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Optimal membrane choice for microdialysis sampling of oligosaccharides

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Abstract

An analytical methodology based on microdialysis sampling, high-performance anion-exchange chromatography and integrated pulsed electrochemical detection for the monitoring of oligosaccharides in bioprocesses is presented. Amylopectin and model maltooligosaccharide standards; glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were used to demonstrate its versatility in view to sampling in enzymatic bioprocesses. The performance and characteristics of membranes with the same cut-off ranging between 3 and 100 kDa, were evaluated with respect to their extraction fraction (EF), permeability factors, temperature stability and protein (enzyme) interaction. All investigated membranes showed some non-specific interaction with enzymes. The EF and non-specific membrane–enzyme interactions were higher for the polysulfone membranes compared with the polyamide and polyethersulfone membranes. For all saccharides, the EF was independent of the concentration even for a 250-fold change in concentration. The EF and morphology of the membranes in their dehydrated state, as observed using scanning electron microscopy did not show any significant difference between membranes exposed to a 90°C temperature for 3 and 24 h indicating their applicability to the study of high temperature bioprocesses. © 1998 Elsevier Science B.V.

Keywords: Microdialysis; Membranes; Carbohydrates; Oligosaccharides

1. Introduction

The utility of enzymes in bioprocesses depends entirely on their purity and hydrolytic properties. Enzyme purification involves rigorous and tedious steps often carried out on very small amounts of enzyme. Further, the process of characterisation of the enzyme's hydrolytic properties or that of an unknown substrate requires analytical methodology that enables handling of small amounts of enzyme/substrate. On-line microdialysis with high-performance anion-exchange chromatography (HPAEC) and integrated pulsed electrochemical detection (IPED) is one such method. This hyphenation of techniques is well suited to characterise hydrolytic properties of enzymes [1–4] or their substrates in small volume (less than 5 ml) bioreactors based on the in situ

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sampling and sample clean-up qualities of a microdialysis probe.

If microdialysis has to be applied routinely in biotechnology, biochemistry, bioengineering or genetic engineering, certain aspects need to be clarified. For example, how significant is enzyme–membrane interaction or the effect of increasing the concentration and molecular size of several analytes in any particular manner during sampling? Do these aspects affect the sampling and if so to what extent? How significant could it be to process optimisation, enzyme or substrate characterisation? The in situ qualities of microdialysis such as the ability to sample nano- or microliter volumes, sample clean-up, on-line dilution in combination with programmable sample injections make it a very attractive and versatile technique. The application of microdialysis in enzymatic bioprocesses in either of the three sampling modes; continuous flow microdialysis sampling (CFMS), stopped flow microdialysis sampling with continuous stirring (SFMSCS) and stopped flow microdialysis sampling with stopped stirring (SFMSSS), were demonstrated recently [3]. This application has been facilitated by the use of an in situ tunable, concentric type of microdialysis probe (see Fig. 1) [5]. The ability to tune (adjust effective dialysis length) the microdialysis probe enables reduction of the detector signal if there is a prolific release of products that could affect the functioning of the detector. Further, the effective dialysable membrane length can easily be tailor-fitted or adjusted for a specific bioreactor volume.

We herein report work carried out using an analytical methodology based on on-line mi-

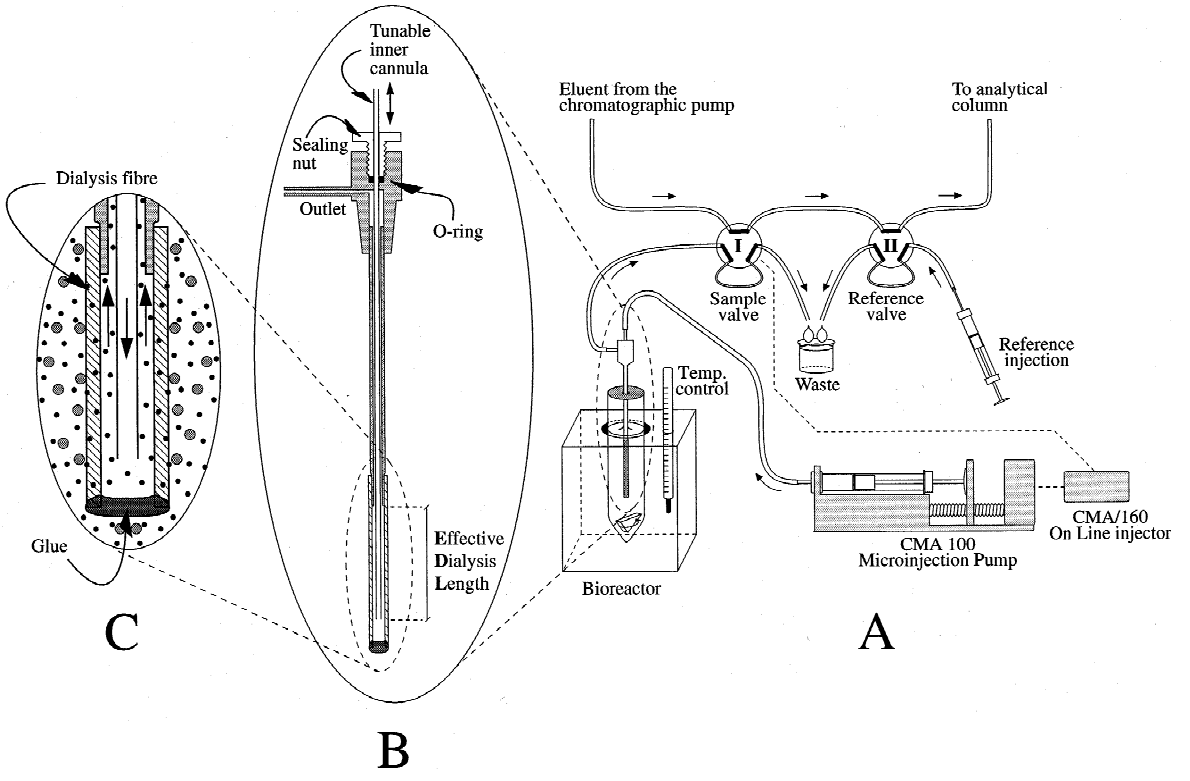


Fig. 1. Schematic representation of the microdialysis probe part of the experimental set-up (A), blow up of the microdialysis probe (B) and the detailed membrane configuration and analyte flow pathway in the probe (C). The construction of the probe is similar to the commercially available ones except for the O-ring and sealing nut which makes it possible to adjust the effective dialysis length (EDL) by either pulling out or pushing in the inner cannula (tuning). The EDL is determined by the length that the inner cannula extends into the membrane. The membrane length is tailor fitted to suit the dimensions of the bioreactor of choice (see B). The hollow fibre membrane is attached to the main probe body and also the tip is closed using glue (see C).

crodialysis–HPAEC and IPED for the monitoring of oligosaccharides (carbohydrates) in view to characterise the hydrolytic properties of enzymes or oligomeric polymers. The performance characteristics of this hyphenation of techniques in complex, high concentration matrix is demonstrated, and also that of membranes supplied by different companies but with the same/different polymeric material and similar cut-off. Membranes were evaluated with respect to their EF, permeability, high temperature stability and their interaction with enzymes (protein). Investigations carried out showed the possibility to enhance the performance of this analytical methodology by optimal choice of parameters and membrane material (and/supplier) for specific sampling conditions.

2. Experimental

2.1. Reagents

Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were obtained from Sigma (St. Louis, MO, USA). The 50% (w/w) NaOH was obtained from J.T. Baker, (Deventer, Netherlands) and NaOAc was obtained from Merck (Darmstadt, Germany). Amylopectin type of potato starch (here referred to as amylopectin) with a degree of branching of 4% as determined by ^{1}H NMR spectroscopy [6], was a gift from Lyckeby Stärkelsen (Kristianstad, Sweden). All reagents were prepared using Millipore water from a Milli-Q system, Millipore (Bedford, MA, USA).

2.2. Enzymes

Four enzymes were used in these investigations; termamyl 120 L (endo-1,4-α-D-glucan, glucanohydrolase, EC 3.2.1.78) was obtained from Novo Industries (Bagsvaerd, Denmark). A heat stable pullulanase (EC 3.2.1.41) obtained from *Bacillus acidopullulyticus* was a gift from Novo Nordisk (Promozyme 200 L). Pullulanase, from *Klebsiella pneumoniae* was purchased from Sigma. The pullulanase's isoelectric point is at pH 4.5 [7]. Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* was a gift from Novo as a crude enzyme originally from Hayashibara Biochemical Labs. (Okayama, Japan). The isoelectric point of the *Pseudomonas* isoamylase is at pH 4.4 [8].

2.3. Enzyme membrane interaction

The solid Sigma pullulanase and Hayashibara isoamylase were dissolved in citrate buffer at pH 6 and pH 3.5, respectively. Enzyme membrane interactions were achieved by inserting the microdialysis probe fitted with a membrane under investigation into an enzyme solution (termamyl 100 μl, Novo pullulanase 1000 μl, Sigma pullulanase 0.12 mg/ml and 1.2 mg/ml for isoamalylase, all enzymes prepared in 5 ml) for 5 min in the case of termamyl, but 20 min for the other enzymes, without perfusion. The membrane was rinsed with pure water and subsequently inserted into the substrate solution and perfused until equilibrium. Injections of the dialysate were made after 30 min and the acquired chromatogram was compared with that of the substrate (maltoheptaose for termamyl and amylopectin for the debranching enzymes). Further investigations were carried out by perfusing the membrane at 10 μl/min to facilitate removal of enzymes in the membrane porous structure.

2.4. Microdialysis experiment

The following hollow fibre membranes were tested: 5 and 30 kDa MWCO polysulfone membranes from Fresenius A/G (St. Wendel, Germany), 3, 5, 10, 30 and 100 kDa polysulfone membranes from A/G Technology (Needham, MA, USA), 10, 30 and 100 kDa MWCO polysulfone membrane from Amicon (Beverly, MA, USA). Two different types of 20 kDa MWCO polyamide membranes and a 20 kDa MWCO polyethersulfone membrane from CMA Microdialysis (Solna, Sweden), and two 6 kDa MWCO polysulfone membranes (one inner and the other outer pressure type) from Nitto Denko (Shiga, Japan) and a 20 kDa MWCO polysulfone membrane from Metallhantering (Stockholm, Sweden) which was already housed on a commercially available microdialysis probe with a 4 mm effective dialysis length, were evaluated.

An in-laboratory fabricated microdialysis probe with an in situ tunable EF (see Fig. 1) was fitted with

membranes having a 10 mm effective dialysis length and was perfused with water over night before carrying out the investigations. Microdialysis experiments were performed by inserting the membrane fitted microdialysis probe into a 5-ml vial housed in a heating and stirring module No. 18971, (Pierce, Rockford, IL, USA). The membranes were perfused at 5 μl/min except for the Amicon membranes which were perfused at 2 μl/min as they had lower EF. The perfusion liquid was delivered by a CMA/100 syringe pump and 20 μl of dialysate were injected by a CMA/160 on-line injector, all from CMA/Microdialysis. All reactions were carried out at 40°C using the CFMS mode.

2.5. Chromatographic system

A Dionex 500 chromatographic system, controlled by PeakNet™ software, Dionex (Sunnyvale, CA, USA) was used to separate and detect the carbohydrates during the investigations. Chromatographic separation was achieved using a CarboPac PA-100 pre- and analytical column (Dionex). The following wave form was used for the detection of saccharides; E_1=0.10 V (t_d=0.20 s, t_1=0.20 s), E_2=0.70 V (t_2=0.19 s) and E_3=−0.75 V (t_3=0.39 s) vs. a Ag/AgCl reference electrode supplied by Antec (Amsterdam, Netherlands) and a gold working electrode.

2.6. Chromatographic conditions

A gradient programme was used for the elution of the saccharides with 150 m*M* NaOH (eluent A) and 250 m*M* NaOAc prepared in 150 m*M* NaOH (eluent B). From 0–3 min, eluent A decreased from 50% to 20%. From 3–5 min, eluent A decreased to 0% until the end of the run where both eluents were readjusted to 50% to enable equilibration of the analytical column. 30% of eluent A and 70% of eluent B were used to separate hydrolysates isocratically during the hydrolysis of maltoheptaose by termamyl.

During the separation of debranched amylopectin, the following gradient was used; from 0–2 min eluent A decreased from 70% to 50%. From 2–20 min, eluent A decreased to 0%, until the end of the run where the 70% A and 30% B composition was maintained to equilibrate the analytical column. For these investigations, eluent B was 500 m*M* NaOAc prepared in 150 m*M* NaOH.

3. Results and discussion

3.1. Performance of the microdialysis membranes

Membranes stipulated to work at a certain pH range would normally do so adequately. Since membranes are bulk polymeric material which are sensitive to their environment [9], microdialysis membranes have to be evaluated in terms of their temperature stability and EF for specific applications. Bungay et al. [10], have described the dialysate extraction fraction as shown in Eq. (1);

$$\mathrm{EF} = \left(C_d^{out} - C^{in}\right)/\left(C_b - C^{in}\right) = 1 - \exp\left[-1/Q_d(R_d + R_m + R_{ext})\right] \quad (1)$$

where EF is the dialysate extraction fraction (sometimes denoted E_d or relative recovery, RR, where $\mathrm{RR} = 100\cdot[C_d/C_b]$), C_d^{out} is the concentration of the analyte in the dialysate, C^{in} is the concentration of analyte in the perfusion liquid, C_b is the concentration of the analyte in the bioreactor, Q_d is the perfusion rate, R_d, R_m and R_{ext} are the resistances to diffusion due to the dialysate, membrane and bioreactor solution, respectively. For a constant perfusion rate, with membranes of equal geometry, EF depends entirely on the membrane resistance (R_m) to diffusion as shown in Eq. (2) [11];

$$R_m = \ln(r_0/r_1)/2\pi L_{edl} D_{eff} \phi_m \quad (2)$$

where r_0 is the membrane outer radius, r_1 is the membrane inner radius, L_{edl} is the effective dialysis length, D_{eff} is the effective diffusion coefficient in the aqueous phase of the membrane and ϕ_m is the aqueous phase volume fraction of the membrane. For membranes with the same L_{edl} and r_0/r_1, permeability will determine EF as the porosity and tortuosity contribute to $D_{eff}\phi_m$. Further, if enzymes or proteinaceous materials interact or are adsorbed on the membrane surface or in the membrane's inner tortuous structure, depending upon their surface energy, D_{eff} might be reduced and thus decrease the EF. Eq. (2) is valid only when there is no interaction of the biomatrix with the membrane. However for

most applications of microdialysis sampling in bioprocesses, an initial decrease (membrane fouling) in EF with time is observed. The EF eventually stabilises, hence the R_m factor in Eq. (1) has to be compensated for by the so called ''Andrade effects'' [12]; Eq. (3).

$$R_m = \ln(r_0/r_1)/2\pi L_{edl} D_{eff} \phi_m + A \quad (3)$$

Eq. (3) describes the membrane's resistance to diffusion in enzymatic bioreactors where the ''Andrade effects'' are experienced. The ''Andrade effects'' will include, amongst other interactions, polymer(membrane)–enzyme or polymer–analyte interactions as the membrane surface responds to the changing aqueous bioreactor environment. This work investigates different membranes as reflected by their EF when sampling model oligosaccharides under different bioreactor conditions. As an attempt to compare the performance of similar membranes, the difference in their EF (ΔEF) is used. The ΔEF is also used to investigate the change in membrane performance after exposure to different temperatures. The Andrade effects will be demonstrated by the effect of bioreactor complexity and as seen by the interaction of enzyme with the membrane surface.

3.2. Evaluation of extraction fraction

3.2.1. 5 kDa membranes

The performance of membranes from different manufacturers with similar MWCO and effective dialysis length was compared by plotting the ΔEF for the saccharides (DP 1–DP 7) at various perfusion rates. The Fresenius 5 kDa MWCO membrane gave higher EFs for lower saccharides (DP 1–4) for all perfusion rates but the A/G 5 kDa membrane had higher EFs for DP 5–DP 7 at all perfusion rates (see Fig. 2a). The Nitto Denko membranes (internal and external pressure type) with the same MWCO (6 kDa) gave lower EFs compared with the A/G and Fresenius 5 kDa MWCO membranes. As shown in Fig. 2b, the internal pressure type membranes, gave higher EFs at 1 μl/min, but they performed comparably at higher flow-rates.

3.2.2. 20 kDa membranes

Fig. 2c shows that the EFs for the two polyamide and the polyethersulfone 20 kDa MWCO membranes are similar although the EF for PA 2 is considerably higher than for the other two at lower flow-rates ≤1 μl/min.

3.2.3. 30 kDa and 100 kDa membranes

With regards to the 30 kDa MWCO membranes, the Fresenius type showed higher EFs for all saccharides especially at decreasing perfusion rates (Fig. not shown). The 10 and 30 kDa MWCO Amicon membranes and all the 100 kDa MWCO membranes were leaking, consequently giving lower EFs. The EFs for the 100 kDa MWCO A/G and Amicon membranes were comparable at higher perfusion rates. Fig. 3a,b shows a comparison of EF for a 3 and 100 kDa MWCO membrane, respectively. These investigations showed the EF for the 3, 5 and 30 kDa membranes to be very comparable at high perfusion rates although they differ considerably at lower perfusion rates. Increasing the MWCO does not seem to increase the EF significantly.

Eq. (1) can be linearised by plotting $-\ln(1-EF)$ vs. $1/Q_d$ which gives the permeability factor ($1/R_d + R_m$) from the slope of a linear regression to this plot [10]. Since the $R_d < R_m$ for dialysis membranes in aqueous solutions, the slope is an indicator of the membrane resistance to diffusion, R_m or the permeability factor, PF ($PF = 1/R_m$). Table 1 presents the EF values evaluated at a perfusion rate of 3 μl/min and the estimated PF for each membrane, for the given saccharides, over the whole perfusion range of 1–9 μl/min. The Fresenius membranes generally show higher permeability as this is confirmed by their EF. From the data, it is evident that as the cut-off increases above 30 kDa, the permeability of the membranes decreases. This is explained by the increased sweating (ultrafiltration effects) by the membranes having larger pores.

3.3. Effect of increasing the concentration of analytes on the EF

High product concentrations or complex bioreactor conditions may affect the EF of single analytes. For the purposes of monitoring carbohydrates in enzymatic bioprocesses where prolific products are produced and product concentrations change drastically, it is necessary to verify the consistency of

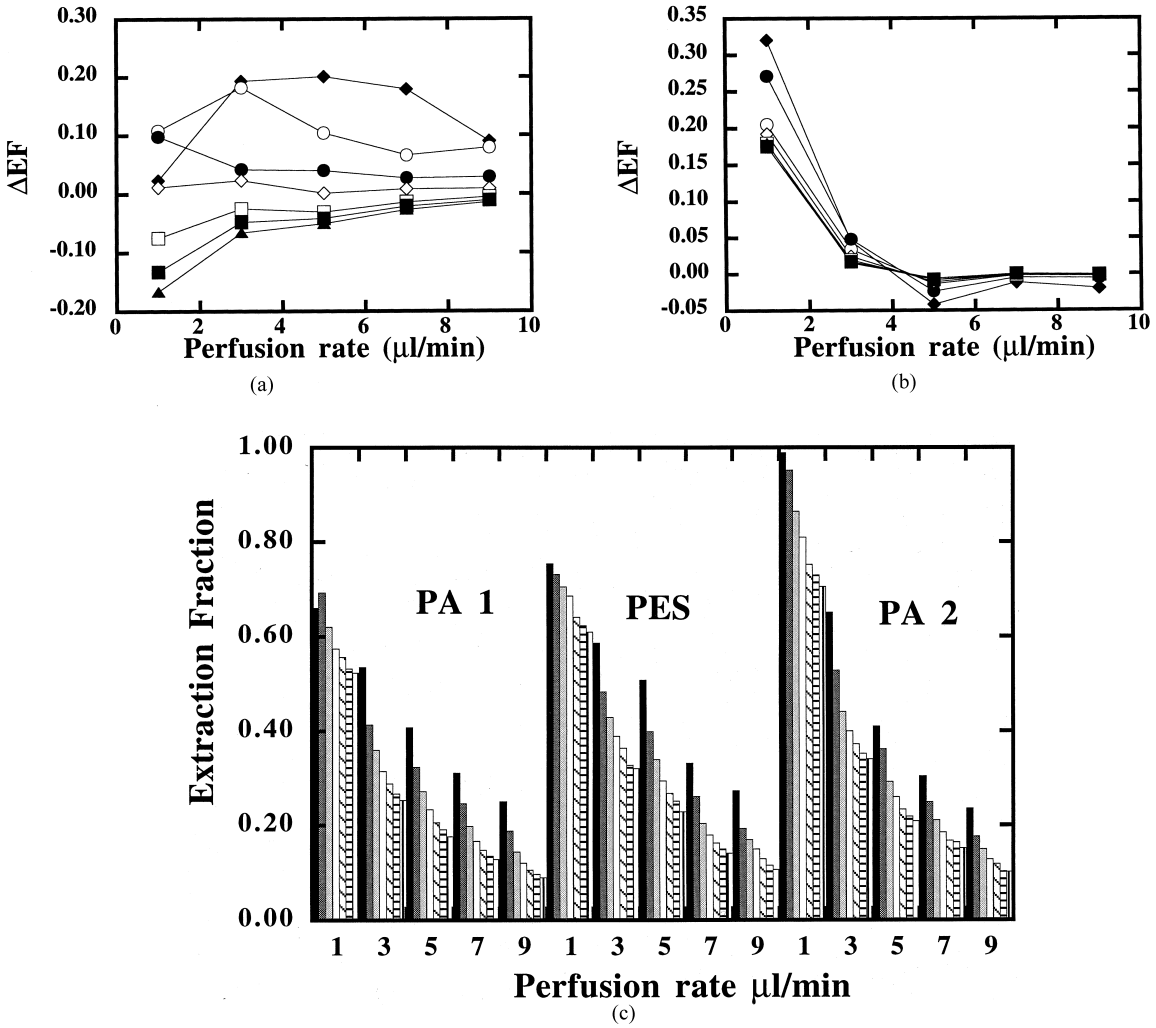
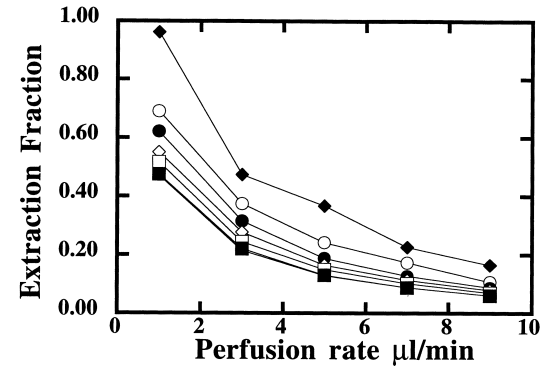


Fig. 2. ΔEF between, (a) Fresenius and A/G 5 kDa MWCO membranes (b) the external and internal pressure type 6 kDa MWCO Nitto Denko membranes, where (♦) represents DP 1, (○) DP 2, (●) DP 3, (◇) DP 4, (□) DP 5, (■) DP 6 and (▲) DP 7. (c) Comparison of EF for 20 kDa MWCO membranes; polyamide (PA 1), polyethersulfone (PES) and polyamide membranes (PA 2) which were perfused between 9 and 1 μl/min, where (■) represents DP 1, (dark grey) DP 2, (light grey) DP 3, (□) DP 4, (▨) DP 5, (▤) DP 6 and (▥) DP 7 (from left to right).

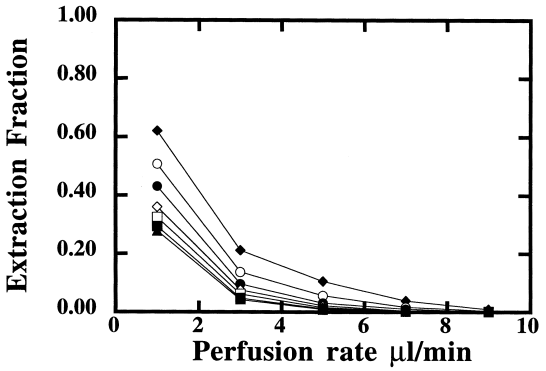
microdialysis sampling within the range of the detector. Table 2 shows EF values for saccharides of DP 1 to DP 7 at three different perfusion rates over a wide concentration range. The EF are very reproducible and independent of the increase in analyte concentration. This observation is consistent with what many workers have predicted and demonstrated with single analytes during pharmacokinetic investigations [13]. However, when the concentration of analytes and other bioreactor metabolites is high, the conditions are more complex in terms of diffusion transport.

3.4. Bioreactor complexity

Microdialysis is a diffusion driven sampling process, consequently an increase in bioreactor complexity could result in hydrodynamic resistance which might decrease D_{eff} of the analytes. In order to investigate the effect of bioreactor complexity, EF



(a)



(b)

Fig. 3. Comparison of the performances of a 3 kDa MWCO membrane (a), and 100 kDa MWCO membrane (b) where; (♦) represents DP 1, (○) DP 2, (●) DP 3, (◇) DP 4, (□) DP 5, (■) DP 6 and (▲) DP 7.

were evaluated at 2, 5, 7 and 9 μl/min as the analyte composition was changed. Fig. 4a,b show EF plots for saccharides perfused at 9 μl/min during which the complexity of the bioreactor was increased from the lower saccharides (DP 1 to DP 7) and higher to lower (DP 7 to DP 1), respectively. The EF is constant for all the saccharides as the complexity is increased. A similar trend was observed for values obtained at 5 and 7 μl/min. The EF values evaluated at 2 μl/min (see Fig. 4c,d) show that the addition of larger saccharides (Fig. 4c) to the bioreactor which already has smaller ones, results in a decrease in the EF of the smaller saccharides.

From the EF plots in Fig. 4, it is evident that the lower saccharides, especially glucose are affected by the increased bioreactor complexity more than the higher ones (e.g., DP 6 and DP 7). This is because as the size of the analyte molecules increases, the diffusion of the larger ones, which is inherently slow will not be affected as significantly as that of glucose. In a more complex bioreactor, the smaller analyte molecules will collide with the larger molecules in a Brownian sense, such that their effective diffusion will decrease and consequently their EF. This phenomenon is more pronounced at lower rather than higher perfusion rates, indicating that the problem of increased bioreactor complexity can be arrested by working at higher perfusion rates if the EF is high.

3.5. Temperature stability

SEM studies were made on each type of microdialysis membrane after perfusing them continuously at 90°C for 3 and 24 h, respectively. The SEM micrographs for the Fresenius polysulfone membranes showed a decreased porosity and a reduction in the pore sizes on the outer supporting layer (see Fig. 5a,b) for both times as compared to the untreated membranes. The same phenomenon was observed for the A/G polysulfone membrane after exposure to high temperature. The CMA polyamide membrane micrographs on the other hand, showed an increase in porosity after 24 h (see Fig. 5c,d). The increased porosity on the membrane has been shown to be a ''suicidal effect'' which results in a decrease in EF [12]. This is consistent with the observed particles on the membrane surface after 3 and 24 h as they block the membrane pores. All SEM micrographs for the membranes in their dehydrated state showed some morphological change after temperature treatment compared to the untreated membranes. However, the SEM micrographs did not show any significant difference between the membranes which were temperature treated for 3 and 24 h, respectively. This observation is very important as it indicates that the membranes could be used as sampling units in bioprocesses where the temperature is as high as 90°C.

Further investigations were carried out using saccharides of DP 1 to DP 7. The EF was evaluated at 40°C, before and after raising the temperature to 90°C for 4 h. The lower the ΔEF values are, the better the membrane withstands the temperature

Table 1
Extraction fractions and permeability factors for different microdialysis membranes

Membrane type	DP 1	DP 2	DP 3	DP 4	DP 5	DP 6	DP 7
PF for A/G 3 kDa	3.48	1.16	0.97	0.79	0.72	0.64	0.64
EF at 3 μl/min	0.48	0.37	0.32	0.32	0.28	0.25	0.21
PF for Fresenius 5 kDa	7.62	1.72	1.26	0.83	0.59	0.43	0.34
EF at 3 μl/min	0.58	0.45	0.31	0.23	0.16	0.12	0.10
PF for A/G 5 kDa	4.18	1.29	0.97	0.81	0.74	0.66	0.61
EF at 3 μl/min	0.48	0.32	0.31	0.23	0.21	0.18	0.17
PF for A/G 10 kDa	3.94	1.45	1.18	1.01	0.90	0.81	0.78
EF at 3 μl/min	0.61	0.40	0.37	0.27	0.25	0.21	0.20
PF for CMA 20 kDa	7.68	3.22	2.07	1.71	1.34	1.42	1.24
EF at 3 μl/min	0.62	0.40	0.33	0.28	0.39	0.21	0.21
PF for Fresenius 30 kDa	6.05	3.63	2.26	1.65	1.47	1.33	1.31
EF at 3 μl/min	0.66	0.58	0.48	0.43	0.40	0.38	0.37
PF for A/G 30 kDa	3.50	2.04	1.52	1.27	1.06	0.92	0.92
EF at 3 μl/min	0.62	0.43	0.36	0.30	0.26	0.23	0.22
PF for A/G 100 kDa	1.08	0.80	0.65	0.51	0.45	0.40	0.38
EF at 3 μl/min	0.12	0.08	0.05	0.04	0.03	0.02	0.02
PF for Amicon 100 kDa	0.15	0.09	0.07	0.06	0.06	0.048	0.05
EF at 3 μl/min	0.03	0.01	0.01	0.01	0.01	0.01	0.01
PF for Nitto Denko NT-3050 6 kDa	1.27	0.90	0.63	0.53	0.47	0.44	0.44
EF at 3 μl/min	0.23	0.13	0.08	0.06	0.05	0.04	0.03
PF for Nitto Denko NT-3306 6 kDa	0.45	0.35	0.27	0.22	0.19	0.16	0.17
EF at 3 μl/min	0.18	0.07	0.05	0.03	0.03	0.02	0.02

The table shows a summary of the permeability factors (PF), EF and cut-off for different membranes. All membranes were made from polysulfone except for the polyamide CMA membrane. The PF were evaluated from a linear regression plot of $\ln(1-E_d)$ vs. $1/Q_d$ for all saccharides at the investigated perfusion rates of 1, 3, 5, 7 and 9 μl/min, for each membrane type and MWCO. The EF values at 3 μl/min give an overall performance of the different membranes.

Table 2
Extraction fraction values for saccharides over a wide analyte concentration range, and different perfusion liquid flow-rates

DP	Concentration range (μ*M*)	Flow-rate (μl/min)		
		2	5	9
1	3.9–250	0.94 (0.06)	0.56 (0.04)	0.36 (0.04)
2	2.5–160	0.72 (0.02)	0.40 (0.01)	0.23 (0.01)
3	1.9–120	0.62 (0.03)	0.34 (0.01)	0.19 (0.01)
4	0.9–60	0.53 (0.03)	0.28 (0.01)	0.153 (0.01)
5	0.8–50	0.46 (0.02)	0.23 (0.01)	0.13 (0.01)
6	0.8–50	0.41 (0.02)	0.20 (0.01)	0.11 (0.01)
7	0.6–40	0.37 (0.02)	0.18 (0.08)	0.10 (0.01)

This table shows the mean and standard deviation (in parentheses) values for the extraction fractions at six different concentrations ($n=6$), in the range given for each saccharide. The values were evaluated individually for a mixture of saccharides, whose concentrations were increased at the same time at the given perfusion rates.

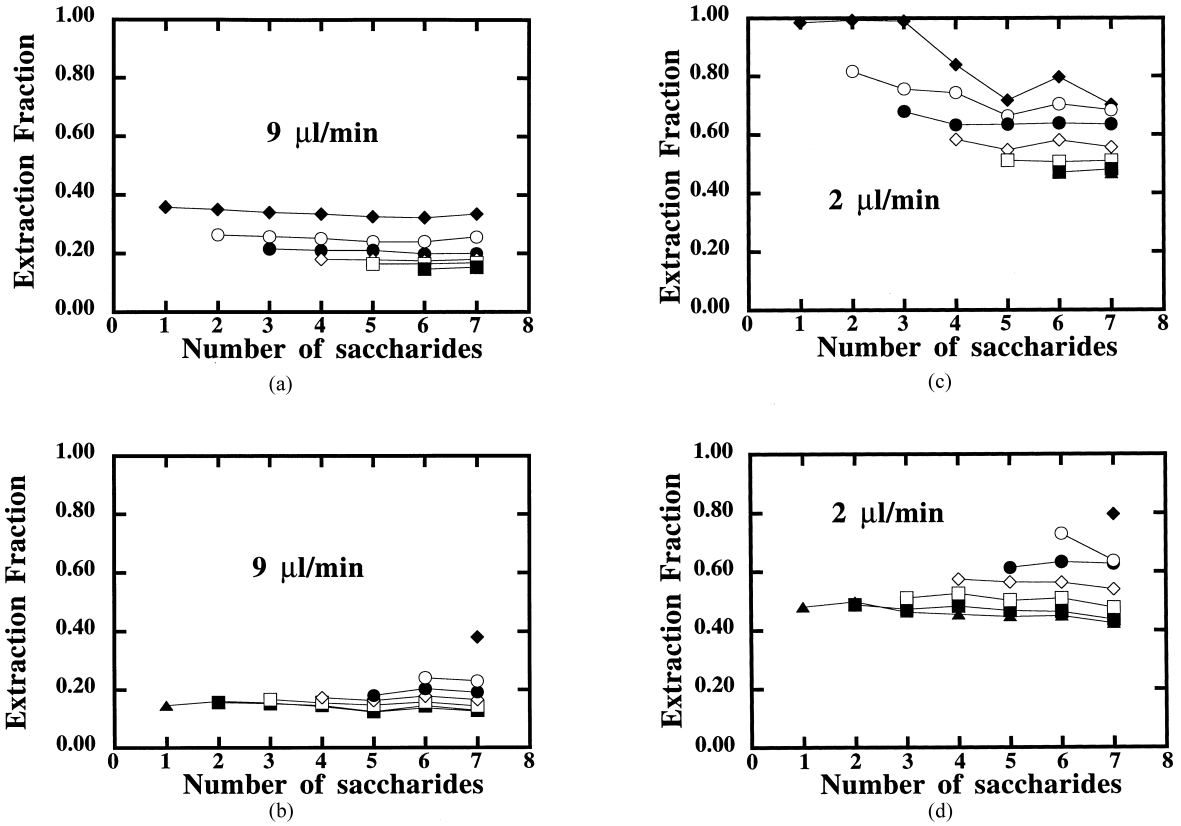


Fig. 4. EF plots demonstrating the interaction of analytes of different sizes as the complexity of the bioreactor is increased by adding larger analytes to smaller ones (a,c), and when the complexity is increased by adding smaller analytes to larger ones (b,d), when perfused at 9 and 2 μl/min, respectively. The symbols represent saccharides as: (♦) DP 1, (○) DP 2, (●) DP 3, (◇) DP 4, (□) DP 5, (■) DP 6 and (▲) DP 7.

treatment. Fig. 6 shows ΔEF for the two polyamide 20 kDa MWCOs (Fig. 6a,b), polysulfone 30 kDa MWCO (Fig. 6c) and polyethersulfone 20 kDa MWCO (Fig. 6d) membranes. Fig. 6a shows a significant change in EF for the polyamide membrane, compared with the other type of polyamide membrane (Fig. 6b). The second polyamide membrane is thus much more suitable for use at high temperature. The polysulfone membrane (see Fig. 6c) has the smallest ΔEF even at low perfusion rates although the EF values have a wider spread compared to those of the polyamide membranes. The EF for DP 1 and DP 2 show a significant change for the polyethersulfone membrane (see Fig. 6d). These investigations showed that the polyamide (see Fig. 6b) and polysulfone (see Fig. 6c) membranes were the most suitable to apply in high temperature bioprocesses.

3.6. Membrane enzyme interaction

The interaction between the membrane and the protein has been of special interest, for example as reflected by the works of Andrade [14–16] and investigations carried out on ultrafiltration membranes [17,18]. When harvesting cells from fermentation broths or during filtration of protein solutions, the proteins are known to foul the membranes by adsorption either in the pores or on the membrane surface. An enzyme/protein layer on the surface of the membrane may affect its hydrophilicity, and consequently affect the EF. It has been demonstrated

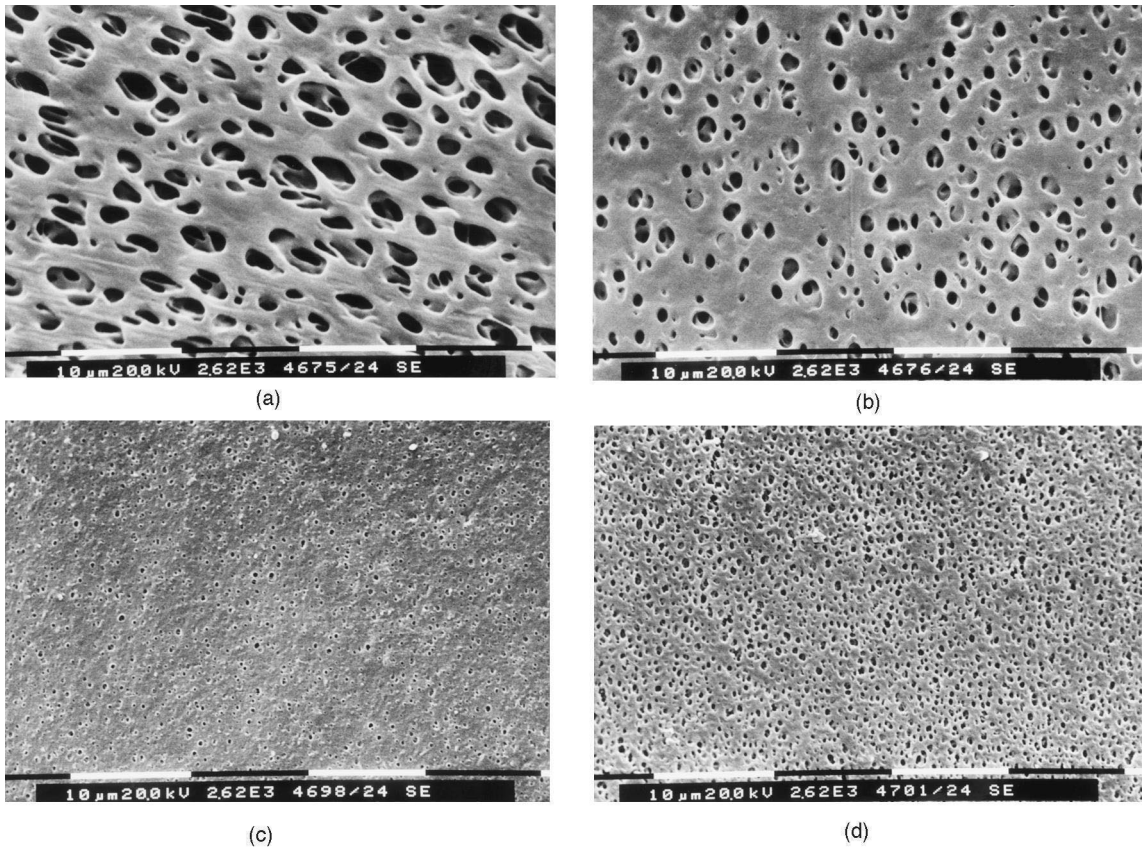


Fig. 5. SEM micrographs for the dehydrated Fresenius polysulfone membrane and the CMA polycarbonate membrane before temperature treatment (a), (c) and after exposure to 90°C for 24 h, (b) and (d), respectively.

that adsorbed protein molecules can behave like flexible polymers, thus their interaction with the membrane will determine their molecular state [19]. The protein contributes to hydrodynamic resistance as it changes its conformation to associate with the membrane surface. The increased hydrodynamic resistance due to the presence of the protein consequently decreases the permeability factor. However, the very low enzyme concentrations used in bioprocesses are not envisaged to cause such type of fouling by adsorption. The fouling of the membrane could occur concomitantly with the adsorption and modification of the enzyme form, thus changing its catalytic activity [20]. To achieve maximum substrate conversion in bioprocesses, and also to characterise unknown enzymes or substrates, it is crucial that the degree and extent of interaction between the enzymes and the membrane material are known. It is of interest to point out that the enzyme could be adsorbed on specific activated sites on the membrane, thus creating a sampling device that is also a biospecific reactor. Although immobilised enzymes are associated with lower activity, they are much more preferred as they can be reused. Membranes are therefore good candidates since they have large inner and outer surface areas which could be utilised for specific protein–membrane immobilisation.

3.7. Non-specific interaction of enzyme with microdialysis membranes

The non-specific interaction of the membranes

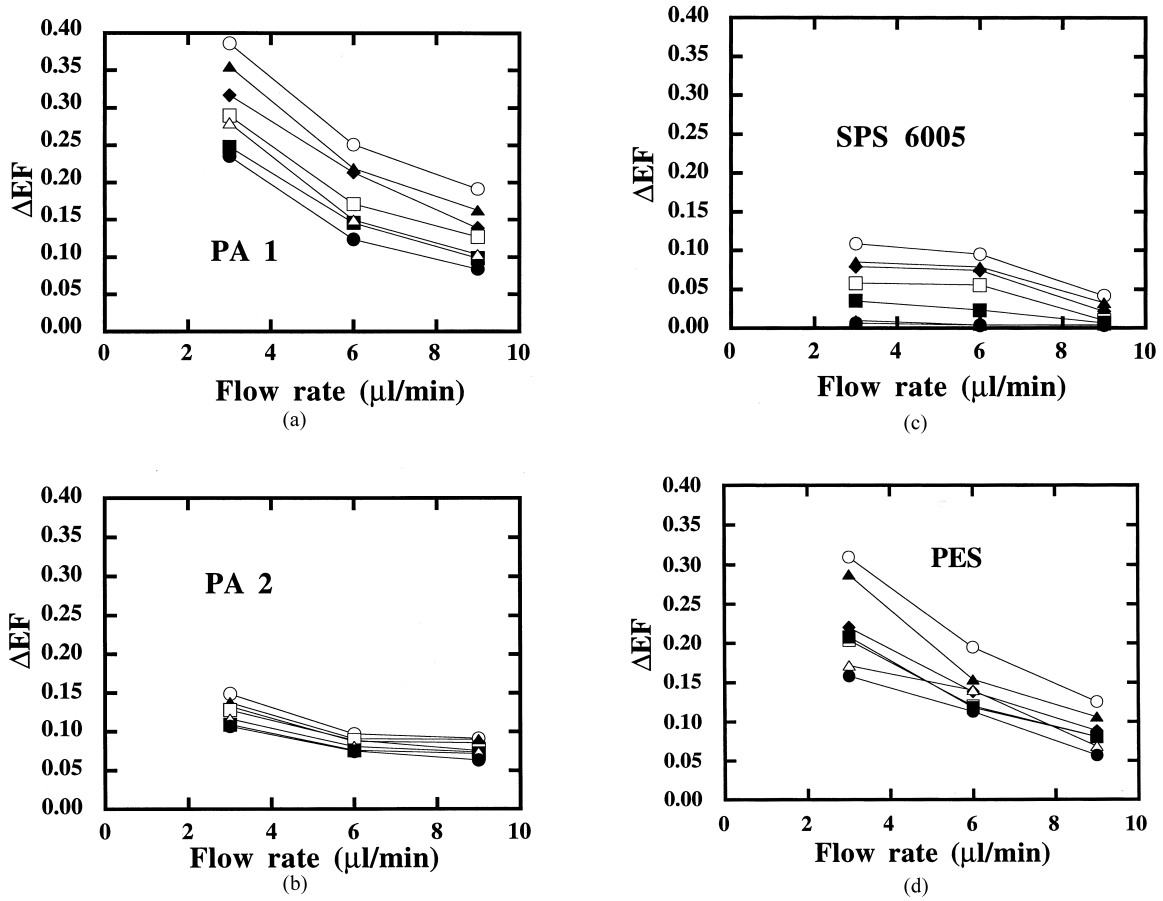


Fig. 6. Performance of the polyamide membranes (a) PA 1, (b) PA 2 and polysulfone membrane, SPS 6005 (c) and polyethersulfone membrane, PES (d) after exposure to 90°C temperature for 3 h. ΔEF values were derived from the difference between EF values before and after temperature treatment (EF values before treatment were higher for all membranes). The symbols represent saccharides as: (♦) DP 1, (○) DP 2, (●) DP 3, (◇) DP 4, (□) DP 5, (■) DP 6 and (▲) DP 7.

with enzymes was investigated with all types of membranes by monitoring hydrolysis products. Termamyl 120 L, which catalyses the hydrolysis of α-1,4-linkages, showed a significant interaction with all the tested membranes as DP 7 was hydrolysed. The exposure of the pullulanase from Novo and Sigma, and that of the isoamylase, all of which catalyse the hydrolysis of α-1,6-D-glucosidic linkages of branched glucans, indicated some interaction of the enzymes with the dialysis membranes. The polysulfone membrane from Fresenius and A/G showed the highest product yield indicating a significant interaction with the enzyme. The polyamide and the Amicon polysulfone membranes exhibited the least interaction with the enzymes. The higher interaction observed for the A/G and Fresenius membranes could probably be due to a high EF and also a more optimal surface which facilitates the interaction with the enzymes especially in the inner spongious layer. From these investigations, the site of enzyme–membrane interaction (inner or outer membrane layer) was not clear. SEM studies have shown a dense porous structure for the Fresenius polysulfone membrane which could possibly ''physically entrap or immobilise'' the enzyme [12]. With this morphological aspect in mind, further investigative

studies were carried out to identify the possible interaction sites.

3.8. Enzyme membrane interaction sites

The Fresenius polysulfone membrane has outer pore diameters as large as 2 μm, and consequently, it is expected that most of the enzyme will be within the porous network structure of the supporting layer. This is so because the molecular mass of the isoamylase [21] is 90 kDa, 58–66 kDa for the pullulanase [22] and 63 kDa for an α-amylase from *Bacillus licheiformis* [23]. Further, the size of albumin in the 60–90 kDa range has been shown to be in the nm-range (14×4 nm) [24]. Moreover, the hydrodynamic radius of the enzymes investigated is of a much smaller order of magnitude compared with the outer pore diameter. The enzyme–membrane interaction was therefore investigated with respect to EF of analytes. A glucose solution was dialysed and injections were made with the on-line injector. After these injections, the interaction with the Sigma pullulanase was effected as before and the resulting enzyme activated membrane was used to dialyse the glucose solution. The enzyme interacting with the membrane did not affect the EF for glucose. If the enzyme is adsorbed inside the separating pores of the membrane, this is expected to reduce the permeability of the membrane to the analytes if the enzyme concentration is high or the hydrodynamic radius is very large. However, for this work, such interactions are not felt by the analytes.

Further experiments were carried out by exposing the membrane to a termamyl solution for 2 s and 5 min, respectively. Fig. 7 shows the hydrolysis profile for DP 7 to lower saccharides when the 5 kDa MWCO Fresenius membrane was exposed to the enzyme for 0 (substrate), 2 s and 5 min. The investigation demonstrates that the enzyme diffused easily into the membrane porous structure within a short time (2 s). It also verifies that some of the enzyme could be ''trapped'' within the porous structure. A further exposure of the membrane to the enzyme for 5 min, with subsequent perfusion (rinsing) at 10 μl/min for 5 and 30 min gave the hydrolysis patterns shown in Fig. 8. Complete hydrolysis of DP 7 was achieved when the membrane

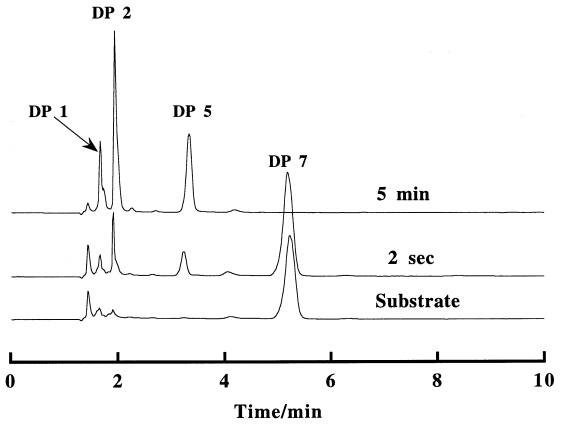


Fig. 7. Chromatogram of the hydrolysis of DP 7 after the membrane was exposed to the enzyme solution for 2 s and 5 min, respectively, demonstrate the rapid rate of enzyme membrane interaction.

was only rinsed from the outside (Fig. 8B), but perfusion of the membrane at 10 μl/min for 5 (Fig. 8C) and 30 (Fig. 8D) min gave partial hydrolysis of DP 7. The high perfusion rate resulted in the sweating of the membrane which gave a back

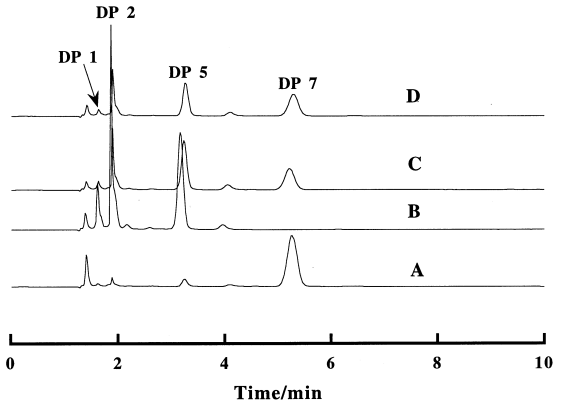


Fig. 8. Chromatograms of DP 7 show that rinsing the outside of the membrane is not sufficient to remove non- or weakly-interacting enzymes since there is total hydrolysis of the substrate. Chromatogram (A) was obtained by dialysing the substrate, (B) chromatogram showing total hydrolysis of DP 7 after an enzyme treated membrane was flushed with water from the outside to remove weakly interacting enzymes, the membrane was perfused at 10 μl/min for 5 (C) and 30 min (D), respectively to remove weakly-interacting enzymes, and consequently, hydrolysis is not complete as some DP 7 is left.

pressure that pushed out all the non- or weakly interactive enzyme from the porous membrane matrix.

4. Conclusion

An analytical methodology for use in the sampling of oligosaccharides has been presented. This hyphenation of techniques is based on the in situ sampling and sample clean-up properties of microdialysis, high resolution separation of HPAEC and the sensitivity of IPED. The present work has demonstrated the performance of various microdialysis membranes with a cut-off ranging between 3 and 100 kDa. Three membrane types; polysulfone, polyethersulfone and polyamide, with different morphology and polymeric structure, supplied by different companies were evaluated. Increasing the complexity of the analytes does not affect their EFs at high perfusion rates as much as at lower perfusion rates. The membranes showed different morphological changes and characteristics after exposure to 90°C for 0, 3 and 24 h. The polysulfone membrane from Fresenius had a significant reduction in porosity, unlike the polyamide membrane which showed an increased porosity. However, the membrane morphology between 3 and 24 h exposure was not significantly different indicating that there is a possibility to apply them at such temperatures. All membranes interacted with the tested enzymes although this interaction did not affect the EF. Such interactions were observed to occur even after a 2 s exposure to an enzyme solution. The observed enzyme membrane interaction, undesirable for some processes, could be used to an advantage in others. This work further demonstrates that optimal membrane choice can enhance the performance of this hyphenation of techniques in sampling of bioprocesses.

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