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Bioselective detection in liquid chromatography by the use of immobilized enzymes*

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Abstract: The combination of liquid chromatography and immobilized enzyme reactors (LC/IMER) is a rapidly developing field of research. The enzymes are used to catalyse chemical reactions and thereby facilitate selective detection. In this instance the chemical derivatizations are performed in the post-column mode. The selectivity is demonstrated with respect to interfering compounds present in complex samples.

Keywords: *Liquid chromatography; immobilized enzyme reactor; amperometric detection; chemically modified electrode; sugars; inositol phosphates; amino acids; fermentation; serum.*

Introduction

There has been an increasing need for the characterization and quantification of analytes such as carbohydrates, amino acids and metabolic intermediates, in chemical reaction media, fermentation broths and clinical samples [1, 2]. The concentrations of amino acids, inositol phosphates and monosaccharides in biological samples can serve as indicators of different diseases making their selective detection important [3, 4]. Fermentation substrates often contain a mixture of saccharides used as the carbon source in numerous fermentation processes, including those for the production of penicillin, enzymes and ethanol [5, 6]. By the selective determination of the sugars in the process, the metabolism as well as the process and process yield can be controlled and monitored, allowing precise optimization of the technical processes [7–9].

Liquid chromatography (LC) is an efficient separation technique that can be coupled to various selective post-column reaction detection devices. A number of immobilized enzymes have been used as components of selective detection devices in LC. The enzyme(s) are immobilized on a solid support, and contained in a packed bed immobilized enzyme reactor (IMER). After separation of the analytes, the column effluent is mixed with a make-up flow containing the substrates necessary for the enzymatic conversion in the IMER.

When the products of the enzyme reaction lack detectable physical or chemical properties, coupled enzyme reactions can be used. Compounds involved in or products of unfavourable equilibria may also be efficiently converted to a detectable species with thermodynamically favourable coupled enzyme reactions. Enzymatic reactions used in this context frequently involve production of H_2O_2 or NADH.

When H_2O_2 or NADH is the final product in the enzymatic reaction, they can be electrocatalytically oxidized at a chemically modified electrode (CME) [10–12]. In this way, the electrochemical reaction can be made selective for the enzymatic product, eliminating a response from a series of potentially interfering compounds present in the sample [13]. Selective detection of H_2O_2 can also be accomplished by further reaction with horse-radish peroxidase to produce a red coloured quinoneimime complex [14]. The usefulness of

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post-column derivatization is illustrated in biochemical and biological samples, and the selectivity and sensitivity of the approach will be demonstrated.

Applications of Immobilized Reaction Detectors in Liquid Chromatography

Sugars in fermentation samples

In the search for alternative energy sources a great deal of effort has been expended in finding solutions through chemical or biochemical processes. Waste water from the pulp industry containing large amounts of sugars (D-glucose, D-galactose, D-mannose, L-arabinose and D-xylose) derived from lignocellulose fractions has the potential of being an inexpensive carbon source for ethanol production through fermentation by *Saccharomyces cerevisiae* [15].

Selective determination of the sugar concentrations in fermentation substrates are of great importance for process monitoring and control [16, 17]. Other compounds in the fermentation broth very often interfere with the qualitative and the quantitative evaluations of the sugars [18]. For efficient control of the process a selective determination of species in the broth can be coupled to fermentation adjustment and feed back control [19].

Saccharides are compounds with no chromophores or fluorophores which make their direct and selective detection difficult in the presence of other compounds. However, by the use of co-immobilized glucose dehydrogenase (GDH), mutarotase (MUT) and galactose dehydrogenase (GADH) contained in one IMER the indirect measurement of a number of aldoses was made feasible by post-column derivatization and electrochemical detection of the NADH formed in the enzymatic reactions, exemplified by the oxidation of β-D-glucose with GDH (equation 1):

$$\beta\text{-D-glucose} + NAD^+ + H_2O \xrightarrow{GDH} \text{D-gluconate} + NADH + H^+. \quad (1)$$

The NADH produced in reaction (1) is then electrocatalytically oxidized at 0 mV vs Ag/AgCl in a sequence of fast reactions at an electrode surface chemically modified with a phenoxazine derivative [10, 20]. The CME is contained in an amperometric flow through cell of the wall-jet type. The net electrochemical reaction is

$$NADH \rightarrow NAD^+ + H^+ + 2e^-. \quad (2)$$

This method has been used in a number of applications [5, 6, 13, 21] and is illustrated here by the analysis of the carbohydrate composition in a fermentation sample. A schematic diagram of the experimental set-up shown in Fig. 1, where the sample clean-up is made on-line. Small disposable Sep-Pak columns containing quaternary ammonium and C_{18} phases [5, 6] were used off-line prior to the analysis for the removal of brown coloured and apolar compounds in the broth.

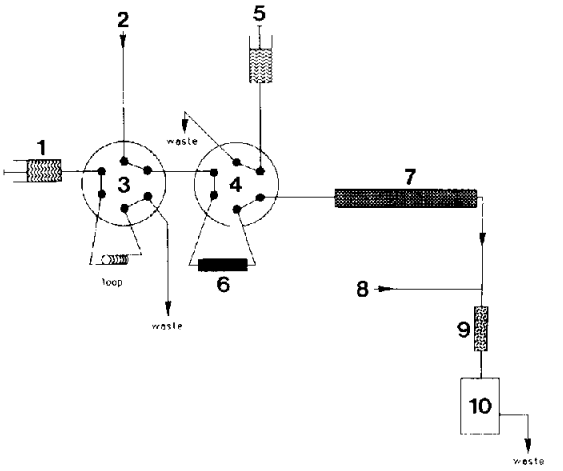

Figure 1
Instrumental set-up used for the catalytic post-column reactor and the post-column reaction for sugars; (1) sample, (2) mobile phase (0.6 ml min^{-1}) millipore water, (3) injector with a 10-μl loop (Rheodyne 7045, Cotati, CA, USA), (4) switch valve (Rheodyne 7000), (5) regenerating solution (1 M HNO_3), (6) clean-up column (Aminex A-29, 6 × 0.4 cm i.d., Bio-Rad, USA), (7) analytical column (Aminex HPX-87P), (8) make-up flow (0.1 ml min^{-1}) containing 14 mM dissolved NAD^+, 7 mM Mg $(NO_3)_2$ and 3.5 mM EDTA in 0.7 M phosphate buffer (pH 7.0), (9) IMER with co-immobilized XI/MUT/GDH/GADH (volume 300 μl, 24 × 4 mm i.d.), (10) amperometric detector comprising a wall-jet flow through cell [13], a potentiostat, and a recorder.

In order to detect the sugars in an ethanol fermentation of hydrolysed lignocellulose [15] an LC system was designed in which the selectivity of the detection step was obtained by combining on-line pre-column clean-up of the sample [18] with a post-column detector consisting of an IMER and a CME [6, 18]. The IMER contained co-immobilized xylose isomerase (XI), MUT, GDH and GADH. Since xylose cannot be fermented directly by *S. cerevisiae*, XI was added to the fermentation to produce D-xylulose [15] (reaction 3). This pentose is a ketose for which no commercial dehydrogenase is available for the formation of

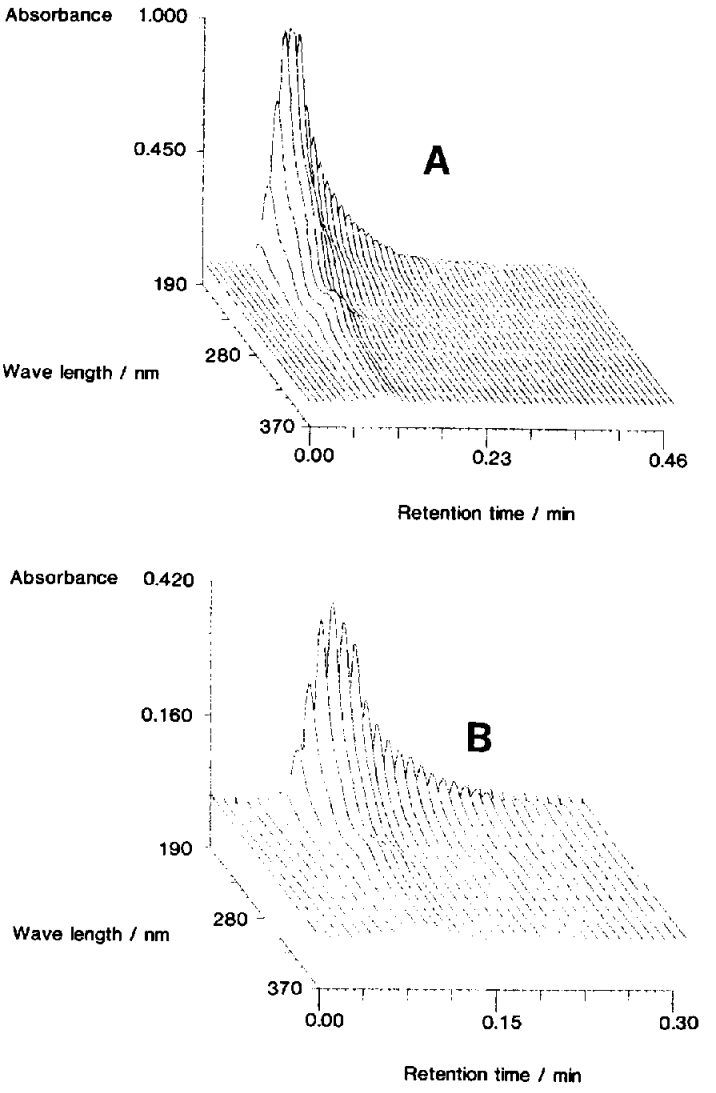

Figure 2
UV-spectra of fermented lignocellulose hydrolysate (a) before (diluted 5000×) and (b) after the clean-up procedure (diluted 50×).

NADH. However, D-xylulose can be detected after isomerization to α-D-xylose with XI (equation 3):

$$\text{D-xylulose} \overset{\text{XI}}{\rightleftarrows} \alpha\text{-D-xylose}. \qquad (3)$$

The α-D-xylose formed is then mutarotated to the β-form (reaction 4), which is the active anomeric form for oxidation by GDH (c.f. reaction 1) thus making xylulose detectable:

$$\alpha\text{-D-xylose} \overset{\text{MUT}}{\rightleftarrows} \beta\text{-D-xylose}. \qquad (4)$$

An example of the selectivity that can be obtained in LC by the combination of on-line pre-column sample pretreatment in conjunction with the use of IMERs and a CME is demonstrated in Figs 2 and 3. Figure 2 shows the UV-spectra of a crude (Fig. 2a, diluted 5000×) and a pretreated (Fig. 2b, diluted 50×) fermentation sample. After pretreatment, the UV spectrum of the sample is greatly decreased in absorbance intensity revealing that most of the UV absorbing constituents, expected to interfere with the separation and detection steps, are removed by the pretreatment step. The spectrum in Fig. 2(b) is magnified compared with the spectrum of the crude sample to illustrate the efficiency of the pretreatment step. Figure 3 shows the resulting chromatogram of the injected sample from Fig. 2(b) from an ethanol fermentation using hy-

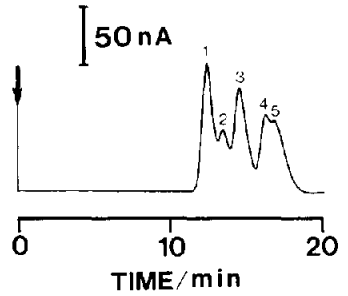

Figure 3
Chromatogram of 10 μl of fermented hydrolysed lignocellulose broth, diluted 100-fold with the standard addition of xylulose. Sample clean-up was performed as described in the text. Detection was performed by a co-immobilized XI, MUT, GADH reactor (300 μl, 24 × 4 mm i.d.) and amperometric detection using a phenoxazine derivative modified electrode. Concentrations found: 80 μM glucose (peak 1), 0.108 mM xylose (peak 2), 83 μM galactose (peak 3), 57 μM arabinose (peak 4) and 2.0 mM xylulose (peak 5).

drolysed lignocellulose as the carbon source [15, 18]. Only the sugars present in the broth are detected and can easily be identified and quantified.

The method is very selective for carbohydrates because the enzymes (XI, MUT, GDH and GADH) only use sugars as their substrates and the electrochemical oxidation of NADH is very selective. The detector response to the sugars in the fermentation sample was shown to have a linear dynamic range of more than two orders of magnitude [6]. No major changes in performance of the IMER were observed after the analysis of more than 60 samples. The operational stability of the IMER in continuous use over a period of 2 weeks was more than 96% with this fermentation broth as the matrix. The stability of these IMERs revealed a remaining activity of 90% after 6 months when stored at 4°C in 0.1 M phosphate buffer at pH 6.0 containing 0.75 M sodium chloride.

The fermentation samples were diluted and pretreated by sterile membrane filtration and then injected into the LC-system. By using an anion-exchange pre-column (Aminex A-29, Bio-Rad, 80 × 4 mm i.d.) on-line (Fig. 1) efficient clean-up of the sample was obtained by sorption of phenolic derivatives and acids [18]. The sample was also desalted before entering the analytical column because the ligand exchange column (Pb^{2+}) used for separation was not compatible with some anions. The clean-up column was regenerated with 1 M HNO_3 followed by equilibration with water. Injections of water showed no evidence of memory effects.

Additional peak broadening in the IMER [5, 6, 21] was kept at a minimum by using a low flow rate of the make-up liquid and by using single bead string reactors (SBSR) for mixing of the effluent of the separation column with the make-up flow. The reactor volume, typically 50–350 μl, and the particle diameter of the support were small in order to further minimize band broadening [22]. Slurry packing the IMERs with standard LC-column packing equipment results in IMERs with high catalytic efficiencies and with improved flow characteristics. The pressure when packing the IMER was <1000 psi to avoid decrease in enzyme activity. However, enzymes are unique and packing procedures should be customized to each particular applicator.

Sugar phosphates

Inositol phosphates are currently receiving considerable attention because of their important rôle as transmitters [4, 23]. Selective analytical detection techniques for inositol trisphosphate (IP3) could make it possible to study the mechanisms of action of drugs and hormones *in vivo* [23, 24].

A flow system incorporating a coupled enzymatic reaction sequence was developed for the analysis of 1,2,6-inositol trisphosphate and the inositol phosphates formed as metabolic intermediates. One particular IMER, containing alkaline phosphatase (AP), was used for the complete dephosphorylation of IP1–IP5 to free inorganic phosphate and myo-inositol [21] up to a concentration of 0.7 mM (equation 5):

$$IPx + nH_2O \xrightarrow{AP} \text{myo-inositol} + nPO_4^{3-}. \quad (5)$$

myo-Inositol dehydrogenase (IDH) catalyses the oxidation of myo-inositol whereby NADH and myo-inosose are formed:

$$\text{myo-inositol} + NAD^+ \overset{IDH}{\rightleftarrows} \text{myo-inosose} + NADH. \quad (6)$$

Since the equilibrium constant for reaction (6) is very unfavourable (1.2×10^{-3} M, pH 8.3) two additional enzymes, lactate dehydrogenase (LDH) and lactate oxidase (LOD) were co-immobilized with IDH and contained in a

second IMER to drive the reactions towards the product side (reactions 6–8) [25].

$$\text{NADH} + \text{pyruvate} + \text{H}^+ \overset{\text{LDH}}{\rightleftarrows} \text{NAD}^+ + \text{L-lactate}, \quad (7)$$

$$\text{L-lactate} + \text{O}_2 \xrightarrow{\text{LOD}} \text{pyruvate} + \text{H}_2\text{O}_2. \quad (8)$$

These coupled reactions yield an overall equilibrium constant for the oxidation of myo-inositol by molecular oxygen to myo-inosose and H_2O_2 of 2×10^{15} M, at pH 8.3. When measured amperometrically at a bare electrode, the produced H_2O_2 shows high background currents due to a high oxidation overpotential. By the introduction of a third IMER containing horseradish peroxidase (HRP) and adding hexacyanoferrate(II) to the carrier stream in an additional line, hexacyanoferrate(III) is formed and can be measured amperometrically at a low potential (0 mV vs SCE) [26].

$$\text{H}_2\text{O}_2 + 2\text{Fe(CN)}_6^{4-} + 2\text{H}^+ \xrightarrow{\text{HRP}} 2\text{Fe(CN)}_6^{3-} + 2\text{H}_2\text{O}, \quad (9)$$

$$\text{Fe(CN)}_6^{3-} + \text{e}^- \xrightarrow{\text{0mV vs SCE}} \text{Fe(CN)}_6^{4-}. \quad (10)$$

At this potential there is a small interference from other constituents, e.g. ascorbic acid and uric acid, normally present in blood serum [26].

Chromatographic separation of myo-inositol and a mixture of inositolphosphates (obtained from enzymatically hydrolysed phytic acid) is illustrated in Fig. 4, using the coupled enzyme reactions and electrochemical detection described above.

Amino acids

As with sugars, the concentration of amino acids in biological fluids may be used as markers of metabolic malfunctions [4, 27]. The concentration of amino acids in cell cultures may also have a regulatory effect on the production yield [28]. Selective measurements of L- and D-amino acids can be made either by introducing the stereoselectivity in the separation step (e.g. using either chiral separation columns or achiral columns with chiral mobile phases [29, 30]) or in the detection step by using enantioselective derivatization or stereoselective enzymes [31].

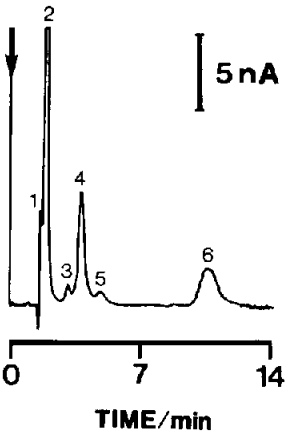

Figure 4
Chromatogram of a 20-μl injection of inositol phosphates on an anion exchanger (Mono Q, HR 5/5, Pharmacia Fine Chemicals, Sweden) monitored by the use of co-immobilized enzyme reactors and amperometric detection at a graphite electrode. Applied potential was 0 mV vs SCE. 10 μM myo-inositol (peak 1), 90 μM IP_1 (peak 2), 65 μM IP_2 (peaks 3–5) and 35 μM 1,2,6-IP_3 (peak 6). This standard solution was made from enzymatically hydrolysed phytic acid.

The stereoselective enzymatic detection of amino acids can readily be made by using L- or D-amino acid oxidases (LAAO and DAAO). An LC method was therefore constructed incorporating two IMERs containing LAAO or DAAO in the post-column reactor [32]. In the presence of molecular oxygen the enzymes oxidize the amino acids forming H_2O_2 and an oxo-acid (equations 11 and 12):

$$\text{L-amino acid} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{LAAO}} \text{2-oxo acid} + \text{NH}_3 + \text{H}_2\text{O}_2, \quad (11)$$

$$\text{D-amino acid} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{DAAO}} \text{2-oxo acid} + \text{NH}_3 + \text{H}_2\text{O}_2. \quad (12)$$

A variety of methods exist for the detection of enzymatically produced H_2O_2 [33]. Here, the H_2O_2 generated in the two AAO-reactors is reduced in a second IMER containing HRP using 4-aminoantipyrine (4-AP) and dichlorophenolsulphonate (DCPS) as the reducing agent to form a red coloured complex (a quinoneimine) that is measured at 514 nm [14]:

$$\text{H}_2\text{O}_2 + \text{4-AP} + \text{DCPS} \xrightarrow{\text{HRP}} \text{quinoneimine} + \text{H}_2\text{O}. \quad (13)$$

Flow injection (FIA) peaks of different L- and D-amino acids are shown in Figs 5 and 6,

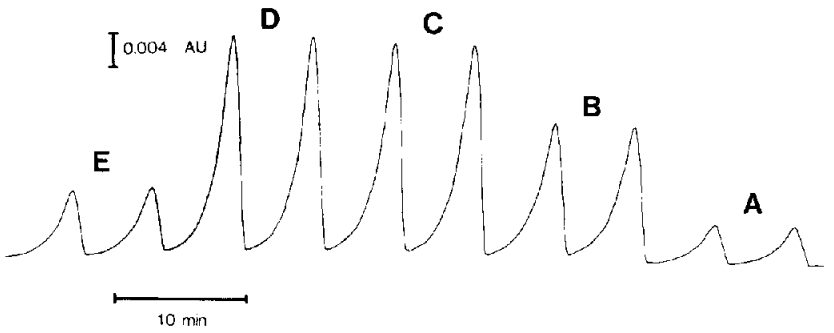

Figure 5
FIA recordings of (A) human serum, (B) L-leucine, (C) L-phenylalanine, (D) L-cysteine and (E) L-histidine. The concentrations of the L-amino acids were 100 μM. The serum was deproteinized and filtrated resulting in a final dilution of 10×. The components of the FIA system were: a carrier of a 0.1 M phosphate buffer at pH 7.0 (0.3 ml min^{-1}), a reagent solution of the same buffer (0.6 ml min^{-1}) with 0.2 mM 4-AP, 4 mM DCPS and 1 mM dichlorophenol [14], an LAAO reactor (85 μl), an HRP reactor (150 μl) and an injection loop (50 μl).

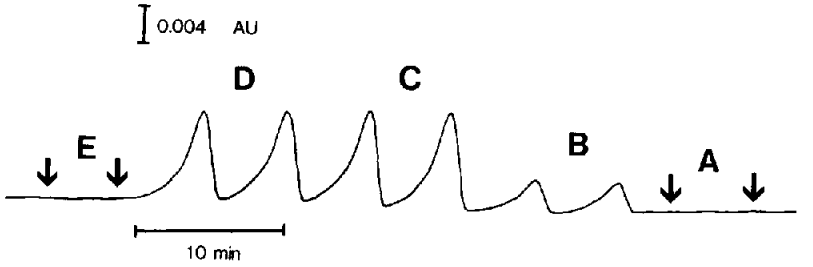

Figure 6
FIA recordings of (A) human serum, (B) D-leucine, (C) D-phenylalanine, (D) D-cysteine and (E) D-histidine. Same conditions as in Fig. 5 except the volume of the DAAO reactor (120 μl).

respectively. Double injections are shown in Fig. 5 of samples (100 μl) of four L-amino acids, L-cysteine, L-leucine, L-phenylalanine and L-histidine, having high conversions in the LAAO reactor [32] as well as of a sample of a deproteinized, filtrated and diluted (10:1) human serum. The response of the serum sample is mainly due to the presence of L-phenylalanine, L-leucine, L-isoleucine, L-tyrosine and L-tryptophan [32] as expected from a normal human serum [34]. Figure 6 shows the FIA responses of double injections of the same concentrations of D-amino acids and the same serum sample when a DAAO reactor was used instead. The conversions of the substrates in the DAAO reactor are lower than those of the substrates in the LAAO reactor because of the combined effects of using enzyme preparations of different activities, different coupling yields of active immobilized enzyme in the two reactors and different selectivity patterns of the two enzymes [32]. As expected no D-amino acids could be found in the serum sample.

Conclusions

The detection selectivity and sensitivity that can be obtained when IMERs are combined with a selective detection of the enzymatic product (either electrochemical or photometric) show great potential for the analysis of complex samples. These types of post-column detectors may open up new possibilities for the analysis of compounds in complex matrices other than those reported here. Most of the applications with LC–IMER coupling have been made in the biomedical and clinical fields [21, 35, 36]. It seems likely that an increase in this research area will be seen in the near future.

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