Qualitative and quantitative carbohydrate analysis of fermentation substrates and broths by liquid chromatographic techniques

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Abstract

Quantitative and qualitative aspects of the column liquid chromatographic analysis of carbohydrates in complex fermentation media are considered. Two fermentations of lignocellulose hydrolysates with Saccharomyces cerevisiae for the production of fuel ethanol were followed. In one spent sulphite liquor and in another an enzymatic hydrolysate of Salix caprea was used as the fermentation substrate. Two of the most commonly used chromatographic set-ups, one ligand-exchange and one ion-exchange system with refractive index and pulsed amperometric detection, respectively, were used to determine the carbohydrates. Some interfering compounds were eliminated by solid-phase extraction prior to sample introduction into the separation system. However, incomplete clean-up of the samples before chromatographic separation resulted in co-elution of matrix compounds with the sugars, introducing quantitative and qualitative errors in the evaluation of the sugar content. In fact, only in 30% of the samples analysed did the results between the two methods agree within 5%. The carbohydrate content of the fermentation samples as given by the two chromatographic methods is presented. Liquid chromatography coupled with thermospray mass spectrometry in both positive- and negative-ion modes was used for the characterization of the molecular ions of glucose, xylose, galactose, arabinose, mannose and well known interfering compounds such as phenolics and related aromatic compounds, and applied to biotechnological fermentation samples for qualitative analysis. Diode-array UV spectrophotometry was used as a complementary detection technique in order to identify unequivocally carbohydrates present in these fermentation media and trace interfering phenolic compounds.

1. Introduction

Since the oil crisis in the mid 1970s, alternative energy sources have been of great importance. For instance, ethanol produced in bioprocesses has been considered as a renewable energy source suitable for usage as a liquid fuel or fuel additive. In North America ethanol is blended with gasoline, and in Brazil ethanol has been used as a fuel for several years [1]. In Sweden, fuel ethanol produced from lignocellulosic material has been suggested as a possible energy source, and it has been successfully employed in 32 buses in Stockholm [2]. These buses have a positive effect on the environment and a further 300–400 ethanol-fuelled buses are planned for Stockholm. As fuel ethanol is a bulk product, the manufacturing process needs to be efficient

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to be cost effective in comparison with gasoline. Factors such as environmental benefits and political decisions will be of great importance to the extent of application of fuel ethanol in the future [1,3].

Fuel ethanol can be produced from different sources of lignocellulose, *e.g.*, agricultural residues, forestry products or municipal waste material [4–7]. The lignocellulosic raw material consists of cellulose, hemicellulose and lignin with variable distributions. The cellulose and hemicellulose fractions can be degraded to a fermentable hydrolysate from which ethanol can be produced. Depending on the raw material and the pretreatment, the lignocellulose hydrolysates will contain different and variable amounts of mono-, di- and oligosaccharides [5]. During the pretreatment not only monosaccharides but also acetic acid, furfural and extractives are released. These substances have an inhibitory effect on the fermenting microorganisms. Other substances with inhibitory action, such as sugar and lignin degradation products, are produced during the pretreatment to variable extents [8–13]. The fermentation process produces not only ethanol but also by-products, *e.g.*, acetic acid, glycerol, succinic acid and xylitol [14]. To ferment the hydrolysates successfully requires a microorganism that can ferment all sugars present to ethanol and also withstand the inhibitors present. Most attention has been given to how the pentose sugars can be fermented to ethanol. Several possible fermentation alternatives have been suggested, *e.g.*, recombinant *Escherichia coli*, *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae* and recombinant *Saccharomyces cerevisiae* [15–17]. Altogether this means that the fermentation substrate has a complex composition which changes throughout the process, and accurate analytical techniques are of utmost importance in developing and optimizing the fermentation process.

The production of fuel ethanol is an example of an industrial fermentation process where a highly complex substrate is used for which analytical techniques have been developed to only a limited extent. In this study we chose to work with two lignocellulose hydrolysates, spent sul-

phite liquor (SSL) and an enzymatic hydrolysate of steam-pretreated *Salix caprea* (EH), the compositions of which are completely different regarding sugar content, inhibitors and the matrix as a whole.

For the analysis of these highly complex fermentation substrates and broths, containing a broad range of lignin breakdown products, intermediates and products, there is a need to find a reliable methodology for accurate qualitative and quantitative analyses for monosaccharides present in these samples. Sample-handling techniques such as solid-phase extraction prior to separation for selective enhancement in chromatographic analysis are commonly utilized. Liquid chromatography (LC) in combination with mass spectrometry (MS) is another powerful method in biotechnological applications. Thermospray (TSP) LC–MS is an appropriate technique for quantification, as demonstrated in the determination of carbamates and chlorinated phenoxy acids, for which positive- and negative-ion detection modes were used [18,19]. TSP as an interfacing system has been found appropriate for quantification purposes at a level of 100 ppb, whereas the absolute detection limit is around a few nanograms under full-scan conditions and below 1 ng in the selected-ion monitoring (SIM) mode. Of the three commercial interfaces available, TSP, electrospray (ES) and particle beam (PB), only TSP and ES were adequate for quantitative purposes. PB showed statistically significant differences in quantification and was inefficient in transporting the analytes into the ion source. The linear dynamic range is 10–1000 ng/ml and the use of SIM is recommended for quantification as the relative standard deviation is lowered to 19%. The advantage of using TSP is that it can be used in both positive-ion (PI) and negative-ion (NI) modes [18,19]. Here, we report on the investigation of qualitative and quantitative evaluations of carbohydrates present in fermentation substrates and broths by using two of the most common LC methods utilized for sugar determination. Mass spectrometry and diode-array UV spectrophotometry are complementary techniques used in the characterization of these processes.

2. Experimental

2.1. Chemicals

High-performance liquid chromatographic (HPLC) grade water, methanol and acetonitrile from Merck (Darmstadt, Germany) were passed through a sterile filter (0.45 μm) (Waters Chromatography Division, Millipore, Bedford, MA, USA) before use. Ammonium formate was supplied by Fluka (Buchs, Switzerland). All carbohydrates, L-(+)arabinose, cellobiose, D-(+)galactose, D-(+)glucose, D-(+)mannose and D-(+)xylose, were of Sigma grade (Sigma, St. Louis, MI, USA) and all phenolic standards were of analytical-reagent grade from Merck. Baker's yeast, *Saccharomyces cerevisiae*, was obtained from Jästbolaget (Rotebro, Sweden). Nutrients added were yeast extract (Difco, Detroit, MI, USA), and analytical-reagent grade ammonium hydrogenphosphate, magnesium sulphate heptahydrate and sodium phosphate (all from Merck). The two fermentation substrates, spent sulphite liquor (SSL) and the enzymatic hydrolysate (EH) produced from fast-growing willow (*Salix caprea*), were kindly supplied by MoDo AB (Örnsköldsvik, Sweden) and the Department of Chemical Engineering I, Lund Institute of Technology, University of Lund (Lund, Sweden), respectively. All other standards were of analytical-reagent grade and purchased from Merck or Sigma. All solutions were prepared by dissolving the substances in water obtained from a Millipore (Bedford, MA, USA) Milli-Q water-purification system or HPLC-grade water.

2.2. Instrumentation

Two chromatographic systems for the determination of sugars were used. The first contained a high-pressure LC pump (Waters Model 600 programmable solvent-delivery module) equipped with a Model 7045 six-port injection valve with a 20-μl loop (Rheodyne, Cotati, CA, USA), a Model 2142 refractive index (RI) detector (LKB, Bromma, Sweden) and a Model 2210

chart recorder (LKB). The analytical column (300 × 7.8 mm I.D.) was a ligand-exchange column in the Pb^{2+} form (Aminex HPX-87P; Bio-Rad Labs., Richmond, CA, USA) heated to 85°C in a chromatographic oven (Waters column heater module, controlled by a Waters temperature-control module). Milli-Q-purified water was used as the mobile phase, pumped at a flow-rate of 0.6 ml/min. This system is referred to as LEC–RI below.

The second chromatographic system consisted of a high-pressure LC pump, an injection valve with a 25-μl loop, a guard column (CarboPac guard 25 × 3 mm I.D.), an analytical column (CarboPac PA1, 250 × 4 mm I.D.) and a pulsed amperometric detection (PAD) system, all from Dionex (Sunnyvale, CA, USA). The mobile phase was 10 mM NaOH, except where stated otherwise, and was pumped at a flow-rate of 1.0 ml/min. The separations were carried out at room temperature. This set-up is referred to as AEC–PAD.

Gel permeation chromatography (GPC) was performed on an LC system consisting of a Model 400 high-pressure pump (Applied Biosystems, Foster City, CA, USA), a Model 6020 injector (Rheodyne) and a Vari-Chrom UV–visible spectrophotometric detector (Varian, Sunnyvale, CA, USA). Analyses of spectra were performed on a Chrom-A-Scope diode-array UV detector (Barspec, Rehovot, Israel). The analytical columns used were a Bio-Beads SX-12 (Bio-Rad Labs.) stainless-steel column (450 × 10 mm I.D.) with dichloromethane as the mobile phase and a Phenogel (Phenomenex; Remuko, Palos Verdes, CA, USA) column (40 × 4.6 mm I.D.) with tetrahydrofuran as the mobile phase.

Reversed-phase separations were carried out on a LiChroCART cartridge column (12.4 cm × 4.0 mm I.D.) packed with 5-μm LiChrospher 100 RP-18 (Merck) using methanol–50 mM acetate buffer (pH 4.2) (20:80) as the mobile phase, followed by a step gradient to a 90% methanol content of the mobile phase run after each separation, using a Chrom-A-Scope rapid-scanning UV detector. The scanning was performed between 190 and 380 nm.

The LC–MS system for qualitative evaluation of sugars consisted of a Model 510 high-pressure pump (Waters Chromatography Division). Injection was carried out using a Model 7125 six-port injection valve with a 20-μl loop (Rheodyne). In flow-injection experiments, a reversed-phase eluent of methanol–water (50:50) containing 0.05 M ammonium formate was used at a flow-rate of 1 ml/min. All mobile phases were degassed in either an ultrasonic bath or by helium gas.

A Hewlett-Packard (Palo Alto, CA, USA) Model 5988A thermospray quadrupole mass spectrometer and a Hewlett-Packard Model 59970C instrument for data acquisition and processing were employed. The temperatures of the TSP were 100, 188, and 270°C for the steam, vapour and ion source, respectively. In all experiments the filament was on. Full-scan conditions were used in most of the experiments, with scanning from m/z 92 to 500 and from m/z 138 to 400 in the PI and NI modes, respectively.

2.3. Fermentation substrate

For fermentation, two different substrates were used; spent sulphite liquor (SSL) and enzymatic hydrolysate (EH) of steam-pretreated Salix caprea. The substrates were supplemented with 2.5 g/l of yeast extract, 0.25 g/l of $(NH)_4HPO_4$ and 0.025 g/l of $MgSO_4 \cdot 7H_2O$ and buffered with 0.1 M sodium phosphate to a final pH of 5.5.

2.4. Fermentation conditions

For fermentation, a 25-ml beaker sealed with a rubber stopper and supplied with a cannula for carbon dioxide removal was used. The beaker was inoculated with compressed baker's yeast (2 g) and either SSL or EH was added to give a final volume of 25 ml. The beaker was slowly stirred at 30°C. Samples (100 μl) were taken after 0, 1, 3 and 24 h of fermentation and the pH was adjusted to 5.5 at each sampling.

2.5. Sample clean-up and fractionation of the fermentation samples

All samples were diluted 50-fold and filtered through a 0.45-μm membrane filter (Schleicher & Schüll, Dassel, Germany) to eliminate particulate contaminants. In the purification by solid-phase extraction (SPE), a diluted sample (1.0 ml) was stirred in a beaker for 5 min with a mixture of polymeric anion- (20 mg) and cation-exchange (10 mg) support (Amberlite; BDH, Poole, UK).

The SSL and EH samples were centrifuged after dilution in a Wifug bench-top centrifuge at 5700 rpm for 1 min, prior to filtration and prior to SPE treatment. Fractionation of SSL and EH samples was performed on the ligand-exchange LC system with the Aminex HPX-87P column and fractions corresponding to each eluting peak (glucose, xylose, galactose, arabinose–ethanol and mannose) were collected.

3. Results and discussion

3.1. Composition of biotechnological samples

The nature of these fermentation samples and substrates is highly complex and the knowledge of their compositions is limited. SSL is a waste material from the pulping industry having a high salt content and produced under harsh conditions in a recycling system. In SSL the sugars solubilized are mainly those from the hemicellulose fraction as the cellulose fraction is utilized in the pulping. The EH was prepared in a two-stage steam-pretreatment process (acidic conditions) combined with enzymatic hydrolysis [20,21]. In the EH, both the cellulose and the hemicellulose can be hydrolysed and thus oligo-, di- and monosaccharides from both fractions are expected to be present. Moreover, SSL is produced from softwood whereas EH is produced from hardwood, and therefore the compositions of the hemicellulose fractions differ [15]. Depending on the conditions of hydrolysis, pentoses and hexoses emanating from the hydrolysed polysac-

charides can easily be converted into different degradation products, e.g., furfural and 5-hydroxymethylfurfural, which not only decrease the amount of usable carbon source for the yeast but also are toxic and may inhibit the fermentation process. In enzymatic hydrolysis the conditions are mild, resulting in a more controlled composition of the product, subsequently used as a substrate. The enzymatic hydrolysis step is specific in contrast to the chemical treatment, which yields a broader range of matrix components. SSL is obtained from an industrial recycling system where interferents can be accumulated. Both substrates will consist of poly-, oligo- and monomers of lignin and various oxidation and breakdown products thereof. Humic substances and browning compounds have also been found in these substrates. Humics are dark-brown, amorphous, colloidal compounds. The major constituents are humic acids, aromatic polymers with a high molecular mass to which a high number of functional groups are attached. Many of the components in the matrix not only interfere in the analysis but also have an inhibitory action on the fermenting microorganism. The fermentation process itself causes a complexity in sample composition as cell mass and nutrients are added to the substrate and different products are produced. A further complication is that the ethanol produced during the process will increase the solubility of more hydrophobic substances in the broth, such as various substituted phenols.

3.2. LC separation systems for carbohydrates

A series of complementary investigations were performed to characterize these highly complex fermentation samples. The analytical procedures used are shown in Fig. 1. These were applied to both the fermentation substrate and the broth, and also to the fractions collected from the LEC–RI system. The crude samples and fractions were independently investigated with four different methods, i.e., UV spectrophotometry, TSP-MS, GPC and reversed-phase chromatography. Two well established LC separation systems for carbohydrate analysis were used for
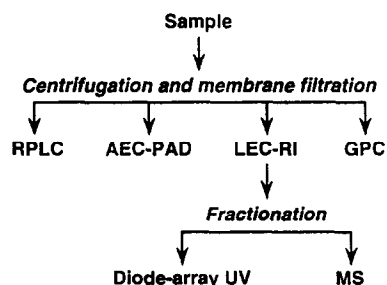

Fig. 1. Illustration of analytical techniques used for the characterization of biotechnological samples. RP-LC = reversed-phase liquid chromatographic system; AEC–PAD = anion-exchange chromatographic system with pulsed amperometric detection; LEC–RI = ligand-exchange chromatographic system with refractive index detection; GPC = gel permeation chromatography; MS = mass spectrometry.

quantitative and qualitative evaluation of these fermentation substrates and broths. In both systems the stationary phase has a poly(styrene-divinylbenzene) matrix, differently derivatized in the two types. One system makes use of ligand exchange as the basis for separation, with Milli-Q-purified water at neutral pH as the mobile phase, while the other utilizes anion exchange with aqueous sodium hydroxide solution of pH 12–13 as the mobile phase. The ligand-exchange system is coupled to a refractive index detector (LEC–RI) and the anion-exchange system to a pulsed amperometric detection unit (AEC–PAD). Polarimetry is the classical way of determining sugar levels and is still used in many industries, but as LC has been introduced for quality control, RI has become the most popular detection method for sugar analysis. This is due mainly to the simplicity of operation of these units. Their lack of selectivity and sensitivity, however, limits their usefulness in analyses of samples with some degree of complexity. This has been shown in several industrial and biological applications [22,23].

The LC systems were investigated with respect to linear response characteristics, repeatability, reproducibility and sensitivity, with five different monosaccharide standards, glucose, xylose, galactose, arabinose and mannose (Table 1). In the concentration range investigated (0.25–5 mM), the LEC–RI system was found to respond

Table 1
Data for the two separation and detection methods

Parameter	LEC–RI	AEC–PAD
Calibration graph concentration range	0.25–5 mM	2–30 μM
Linearity:		
Glucose	$y = 21.9x + 4.6$ ($R = 0.9943$)	$y = 2.1x + 0.86$ ($R = 0.9989$)
Xylose	$y = 20.2x + 4.2$ ($R = 0.9944$)	$y = 2.0x + 0.43$ ($R = 0.9990$)
Galactose	$y = 19.6x + 4.3$ ($R = 0.9942$)	$y = 2.0x + 1.5$ ($R = 0.9992$)
Arabinose	$y = 18.0x + 3.9$ ($R = 0.9931$)	$y = 2.3x + 0.77$ ($R = 0.9994$)
Mannose	$y = 10.2x + 2.4$ ($R = 0.9940$)	Not determined
Limit of detection (LOD)[a]	0.1 mM (mannose 0.15–0.2 mM)	0.1–1 μM (mannose 5–20 μM)
Repeatability:		
Retention time	0.34%[b] ($n = 14$)	0.1–3%[c] ($n = 4$–5)
Peak height	<1%[d] ($n = 7$)	5–6% ($n = 4$)
Reproducibility[c]	ca. 1%	Not determined

[a] For AEC–PAD, this depends on the condition of the PAD detector. LOD is defined as 2–3 times the noise level.
[b] Five calibration solutions and nine real samples.
[c] 0.1% = four identical samples; 3% = five calibration solutions.
[d] Earlier investigations [25].

linearly up to about 2–3 mM with regression coefficients (R) very close to 1.0. Including the response obtained for a 5 mM concentration in the linear regression calculations lowered the R values to about 0.994, indicating a small deviation from linearity. With the AEC–PAD system, the calibration graph ranged between 2 and 30 μM. High R values (0.9989–0.9994) were obtained over the entire interval, indicating good linearity. No response for mannose, however, appeared until a concentration of 20 μM was injected and subsequently this sugar was not used for the calibration graph. The limit of detection (LOD) is normally low for sugars in this system 0.1–1.0 μM (Table 1). Our experience is that the performance of the PAD unit determines the LOD. Poisoning of the electrode will eventually occur after repetitive injections of these complex samples, although the surface of the electrode is cleaned after each measurement [24,25] (see below). We found that after running the system for 24 h, a drastic increase in response was obtained by flushing the detector cell with 0.2 M NaOH for 1 h. A lowering of the detection limits by a factor between 5 and 10 was feasible with this cleaning step, indicating that matrix compounds causing electrode fouling

were eliminated from the electrode surface. The LOD value for all sugars except mannose was therefore found to be about 1 μM for an "old" cell surface and about 0.1 μM for a "fresh" one. As mentioned above, the response for mannose is lower, giving LODs of between 5 and 20 μM depending on the condition of the detector. In the LEC–RI system, the limits of detection were ca. 0.1 mM except for mannose, which was about twice as high. The lowering of the response is due to the high k' value of mannose and its lower RI factor, which is about half that of the other sugars.

The AEC–PAD system thus offers an LOD two to three orders of magnitude lower than that of the LEC–RI system. Sensitivity, however, is not a critical parameter in these applications during most of the process time, owing to the relatively large amounts of the analytes. Baseline separation of the five sugars is obtained with the AEC–PAD system, in contrast to the LEC–RI system, in which the peaks of arabinose and mannose partly overlap. The improved separation is due to the formation of the enolate form of the sugars at this high pH. As anions, they are more easily separated compared with the separation system based on ligand exchange, where

the sugars are uncharged. In the ligand-exchange mode, the forces of interaction are obviously much weaker, resulting in lower resolution. On the other hand, the repeatability and reproducibility of the LEC–RI system are better, regarding both retention time and peak height (Table 1). In fact, the repeatability of retention times of the AEC–PAD system can be very poor. We found that the electrochemical detector was sensitive to changes in pH resulting from dissolution of carbon dioxide into the NaOH-based mobile phase, which also changes its ionic strength and thus affects the chromatographic separation.

A difference between the samples analysed by the two systems was the utilization of an SPE step prior to injection into the LEC–RI system. A mixed anion- and cation-exchange SPE support was used in order to exclude Cl^- ions and cations such as metal ions present in the samples. The presence of these ions will otherwise ruin the performance of the analytical column. In addition, we have found in other studies that these polymeric SPE supports eliminate interfering matrix compounds to a varying extent, aromatic lignin breakdown products being removed very efficiently [26,27]. Typical recovery values for the five sugars were found to be 88–94%. This clean-up step should also be taken into consideration in evaluating the stability of the

two systems. The better stability found for the LEC–RI system could well be due to the less complex samples handled by the analytical column and the detector, while all matrix components were introduced into the AEC–PAD system.

The amounts of each sugar in the two hydrolysates and the corresponding chromatograms are shown in Table 2 and Fig. 2. As is evident from Table 2, some results obtained with the two methods for the contamination of the sugars agree very well whereas others differ. In only 30% of the cases (12 out of 40) did the results of the two methods agree within 5%. There are several reasons for these differences. First, with the LEC–RI system, it is sometimes difficult to determine small amounts owing to the elution of interfering matrix compounds (see Fig. 2A). The samples run using the AEC–PAD system had to be more diluted so as to fall within the linear concentration range. Any interfering compounds were then equally diluted. Indeed, these chromatograms appear very clean (see Fig. 2C), resembling those obtained when running standard carbohydrate solutions, and are accordingly easier to quantify. The chromatograms (Fig. 2C and D) also reflect the superior discrimination of the amperometric detector where the chosen operating potential seems to be optimum with respect to the overwhelming number of matrix

Table 2
Determination of sugars (mM) in fermentations of SSL and EH using ligand-exchange chromatography with refractive index detection and anion-exchange chromatography with pulsed amperometric detection

Method	Sugar	SSL 0	SSL 1	SSL 3	SSL 24	EH 0	EH 1	EH 3	EH 24
LEC–RI	Glucose	28	9.0	0	0	390	160	4.4	0
	Xylose	66	66	54	44	54	30	48	26
	Galactose	29	32	26	21	19	14	4.2	3.0
	Arabinose	10	14	10	8.5	12	0	0	0
	Mannose	120	89	0	0	11	0	0	0
AEC–PAD	Glucose	25	6.7	2.6	0	357	165	4.0	0
	Xylose	63	66	52	36	55	30	47	26
	Galactose	32	31	26	17	6.8	2.8	6.0	2.4
	Arabinose	14	15	12	8.7	4.9	4.0	3.7	2.1
	Mannose	85	48	5.8	2.2	11[a]	0	0	0

SSL = spent sulphite liquor; EH = enzyme hydrolysate; 0, 1, 3 and 24 = time (h) after start of fermentation.
[a] This value was determined in a separate analysis which does not correspond to the chromatogram shown in Fig. 1D.
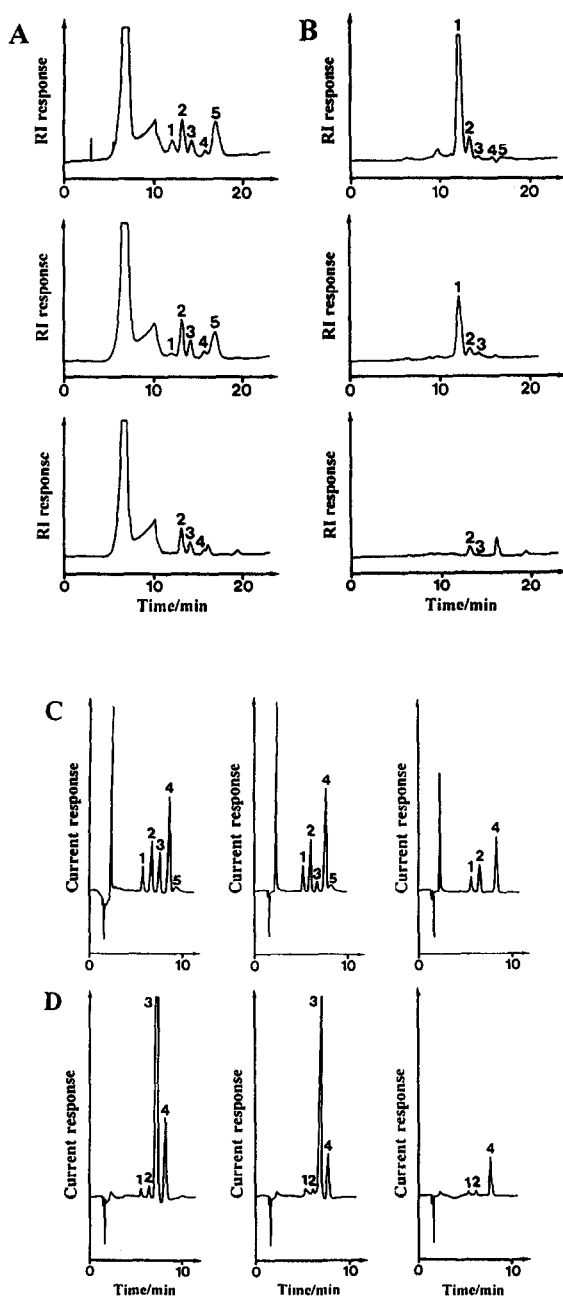
Fig. 2. Chromatograms of samples taken after 0, 1 and 24 h of fermentation in SSL and EH using two different separation and detection principles. (A) LEC–RI of SSL; (B) LEC–RI of EH; (C) AEC-PAD of SSL; (D) AEC-PAD of EH. Peaks (LEC–RI): 1 = glucose; 2 = xylose; 3 = galactose; 4 = arabinose; 5 = mannose. Peaks (AEC–PAD): 1 = arabinose; 2 = galactose; 3 = glucose; 4 = xylose; 5 = mannose.

compounds in these crude samples. Second, as the PAD is more sensitive with lower LOD values, the determination of smaller amounts can be made more accurately.

The sugar initially having the highest concentration in SSL is mannose, followed by xylose, about equal amounts of glucose, galactose, and arabinose (see Fig. 2A and C). In EH, glucose is the totally dominant sugar and only small amounts of the others are traceable (Fig. 2B and D). When *Saccharomyces cerevisiae* is used in fermentation, glucose and mannose are the first two fermentable carbohydrates to be metabolized. The two methods give very similar values for the concentration of the virtually non-fermentable pentose xylose (see Table 2), whereas the largest discrepancies between the methods are found for mannose in the SSL fermentation. A reliable comparison cannot be made for arabinose owing to the lower sensitivity of the ligand-exchange system. Galactose and arabinose seem also to vary at the beginning of the process when EH is fermented. It was found in another study that, when the ligand-exchange system was used, unidentified peaks appeared in the chromatogram during the process [22]. In addition, some of the sugar peaks did not decrease in the order expected during the production of ethanol [27].

The variation in the evaluated concentrations can be explained partly by the different separation mechanisms of the two systems, although the largest difference lies in the detection methods. RI detection measures the difference in refractive index between the solutes eluting through the flow cell and the eluent present in the reference cell. Compounds eluting through the cell with either a higher or a lower refraction index than that in the reference cell will result in a positive or a negative peak. However, if several analytes pass simultaneously, an additive multi-component signal will be obtained. This is the case if the resolution is incomplete in the chromatographic separation. The corresponding peak will be positive if the sum of the RI factors of the solutes in the unresolved peak is value higher than that in the reference cell and negative if the sum is lower, and a flat baseline is

obtained if the sum is zero or close to zero. The last case has been found to occur in certain industrial applications [22]. The RI detector is thus much affected by the presence of interfering substances, to which the PAD does not necessarily respond.

Oxidation of sugars at electrode surfaces often suffers from electrode fouling due to electrochemical formation of products that adsorb on the electrode surface [24]. Electrochemical detection principles such as PAD, pre- and post-column derivatization systems with various chemical reagents and enzymes as catalytic reagents have been intensively studied in order to circumvent the irreversible fouling effects of the electrode [22,25].

3.3. Characterization by gel permeation and reversed-phase chromatography

Samples were further characterized by GPC and reversed-phase chromatography. GPC in combination with diode-array detection provided complementary information on the distribution of molecular size and shape of the components present in the samples withdrawn from the process 0, 1, 3 and 24 h after the initiation of the fermentation process. Fingerprint spectra can be obtained for the specific compounds present in each substrate and broth [28,29], and also for each resulting chromatographic peak. These data provide information about changes in matrix composition and possible indications of the presence of substituted phenolics and structurally related molecules, as breakdown of lignin oligo- and polymers can give products of these types.

Typical chromatograms obtained from two of the samples using this technique are shown in Fig. 3, which indicates the distribution of predominantly mono-, di- and oligomeric breakdown products of lignin present in the broth. It was found that the composition of these lignin breakdown products differed widely depending on the time the sample was taken during the fermentation. The GPC column used in this instance (SX-12) has an upper molecular mass exclusion limit of 400, which means that the compounds eluting after the front peaks are
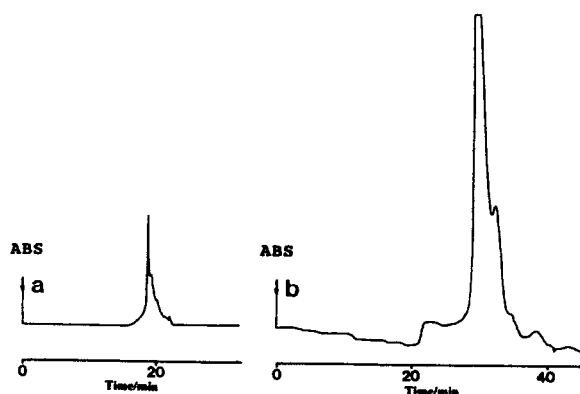

Fig. 3. Gel permeation chromatographic separations of (A) fermented SSL diluted 1000-fold on a Phenogel separation column using tetrahydrofuran as the mobile phase and (B) fermented SSL diluted 1000-fold on an SX-12 separation column using dichloromethane as the mobile phase. Flow-rate, 1 ml/min; injection volume, 20 μl; UV detection 280 nm, 0.1 AUFS.

predominantly of molecular mass below 400. The front will contain lignin breakdown products with a molecular mass higher than the exclusion limit of the column, whereas the last peak will typically contain, among all other fermentation products and intermediates, lignin monomers, mono- and disaccharides, small oligosaccharides, furan aldehydes and the alcohol produced. In the chromatogram shown in Fig. 3B, where the molecular mass exclusion limit of the column used (Phenogel) is 1000, the front contains all excluded compounds with molecular mass above 1000. The rest of the sample is seen as eluting peaks with a poorer resolution. These results clearly show the presence of compounds having a range of different molecular masses in the samples, despite pretreatment by filtration, centrifugation and SPE.

Isocratic reversed-phase separations were utilized to separate and determine furan aldehydes. The level of furfural and 5-hydroxymethylfurfural can be correlated with the efficiency of the hydrolysis steps, as these two aldehydes are products originating from hexoses and pentoses. Low levels of 5-hydroxymethylfurfural present in the substrates will reflect optimum hydrolysis conditions with high hexose levels. This was of particular interest as the low molecular masses of
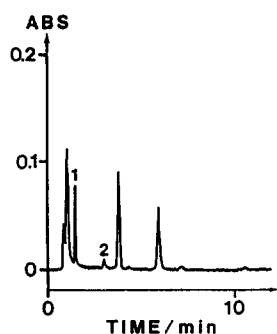
Fig. 4. Reversed-phase separation from SSL (*t* = 3 h) diluted 200-fold on a LiChrospher 100 RP-18 analytical column. Mobile phase, methanol–50 m*M* acetate buffer (pH 4.2) (20:80); flow-rate, 1 ml/min; injection volume, 20 μl; UV detection at 280 nm, 0.05 AUFS. Peaks: 1 = 5-hydroxy-methylfurfural (40 μ*M*); 2 = furfural (0.6 μ*M*).

the two aldehydes did not permit analysis with TSP-MS (see below). Fig. 4 illustrates a reversed-phase separation in which both furfural and 5-hydroxymethylfurfural can be identified. The mobile phase was chosen to contain smaller amounts of organic additives such as methanol [methanol–10 m*M* acetate buffer (pH 4.2) (20:80)] in this particular case in order to achieve a good resolution. A step gradient to a 90% methanol content of the mobile phase was run after each separation (data not sown) to clean the column from strongly bound hydrophobic lignin breakdown products. The column was equilibrated with the mobile phase (10 ml) before the next injection was made.

3.4. Fractionation from the ligand-exchange system

UV spectra of the fractions obtained by LEC–RI showed that they contained not only sugars but also other compounds. This is clearly seen for both the hexoses and the pentoses by comparison of the spectra from a pure glucose solution (Fig. 5A), the glucose and xylose fractions from SSL (Fig. 5B and C) and arabinose and mannose from EH (Fig. 5D and E). The UV spectra from pure hexose and pentose solutions appear almost identical. As is clearly seen, there is a marked difference when comparing the pure standard solution with the fractionated samples.
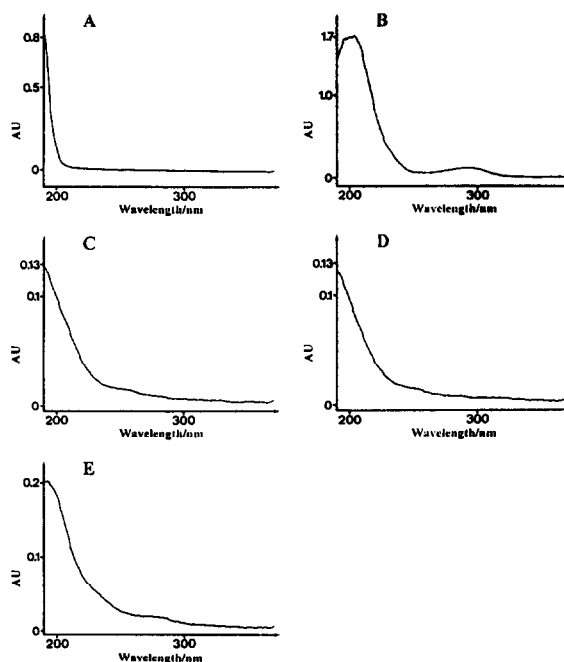

Fig. 5. Diode-array UV spectra of (A) a pure glucose standard, (B) a fractionated glucose peak present in SSL, (C) a fractionated xylose peak present in SSL, (D) a fractionated arabinose peak present in EH and (E) a fractionated mannose peak present in EH.

In Fig. 5B and E there are similar peak maxima around 220 and 280 nm similar to those in the spectra of many of the phenolic and related aromatic compounds. In fact, the spectra obtained from these fractionated chromatographic eluates resemble those of some of the fermentation broths previously investigated after liquid–liquid extraction [28].

3.5. LC–MS

Sugars are a class of compounds difficult to handle by MS. They are not amenable to study directly by GC–MS and chemical derivatization is needed. In order to characterize the sugars directly, either chemical ionization mass spectrometry (CI-MS) (using methane) [30] or different combinations of LC–MS [31–33] have been suggested. Advantages claimed in the use of LC–MS for sugars include ease of sample preparation, speed of analysis, similar sensitivity and

good reproducibility. In CI-MS the $[MH]^+$ ion was usually present together with a pattern of fragmentation that permitted the deduction of the sugar structures [30]. When LC–MS was used with a moving belt interface, xylose, fructose, glucose and sucrose being characterized by ammonia CI-MS, the $[M + NH_4 - H_2O]^+$ and $[M + NH_4]^+$ ions were major peaks in the spectrum [31]. It should be noticed that with LC–TSP-MS, the $[M + NH_4]^+$ ion was the base peak for glucose [32]. However, when the TSP probe temperature is increased above 230°C, a slight increase in the relative abundance of the dehydrated $[M + NH_4 - H_2O]^+$ ion is noticed (usually it gives a 10% relative abundance). Although

most of the experiments performed under LC–MS conditions were carried out in the PI mode, one example reported in the literature indicated that LC–TSP-MS in the NI mode was feasible for glucose, thus giving $[M - H]^-$ as the base peak [33], with a sensitivity similar to that of GC–MS.

The objective of our work was to use LC–TSP-MS for the characterization of the different sugars in the PI and NI modes. From the results with the sugars shown in Table 3, it can be seen that all the compounds behave similarly in the PI and NI modes of detection, and that degradation does not occur. The $[M + NH_4]^+$ ion is always the base peak in the PI mode, as expected for

Table 3
Important fragments, relative abundances (RA) and arbitrary units (AU) given in an absolute scale of units in mass spectrum observed in LC–TSP-MS in positive-(PI) and negative-ion (NI) modes and filament-on mode of operation for sugars

Molecular mass	Compounds and ions (m/z and tentative identification)	RA		AU
		PI	NI	Base peaks
180	Glucose			
	180 $[M + NH_4 - H_2O]^+$	10		
	198 $[M + NH_4]^+$	100		150
	179 $[M - H]^-$		20	
	225 $[M + HCOO]^-$		100	40
150	Xylose			
	150 $[M + NH_4 - H_2O]^+$	10		
	168 $[M + NH_4]^+$	100		60
	149 $[M - H]^-$		1	
	195 $[M + HCOO]^-$		100	15
150	Arabinose			
	150 $[M + NH_4 - H_2O]^+$	25		
	168 $[M + NH_4]^+$	100		20
	149 $[M - H]^-$		1	
	195 $[M + HCOO]^-$		100	15
180	Galactose			
	180 $[M + NH_4 - H_2O]^+$	25		
	198 $[M + NH_4]^+$	100		2
	179 $[M - H]^-$		5	
	225 $[M + HCOO]^-$		100	4
180	Mannose			
	180 $[M + NH_4 - H_2O]^+$	15		
	198 $[M + NH_4]^+$	100		12
	179 $[M - H]^-$		5	
	225 $[M + HCOO]^-$		100	4

Amount injected: 1 μg.

TSP. In contrast, when LC–MS was used with a moving belt interface or the sugar solution was spotted directly on the belt, the ratio between the $[M + NH_4 - H_2O]^+$ and $[M + NH_4]^+$ ion abundances changed. Generally, with direct spotting the relative abundance of the $[M + NH_4]^+$ ion increased whereas that of $[M + NH_4 - H_2O]^+$ decreased, suggesting that during moving belt LC–MS some thermal degradation occurred [31]. In our experiments, the abundance of $[M + NH_4]^+$, similar to that reported by other workers using TSP conditions [32], was also similar to that reported with direct spotting, indicating that LC–TSP-MS offers a better approach than the moving belt as it avoids thermal degradation. One reason for the abundance of degradation under TSP conditions in this study is that TSP temperatures below 230°C were employed, as recommended by other workers [32].

With TSP-MS in the NI mode, the common base peaks correspond to the adduct ion with the eluent additive formate, and as the second peak the proton abstraction peak ($[M - H]^-$ ion). In this respect these data differ from the TSP-MS of glucose in the NI mode in that we obtain as the base peak the adduct with formate, whereas Reid et al. [33] did not report adduct formation with their additive, ammonium acetate. This may be explained in this work by the use of a different source; the instrument used usually gives much more adduct formation under NI TSP-MS conditions, as reported [34]. The different eluent additive, formate in our experiments, forms adducts more readily than acetate [33]. The formation of both ions in TSP-MS in the NI mode indicates an intermediate behaviour and shows that adduct formation is always more important than proton abstraction, as has been noted for various phenoxyacetic acid herbicides [35]. By calculating the relative abundance (RA) values (see arbitrary units) as shown in Table 3 (the percentage of each ion in the spectrum), comparison of the spectra is feasible. It is possible to overcome fluctuations and experimental variations in the instrumental performance by use of these calculated values. It is readily seen from Table 3 that glucose and xylose gave signal responses at least one order of magnitude better

than those given by galactose and mannose. This implies that the LODs for glucose and xylose will be lower than those for galactose and mannose.

The relative abundances of the important ions obtained from different phenolics in TSP-MS can be found, as indicated in Table 4. The fragmentation pattern for the different phenolic compounds under NI TSP-MS conditions follows expectations [34,36] and gives, in general the $[M - H]^-$ and $[M + HCOO]^-$ ions as the main peaks. In some instances $[M - H]^-$ is not observed for a series of compounds, e.g., 4-hydroxybenzoic acid, 2,4-dihydroxybenzaldehyde and benzoic acid (see Table 4); this is due to the fact that the scan range started at m/z 138, so that it was impossible to detect their proton abstraction peak for a compound with a lower molecular mass. It is interesting to note that when an ethoxy group is introduced, as in 3-ethoxy-4-hydroxybenzaldehyde, the compound gives a response under PI conditions, which indicates the enhancement of the proton affinity of this compound by the addition of an ethoxy group to the molecule. With hydroxycoumarin, the behaviour is reversed; when a hydroxy group is introduced, the gas-phase basicity increases and therefore the hydroxy compound gives a response in the NI mode, whereas coumarin does not. Benzoic acid derivatives can only stabilize the negative charge by electron delocalization in the aromatic ring and so give good responses in TSP-MS in the NI mode.

To distinguish between sugars, the use of MS–MS will be needed, as has been demonstrated for triazine herbicides [37]. The identification of m-coumaric acid was also feasible in an SSL fraction (from flow-injection chromatograms; see Fig. 6). All the individual peaks correspond to different injections and each peak at m/z values of 163 and 209 also matches the total ion chromatogram. The use of the NI mode permitted SIM using the two main ions in the isolated fraction. This technique was found to be useful for identification in these complex samples, as this mode of operation enhances the signal in the total current trace. This is illustrated in Fig. 6 (peaks between 12 and 17 min) by the definite identification of the presence of this compound

Table 4
Important fragments, relative abundances (RA) and arbitrary units (AU) given in an absolute scale of units in mass spectrum observed in LC–TSP-MS in positive-(PI) and negative-ion (NI) modes and filament-on mode of operation for substituted phenols

Molecular mass	Compounds and ions (m/z and tentative identification)	RA		AU
		PI	NI	Base peaks
138	4-Hydroxybenzoic acid			
	183 $[M + HCOO]^-$	n.d.[a]	100	3
154	3,4-Dihydroxybenzoic acid			
	153 $[M - H]^-$	n.d.	100	1
	199 $[M + HCOO]^-$	n.d.	100	1
154	3,5-Dihydroxybenzoic acid			
	153 $[M - H]^-$		10	
	199 $[M + HCOO]^-$	n.d.	100	1
166	3-Ethoxy-4-hydroxybenzaldehyde			
	167 $[M + H]^+$	100		3
	184 $[M + NH_4]^+$	30		
	165 $[M - H]^-$		100	5
	211 $[M + HCOO]^-$		60	
138	2,4-Dihydroxybenzaldehyde			
	183 $[M + HCOO]^-$	n.d.	100	5
138	3,4-Dihydroxybenzaldehyde			
	183 $[M + HCOO]^-$	n.d.	100	11
122	Benzoic acid			
	167 $[M + HCOO]^-$	n.d.	100	6
154	2,5-Dimethoxyphenol			
	155 $[M + H]^+$	100		4
	199 $[M + HCOO]^-$		100	1
126	5-Hydroxymethylfurfural			
	144 $[M + NH_4]^+$	100	n.d.	100
148	Cinnamic acid			
	147 $[M - H]^-$	n.d.	10	
	193 $[M + HCOO]^-$	n.d.	100	3
146	Coumarin			
	147 $[M + H]^+$	5	1	164
	164 $[M + NH_4]^+$	100		
162	4-Hydroxycourmarin			
	163 $[M + H]^+$	30	5	180
	180 $[M + NH_4]^+$	100		
	161 $[M - H]^-$		100	3
	207 $[M + HCOO]^-$	25	1	
162	7-Hydroxycoumarin			
	163 $[M + H]^+$	7		
	180 $[M + NH_4]^+$	100		140
	161 $[M - H]^-$		100	8
	207 $[M + HCOO]^-$		90	
164	m-Coumaric acid			
	163 $[M - H]^-$	n.d.	3	
	209 $[M + HCOO]^-$	n.d.	100	5

Amount injected: 1 μg.
[a] n.d. = Not detected. Other phenolics not reported in this table gave no signal under the experimental conditions used.
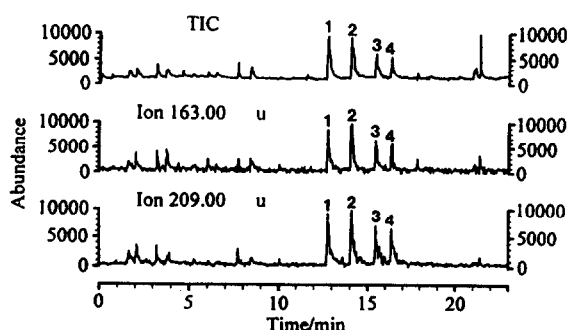
Fig. 6. Total ion current and selected-ion chromatograms of a ligand-exchange chromatographic fraction of SSL under NI TSP-MS conditions. Peaks 1 and 2 are from SSL 1 h and peaks 3 and 4 are from SSL 3 h. Ions monitored correspond to $[M - H]^-$ and $[M + HCOO]^-$ with m/z values of 163 and 209, respectively. Carrier stream, methanol–water (50:50) containing 0.05 M formate at 1 ml/min.

in the sample. Although only *m*-coumaric acid could be identified in these samples there are many other peaks with various masses. These ions are seen after background correction and in many instances have high molecular masses, above 2000. Hence it cannot be qualitatively proved that these compounds are breakdown products of lignin, with aromatic structures as shown in earlier investigations [28]. Studies of the use of LC–TSP-MS for identification and quantification purposes with phenolics in these types of complex samples are continuing. In this context, the use of new interfacing systems such as electrospray [38] has been demonstrated to be useful for the determination of phenolics in waste streams, in many instances in combination with MS–MS. The combination of TSP and ES may also be very powerful in this field.

4. Conclusions

This study has clearly shown the problems encountered in the determination of sugars in fermentation substrates and broths. These problems are alleviated using lignocellulose hydrolysates as a model substrate, utilizing the most commonly used LC techniques. Today, many processes are operated with subsequent analysis of these or similar carbohydrates; however, very

little information is available regarding their qualitative and quantitative validation.

For the proper utilization of these lignocellulose hydrolysates it is essential that a true picture of the distribution of the various sugars is known, in addition to their individual concentrations. Different hydrolysis methods or small changes in the parameters of the process may cause alterations in the sugar yield, which may in turn cause changes in the chemical environment with which the microorganism has to cope in the fermenter. This is of special importance when new strains of microorganisms are tested as candidates for possible utilization in biotechnological processes or in understanding the metabolic pathways of different (*e.g.*, recombinant) species. These applications using a combined analytical methodology show advantages for the characterization of carbohydrates and phenolic compounds possibly present in fermentation substrates and broths interfering with the chromatographic evaluation.

It is also envisaged that ES and TSP will be used as ionization methods in conjunction with MS for quantitative purposes in the analysis of carbohydrates in complex samples. Both interfaces will be compared in the PI mode for sugars and in the NI mode for phenolics. This approach will be useful for scientists working in the biotechnological field for identifying and determining compounds present in the complex fermentation processes. The technique will also be complementary to current analytical methods used in the biotechnological field.

There are methods of avoiding interferences from matrix components in these samples, such as the introduction of small precolumns in a coupled column system in order to eliminate interfering compounds on-line in a chromatographic system. The combination of a coupled-column clean-up step may be combined with selective detection utilizing enzyme-based detection systems, specially developed for these types of fermentation samples. LC sensors based on chemical recognition as opposed to those based on physical recognition is currently under study due to the lack of such detection units. Several papers have reported the use of biological recog-

nition in sensor technology. Immobilized enzyme reactors are currently marketed by a few companies for use in pre- or postcolumn derivatization of substrates inherently difficult to monitor selectively. Enzyme electrodes have been studied mainly for the selective determination of a single substrate and have therefore found only few applications in LC. However, most enzymes are specific to a group rather than a single substrate and there are many enzymes with a broad selectivity pattern. Therefore, in this context, with recent knowledge of enzyme stabilization and organic-phase enzymology, it is expected that biological recognition will in the future have a great impact in sensor technology for LC applications.

5. Acknowledgements

6. References

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