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On-line sample clean-up of fermentation broths and substrates prior to the liquid chromatographic separation of carbohydrates

G. MARKO-VARGA*
Department of Analytical Chemistry, University of Lund, P.O. Box 124, 221 00 Lund (Sweden)
E. DOMINGUEZ[a] and B. HAHN-HÄGERDAL
Department of Applied Microbiology, University of Lund, P.O. Box 124, 221 00 Lund (Sweden)
L. GORTON
Department of Analytical Chemistry, University of Lund, P.O. Box 124, 221 00 Lund (Sweden)
and
H. IRTH, G. J. DE JONG, R. W. FREI[b] and U. A. Th. BRINKMAN
Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)
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ABSTRACT

A coupled precolumn set-up is described for the clean-up of fermentation broths and substrates of hydrolysed lignocellulose prior to the analysis of carbohydrates. Matrix components cause severe problems owing to irreversible adsorption, increased back-pressure in the analytical column and interferences with the detection of the analytes. Sep-Pak (quaternary ammonium and amino) phases were used for the removal of brown reaction compounds and aldehydes. Non-polar (aromatic) compounds, phenolic acids and their derivatives and metal ions were removed using the hydrophobic polymer PLRP-S and a mixed cation–anion-exchange phase, respectively. Breakthrough volumes on the four precolumns were measured for fifteen model compounds normally present in hydrolysed lignocellulose. Applications to real samples illustrate the increased detectability in refractive index and UV detection and in detection systems using immobilized enzyme post-column reactors with UV and electrochemical detection.

INTRODUCTION

The increasing demand for the characterization and quantification of substrates and metabolites in complex biological matrices and for the screening of large numbers of samples has stimulated progress with new detection principles in these areas. The selectivity and sensitivity in column liquid chromatographic (LC) detection

[a] On leave from the Department of Pharmacy, Food Analysis and Nutrition, University of Alcala de Henares, Madrid, Spain.
[b] Author deceased.

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modes are crucial factors that have to be optimized in the study of sample composition in fermentation processes [1–3]. The concentration levels of interfering compounds vary strongly in the fermentation process as a result of the biological activity in the cells and the media. Often obtaining good resolution is the most time-consuming step in setting up a chromatographic system. There is therefore an increasing tendency to introduce selectivity in the sample preparation and/or in the detection step in order to facilitate the separation procedure [4]. A precolumn sample clean-up step before injection can be introduced by the use of solid-phase extraction [5–7] on either disposable or reusable precolumns in a coupled column set-up. With complex samples both pre- and post-column selectivity are required and can correspondingly be introduced.

Sample clean-up can be accomplished either (i) by sorption of the analyte on the clean-up phase and elution of most of the interfering compounds, which can be performed in one step or in several steps in which the eluent strength gradually increases linearly or in a step gradient, the strongest eluent desorbing the analyte, or (ii) by sorption of the interfering compounds on the precolumn, while the analyte has no affinity to bind to the column material and elutes directly. If there is a large variety of interfering compounds, several clean-up columns with different sorption characteristics have to be used.

Waste water from the pulp industry contains a hemicellulose fraction with high levels of lignocellulose, oligosaccharides and various monosaccharides as the major sugar constituents [8,9]. This fraction can be used as a substrate for ethanol production by fermentation with *Saccharomyces cereviseae* [10]. The acid hydrolysis of wood will produce a complex mixture, containing a wide variety of compounds present in the technical substrate used in the fermentation process (see Fig. 1). Moreover, during this fermentation step, the level of toxic compounds may be decreased or even reduced to zero, which is also beneficial from an environmental point of view. This paper describes the removal of a large number of interfering compounds present in a hydrolysed lignocellulose substrate and in fermentation broths that would otherwise severely disturb the determination of the carbohydrate composition. Fifteen test compounds (for structures, see Fig. 2) are sorbed in a coupled pre-column set-up.The precolumns used are both disposable cartridges and small stainless-steel columns packed with silica and/or polymer-based stationary phases. After passage through four precolumns, the sugars in hydrolysed waste water and in the fermentation broths can be introduced into a conventional LC system.

The sugars are monitored either directly with refractive index (RI) or UV (195 nm) detection or with a post-column detection [11,12] system using a co-immobilized enzyme reactor (CIMER) [2] and UV or electrochemical detection.

EXPERIMENTAL

Apparatus

A schematic diagram of the experimental LC set-up is given in Fig. 3. The apparatus consists of the three high-pressure pumps (two Model 2150 from LKB, Bromma, Sweden, and one Model 600 from Waters Assoc., Milford, MA, U.S.A.), four six-port valves [one Waters Assoc. U6K, one Rheodyne (Cotati, CA, U.S.A.) Model 7000 and two Rheodyne Model 7045], denoted I, II, III and IV in Fig. 3, two

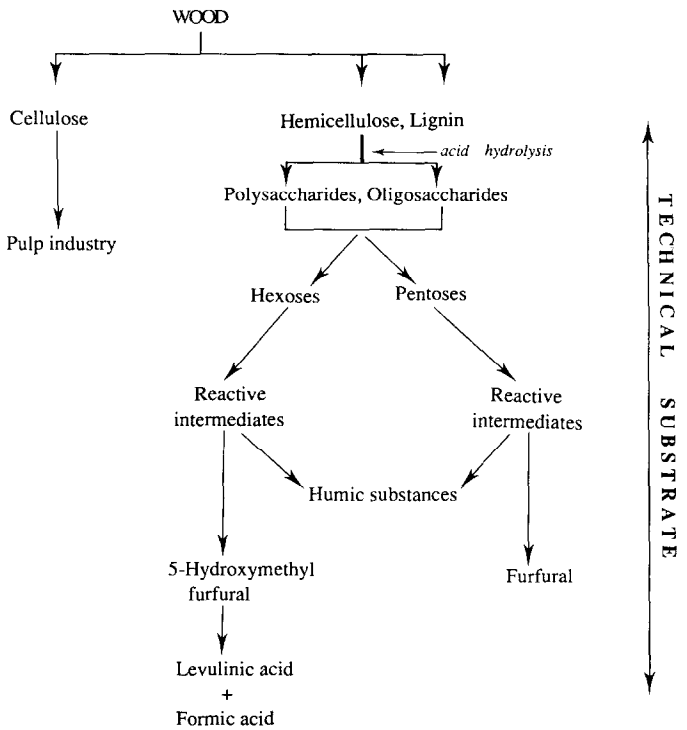

Fig. 1. Breakdown scheme of the acid hydrolysis of lignocellulose.

injection loops (1000 and 10 μl), a low-dead-volume mixing T-piece, a recorder (LKB 2210), an RI detector (Model 2142, LKB) and a UV spectrophotometer (Model SPD-6A, Shimadzu, Kyoto, Japan). The laboratory-built amperometric flow cell was a three-electrode cell of the wall-jet type using a modified graphite working electrode, a platinum auxiliary electrode and an Ag/AgCl reference electrode (Model K 801, Radiometer, Copenhagen, Denmark), kindly provided as a gift by Dr. T. Buch-Rasmussen (Radiometer). The working electrode was a chemically modified electrode (CME) modified with a phenoxazine derivative [13]; the mediator was immobilized by adsorption as described previously [14,15]. The applied potential used throughout this work was 0 mV *vs.* Ag/AgCl, controlled by a potentiostat (Zäta Elektronik, Lund, Sweden). The eluent and the reagent solution were pumped at a flow-rate of 0.6 and 0.1 ml/min, respectively, unless indicated otherwise. The CIMER was operated at room temperature; it has a volume of 295 μl (23.5 × 4.0 mm I.D.).

The analytical column (300 × 7.8 mm I.D.), a ligand-exchange column in Pb(II) form (Aminex HPX-87 P), was run at 65°C and was prethermostated as described previously [7]. Precolumn 3 (PLRP-S, Polymer Labs., Church Stretton, Shropshire, U.K.; particle size 10 μm, pore size 100 Å, 50 x 4.5 mm I.D., packed under high pressure) and precolumn 4 containing a 1:1 mixture of an anion- and cation-exchange support (Aminex A-29, Bio-Rad Labs., Richmond, CA, U.S.A., and Aminex HPX-87H, both with 9-μm particle size, 50 × 4.0 mm I.D., packed under

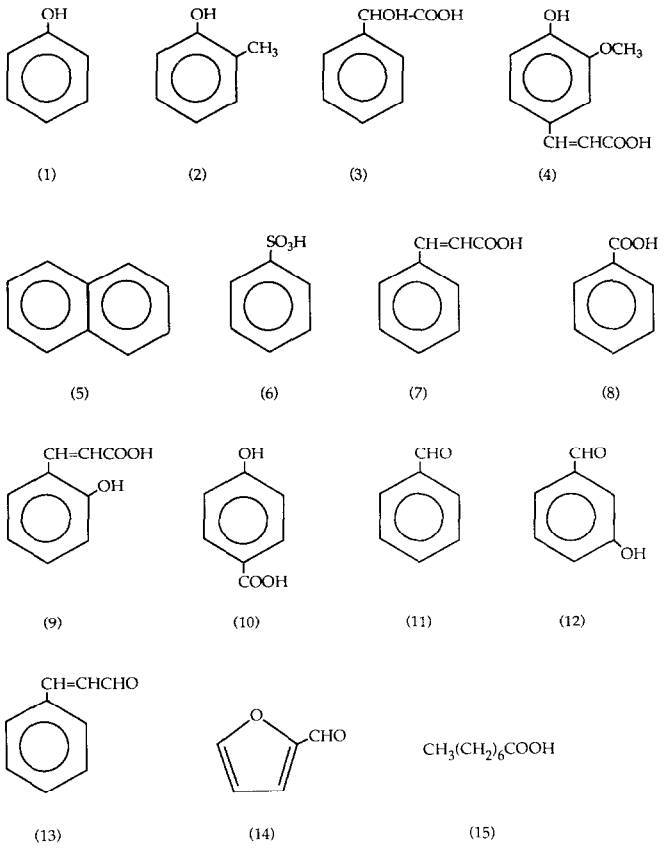

Fig. 2. Structures of the fifteen model compounds investigated. 1 = Phenol; 2 = *o*-cresol; 3 = mandelic acid; 4 = ferulic acid; 5 = naphthalene; 6 = benzenesulphonic acid; 7 = cinnamic acid; 8 = benzoic acid; 9 = *o*-coumaric acid; 10 = *p*-hydroxybenzoic acid; 11 = benzaldehyde; 12 = *m*-hydroxybenzaldehyde; 13 = cinnamaldehyde; 14 = furfural; 15 = caprylic acid.

high pressure) were all packed with polymer-based materials. Precolumns 1 and 2 were Sep-Pak (Waters–Millipore, Milford, MA, U.S.A.) cartridges packed with a quaternary ammonium (QMA) and a primary amino-type silica-based stationary phase, respectively, both with a particle size of 40 μm. Breakthrough curves for the different precolumn stationary phases were recorded using UV detection at 225 nm (standard solutions of the model compounds) or 600 nm (spent sulphite liquor).

Preparation of the CIMER

The co-immobilization of mutarotase [MT, E.C. 5.1.3.3, from porcine kidney, Sigma (St. Louis, MO, U.S.A.), cat. No. M-4007], glucose dehydrogenase [GDH, E.C. 1.1.1.47, from *Bacillus megaterium*, Merck (Darmstadt, F.R.G.), cat. No. 13732] and galactose dehydrogenase (GADH, E.C. 1.1.1.48, from recombinant *Escherichia coli* using *Pseudomonas fluorescens* gene, Sigma, cat. No. G-6637) was done covalently with glutaraldehyde as described previously [15]. The support used was a CPG-10 glass (particle size 37–74 μm, pore size 500 Å; Serva, Heidelberg, F.R.G.). In the

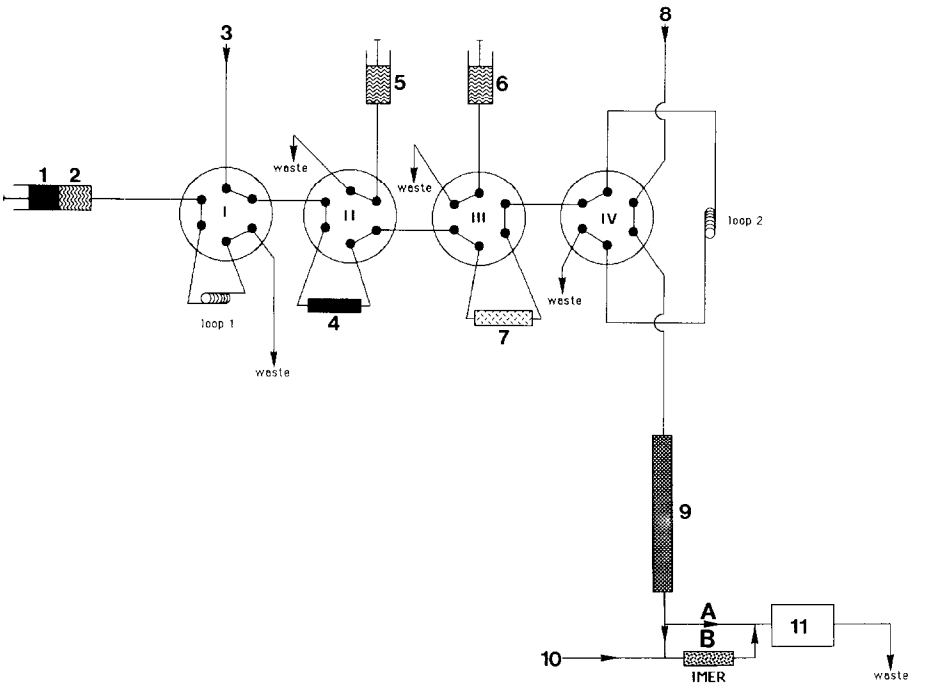

Fig. 3. Scheme of the precolumn and analytical system with (A) direct RI or UV monitoring and (B) CIMER post-column reaction detection. 1, Sep-Pak QMA precolumns; 2, Sep-Pak amino precolumn; 3, preconcentration pump; 4, PLRP-S precolumn; 5, regeneration solvent [methanol–water (80:20)]; 6, regeneration solvent (nitric acid); 7, mixed cation–anion-exchange precolumn; 8, LC pump; 9, analytical column; 10, reagent pump; 11, detector; I and IV, injectors; II and III, switch valves.

CIMER the sugars react with the cofactor NAD^+ (nicotinamide adenine dinucleotide), yielding stoichiometric amounts of NADH, reflecting the sugar levels in the sample [2]. The enzymatically produced NADH is monitored either by UV absorbance (340 nm) or by electrochemical detection, which involves two selective steps. In the amperometric detector cell, NADH is electrocatalytically oxidized at 0 mV *vs.* Ag/AgCl at an electrode chemically modified with a phenoxazine derivative [16].

Fermentation conditions

A slowly stirred 250-ml beaker sealed with a rubber stopper was inoculated with 145 g/l of baker's yeast, *Saccharomyces cereviseae* (dry weight). Spent sulphite liquor (SSL), sodium based, was kindly supplied by Modo (Örnsköldsvik, Sweden). The only pretreatment of the substrate was the adjustment of the pH to 6.0 with sodium hydroxide and the addition of fermentation nutrients: 0.25% (w/v) yeast extract (Difco, Detroit, MI, U.S.A.) and 0.025% (w/v) $(NH_4)_2HPO_4$. After 30 min and 4 h, samples were taken, diluted and filtered prior to analysis.

Clean-up procedure

The samples were diluted 25–200-fold with water before adjusting the pH to 6–7 with sodium hydroxide. The sample was then filtered through a 45-μm sterile mem-

brane filter to remove solids. Loop I (1000 μl) was filled by flushing a 1.5–2 ml sample through the QMA (precolumn 1) and amino (precolumn 2) clean-up columns (see Fig. 3). By switching valve I the sample was transported on-line onto the PLRP-S column (precolumn 3) and then onto the mixed anion–cation-exchange column (precolumn 4). The purified sample was trapped in a 10-μl loop connected to valve IV and injected into the analytical system.

Chemicals

HPLC-grade water was produced in a Milli-Q system (Millipore, Bedford, MA, U.S.A.) and was used throughout this work. Water was used as the mobile phase in the coupled clean-up precolumns and in the separation column. A 0.7 *M* phosphate buffer (pH 7.0) containing 14 m*M* NAD^+ and 7 m*M* EDTA was used as the reagent stream for the enzymatic reaction.

Model compounds

The model compounds and test mixtures were prepared by dissolving appropriate amounts in water and in water–ethanol mixtures. NADH was diluted with buffer (0.1 *M* phosphate buffer, pH 7.0) for the preparation of standard solutions that were run in the flow-injection mode. The NADH concentrations were determined by UV absorption spectrometry at 340 nm.

RESULTS AND DISCUSSION

Nature of the fermentation broth and substrate

The technical substrate, spent sulphite liquor (SSL), is a by-product from the sulphite pulping process. In this investigation it was used as a model substrate for the fermentation of lignocellulose hydrolysates, as it contains soluble sugars. SSL is in some ways comparable to other wood hydrolysates with a complex composition [17–20]. Lignin has a complicated aromatic polymer structure and consists of dehydrogenated polymers of coriphenyl, sinapyl and *p*-coumaryl alcohol. Its acid hydrolysis yields a wide variety of phenolic derivatives [20–22]. Further, the fermentation process itself can cause, depending on the microorganisms involved, a considerable increase in the complexity of the sample.

During the hydrolysis of wood, humic substances are produced, which are believed by some workers to be related to a direct decomposition product of lignin and by others to be compounds produced by microorganisms [20,23–25]. They are dark brown, amorphous and colloidal compounds with a complex composition and incompletely known structure. The major constituents are humic acids, which are polymers of hydroxyphenols and hydroxybenzoic acids, *i.e.*, aromatic polymers of high molecular weight to which an unusually large number of functional groups are attached. Humic substances are also coupled to proteins, peptides, amino acids and aliphatic and aromatic compounds [24].

The hydrolysis of wood further results in browning reactions, which are also very common in fruit juices, milk products, sugar cane molasses and microbial growth media. The compounds formed during browning reactions give rise to the dark brown colour of the SSL and, later, also of the broth. The presence of browning-reaction compounds is known to affect the growth and metabolism of various mi-

croorganisms [20,26]. Furfural, maltol and 5-hydroxymethylfurfural are examples of browning-reaction products known to inhibit fermentation processes [23]. Reactions of amines, amino acids and proteins with sugars, aldehydes and ketones, known as Maillard reactions, also produce browning compounds [27,28]. They involve a condensation reaction between the carbonyl groups of carbohydrates and the α-amino groups of amino acids or proteins. This initiates the formation of Schiff's bases, which further undergo a cyclization to the corresponding N-substituted glycosylamines [29]. These reactions can take place in both acidic and alkaline media. The browning reactions are mainly caused by monosaccharides, but can also proceed in the presence of reducing disaccharides such as maltose and lactose. Non-reducing sugars, oligosaccharides and polysaccharides cannot participate unless the glycosidic bonds are cleaved, releasing reducing (mono)saccharides [30]. Further series of rearrangements of the N-substituted glucosamine can occur and lead to 1-amino-1-deoxy-2-ketose derivatives, causing the formation of brown pigments. Caramelization is another process in which sugars, in the absence of proteins and amino acids heated over their melting-points, darken to a dark brown colour. These reactions take place under both alkaline and acidic conditions. The chemical composition of the reaction products is complex and little understood. Phenolic compounds can be catalysed enzymatically to form brown melanins by polyphenolic oxidase, also known as phenolase (E.C. 1.10.3.1).

Precolumn system

The matrix components in SSL cause major problems in sugar determination. Apart from obscuring the sugar peaks in both UV and RI detection, they cause severe problems due to irreversible adsorption and increased back-pressure and they also have an inhibitory effect on the enzyme activity in the CIMER [16]. We have developed a precolumn clean-up procedure in order to remove these matrix components. Fifteen model compounds known to be breakdown products in lignin hydrolysates were chosen to determine breakthrough volumes on the stationary phases investigated (for the overall scheme, see Fig. 4).

Removal of brown compounds (precolumn 1). The selective clean-up of humic-containing waters on both reusable [31] and disposable [32,33] precolumns has been reported. The removal of disturbing brown components such as humic substances and lignin breakdown products must essentially be complete. These compounds adsorb strongly to the polymeric stationary phase in the analytical column and interact with the analytes in an unpredictable way, which causes an increasing back-pressure and errors in quantitative evaluations.

The sorption capacity of the compounds causing the brown colour in SSL, which has a dry weight of 5 mg/ml, was determined for different disposable clean-up columns (Sep-Pak); the results are given in Table I. The anion-exchange material was found to have the highest capacity (27 ml/g), indicating the anionic characteristics of the brown compounds at pH 6.0. The smaller capacities for the QMA phase at lower dilution factors (50–100) can be explained by the fact that, despite the high dilution, overloading of the stationary phase rather than insufficient retention determines the breakthrough. The other ten Sep-Pak phases with varying functionalities did not exhibit sufficient affinity for the browning-reaction compounds ($V_b < 2$ ml, see Table I).

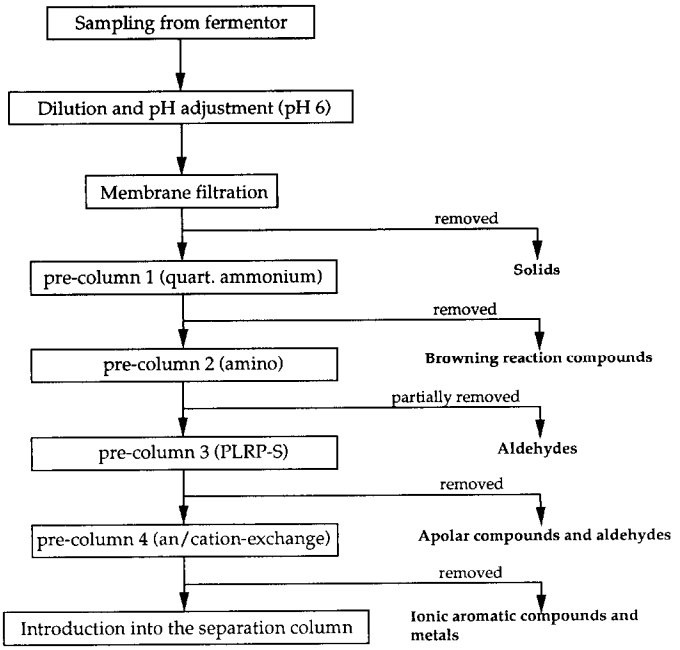

Fig. 4. Scheme of the clean-up procedure.

Sorption of aldehydes on the amino phase (precolumn 2). Many aldehydes have an inhibitory effect on the enzyme activity and also may interfere with the separation and UV or RI detection of the carbohydrates. Aromatic aldehydes are most often separated by reversed-phase chromatography using silica-based C_{18} supports [34,35]. They can also be oxidized on-line on a polymeric anion-exchange resin in the permanganate form [36], or reduced by immobilized borohydride on the same support [37]. These solid-phase extraction columns are used in the precolumn mode.

As aldehydes preferentially bind to amino-modified stationary phases, probably owing to Schiff's base formation, we investigated the use of a Sep-Pak amino-bonded stationary phase for their removal. This step was introduced to increase the sorption properties of interfering aldehydes. The breakthrough volumes on a precolumn (30 × 4.0 mm I.D.) packed with this stationary phase were 3–5 ml for all the aldehydes (0.1 m*M*) investigated (see Table II). No significant differences were found in the breakthrough volume using 1 m*M* concentrations of aldehydes, indicating that this breakthrough is caused by chromatographic elution.

Sorption of model compounds on the PLRP-S phase (precolumn 3). Breakthrough experiments were run with fifteen model compounds (see Fig. 2) in either pure water (the water-soluble compounds) and/or in ethanol–water (1:99 or 5:95, v/v) (all compounds). In actual practice, the ethanol content after fermentation is about 5%; consequently, the breakthrough was investigated under these conditions. The breakthrough volumes are given in Table II. The breakthrough volume of most of these compounds decreases when the alcohol level increases. All model compounds except caprylic acid have an aromatic skeleton with various functional groups at-

TABLE I

BINDING CAPACITY OF BROWNING-REACTION COMPOUNDS IN SSL ON DIFFERENT SEP-PAK STATIONARY PHASES

Column, 30 × 4.0 mm I.D.; particle size, 40 μm; flow-rate, 1.0 ml/min; pH of the sample solution, 6.0; relative standard deviation, 3–8% ($n = 5$).

Column type	Capacity (ml/g stationary phase)	Dilution factor of SSL sample
QMA	27	200
	14	100
	6	50
C_{18}	1	100
Primary amino	2	100
Florisil	1	100
Carboxymethyl	1	100
Silica	1	100
Cyano	1	100
Diol	1	100
Alumina A	0	100
Alumina B	1	100
Alumina N	1	100

tached to it. Benzenesulphonic acid is the only compound that has no affinity for the hydrophobic phase regardless of the eluent composition, *i.e.*, it is too strong an acid although it contains an aromatic ring. *o*-Cresol, with an additional methyl group, is more hydrophobic than phenol and is therefore retained more strongly on PLRP-S. *p*-Hydroxybenzoic acid, *o*-coumaric acid, mandelic acid and ferulic acid all contain a phenol structure with an additional carboxylic group. The introduction of a charged group increases the polarity of the molecule and decreases the binding strength. Mandelic acid contains an ethyl group between the aromatic ring and the carboxylic group which, as the breakthrough volumes show, does not seriously influence the hydrophobicity of the molecule compared with *p*-hydroxybenzoic acid. However, *o*-coumaric acid and ferulic acid are more strongly sorbed owing to the aliphatic double bonds in these molecules. The additional methoxy group in ferulic acid does not increase the hydrophobicity. The breakthrough volumes further show that the aldehyde group does not contribute as much to the polarity of the molecule as an alcohol or carboxylic group; all aldehydes except furfural show good binding properties for PLRP-S. As could be expected, naphthalene, which represents the hydrophobic class of compounds present in the broths, is the most strongly bound of all the compounds investigated.

Sorption of model compounds and metal ions on polymeric anion–cation-exchange resin (precolumn 4). All phenolic derivatives and acids except benzenesulphonic acid have pK_a values around 4 or higher (see Table II). When using pH 6 in our experiments, all acids will be deprotonated; consequently, they are well retained on the anion-exchange phase, as shown in Table II. As is to be expected, increasing the ionic strength in most instances decreased the sorption capacity owing to a competitive elution mechanism. The breakthrough volume for naphthalene and all aldehydes on this phase is low.

TABLE II

BREAKTHROUGH VOLUME (V_b) OF FIFTEEN MODEL COMPOUNDS ON PRIMARY AMINO, POLYMERIC (PLRP-S) AND POLYMERIC ANION-EXCHANGE SUPPORTS

Column size, 30 × 4.0 mm I.D.; analyte concentrations were 0.1 m*M*.

Analyte	pK_a	V_b (ml)						
		Amino[a]	PLRP-S			Aminex A-29		
			A[b]	B[c]	C[d]	A[b]	B[c]	D[e]
Phenol	9.98	–[f]	13	11	8	3	2	1
o-Cresol	10.20	–	37	30	26	2	1	1
Ferulic acid		–	n.d.[g]	24	13	n.d.	352	64
Mandelic acid	3.85	–	4	4	2	412	380	50
Benzenesulphonic acid	0.70	–	0	0	0	136	80	39
Cinnamic acid	3.89	–	n.d.	78	60	n.d.	486	148
o-Coumaric acid	4.61	–	n.d.	26	16	n.d.	324	62
Benzoic acid	4.19	–	n.d.	20	13	n.d.	209	106
p-Hydroxybenzoic acid	4.48/9.32	–	n.d.	2	1	n.d.	352	150
Caprylic acid	4.85	–	n.d.	20	10	n.d.	33	18
Benzaldehyde		5	n.d.	40	19	n.d.	0	0
Furfural		3	5	3	2	0.	0	0
m-Hydroxybenzaldehyde		4	n.d.	4	3	n.d.	2	1
Cinnamaldehyde		5	n.d.	62	28	n.d.	0	0
Naphthalene		–	n.d.	392	309	n.d.	3	3

[a] In water–ethanol (99:1, v/v) containing 1 m*M* sodium sulphite and 1 m*M* sodium acetate.
[b] In water.
[c] In water–ethanol (99:1, v/v).
[d] In water–ethanol (95:5, v/v).
[e] In water–ethanol (95:5, v/v) containing 1 m*M* sodium sulphite and 1 m*M* sodium acetate.
[f] Not determined.
[g] Not dissolvable.

Metal ions in the samples have to be quantitatively removed becaused the analytical column is a ligand-exchange column with Pb(II) ions bound on the sulphonated polystyrene–divinylbenzene support. If other cations such as (Ni(II), Fe(II) or Co(II), or higher concentration of H^+, are present, ion exchange will take place, which will result in a mixed ligand-exchange phase. This will influence the capacity factors of the analytes in an unpredictable way. In most instances the capacity factors will decrease and the support will swell, which results in an increased back-pressure [16]. The binding capacity of the cation exchanger packed in precolumn 4, which was determined by atomic absorption spectrometry, was 2.0 mmol/g phase for Fe(II), 2.1 mmol/g for Ni(II) and 2.2 mmol/g for Co(II). These values agree with the retention order found by others using polymeric supports for the ion chromatographic separations of metal ions [38]. The background levels of Ni(II), Fe(II) and Co(II) in the lignocellulose substrate used were 0.25, 3.6 and 0.5 ppm, respectively, which would theoretically allow more than 2400 injections (1000 μl) of undiluted substrate before regeneration of the ion-exchange column with nitric acid is required.

Performance of the clean-up procedure

The total analytical system including the CIMER was used to test the robustness of the coupled precolumn set-up (see Fig. 4). The diluted sample was introduced into loop 1 (1000 μl) by flushing 1.5–2.0 ml of sample through the QMA (precolumn 1) and amino (precolumn 2) clean-up columns. By switching valve I the sample is eluted on-line into the PLRP-S column (precolumn 3), where the more hydrophobic compounds are bound, and then into the mixed anion–cation-exchange column (precolumn 4) where the phenolic derivatives, acids and metal ions are retained. The purified sample is trapped in a 10-μl loop (loop 2) connected to valve IV and injected into the analytical system. Fig. 5 shows the peak profiles of different injection volumes which were recorded by placing an RI detector in series with the precolumn system. When the maximum sugar concentration was reached, the injection was performed. Depending on the complexity of the matrix, the QMA and amino Sep-Pak precolumns were exchanged after 3–10 injections. An attempt was made to regenerate the QMA phase, which was loaded with brown components, by washing with ethanol and phosphoric acid. The results were not encouraging: only about 15–30% of the sorbed material could be removed. Regeneration with more strongly acidic or alkaline solutions might destroy the bonded-phase material and was therefore not attempted. The PLRP-S (precolumn 3) was regenerated with 3 ml of methanol–water (80:20, v/v) and the mixed anion–cation exchanger (precolumn 4) by flushing with 1 ml of 1 *M* nitric acid. After each run the columns were equilibrated with 15 ml of water before re-use.

Table II shows that all the acidic test compounds investigated have breakthrough volumes higher than 10 ml for at least one of the stationary phases used. In all instances except phenol the breakthrough volume is even higher than 30 ml. As the total volume injected is only about 1 ml, a very efficient clean-up is obtained. For real samples, the efficiency of the sample clean-up procedure was studied by means of UV spectrometry. The samples that were analysed were fermented and unfermented hydrolysed lignocellulose (SSL). Untreated samples exhibited strong absorbance in the region of 230–270 nm. After the clean-up procedure, the absorbance in this wavelength range diminished by more than two orders of magnitude for both types of

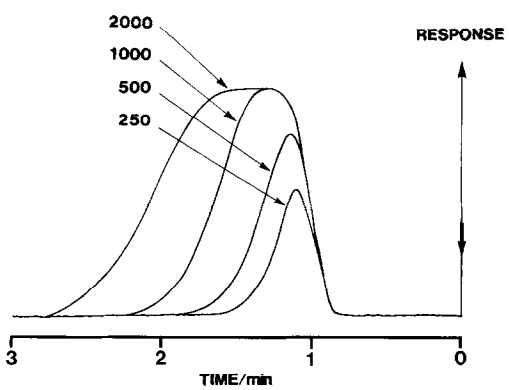

Fig. 5. Peak profiles of different injection volumes (250, 500, 1000 and 2000 μl) recorded by placing an RI detector (attenuation 64) in series with the precolumn system.

samples, which indicates that the aromatic sample constituents are efficiently removed.

The recovery of the whole procedure for cellobiose, glucose, xylose, galactose, arabinose and mannose (30 μM–25 mM) was 91% (relative standard deviation = 2–6%, $n = 30$) when comparing a direct injection of a SSL standard into valve 4 with a sample injected in valve 1 and running through the whole precolumn set-up. The resulting chromatograms for standard solutions are shown in Fig. 6. When using an RI detector several unknown peaks appear in the chromatogram, probably originating from the ion-exchange and amino precolumns, as they also appear when analysing reference solutions (see Fig. 6b). However, these peaks do not interfere with the analyte peaks; therefore, no attempt was made to eliminate this problem. Fig. 6 also demonstrates that, except for a shift in retention times, there is no major difference between direct injections and injections following the clean-up procedure.

Determination of sugars in fermentation broths and substrates

The usefulness of the precolumn clean-up procedure was investigated for the various detectors, *viz.*, RI and UV (190 nm) detection and post-column CIMER coupled to either a UV or an electrochemical detector. On comparing the chromatograms for crude and purified samples it is obvious that the clean-up is of major importance in RI detection. If the clean-up system is omitted, matrix components of the fermented sample interfere with the glucose and xylose peaks (see Fig. 7a); after clean-up, quantification of these sugars is possible (Fig. 7b). The peak heights for galactose and mannose without clean-up (see Fig. 7a) are much higher than those found after clean-up (Fig. 7b), which indicates that these peaks also contain components other than the sugars.

The sugars show only weak UV absorption below 200 nm. At 195 nm, impurities present in the eluent, including dissolved oxygen, cause severe problems with

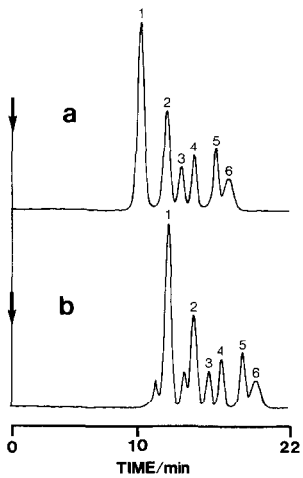

Fig. 6. Separation and RI detection (attenuation 4) of a standard sugar mixture. (a) 10-μl direct injection; (b) 10-μl injection after precolumn clean-up. Peaks: 1 = cellobiose, 20 mM; 2 = glucose, 2 mM; 3 = xylose, 1 mM; 4 = galactose, 1 mM; 5 = arabinose, 1 mM; 6 = mannose, 1 mM. LC conditions: flow-rate, 0.6 ml/min; mobile phase, water; temperature, 65°C; for other conditions, see Experimental.

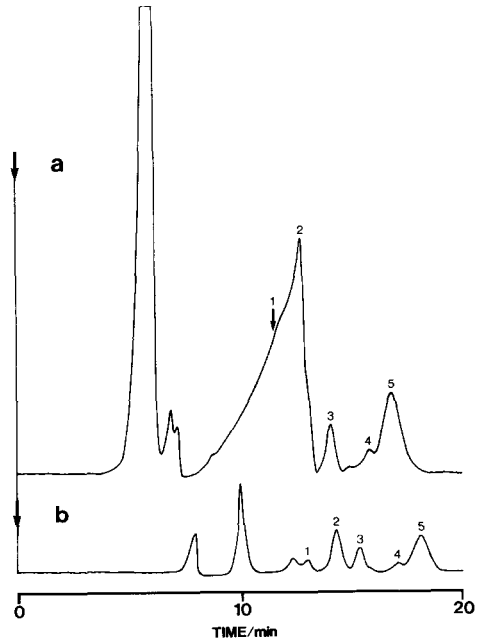

Fig. 7. Separation and RI (attenuation 2) detection of sugars present in fermented SSL diluted 25-fold, (a) before and (b) after precolumn clean-up. Peaks: 1 = glucose, 200 μM; 2 = xylose, 380 μM; 3 = galactose, 190 μM; 4 = arabinose, 75 μM; 5 = mannose, 220 μM. Conditions as in Fig. 6.

baseline stability, which reduces the detection sensitivity. When injecting crude samples it is impossible to quantify or even detect the sugars (Fig. 8a). However, when using the pre-column set-up, quantification is possible (Fig. 8b).

The precolumn set-up was also evaluated using the CIMER post-column detection mode (see Fig. 9) [39,40]. This combines the specificity of enzyme catalysis with the sensitivity of amperometric or UV (340 nm) detection of NADH. Amperometric detection is often preferable to UV detection as it is inherently more selective. After enzyme catalysis the chemically modified electrode (CME) eliminates many of the difficulties encountered in spectrophotometric detection, *e.g.*, overlapping of absorbing peaks, refractive index effects and turbidity. The performance of the CIMER is described in ref. 16.

All six sugars for which the CIMER was active showed a linear response over more than two orders of magnitude (30–200 μM to 5–25 mM). The upper linear range is determined by the amount of bound enzyme in the reactor and the NAD^+ concentration. For a substrate for which the enzyme has a high activity such as glucose it was $5 \cdot 10^{-3}$ M and for a substrate with a low activity such as mannose it was $25 \cdot 10^{-3}$ M. The limits of detection were determined by the noise of the CME and were $3 \cdot 10^{-5}$ M for glucose and $2 \cdot 10^{-4}$ M for mannose; all sugars had almost the same linear range as described previously [15]. When using the postcolumn detection modes, the band broadening increases only moderately compared with direct RI detection [7]. A comparison of CIMER–UV and CIMER–EC (see Fig. 9) with direct RI or UV detection (Figs. 7 and 8, respectively) shows the advantage of the former detection modes regarding selectivity and sensitivity.

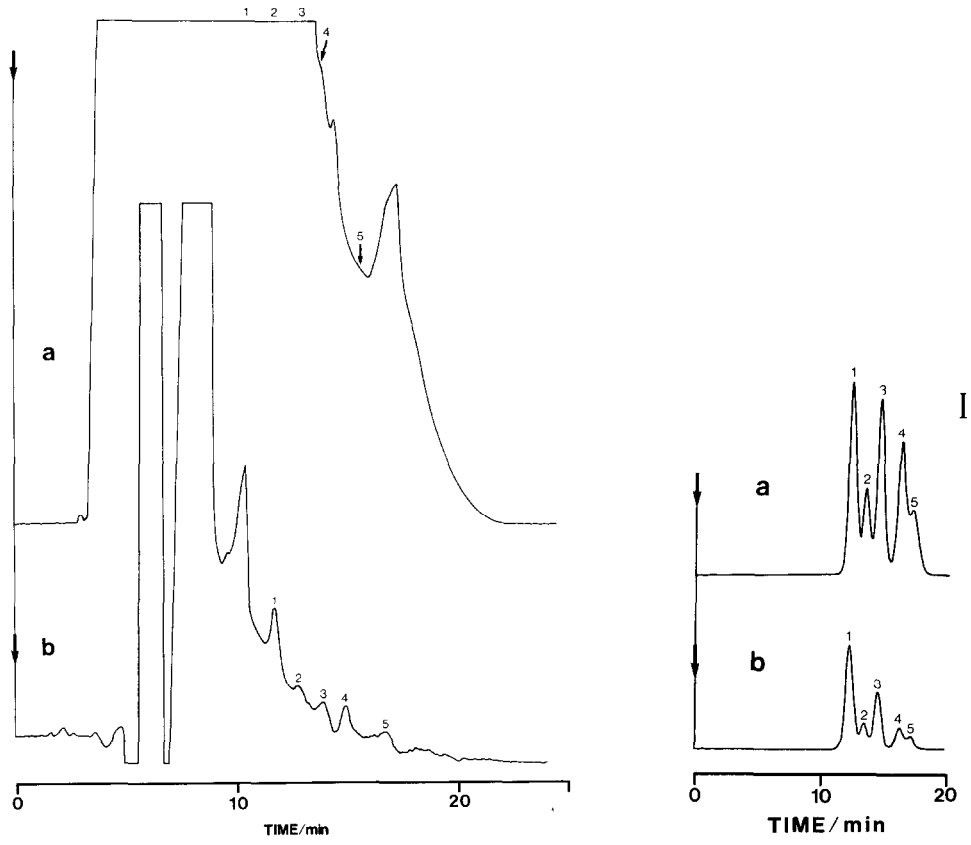

Fig. 8. Chromatogram of unfermented SSL diluted 100-fold using UV detection (195 nm; attenuation, 0.01 a.u.f.s.) (a) before and (b) after precolumn clean-up. Peaks: 1 = glucose, 150 μM; 2 = xylose, 170 μM; 3 = galactose, 200 μM; 4 = arabinose, 120 μM; 5 = mannose, 400 μM. Conditions as in Fig. 6.

Fig. 9. Chromatogram of fermented SSL diluted 200-fold using (a) CIMER–electrochemical detection at the start of the fermentation process and (b) CIMER–UV (340 nm, 0.1 a.u.f.s.) detection at the end of the fermentation process. Conditions: LC flow-rate, 0.7 ml/min; reagent flow-rate, 0.15 ml/min; amperometric oxidation potential at CME, 0 mV *vs.* Ag/AgCl. Peaks: (a) 1 = glucose, 65 μM; 2 = xylose, 180 μM; 3 = galactose, 78 μM; 4 = arabinose, 57 μM; 5 = mannose, 280 μM; (b) 1 = glucose, 100 μM; 2 = xylose, 190 μM; 3 = galactose, 97 μM; 4 = arabinose, 34 μM; 5 = mannose, 110 μM.

CONCLUSIONS

An on-line precolumn set-up has been designed which features four precolumns containing anion- and cation-exchange, amino-bonded silica and hydrophobic PLRP-S materials. If this set-up is combined with a CIMER postcolumn derivatization system, rapid analytical information on the carbohydrate composition in fermentation processes can be obtained. The whole set-up is simple to operate and can provide selective, rapid and reproducible process data.

Although one cannot readily conclude this from all the chromatograms in this paper (*cf.*, Figs. 7b and 9), the inherent selectivity of the CIMER post-column detec-

tion modes often is superior to RI and low-wavelength (190-nm) UV detection in the analysis of fermentation substrates and broths of widely different composition. The use of the precolumn clean-up system allowed the injection of at least 150 samples during a 3-week period with only minor (10%) decreases in enzyme activity, which compares favourably with previous experiments [16].

Further work on real samples with the described method is in progress.

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REFERENCES

1 H. Senn, *Doctoral Thesis*, University of Basle, Zürich, 1989.
2 G. Marko-Varga, *Anal. Chem.*, 61 (1989) 831.
3 H. Engelhardt and P. Ohs, *Chromatographia*, 23 (1987) 657.
4 R. W. Frei, *Acta Pharm. Suec.*, 25 (1988) 97.
5 M. W. F. Nielen, A. J. Valk, R. W. Frei, U. A. Th. Brinkman, Ph. Musscle, R. de Nijs, B. Ooms and W. Smink, *J. Chromatogr.*, 393 (1987) 69.
6 L. Yago, *Int. Lab.*, Nov./Dec. (1985) 40.
7 G. Marko-Varga, *J. Chromatogr.*, 408 (1987) 157.
8 F. Tjerneld, I. Persson, P. Å. Albertsson and B. Hahn-Hägerdal, *Biotechnol. Bioeng.*, 15 (1985) 40.
9 T. Linden and B. Hahn-Hägerdal, *Biotechnol. Tech.*, 3 (1989) 189.
10 K. Skoog and B. Hahn-Hägerdal, *Enzyme Microb. Technol.*, 10 (1988) 66.
11 R. W. Frei, *Chromatographia*, 15 (1982), 161.
12 I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986.
13 L. Gorton, B. Persson, M. Polasek and G. Johansson, in A. Ivaska (Editor), *Proceedings of Electro-FinnAnalysis (1988)*, Plenum Press, New York, in press.
14 R. Appelqvist, *Doctoral Thesis*, University of Lund, Lund, 1987.
15 R. Appelqvist, G. Marko-Varga, L. Gorton, A. Torstensson and G. Johansson, *Anal. Chim. Acta*, 169 (1985) 237.
16 G. Marko-Varga, E. Dominguez, B. Hahn-Hägerdal and L. Gorton, *J. Chromatogr.*, 506 (1990) 423.
17 B. Sanchez and J. Bautista, *Enzyme Microb. Technol.*, 10 (1988) 315.
18 P. A. Pfeifer, G. Bonn and O. Bobleter, *Biotechnol. Lett.*, 6 (1984) 541.
19 A. V. Tran and R. P. Chambers, *Enzyme Microb. Technol.*, 8 (1986) 439.
20 C. H. Fuchsman, *Industrial Chemistry and Technology*, Academic Press, New York, 1980.
21 E. Burtscher, O. Bobleter, W. Schwald, R. Concin and H. Binder, *J. Chromatogr.*, 390 (1987) 401.
22 R. W. Thring, E. Chornet and R. P. Overend, *J. Chromatogr.*, 467 (1989) 441.
23 T. A. Clark and K. L. Mackie, *J. Chem. Technol. Biotechnol.*, 34B (1984) 101.
24 G. G. Choudry, *Humic Substances*, Gordon and Breach, New York, 1984.
25 H. L. Bohn, M. McNeal and G. A. O'Connor, *Soil Chemistry*, Wiley, New York, 1979.
26 N. Banerjee, R. Bhatnager and L. Viwanathan, *Enzyme Microb. Technol.*, 3 (1981) 24.
27 N. A. M. Eskin, H. M. Handerson and R. J. Townsend, *Biochemistry of Food*, Academic Press, New York, 1971.
28 L. C. Maillard, *C. R. Acad. Sci.*, 154 (1912) 66.
29 S. M. Partridge and R. C. Brimley, *Biochem. J.*, 51 (1952) 628.
30 M. Karel and T. P. Labuza, *J. Agric. Food. Chem.*, 16 (1968) 717.
31 G. Marko-Varga, I. Csiky and J. Å. Jönsson, *Anal. Chem.*, 56 (1984) 2066.
32 I. Csiky, G. Marko-Varga and J. Å. Jönsson, *Anal. Chim. Acta*, 178 (1985) 307.
33 E. Hoffmann, G. Marko-Varga, I. Csiky and J. Å. Jönsson, *Int. J. Environ. Anal. Chem.*, 25 (1986) 161.

34 E. Burtscher, H. Binder, R. Concin and O. Bobleter, *J. Chromatogr.*, 252 (1982) 167.
35 A. P. Cook, T. M. Macleod, J. D. Appleton and A. F. Fell, *J. Chromatogr.*, 467 (1989) 395.
36 K.-H. Xie, C. T. Santa Maria, I. S. Krull, U. Neue, R. King, B. Bildingmeyer and A. Newhart, *J. Liq. Chromatogr.*, 6 (1983) 2109.
37 I. S. Krull, K-H. Xie, S. Golgan, U. Neue, R. King and B. Bildingmeyer, *J. Liq. Chromatogr.*, 6 (1983) 605.
38 D. T. Gjerde and J. S. Fritz, *Ion Chromatography*, Hüthig, Heidelberg, 2nd ed., 1987.
39 K. Shimada, T. Oe and T. Nambara, *J. Chromatogr.*, 492 (1989) 345.
40 L. D. Bowers, in I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, pp. 195-222.