles, by the hydrogen bonding with the –OH groups cannot be ignored.

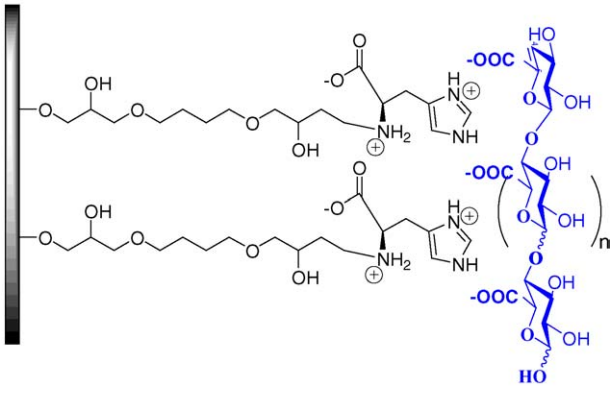

Fig. 2. Proposed mechanism of interactions between oligoglucuronates and histidyl-PEVA membrane.

3.2. Influence of the O-acetylester and DP on adsorption of ΔOGUac

The influence of the degrees of polymerization and acetyl substitution on the adsorption onto His-PEVA were studied in order to compare and differentiate this adsorption mechanism from a conventional ionic exchange column. To start with, we have to note that the very fact is that the adsorption is maximal at pH 4.8. However, a classical ion exchange mechanism was excluded. We preferred the ammonium acetate/acetic acid buffer (50 mM, pH 4.8) because of its volatile character in this part of the study after verifying that this buffer composition did not have any significant difference, comparably to the ones studied above.

The pure oligoglucuronate (ΔOGU_3) and a mixture of acetylated oligoglucuronates with an average DP of 6.5 (ΔOGUac) were chosen to determine the maximum adsorption capacity. From 16 mg of ΔOGU_3 and ΔOGUac injected, 4.7 mg (42.7 μg/cm^2) and 4.5 mg (40.9 μg/cm^2) were purified, respectively (Fig. 3). As can been observed, a maximal specific retention around 30% was obtained. Unexpectedly, the un-*O*-acetylated fraction ΔOGU_3 was retained more strongly and hence, eluted with a higher ionic strength (2 M) than the *O*-acetylated species ΔOGUac, perhaps due to possible induced dipoles. Other possible hypotheses could be: firstly, the low DP could facilitate interactions between entities because of the high mobility of oligomers; secondly, the acetylations of ΔOGUac decreased the strength of the interactions but not the adsorption capacity. Effectively in numerous cases, substituents on poly- and oligosaccharides act often as a frontier between the saccharidic compound and its environment. In fact, several examples described that substituents, independently of the backbone, play a role on interchain and/or intrachain molecular interactions [16]. This phenomenon could be extrapolated to interactions between oligouronides and the L-histidines of the support. Moreover, it was described that the polarity of the polysaccharides varies depending to the presence of substitutions as acetates, sulfates or others [16]. The small module which allowed a recovery only of microgram quantities of the oligouronans, it was difficult to undertake more specific and precise analysis of the chromatographic fractions, which in turn will give the substitution dependent pattern of retention. To obtain larger quantities allowing a more precise analysis, and to attribute the role of acetate substituents on the retention mechanism, preparative studies were operated on an L-His-PEVA 10 times bigger (0.1 m^2). Chromatographic system was used under the same chromatographic conditions as described above but oligomers were eluted in backflushing mode by a linear gradient ammonium acetate/acetic acid between 0.05 and 2 M. To evaluate the potential influence of acetate substituents on oligoglucuronates retentions, the fraction ΔOGUac was chemically deacetylated by alkaline treatment to obtain the mixture ΔOGU. The complete deacetylation was controlled by ^{1}H NMR analysis (Fig. 1c). These two oligoglucuronic samples were applied onto the module. The elution profiles were globally similar for both fractions with presence of two distinct retaining fractions eluted with gradient ionic forces of the same buffer, but the peaks were small and spread out due to high dilution of the injected sample (data not shown).

Nevertheless, it was interesting to note that *O*-acetyl substituents seemed to favour specific adsorption of acetylated oligomers, higher (18%) compared to the adsorption capacity of deacetylated ones (14%). The lower retention capacities compared with the precedent module were attributed to a lower distribution of histidines on this support. It was highly probable that acetates substitution conferred to oligosaccharides a coherent

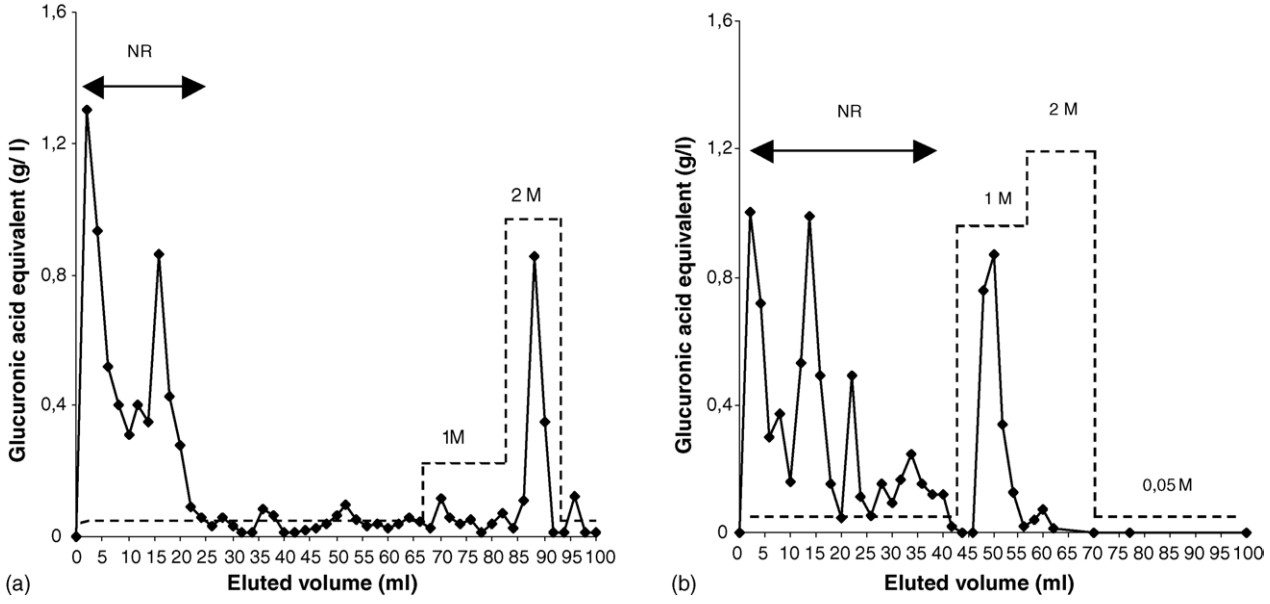

Fig. 3. Elution profile of (a) ΔOGU_3 and (b) ΔOGUac on L-histidine-PEVA membrane (110 cm^2). Oligosaccharide solution (4 g/l in ammonium acetate/acetic acid buffer, 50 mM at pH 4.8) was injected 2 h in closed circuit at the cross flow elution rate of 0.4 ml/min. After module washing (NR: non retained fraction), elution was realized with 1 and 2 M acetate buffer. Fractions (2 ml) were collected and uronic oligosaccharides were assayed.

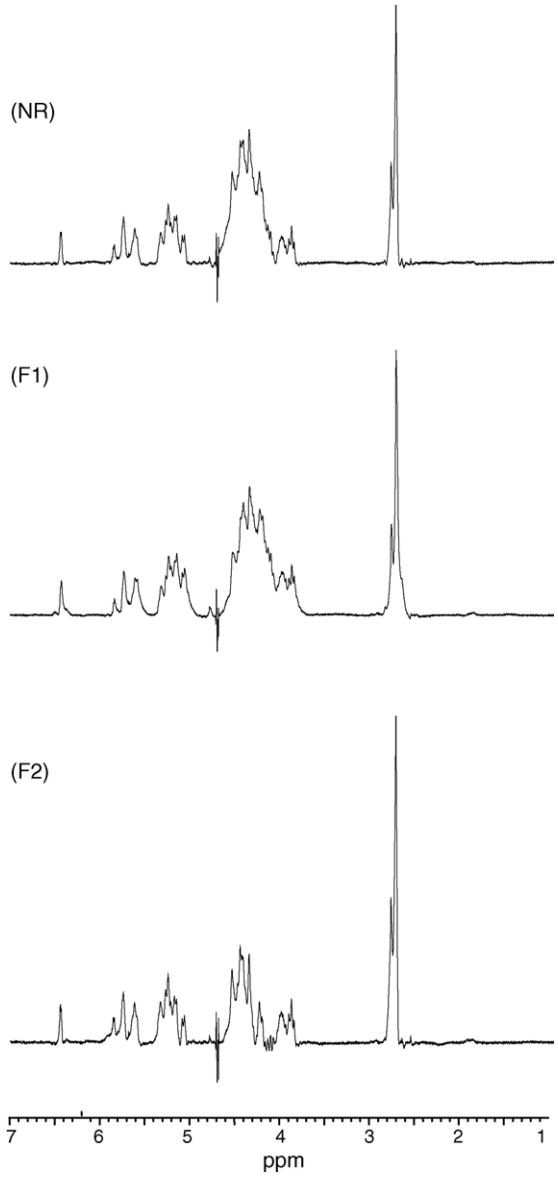

Fig. 4. ^{1}H NMR spectra of: non-retained (NR) ΔOGUac fraction and (F1), (F2) retained ΔOGUac fractions on L-histidine-PEVA module.

three-dimensional conformation which implicated more level of interactions by a specific orientation toward histidine-liganded onto PEVA module. The structural features degrees of ΔOGUac injected fractions, ΔOGUac non-retained fraction and ΔOGUac eluted fraction (F1 and F2) were investigated by ^{1}H NMR spectroscopy (Fig. 4) to evaluate global and specific acetylations degrees (Table 2). In regard to homologies in substitution degrees of all fractions retained or not by the pseudobioaffinity support, no selectivity was identified depending to *O*-acetyl positions. Moreover, after deacetylation of these fractions by alkaline treatment and DP evaluation by spectroscopy ^{1}H NMR, all fractions had a similar DP compared to the loaded one. Then, we can conclude to a selectivity of column depending first to DP (strength of interaction between oligouronides and L-histidines) and second to acetylation rate that increase the fixation capacity.

Table 2
Distribution as a function of degrees of substitution of acetate (DS) and molar proportions of acetyl substituents of retained (F1 and F2) and non-retained ΔOGUac[a] fractions on L-histidine-PEVA membrane

	Starting material	Non retained fraction	F1	F2
Acetate substitution degrees	44.0	42.5	36.0	38.0
2-*O*-Acetyl GlcpA	5.0	5.0	6.0	7.0
3-*O*-Acetyl GlcpA	28.0	26.0	24.0	27.0
Unacetylated GlcpA	67.0	69.0	70.0	66.0

F1: fraction eluted in first step gradient (1 M); F2: fraction eluted in second step gradient (2 M).
[a] Unsaturated acetylated oligoglucuronan.

4. Conclusions

This work showed that the amino acid L-histidine immobilized onto PEVA hollow-fiber membranes constituted a high potential chromatographic tool for oligouronates purification at preparative scales. The oligoglucuronates adsorption was influenced by buffer ions and the interaction carbohydrate/histidine was essentially governed by induced ionic force between the oligosaccharides and histidine residual charge. Nevertheless, other thermodynamics and structural parameters with resultant multiple interactions, such as hydrogen bond and/or hydrophobic associations, seem to play an important role in the "binding" processes. This pseudobioaffinity chromatography development open the way to an "integrated process development" for large-scale purification of oligouronates allowing to valorise industrial applications using a continuous glucuronan depolymerization system with an immobilized glucuronan lyase (GL2) coupled on line to PEVA-histidine module.

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