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# Improvement purification of sulfated oligofucan by ion-exchange displacement centrifugal partition chromatography

Lionel Chevolot[a,b,*], Alain Foucault[a,b], Sylvia Colliec-Jouault[a], Jacqueline Ratiskol[a], Corinne Sinquin[a]

[a]*Unité de Recherche Marine 2: Laboratoire Biochimie et Molécules Marines, IFREMER, Rue de l'Ile d'Yeu, BP 21105, F-44311 Nantes Cedex 3, France*

[b]*Unité de Recherche Marine 2: Laboratoire de Recherches sur les Macromolécules (Unité Mixte de Recherche CNRS 7540), Université Paris Nord, Av. J.B. Clément, F-93430 Villetaneuse, France*

**Abstract**

Centrifugal partition chromatography in ion-exchange displacement mode was used to fractionate mixtures of sulfated oligofucans obtained by partial depolymerization of brown seaweed fucoidans. Diluted (10%, v/v) protonated LA2 (a lipophilic secondary amine) is used as a weak exchanger. In an attempt to improve this method, several solvents (methyl isobutyl ketone, methyl *tert*.-butyl ether, BuOH) were tested to dissolve $LA2H^+$. MtBE produced less bleeding than MiBK, whereas BuOH proved unsuitable. The sample injected needs to be highly diluted in water to ensure participation in the chromatographic process. A comparison of data (NMR, composition, molecular mass) indicated the homogeneity of the fractions obtained as well as the differences between them. 

*Keywords:* Centrifugal partition chromatography; Oligofucans; Fucans; Polysaccharides

## 1. Introduction

Fucoidans, a unique class of high-molecular-mass sulfated fucans extracted from brown seaweeds, manifest various biological activities (e.g. anticoagulant, antithrombotic, antiviral, antiproliferative, antifertilizing and antitumoral) [1] probably related to different sites along the polymeric chain. Low-molecular-mass fucans (LMWFs) can be obtained as mixtures of oligosaccharides by acid hydrolysis [2] or radical depolymerization [3]. To fractionate such mixtures, we are developing a new method involving centrifugal partition chromatography (CPC) [4]. CPC is a liquid–liquid chromatographic technique using immiscible solvents or solutions as stationary and mobile phases (e.g. a water-immiscible organic solvent and an aqueous solution). The stationary phase is retained by centrifugal force, while the mobile phase passes through and is mixed by the turbulence produced by the flow. One of the main advantages of CPC over high-performance liquid chromatography (HPLC) is the high selectivity provided by a suitable solvent system [5]. At the present time, this method is mainly used to purify natural products [5–8]. Only a few works have been devoted to separation of highly negatively charged organics such as LMWFs [4] or glycosaminoglycans [9], which are only soluble in water. However, a lipophilic cation (or retainer ion) can be used as a counter-ion to increase LMWF solubility in organic solvents and thus allow

*Corresponding author. Tel.: +33-240-374-057; fax: +33-240-374-071.

*E-mail address:* chevolot@ifremer.fr (L. Chevolot)

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partition between both phases. The theory behind this approach and the first results were reported in a study using methyl isobutyl ketone (MiBK) as solvent, protonated LA2 (a secondary fatty amine) as retainer and $OH^-$ as displacer ion (displacement elution was preferred because of its preparative nature) [4]. These initial experiments revealed two drawbacks: relatively large bleeding (4–5%) and loss of part of the injected compound (a sulfated fraction mistakenly considered to be unsulfated [4]). Further experiments described in the present study were conducted to check the efficiency of the method (by chemical analyses and NMR studies) and eliminate the drawbacks by using other solvents and modifying injection conditions.

## 2. Experimental

### 2.1. Materials

Fucoidans were extracted from the brown seaweed *Ascophyllum nodosum* [10] and partially depolymerized either by acid hydrolysis [2,11], (FH fraction, identical to $\mathbf{H^3 5}$ in the last cited paper) or a radical process (FR fraction) [3]. On the basis of previously reported analytical methods [11], the characteristics of FR and FH were respectively: weight-average molecular mass: 10 000 (polydispersity: 1.7) and 5200 (polydispersity: 1.4); fucose content: 35% and 41% (w/w); and sulfate content: 34% and 30.5% (w/w).

### 2.2. Centrifugal partition chromatography

The HPCPC Series 1000 apparatus (Sanki Engineering, Nagaokakyo, Kyoto, Japan) was used as previously described [4]. The organic stationary phase was a 10% (v/v) solution of Amberlite LA2 (0.225 mequiv. $ml^{-1}$) protonated by HCl (Amberlite LA2 is an oil-soluble secondary fatty amine manufactured by Rohm & Haas, Philadelphia, PA, USA) in MiBK or methyl *tert*.-butyl ether (MtBE). FR or FH (1.5 to 2 g dissolved in 10 or 60 ml of water saturated with organic solvent) was injected into the chromatograph and eluted in displacement mode, with an aqueous mobile phase of 0.05 *M* of NaOH saturated with a 10% solution of unprotonated LA2 in MiBK or MtBE. The effluent (2 ml $min^{-1}$ flow) was monitored with a Microflow pH sensor (Broadley–James, Santa Ana, CA, USA). Fractions (20 ml) were tested for the presence or absence of sugar (Dubois test [12]). Unretained sugars present when samples were injected within concentrated solutions were eluted in the first fractions. Only NaCl was eluted subsequently. The first retained sulfated fucans appeared when pH increased. This material was eluted at the expected volume corresponding to column capacity (Fig. 2), at which time 4 ml fractions were collected. Each fraction was supplemented with $NH_3$, extracted three times with pure solvent (to facilitate LA2 removal), evaporated and then weighed. Each fraction weighed between 40 and 65 mg, and the sum of the weights gave the retained mass. Some selected fractions were analyzed for sugar composition, sulfur content and molecular mass determination using previously described methods [11].

### 3.1. Distribution isotherms of FR in MiBK or MtBE

This experiment was performed only with FR. The organic phases were 10% (v/v) solutions of protonated LA2 in MiBK, MtBE or BuOH equilibrated with water. Twenty milligrams of FR were dissolved in water (1 ml) and then shaken with the organic phase (1 ml). After centrifugation, the FR concentration in aqueous phase $(FR)_{aq}$ was determined by high-performance size-exclusion chromatography (HPSEC) with refractometric detection [4]. The concentration in organic phase $(FR)_{org}$ was deduced as the difference. Further additions of FR allowed the curve, $(FR)_{org}=f[(FR)_{aq}]$, to be plotted. Linearization was obtained by plotting $1/(FR)_{org}=f[1/(FR)_{aq}]$ as a linear function, $1/(FR)_{org}=1/K(FR)_{aq}+1/C$, in which $K$ was the conditioning distribution constant and $C$ the maximum concentration in the organic solvent.

### 3.2. Proton NMR spectroscopy

One-dimensional $^1H$ NMR spectra were recorded on a Bruker DRX-400 spectrometer equipped with an indirect 5 mm $^1H\{BB\}$ gradient probehead at a

probe temperature of 298°K. Prior to analysis, samples were exchanged twice in $^2H_2O$ (99.9 atom% $^2H_2O$, Euriso-top, Gif sur Yvette, France), with intermediate freeze-drying or evaporation (Speed-Vac), and then redissolved in 99.96 atom% $^2H_2O$ (Merck). Chemical shifts are expressed in ppm by reference to an external standard (trimethylsilylpropionic acid). The one-dimensional spectra were recorded with a spectral width of 4111 Hz in a 32 K dataset. No suppression of the $HO^2H$ signal was performed. Double-quantum-filtered COSY spectra (DQF-COSY) were recorded by collecting 512 (F2)*256−300 (F1) data points zero-filled to 512 (F1) using a spectral width of 2100 Hz (400 Mhz), with a repetition time of 2 s.

## 4. Results and discussion

### 4.1. Method efficiency

In our previously reported experiment, 800 mg of FR were injected and fractionated by CPC [4]. To evaluate the preparative performances of this method, two larger samples of FR (1.7 g) and FH (1.5 g) were injected in 10 ml of water and fractionated, using MiBK as solvent (see Section 2). The analytical results shown in Table 1 correspond to the injection of FH. For this experiment, the first and last retained fractions were the 26th and 46th, respectively. For both samples, a significant part of the injected matter (30–40%) was unretained and eluted very early despite being sulfated (sulfur content ≈7% and 9% for FH and FR, respectively). For retarded fractions, as expected, sulfur content increased with elution time (from 10.3 to 13.6% in case of FH), indicating that separation occurred as a function of the negative charge. Concerning composition, the unretained and first retained fractions showed relatively high content of sugars other than fucose, which was the only present in the more retarded fractions (Table 1). This indicated a significant improvement in their homogeneity in comparison with the starting material, as confirmed by their low polydispersity (≈1.25, Table 1). Similar results were observed with the FR sample: the sulfur content of retained fractions increased from 7.1 to 12%, but the polydispersity of each was less improved remaining around 1.4–1.5.

### 4.2. Solvent selection

As a suitable solvent should be water-immiscible, sufficiently polar to ensure correct solubilization of the ionic pair formed by LMWFs and $LA2H^+$, and relatively inexpensive for use on a preparative scale, MiBK appeared to be a good choice. Two other solvents were tested: BuOH, which is protic and very polar in order to retain the organic pairs more efficiently and avoid flushing of a large quantity of material in the first fractions; and MtBE, which has lower density than MiBK (0.74 vs. 0.8) in order to

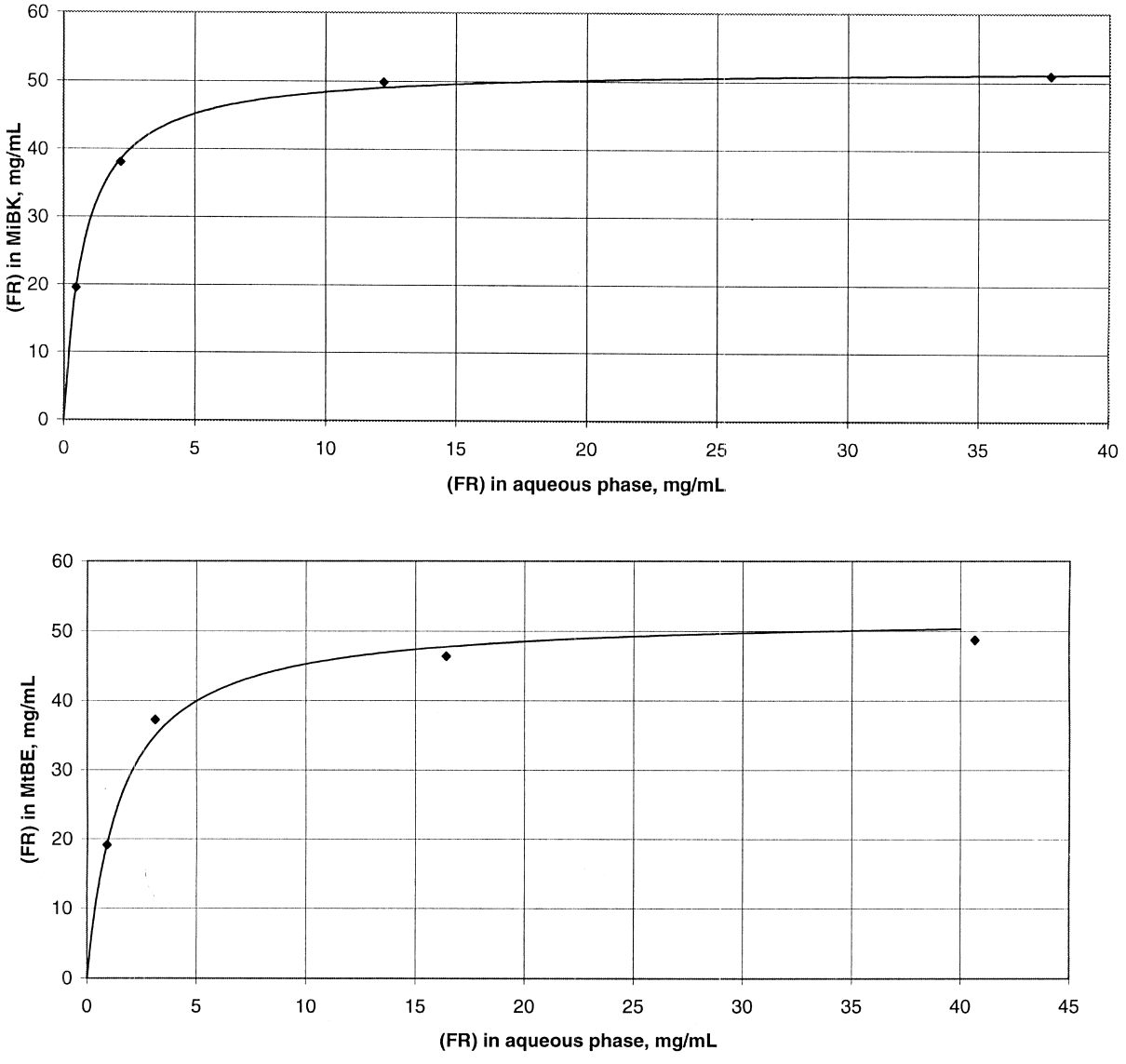
Table 1
Composition (in %, w/w) and molecular mass of FH (starting material) and seven fractions obtained from it by centrifugal partition chromatography (CPC)

| | FH | Unretained fraction | Retained fractions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 27 | 30 | 34 | 38 | 42 | 44 |
| $M_w$ [a] | 5200 | 4230 | 3500 | 3920 | 4510 | 5600 | 5400 | 5500 |
| $M_n$ [a] | 3700 | 2820 | 2500 | 2900 | 3450 | 4400 | 4300 | 4500 |
| Polydispersity | 1.4 | 1.5 | 1.4 | 1.35 | 1.31 | 1.27 | 1.24 | 1.25 |
| Sulfur content | 9.4 | 7.1 | 10.3 | 10.9 | 11.7 | 13 | 12.7 | 13.6 |
| Fucose | 41 | 33 | 48 | 47 | 50 | 43 | 40 | 40 |
| Galactose | 1.5 | 4 | 1 | *[b] | * | * | * | * |
| Xylose | 2 | 4.5 | * | * | * | * | * | * |
| Uronic acid | 7.6 | 12 | 4 | * | * | * | * | * |

[a] The 27th and 44th were the second and antepenultimate retained fractions. Chemical analysis had a SD of 5–15% [10]. $M_w$=Weight-average molecular mass; $M_n$=number-average molecular mass.

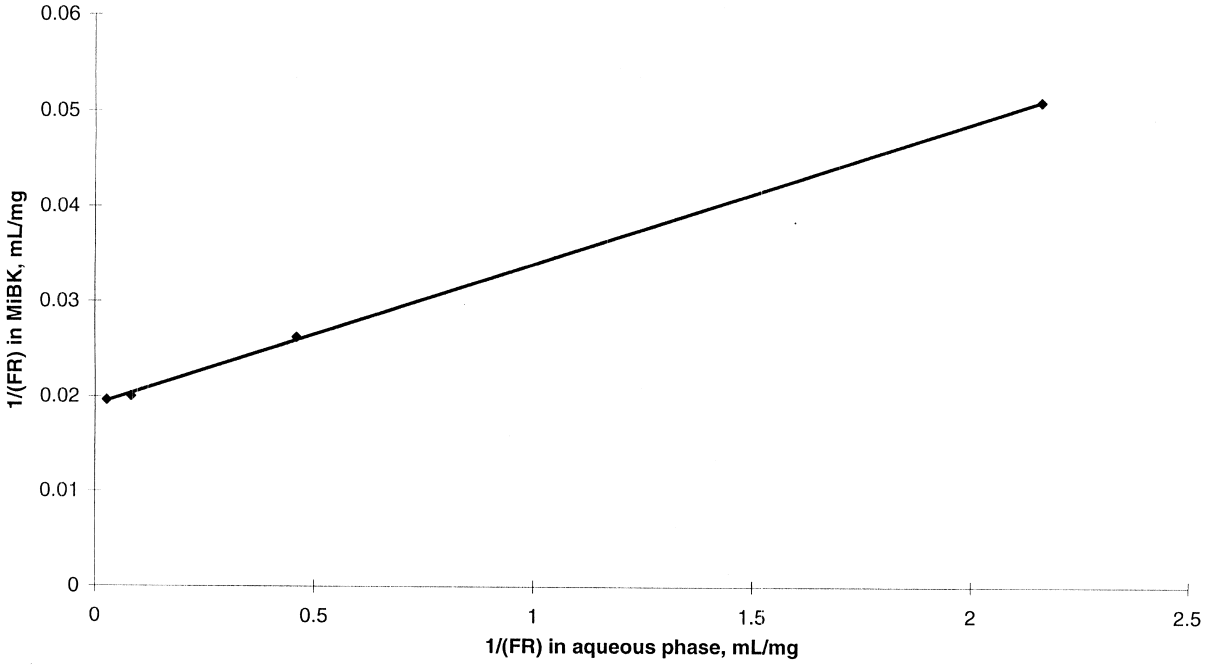
[b] Less than 1%.

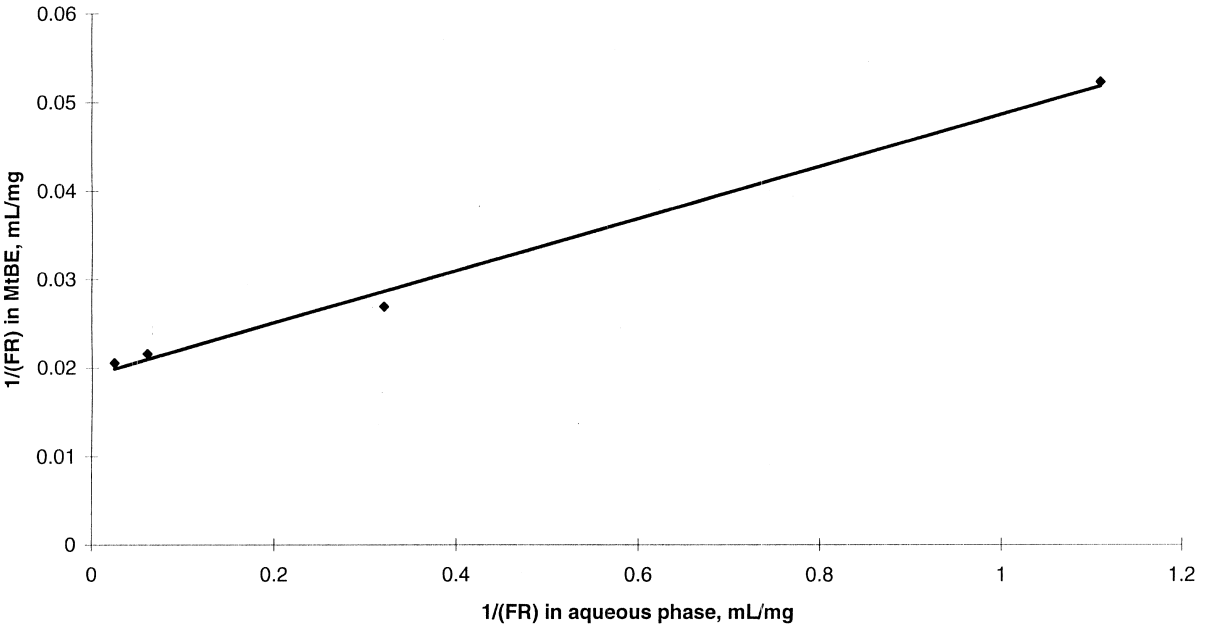
speed up decantation and reduce bleeding. Other water immiscible solvents seem less convenient because they are or not polar enough or denser or too inflammable (e.g. $Et_2O$) for use on a preparative scale. In preliminary tests, distribution isotherms (FR concentration in organic phase vs. FR concentration in aqueous phase) were plotted to select the best solvent (Fig. 1a). For MiBK and MtBE, these curves had their customary shape, with a broad plateau corresponding to the maximum concentration in




(a)

Fig. 1. (a) Plots of FR concentrations in organic $(FR)_{org}$ vs. in aqueous phase $(FR)_{aq}$ for two solvents (MiBK, MtBE): (b) Corresponding linear transformation plots $(1/(FR)_{org}$ vs. $1/(FR)_{aq})$. The respective regression lines were $1/(FR)_{org}=0.0149/(FR)_{aq}+0.0192$ ($r^2=0.999$) and $1/(FR)_{org}=0.0296/(FR)_{aq}+0.0191$ ($r^2=0.994$), with mg $ml^{-1}$ as concentration unit. The maximum concentration in organic phase was calculated. From the *y*-intercepts ($1/C$): 52 mg $ml^{-1}$ or 0.224 mequiv. $ml^{-1}$ in sulfate equivalent. Global conditional distribution constants were deduced from the slope ($1/K$): $K=67$ and $K=34$ respectively for MiBK and MtBE.




(b)

Fig. 1 (*continued*).

organic phase. Linearization (plotting $1/(FR)_{org} = f[1/(FR)_{aq}]$, Fig. 1b) gave exactly the same *y*-intercept ($1/C$) for both solvents, corresponding to the maximum concentration $C$ in the organic phase: 52 mg $ml^{-1}$ or 0.224 mequiv. $ml^{-1}$ sulfate (which was very close to the theoretical maximum concentration of 0.225 mequiv. $ml^{-1}$). The calculated global conditional distribution constant $K$ was 67 for MiBK and 34 for MtBE. Both solvents appeared to be suitable in this respect and capable of retaining LMWFs efficiently in the CPC apparatus. Unexpectedly, when BuOH was used, the FR concentration in the organic phase increased and then decreased when more FR was added. This phenomenon was probably due to the formation of micelles in the BuOH, since this solvent is relatively polar and dissolves a large amount of water. When micelles reached a critical size (i.e. the lipophilic parts of LA2 were sufficiently masked by sulfated fucoses), they became more soluble in water than in BuOH. This did not occur for other solvents because they are much less polar. It was concluded that BuOH was not a suitable solvent.

### *4.3. Improvement of the method*

To compare MiBK and MtBE, a new experiment was conducted by injecting around 2 g of FR dissolved in 10 ml of water and 10 ml of MtBE that was used as the organic solvent for $LA2H^+$ (see the Experimental section). Bleeding was lower with MtBE (2%) than with MiBK (4–5%). Thus, as expected, there was significant improvement in this respect, but LMWF retention was not better with MtBE, because around 50% of the material was still unretained. As concentrated solutions of FR or FH are very viscous, a plausible explanation for these results is that exchanges between both phases did not

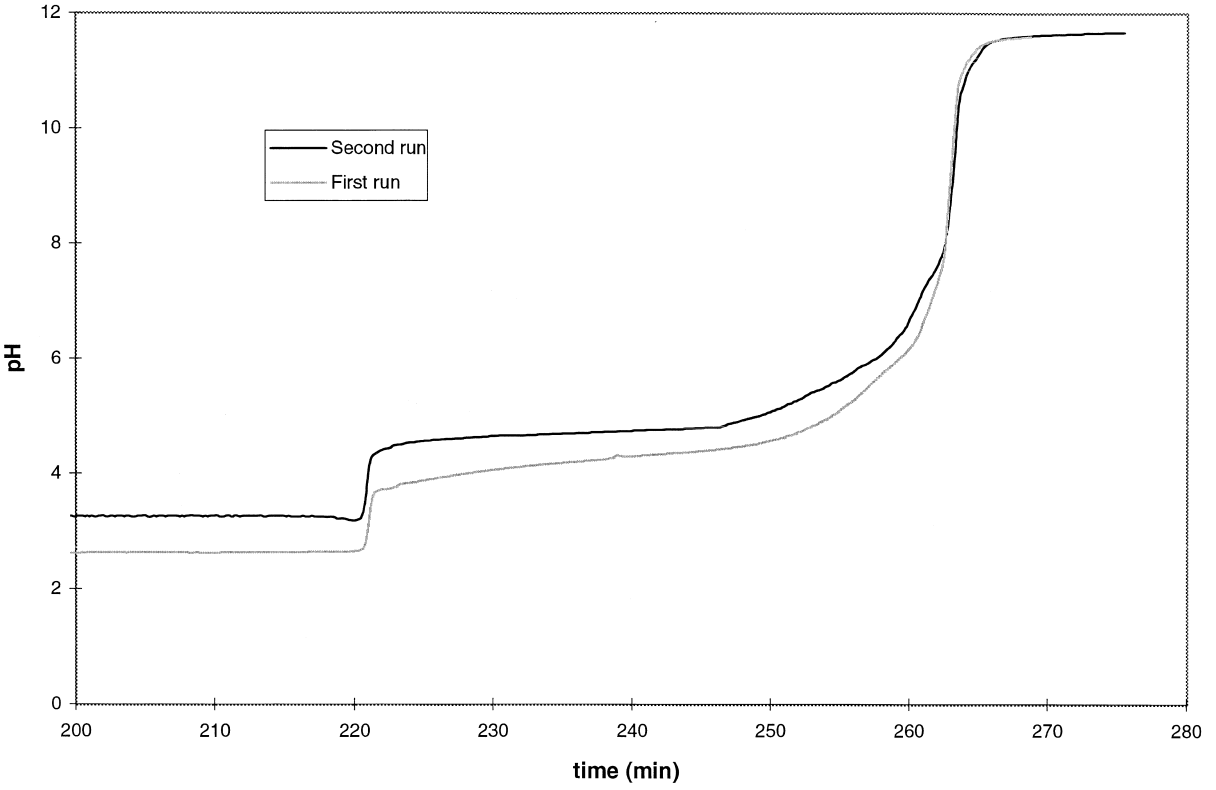



Fig. 2. Traces of oligofucan (FR) separations, pH=*f*(time), using MtBE as solvent. The grey curve corresponds to an injection of crude FR, while the black curve represents the trace obtained by rechromatography of pooled fractions coming from the first four runs and eluted at pH higher than 4.6. Note the very flat plateau observed in this case.

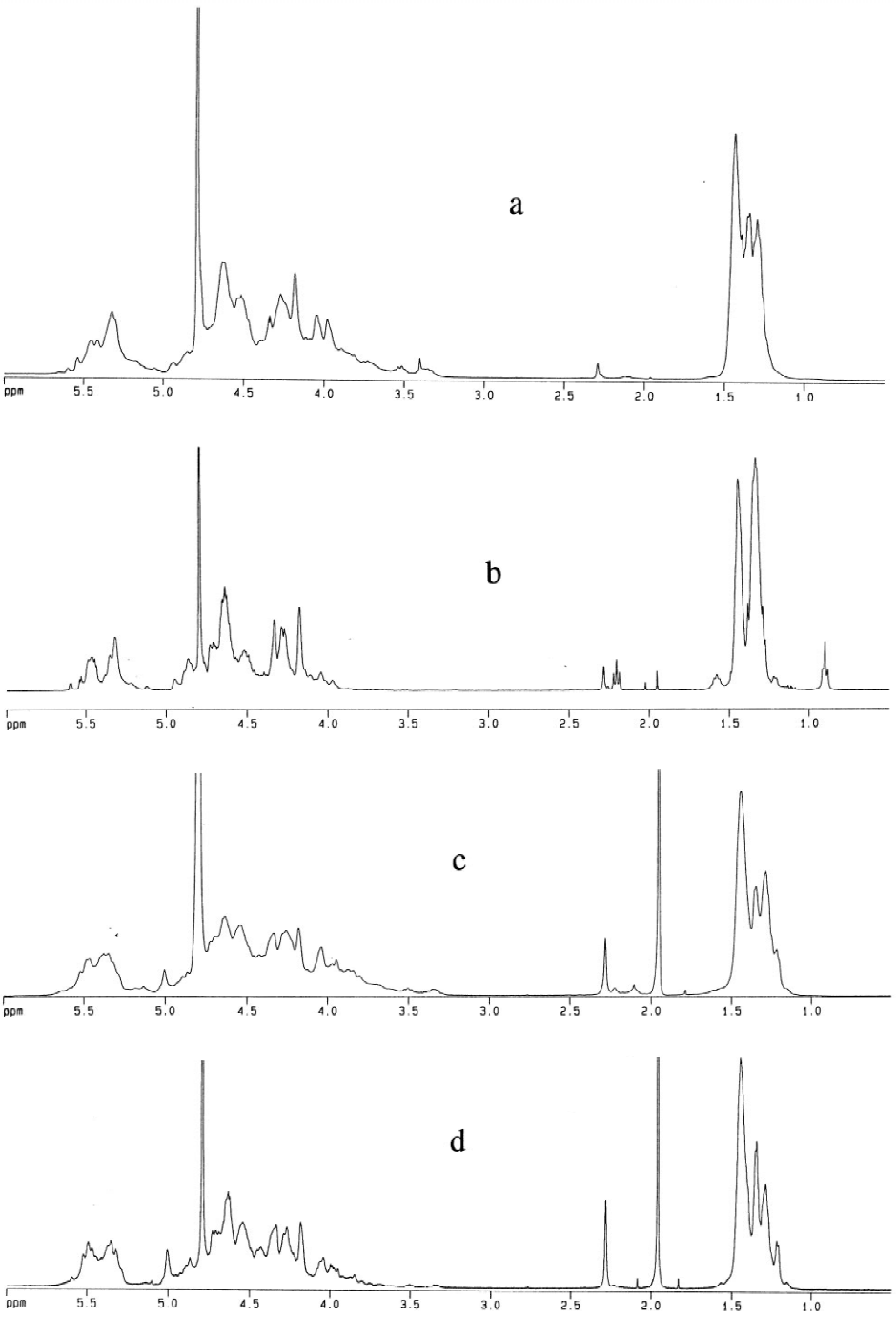



Fig. 3. One-dimensional $^{1}H$ NMR spectra of four LMWFs (crude or purified by CPC): (a) crude LMWF obtained by acid hydrolysis (FH); (b) FH fraction purified by CPC using MiBK as solvent; (c) crude LMWF obtained by radical depolymerization (FR); (d) FR fraction twice purified by CPC using MtBE as solvent. Note the differences between (a) and (b) on the one hand and the relative similarity between (c) and (d) on the other.

occur correctly in these conditions. When the sample was injected as a diluted solution (40 mg $ml^{-1}$), the problem was no longer apparent. Dubois tests were constantly negative until pH increased abruptly once the first materials appeared (Fig. 2). pH then varied very gradually according to the composition of the eluate, without reaching a plateau. When NaOH appeared, pH quickly became very basic. The fractions collected between these two pH events were weighed after elimination of LA2 (as described in Section 2), and the overall mass corresponded to 80–95% of the injected LMWFs. The mass of the first fractions was around 65 mg and then decreased slowly to 50 mg as sulfate content increased. Elution profiles with both solvents (regardless of the sample) were very similar, and there was no obvious improvement in resolution with MtBE versus MiBK, which is not surprising since their solvation properties were not significantly different. To obtain greater homogeneity, fractions appearing at a pH above 4.6 were pooled and rechromatographied in the same conditions (injection of 1.5 g diluted in 60 ml, using MtBE as solvent). The first fucan appeared at a higher pH (around 4.5), and pH then varied much less, indicating that the fractions corresponding to the flat plateau were very homogeneous and that the first purification was efficient (Fig. 2).

### *4.4. Comparison of one-dimensional NMR spectra of crude and purified fractions*

The one-dimensional NMR spectra of crude and purified FH fractions were significantly different (Fig. 3a and b). First, there was almost no signal between 3.5 and 4.1 ppm after purification. As this region corresponds to carbon-linked protons of $CH_2O$ and CHO in unsulfated positions, the lowest sulfated molecules were eliminated. Secondly, the sharpness of other signals was improved because fewer residues displaying similar but not superimposable signals were present. In COSY spectra (not shown), the number of H1-H2 cross-peaks (each corresponding to a different fucose unit) was significantly reduced, which is an additional evidence that purification occurred. However, differences between crude and twice-purified FR fractions were similar and obvious but less striking (Fig. 3c and d), even for COSY spectra (not shown). In fact, unsulfated positions were less abundant in the crude FR than in the FH fractions as it obvious by comparing NMR spectra. It is well known that acid hydrolysis removes some of the sulfate function and produces consequently heterogeneous LMWFs, whereas the radical depolymerization process removes no sulfate and provides a more homogeneous mixture of similarly sulfated oligofucans. Obviously, such a mixture is more difficult to fractionate by pure ion-exchange chromatography, and $LA2H^+$-sulfated fucans interactions are, a priori, only ionic. Further work is in progress to improve this method by using more specific retainers.

## 5. Conclusion

The method described here is efficient for purifying LMWFs sulfated differently and probably useful for fractionation of other anionic compounds such as low-molecular-mass heparin and chondroitin or dermatan sulfate. MtBE induces less bleeding than MiBK and seems generally more suitable. It is very important to inject the sample in diluted solutions to ensure good retention. Fractions derived from FH or FR are pure enough for NMR studies (some new structural data are currently being published [11]).

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