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Review

Biospecific detection in liquid chromatography

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Abstract

This review intends to cover the area of biospecific detection principles in mainly column liquid chromatography (CLC). However, due to the rapid developments of biospecific detection in related flow systems such as flow injection (FI), sequential injection analysis (SIA) and capillary zone electrophoresis (CZE), these are included to some extent. The main topics are enzyme and antibody/antigen based system that have been used to measure a large number of analytes in relation to these different flow techniques. Finally this review attempts to stress important factors that influence these bioselective techniques when applied to real problems in clinical, biotechnological, and environmental applications. Aspects on future trends and possible areas of application are also reported.

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1. Introduction

This review mainly focuses on scientific developments after 1991 as a follow-up on an earlier review [1]. However, as we intend to broaden the perspectives on biodetecting principles as well as report new possible application areas, some earlier work will also be included.

Analytical measurements can be performed in various ways all depending on the objectives of a particular laboratory or industry. Still the most common way to perform a chemical or biological reaction is in the batch mode, e.g. the reactants are allowed to react to some measurable product in a stirred beaker and the reaction product(s) are subsequently measured by some available analytical procedure. Major chemical companies, e.g. Sigma, Boehringer-Mannheim, Worthington, Pierce etc., commercialise enzyme kits for spectrophotometric determination of several analytes. However, since these batch methods are both tedious and costly, cheap, fast, sensitive, and continuous analysis in flowing liquids is desired.

Analytical flow measurements can be classified and divided into two categories [2]: (i) laboratory and industrial analysers, and (ii) continuous flow analysers (CFA) and analysers of discrete analyte zones in flowing liquids; the latter (analysers of discrete analyte zones) can be further divided according to the technique applied into, e.g. segmented flow analysis (SFA), flow injection (FI), and column liquid chromatography (CLC).

CLC is a well established method in most laboratories and its relation to FI has been debated through the years [3,4]. At first glance, the only difference is that FI lacks a separation column. A more fundamental difference is the purpose of the methods and of course the fact that CLC is most often performed at high pressures and FI at low, which in turn imposes different requirements on the operating equipment. So far the purpose of CLC has been to measure many analytes in one single injected sample by separating them from each other before being detected. This imposes a limit on the choice of the detection system which has to be able to measure the same property of several different analytes and consequently results in limited sensitivity and selectivity. In many ways, the goal has been to minimise the separation column, leading to higher sample through-put and lower time. Ultimately, the goal would be to remove the separation column completely, leading more or less to a FI-type configuration. The possibilities of FI are still not fully explored but one purpose is clearly to be able to perform many different chemical reactions with simplicity, high sample throughput, high sensitivity and selectivity. A disadvantage compared with CLC is that in most cases only one single analyte is

analysed at a time. However, interesting attempts to broaden the spectrum of analyte detection in FI have been made. Ruzicka and coworkers [4–6] proposed a new FI technique, sequential injection analysis (SIA), which is based on the use of a sinusoidal flow profile, rather than a constant flow-rate, in combination of multi-wavelengths detection.

One of the key problems in analytical chemistry is selectivity, particularly at low analyte concentrations and in the presence of interfering substances. In most cases conventional CLC detectors e.g. UV, refractive index (RI), and electrochemical detectors lack this necessary selectivity. This is where biospecific detection has proved to be of great potential to the analytical chemist.

The term biospecific detection can include any type of biochemical recognition reaction which can be transformed by an appropriate physical transducer to an electrical signal of some sort. These biochemical components can be of enzyme-, whole cell-, whole tissue-, nucleic acid-, receptor-, or antibody origin. In this review, the field of biospecific detection will be devided into two distinct areas, i.e. bioreactors and biosensors (see Fig. 1A,B). The figure illustrates the biological recognition of biochemical transducers at a biosensor and a bioreactor. The immobilised species can be of enzyme or antibody/antigen origin, where the substrate or antigen/antibody fits in a perfect key-and-lock configuration.

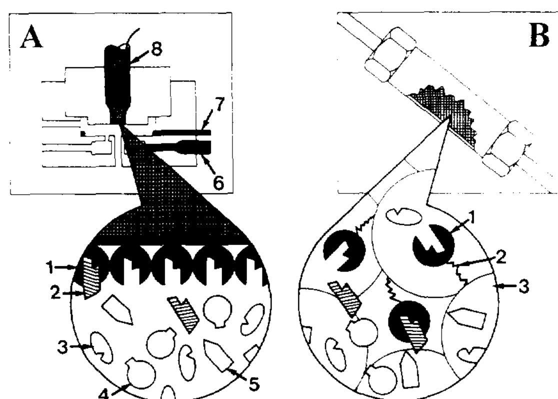


Fig. 1. Illustration of an amperometric biosensor and a bioreactor. (A) Analytical flow cell; 1 = enzyme or antibody bound to an electrode surface, 2 = substrate or antigen with a perfect fit, 3,4,5 = other matrix biomolecules present in the sample, 6 = reference electrode, 7 = auxiliary electrode, 8 = contacting Ag-wire. (B) Enzyme (1) or antibody is bound with a spacer arm (2) on a solid support (3) in a bioreactor.

There is an ongoing debate in the research community on how to define a biosensor. Here, a biosensor is referred to as a system of two transducers, biochemical and physical, in intimate contact with each other, which relate the concentration of an analyte to a measurable electric signal (Fig. 1A). A bioreactor is referred to as a configuration where the immobilised biochemical transducer is separated from the physical transducer (Fig. 1B). The physical transducers referred to can be e.g. electrochemical- (amperometric or potentiometric), optical-, piezo electric-, field effect transistor- (FET), or thermometric sensors.

The following chapters describe the possibilities and limitations of using biological recognition in a direct or indirect way (post- or precolumn) to obtain sensitive and/or selective detection systems. Essentially three major areas are covered, where the first part deals with biological recognition by enzymes and antibody/antigen reactions followed by how these can and have been used for analytical purposes (sections 3–6). The second part discusses some very important aspects of interfacing biological recognition reactions in combination with chromatographic techniques in real life applications (section 7). The third part deals with the possible future of biological recognition as an analytical tool (section 8).

2. Biospecific detection

2.1. Flow injection (FI)

So far it seems that single-line flow injection (FI) combined with enzyme-based biospecific detection units is the predominant technique used and a number of reviews on this subject have been published [7–9], see Fig. 2A1 and A2 exemplified for enzyme-based systems. Lately, much effort has been spent to broaden the

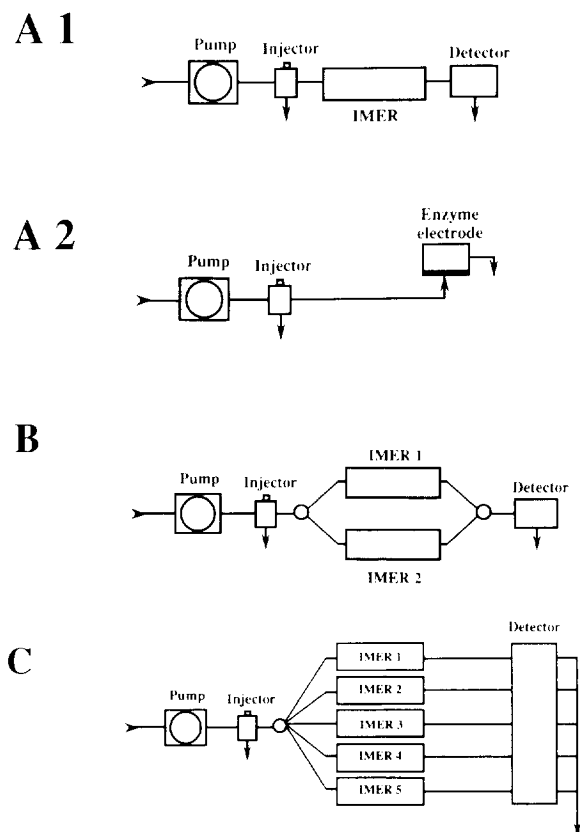


Fig. 2. The most common FI configurations with immobilised enzyme-based detection systems. (A1) Single line system with IMER, (A2) single line system with a biosensor, (B) dual line IMER system and (C) multiline parallel flow system with several IMERs.

spectrum of detected analytes in a single injected sample containing several analytes of interest (Fig. 2, B and C). One way has been to combine several enzymes, which all produce the same enzymatic product [e.g. oxidases which produce hydrogen peroxide (H_2O_2)] with a single physical transducer (e.g. an electrode for H_2O_2 detection or spectrophotometrically). Swindlehurst and Nieman [10] used multi-channel FI with immobilised enzyme reactors (IMERs) for the detection of fructose by immobilised glucose isomerase, sucrose by immobilised invertase, maltose by immobilised amyloglucosidase, and lactose by immobilised β-galactosidase. All these enzymatic reactions resulted in the production of glucose which subsequently was detected by using co-immobilised mutarotase and glucose oxidase. The resulting H_2O_2 was detected by chemiluminescence. Spohn et al. [11], determined glutamine, ammonia, lactate, glucose, and glutamate for fermentation control, by using dehydrogenase-based IMERs coupled in parallel with the fluorimetric detection of NADH. Similar work was performed by several other groups [12–16].

There are a few examples of biosensor arrays with two or more enzyme-modified electrodes used simultaneously for the determination of several analytes [17–20]. A thin-layer flow cell incorporating two biosensors (Fig. 3) was used for simultaneous CLC separation and detection of alcohols and sugars to be used with an ethanol fermentation [21] (Fig. 4).

2.2. Column liquid chromatography (CLC)

The bioselective detection devices used in FI are in many cases further optimised to meet the requirements for their compatibility with CLC. Recent reviews and overviews of the area are given [1,22–29]. The first papers on CLC–IMER studies appeared already in 1979 and 1980 for the determination of bile acids and oxidised steroids [30,31]. Another early work by

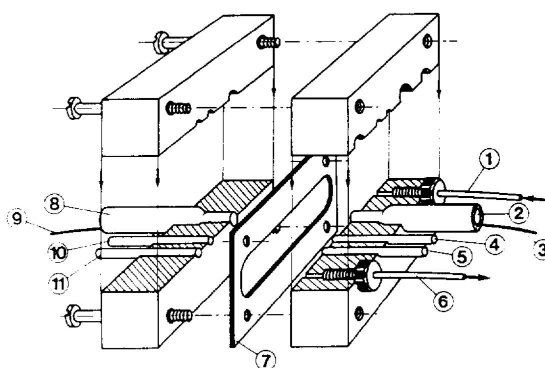


Fig. 3. Schematic drawing of a dual amperometric flow cell. (1) Inlet flow, (2) biosensor I, (3) Ag-wire for electric contact I, (4) reference electrode I (Ag/AgCl), (5) auxiliary electrode I (Pt), (6) outlet flow, (7) spacer, (8) biosensor II, (9) Ag-wire for electric contact II, (10) reference electrode II (Ag/AgCl), (11) auxiliary electrode II (Pt).

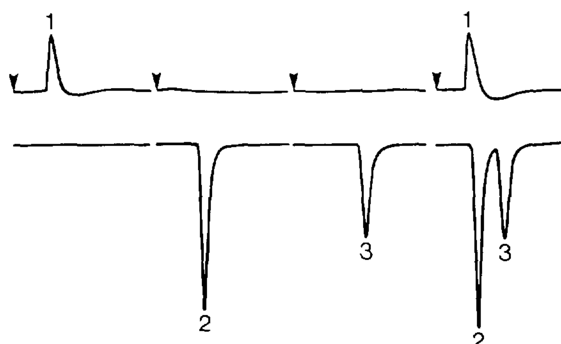


Fig. 4. Resulting chromatograms using the flow cell depicted in Fig. 3. (1) 0.5 mM glucose, (2) 0.25 mM methanol and (3) 1.0 mM ethanol. The three first injections represent single injections of 1, 2 and 3. The last injection represent the separation of a mixture containing 1, 2 and 3 [21].

Takeuchi [32] illustrates the high potential of enzymatic post-column derivatisation of bile acids in blood (Fig. 5).

Reversed-phase chromatography is the most frequently used mechanism for separation in CLC using an organic–aqueous mobile phase. When bioselective detection units are to be used in these separations, the stability of the biochemical transducer toward organic modifiers has to be investigated. The separation is generally optimised towards the use of a low-concentration organic modifier in the mobile phase since in most cases biochemical transducers cannot withstand higher levels of organic solvents. Bowers and Johnson [33] studied the behaviour of a number of immobilised enzymes in organic solvents such as acetonitrile, ethyl glycol, ethanol and methanol. A fraction of 25% of the organic solvent was found to be the upper limit that can be used with respect to enzyme activity. The compatibility with the enzyme activity was found to decrease with increasing hydrophobicity of the solvent. Irth et al. [34] studied the influence of organic modifiers on the binding efficiency of antibodies to an immobilised an-

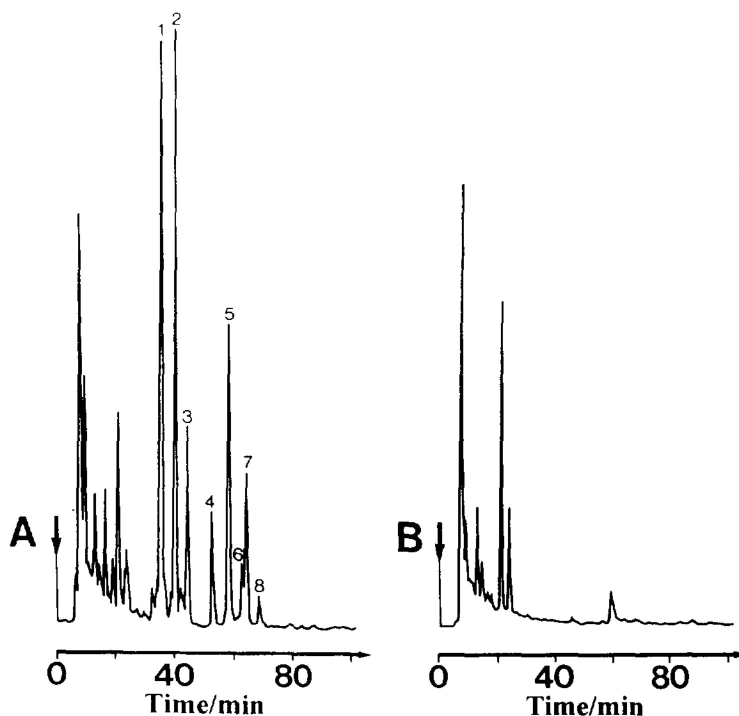


Fig. 5. Chromatogram using fluorometric detection of bile acids contained in calf serum with (A) 3α,β-hydroxysteroid dehydrogenase post-column IMER, (B) without IMER. Reproduced with permission from Ref.[32].

tigen support used as a post-column for CLC. The acceptable levels of methanol and acetonitrile were 30 and 15%, respectively.

Bioreactors are used either in the pre- or post-column mode [1,35]. Only recently have enzyme-based biosensors been used as detection units in CLC [17], the reason probably being that the amount of biocomponent which can be immobilised in a bioreactor is much larger than in a biosensor configuration, leading to significantly higher conversion or binding efficiency. In Fig. 6, some possible configurations for combining biospecific detection units with CLC are depicted.

A pioneer study was performed by El-Rassi and Horváth [36], who inserted a pre-column IMER in a complicated chromatographic system for oligonucleotide synthesis. The oligonucleotides were synthesised by immobilised ribonuclease, fully compatible with the preparative-scale displacement chromatographic separation by which the enzyme product was separated from unreacted reactants.

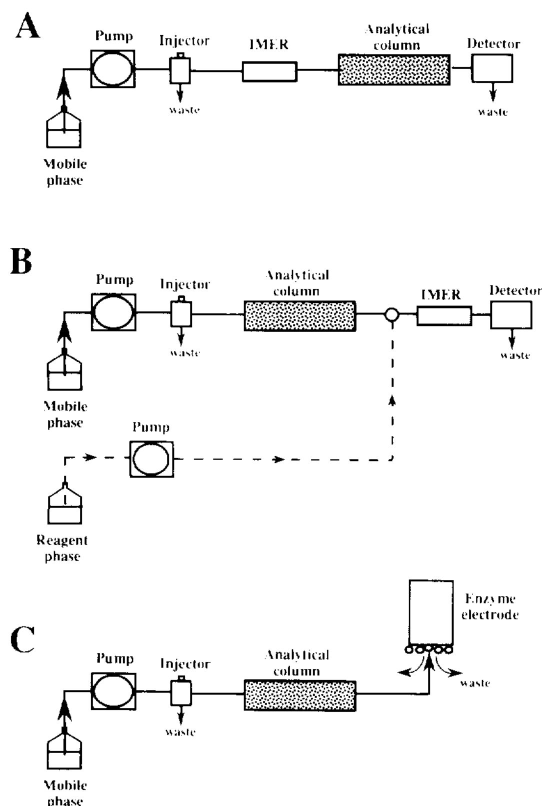
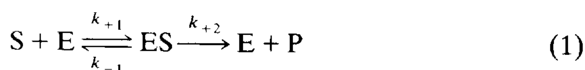


Fig. 6. The most common CLC flow configurations with immobilised enzyme-based detection systems. (A) Pre-column IMER–CLC system, (B) post-column CLC–IMER system. Dotted line indicates additional reagent stream, and (C) CLC system with biosensor detection.

In CLC applications, the biocomponent should ideally be able to detect several analytes present in the sample. This can be achieved either by using group-specific biochemical transducers, e.g. L- and D-amino acid oxidase [17,37], oligosaccharide dehydrogenase [38,39], pyranose oxidase [40,41], antibodies which show cross-reactivity for analytes with related structures [34,42] and by the use of several immobilised biochemical transducer co-immobilised or in series [43–47].

3. Enzymes as analytical tools

Enzymes are proteins that speed up chemical equilibria. The selectivity obtained through this catalysis makes enzymes suitable reagents for chemical analysis. The analyte is transformed in the biochemical reaction and the monitoring of this process may be correlated to the concentration of the analyte. The simplest enzyme-catalysed reaction obeying the Michaelis–Menten model is the one where only one substrate participates, according to reaction (1):



$$S + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \qquad (1)$$

where S is the substrate, E is the enzyme, ES is an intermediate complex between enzyme and substrate, P is the product, and k_{+1}, k_{+2}, k_{-1} are reaction-rate constants for the forward respective backward reactions. From the rate equation $d[S]/dt = V_{max}[S]/(K_M + [S])$ where K_M $[=(k_{-1} + k_{+2})/k_{+1}]$ is the Michaelis–Menten constant and V_{max} is the maximum reaction rate at a particular enzyme concentration for $[S] \gg K_M$, the expression describing the fractional conversion (X) in an IMER can be derived, reaction (2):

$$-\ln(1 - X) = V_{max}t/K_M - [S_0]XK_M \qquad (2)$$

where t expresses the residence time of the substrate in the IMER and $[S_0]$ is the initial substrate concentration [48].

As analytical reagents, enzymes show some characteristics that have to be considered. They are flexible molecules with a large variety in molecular mass and a complex conformation, generally linked to their catalytic activity. One enzyme may be obtained and purified from several biological sources, resulting in preparations with different properties. This is demonstrated for three alcohol oxidases from different biological sources immobilised in carbon-paste electrodes, showing substantially different substrate specificities [49] (Table 1). Enzymes demand defined operation and storage conditions, which limit their activity and stability. They are water soluble and water, to a variable extend, is always required for their activity and solubilisation. The possibility to dissolve and improve the stability of enzymes in organic environments has been demonstrated in several recent papers by chemically modification of enzymes with polyethylene glycol (PEG) [50,51]. Solubilisation of enzymes in both aqueous and various organic solvents of different hydrophobicity was possible with improved stability and activity of the modified enzyme compared to the unmodified enzyme. Enzymes have very well-defined binding sites for the substrate (analyte). Many of them require the presence of cofactors which either are firmly attached to the protein (prosthetic group) or have to be added (co-enzymes). They may be easily activated/inactivated by the presence of other compounds.

Immobilisation of an enzyme usually leads to a

Table 1
Relative response (%) in FI analysis for various substrates with three different carbon-paste electrodes containing alcohol oxidase from different sources [49]

Sample	Alcohol oxidase source		
	Candida boidinii (Serva)	Pichia pastoris	Candida boidinii (Sigma)
Ethanol	100	100	100
Hydrogen peroxide	75	189	866
Methanol	540	282	252
Formaldehyde	22	99	58
Acetaldehyde	<1	<5	10
Propionaldehyde	15	94	163
Butyraldehyde	29	652	378
Acetic acid	<1	19	34
Propionic acid	7	56	51
Butyric acid	37	41	44
Monochloracetic acid	2	11	60
Malic acid	<1	<5	31
Inositol triphosphate	151	159	217
Allylacohol	68	55	46
2-Butenol	79	470	377
2-Chloroethanol	22	24	15
Dihydroxyacetone	5	57	57
Paracetamol	–	–	–
Adenosine	–	–	–
Lactate	–	–	62
L-Phenylalanine	–	–	–

loss of absolute activity. However, in the immobilised state, the enzyme/substrate ratio is usually much higher compared with batch methods, the apparent stability is improved and the enzyme can be re-used multiple times. Since there is no general rule for an optimum immobilisation condition, an empirical approach is always required [52].

The configuration of an immobilised enzyme can have great influence on analyte specificity, which is demonstrated for L-amino acid oxidase in solution, immobilised in an IMER, and immobilised in a carbon-paste electrode (Table 2). In the carbon-paste electrode the enzyme shows response for 19 out of 20 amino acids tested, in the IMER configuration 14 amino acids show response whereas in solution only eight of the amino acids are active with the enzyme.

The extended use of enzymes as routine reagents shows that all the above considerations have not, since the first described analytical application of an urease preparation in 1951 [53], limited their utility as analytical reagents. On the contrary, the new instrumentation and the increased research in this area prove the new perspective and success of enzymes in solving analytical problems in biotechnology, environmental care, clinical diagnosis, and process control.

3.1. Redox enzymes

Oxidases and dehydrogenases, members of the large group of oxidoreductases ("redox enzymes"), are the most commonly used enzymes as analytical reagents. These enzymes catalyse the oxidation or reduction of their substrate. Usually oxidases are most frequently used because the final electron acceptor, molecular oxygen, is readily available in the eluent and because the involved co-factors are strongly attached to the enzymes. As an electron transfer

Table 2
Relative response (%) to L- and D-amino acids using L- and D-amino acid oxidase, respectively, in solution, immobilised in an IMER and in a carbon-paste electrode

Amino acids	L-AAOD Soluble	L-AAOD IMER	L-AAOD Biosensor	D-AAOD IMER	D-AAOD Biosensor
Glycine	0	0	20.2	0	0
Alanine	3.8	1	20.1	94.0	24.6
Valine	0	5.1	35.2	81.4	105.2
Leucine	50	100.2	112.5	100.1	117.4
Isoleucine	6.4	56.5	45.5	137.7	136.9
Serine	0	0	35.7	16.0	16.5
Threonine	0	0	26.9	0	0
Aspartic acid	0	0	14.1	9.6	0
Asparagine	0	4	22	0	0
Glutamic acid	0	0	28.6	11.5	0
Glutamine	0	6.8	28.3	0	0
Lysine	0	3.5	34	4.5	0
Histidine	2.6	29.6	38	2.3	7.4
Arginine	0	6.1	28	8.4	0
Phenylalanine	100	100	100	100	100
Tyrosine	125	89.7	91.8	10.1	35.2
Tryptophan	87.2	86.3	64.8	174.8	0
Cysteine	0	58.7	O×	150.3	148.5
Methionine	85.3	88.4	133.2	–	–
Proline	0	0	32.5	–	–

reaction is involved in the natural cycle of the redox enzymes, enzymatic redox reactions are particularly closely related to electrochemical transducers. This is the reason for the tremendous interest in the construction of "enzyme electrodes", particularly amperometric enzyme electrodes.

3.1.1. Dehydrogenases

The dehydrogenases are characterised by their independence of and insensitivity towards molecular oxygen. One can differentiate between different groups of dehydrogenases; those with bound cofactors and those dependent on a soluble cofactor acting as a cosubstrate in the enzyme cycle. The group depending on a bound cofactor can be further subclassed, e.g., into flavoproteins having, flavin adenine dinucleotide (FAD) or flavin mono nucleotide (FMN) as cofactor or the relatively recently discovered pyrrolo-quinoline quinone (PQQ) dependent dehydrogenases [54–57]. Examples of flavoprotein dehydrogenases are e.g. cytochrome b_2 and diaphorase, both enzymes also containing additional redox-active functionalities (heme groups). The PQQ-dehydrogenases have lately attracted a lot of interest in biosensor configurations partly because of their insensitivity to molecular oxygen in contrast to the oxidases. Direct electron transfer between the dehydrogenases and electrodes has been confirmed. However, the major route of coupling these enzymes to electrochemical transducers has been through the use of redox mediators, shuttling the electrons most often from the reduced form of the enzyme to the electrode [58].

The second type of dehydrogenases are the various dehydrogenases dependent on either nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) as soluble cofactor. Most commonly an $NAD(P)^+$-dependent dehydrogenase is used to oxidise a substrate for the production of a stoichiometric amount of NAD(P)H, which then is measured either amperometrically by electrochemical oxidation, spectrophotometrical monitoring at 340 nm [59], or by fluorescence (excitation at 340 nm, emission at 465–470 nm) [60].

The NAD(P +)-dependent dehydrogenases constitute the largest group of redox enzymes known today (≈ 500), which is not reflected by the number of publications making use of these enzymes in biospecific detection systems. Three major drawbacks of these enzymes constitute the reasons for this. The first reason is that, for their activity, these enzymes rely on a soluble cofactor acting as a co-substrate and as such it needs to be added separately to the sensing system. The cofactor is expensive and for practical use, especially for routine analysis, it may turn out to be much too costly to be continuously added. The second reason is that the formal potential ($E^{\circ\prime}$) of the $NAD(P)^+/NAD(P)H$ redox couple is low with a value of −560 mV vs. SCE (at pH 7.0) [61]. This means that $NAD(P)^+$ has a very low oxidising power compared with the $E^{\circ\prime}$ values of most of the dehydrogenase substrates. To be able to use these systems analytically, a second reaction step is usually necessary to push the equilibrium to the product side. This can be achieved in a number of ways. One way is to couple a second purely chemical or enzymatic step to the dehydrogenase step, in which either the initial NADH produced or the product is consumed. Another way to accomplish this would be to electrochemically oxidise the NADH formed and thus also measure the current as a response signal proportional to the substrate concentration [62–66]. However, and here we now deal with the third obstacle with the $NAD(P)^+$-dependent dehydrogenases, the conversion of both redox forms of the cofactor on naked electrodes is subjected to very pronounced irreversible electrochemistry and for high NADH concentrations (>0.1 mM) further complicated by side reactions and risk for electrode poisoning or fouling [57,58,61,67–69]. Much work has therefore been devoted to the finding of good mediators catalysing the electrochemical oxidation of NAD(P)H within the optimal potential range (around 0 mV vs. SCE) and with enzymatically active $NAD(P)^+$ as the product [57,58,61,67–69]. Fig. 7 shows a dehydrogenase reaction cycle at a mediator modified electrode, catalysing the oxidation of a substrate (S) to a product (P). The role of the mediator

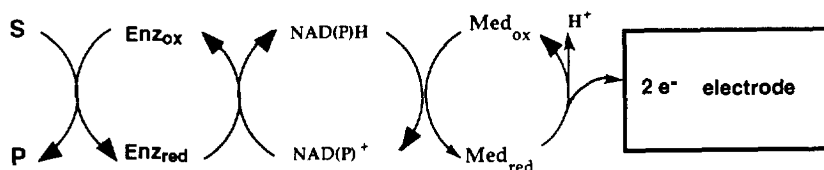


Fig. 7. Reaction sequence at an amperometric electrode for a mediated dehydrogenase catalysed reaction. For more details see text.

(Med_{ox}) is first to oxidise the enzymatically reduced co-factor NAD(P)H back to the enzymatically active $NAD(P)^+$ and then the reduced mediator (Med_{red}) is oxidised back to the Med_{ox} form by the electrode material so that the reaction cycle can start again.

3.1.2. Oxidases

All oxidases depend on a cofactor strongly bound within the enzyme structure. The structure of the redox cofactor is either of the flavin type (FAD or FMN) [58] or a copper ion containing group [55,56]. The enzyme may also contain additional metal ions (e.g., Fe or Mo), which have a rather unclear function. All oxidases make use of molecular oxygen as the reoxidation agent in the catalytic cycle. Depending on the ability of the enzyme to donate two or four electrons to molecular oxygen, either hydrogen peroxide or water is the final product. Due to the high oxidation power of molecular oxygen, all oxidase catalysed reactions can be regarded as chemically irreversible when using its natural electron acceptor. Artificial electron acceptors have also been used. An oxidation reaction catalysed by an oxidase is depicted in Fig. 8. The substrate (S) is oxidised whereby a product (P) is formed and the enzyme-bound cofactor is reduced. Molecular oxygen serves as the reoxidising agent for the reduced cofactor whereby hydrogen peroxide or water is produced depending on the type of cofactor and enzyme. As is easily realised from Fig. 8 an oxidase-based reaction can be electrochemically followed either by the decreased oxygen content in the solution at a Clark-type oxygen electrode as was done in the very first enzyme electrode [70] or if hydrogen peroxide is the end product, by its direct electrochemical oxidation (or reduction) at the electrode [71–79]. However, both these ways of following the enzyme reaction have their drawbacks. The use of the oxygen electrode is based on following a decrease in response current, which is always a drawback. The electrochemical reduction of oxygen calls for a membrane covered electrode (Clark-type) to prevent interfering reactions to occur at the electrode surface, which largely decreases the sensitivity. Additionally, a substantially negative electrode potential (≈ -0.6 V vs. Ag/AgCl) is necessary, leading to a noisy background current.

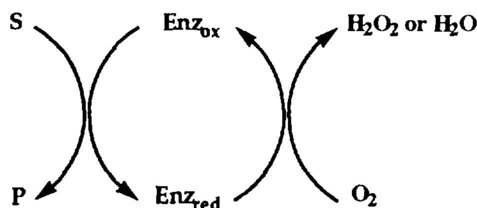


Fig. 8. Reaction sequences for an oxidase catalysed reaction using O_2 as the electron acceptor and using an artificial electron acceptor.

3.1.2.1. Detection of H_2O_2

Due to the drawback of measuring the decrease in the oxygen tension much work has been focused on the detection of hydrogen peroxide. A variety of methods have been worked out and because it is such an important substrate to be selectively determined, new detection systems are likely to appear. It can be detected through direct amperometry or through further reaction chemistry (see below).

Electrochemical oxidation of hydrogen perox-

ide occurs at high over voltages (≈ +0.6–0.7 V vs. Ag/AgCl, pH 7) at metal electrodes and at even higher potentials at carbonaceous electrodes (+0.9–1.15 V). Quite sensitive detection systems can be constructed through this detection principle. However, the necessary high potential opens up the detection system for a variety of interfering reactions mainly for easily oxidisable species such as ascorbate, urate, paracetamol, and neurotransmitters common in clinical samples. Much work has been devoted trying to find alternative electrode materials or electrode modifications to circumvent the drifting response signal and the high applied potential.

Spectrophotometric determinations of H_2O_2 are numerous [80]. Especially the reaction of H_2O_2 with a luminescent or fluorescent dye gives a very sensitive photometric determination of H_2O_2.

3.1.3. Peroxidases

Peroxidases have been used for a long time for the determination of hydrogen peroxide and small organic peroxides. Peroxidase is used to further react the hydrogen peroxide formed as a product in many oxidase reactions particularly to form a coloured [81], a fluorescent [82], or a luminescent [82,83] species that can be detected spectrophotometrically, but also to form electrochemically active compounds more easily detected than hydrogen peroxide itself [84,85].

Peroxidases are generally small, often glycosylated, redox enzymes with protoporphyrin IX (or a related structure) as the strongly bound cofactor. They are all active for the reduction of hydrogen peroxide and structurally related small organic peroxides. In the reaction with peroxide the native form of the enzyme becomes oxidised in a single two-electron step into a state usually denoted compound I. The rereduction back to the native form occurs in two single one-electron steps with an intermediate form denoted compound II. Depending on the peroxidase enzyme a number of different compounds may work as electron donors. With the most commonly used peroxidase, horseradish peroxidase (HRP), virtually any reducing agent may work in this respect, e.g., ferrocyanide, phenol, *ortho*- and *para*-phenylenediamines, iodide, ascorbate, etc. The oxidised reaction product can in turn be electrochemically reduced at an applied potential substantially lower than that used in the direct oxidation of hydrogen peroxide. Peroxide-modified carbon-paste electrodes have therefore been used for the determination of hydrogen peroxide and organic peroxides with the use of soluble or immobilised electron donors acting as mediators. Recently a report has been published on what appears to be a direct electron transfer reaction from electrodes to compounds I and II of HRP and structurally related peroxidases immobilised directly on electrode surfaces without the deliberate attachment of mediators on the electrode surface [86]. In this laboratory peroxidase-based carbon-paste electrodes have been used in a mediatorless fashion and together with hydrogen peroxide producing oxidases. The reaction sequence for such a coupling is illustrated in Fig. 9.

3.2. Non-redox enzymes/coupled enzyme systems

Many enzymatic reactions result in products which are not easily detectable with conventional techniques. Sometimes these products can be transformed in a second (or in several) enzymatic step to detectable species of some sort. Common enzymes used for this purpose are hydrolases, transferases, lyases, isomerases, and ligases. The field has been reviewed by Marko-Varga and Domínguez [87] and Scheller and co-workers [8,9].

4. Enzyme-based detection systems

4.1. Post-column detection of alcohols

The main application area for detection of alcohols is in the biomedical field in monitoring blood alcohol in drivers and in cases of acute alcohol intoxication. Drunk driving and acute ethanol and methanol intoxication require fast and reliable methods with high sensitivity and selectivity. The toxic levels in blood are above 1 mg/ml for ethanol and 0.2 mg/ml for methanol

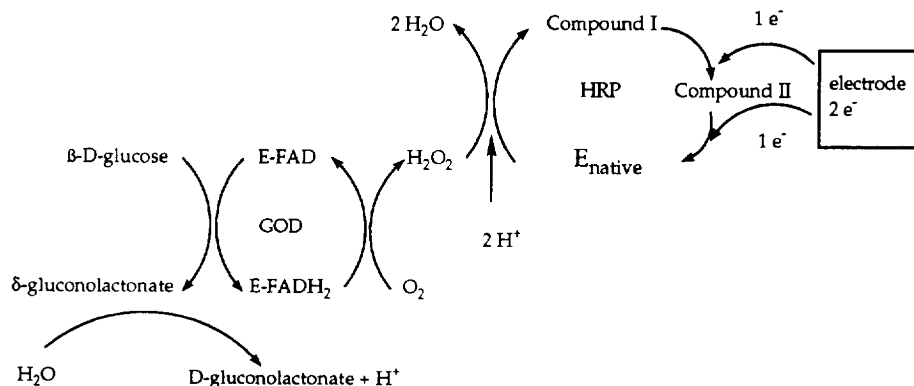


Fig. 9. Schematic representation of a coupled enzyme reaction sequence at an electrode. Glucose oxidase oxidises glucose forming hydrogen peroxide, which is a substrate for peroxidase (HRP). HRP is then electrochemically rereduced to its native form in an apparent electrontransfer reaction.

whereas lethal levels are higher than 3.5 and 0.9 mg/ml, respectively. Determination of alcohol is also interesting in monitoring of alcohols in spirits and wines. Most of the existing analytical methods lack the ability to distinguish between methanol and ethanol, which was one reason why an "epidemic" intoxication caused by adulturated wine could not be identified.

Both oxidases and dehydrogenases from various microorganisms have been used in both IMER and biosensor configurations for the development of detection systems for alcohols. However, as in many other cases most of the work has been done in the FI mode.

Tagliaro et al. [88] reported the use of a post-column alcohol oxidase (AOD) IMER for the detection of methanol and ethanol in microlitre volumes of biological samples such as tears and capillary blood. The alcohols were first separated with two HEMA-S 1000 reversed-phase columns coupled in series using a phosphate buffer (pH 7–9.5) as the mobile phase. The eluting alcohols were then passed into an AOD-IMER. The alcohols were enzymatically oxidised to their corresponding aldehydes and stoichiometric amount of H_2O_2, which subsequently was amperometrically detected at a Pt electrode at +500 mV vs. Ag/AgCl. The paper discusses the problems encountered in the development of IMERs, using a delicate and easily deactivated enzyme like AOD. Immobilisation on both silica- and polymer-based support materials were performed and compared with respect to the influence of different immobilisation chemistries on the activity and the influence of reactor volume and particle size on post-column band-spreading. The results suggested that polymeric supports were superior to silica, the best support in terms of both sensitivity and influence on band-spreading being a polymer-based epoxy-derivatized Separon HEMA-BIO 1000, with an absolute limit of detection (ALD) of 1–5 nmol for methanol (5 or 20 μl).

Pacáková et al. [89] reported a similar post-column IMER system for determination of ethanol in serum. Serum spiked with ethanol was first separated on a Separon SGX C_{18} column with phosphate buffer pH 8.2 as the mobile phase. The eluting ethanol was then oxidised in an IMER packed with AOD immobilised on HEMA Bio 1000 EH epoxy activated support, as described above. The enzymatically produced H_2O_2 was then amperometrically determined at a carbon-fibre array working electrode at +0.9 V vs. Ag/AgCl. The limit of detection (LOD) of ethanol in serum was 0.17 nmol (20 μl), which was one order of magnitude higher than in an aqueous solution.

2-Chloroethanol is an alcohol of great importance generated by fumigation of foodstuffs with ethylene oxide and is a substrate for AOD. Galensa [23] determined 2-chloroethanol in a

post-column CLC–IMER set-up with amperometric detection of the enzymatically produced H_2O_2.

Amperometric biosensors for alcohol determinations, based on immobilising AOD containing whole cell (yeast [90]) or tissue (tomato seed [91]) into carbon-paste electrodes, have been developed as detection units in CLC.

In our laboratory, a carbon-paste biosensor with AOD co-immobilised with HRP was used as a detection unit for a CLC separation of aliphatic alcohols, organic acids, and aliphatic and aromatic aldehydes. For more details see section 7.2.

The equilibrium of the enzymatic reaction for alcohol dehydrogenase (ADH) favours the substrate rather than the product side. Recently, this has been addressed in papers describing ADH-modified carbon-paste electrodes also including NAD^+ and an aqueous insoluble redox mediator, where the reaction is driven towards product formation because the enzymatic production and the mediated electrochemical removal of NADH are localized in close proximity to each other [92]. However, detection systems based on ADH have not yet been used in conjunction with CLC.

4.2. Post-column detection of mono-, di-, oligo- and polysaccharides

Carbohydrates in plant energy storage products are of crucial importance for human nutrition. Glucose is by far the most abundant organic molecule on earth if the glucose molecules in starch, cellulose and other polysaccharides are taken into account. Glucose is also a central and regulating molecule in the metabolism of most organisms.

In humans the great impetus of metabolic and endocrinological disorders, such as diabetes mellitus, galactosuri and lactose intolerance, is the driving force for new developments in sugar analyses. The sugar composition in normal blood and serum is also altered in a series of other disorders and diseases, e.g. cancer and alcoholism.

Receptor sites on cell membranes usually have a carbohydrate component interacting with pathogenic agents. Recent research on e.g. urinary infections has resulted in new carbohydrate drugs interacting with the pathogenic agent thus blocking the possibility for infection. Other examples from the clinical-medical fields are the use of polysaccharides, e.g. of hyaluronates in eye surgery, dextrans as a blood substitute and starch as common filling in pharmaceutical preparations.

The structure and molecular mass are crucial properties of many industrially used polysaccharides. For the manufacturing of modified starch for paper coatings or flame-safe paints, the ratio of amylopectin/amylose in native starch is important. Pollution and the risk for shortage of petrol-based energy sources have forced economists, politicians, and scientists to consider alternative and renewable energy sources. These can be obtained directly from fast growing trees, e.g. *Salix sp.*, but also from garbage dumps or from waste product from paper pulp industries. Cellulose, lignocellulose, and hemicellulose hydrolysates are possible candidates as renewable energy sources through fermentation to ethanol [93].

In all these areas there is the need for robust, selective and sensitive methods for carbohydrate determination. As carbohydrates constitute such a large and heterogeneous group of compounds, the analysis of the various types of small (mono-, di- and oligosaccharides) or big (polysaccharides) molecules gives considerable analytical problems in various fields.

Carbohydrates lack most physical and chemical properties that would make them easily detectable with common detection principles. They are uncharged and have a low absorptivity in the visible and UV regions. RI-, chiral- or evaporative light scattering detection (ELSD) are possible alternatives. However, these techniques lack the necessary selectivity and sensitivity to meet the requirements of modern applications.

The presence of a carbonyl group provides sugars with a certain reducing power. The formal potential ($E^{\circ\prime}$) for the oxidation of glucose is -0.604 V vs. SCE (pH 7) theoretically indicating

that amperometry would be a possible means of detection. However, direct electrochemical oxidation of sugars is complicated by side reactions, resulting in passivation or fouling of the electrode surface. The development of pulsed amperometric detection (PAD) for carbohydrate analysis by the group of Hughes and Johnson [94] has proved to be a powerful tool to overcome the fouling and selectivity problems usually encountered with direct electrochemical oxidation.

Enzymatic oxidation of sugars, particularly in conjunction with amperometry, provides the necessary sensitivity and selectivity for most applications. The commonly used oxidoreductases are often used in conjunction with other enzymes in order to broaden the substrate detectibility and to drive equilibrium reaction towards completion. Several reviews on applications of biospecific detection of carbohydrates have been reported [26,29,95].

The most common approach is to use post-column IMERs for carbohydrate analysis. Only a few examples of biosensor detection combined with CLC for carbohydrate analysis have been presented [18,96] (Table 3).

4.2.1. Mono- and disaccharides

Kiba and co-workers used a sorbitol dehydrogenase IMER as a post-column detection device for the cation-exchange chromatographic separation and detection of the ketoses fructose and sucrose [97] and the alditols D-xylitol, D-sorbitol and D-ribitol, respectively [98]. Sorbitol dehydrogenase catalyses the reduction of ketoses and oxidation of alditols in the presence of NAD^+ or NADH, respectively. However, the ketose reduction reaction is strongly favoured, which means that the systems rely on the spectrofluorimetrical disappearance or appearance of NADH, respectively. The linear working range and LOD for ketoses were: for sucrose, 50–500 μM and 10 μM; for fructose, 25–250 μM and 5 μM (50 μl inj.). The linear working range and LOD for alditols were: for xylitol, 2–500 μM and 1 μM; for sorbitol, 5–800 μM and 3 μM; for ribitol, 2–500 μM and 1 μM (50 μl inj.).

Maes and Nagels [47] developed a system using a hexose oxidase and/or a glucose oxidase (GOD) reactor for detection in a cation-exchange separation of cellobiose, maltose, glucose, xylose, galactose and mannose. The enzymatically produced H_2O_2 was detected at a Pt electrode at +750 mV vs. SCE. The substrate specificities of the two enzymes were different and by combining the two, the substrate specificity could be broadened.

A post-column enzyme reactor with co-immobilised invertase, mutarotase, and GOD was used for the detection of glucose and sucrose in pear juice after separation of the sugars on a C_{18} reversed-phase column [27]. Sucrose is hydrolysed by invertase to α-D-glucose and β-D-fructose. Mutarotase (MUT) converts α-D-glucose to β-D-glucose, since GOD is only active on the β-form of glucose under the formation of H_2O_2, which is amperometrically monitored at +600 mV vs. Ag/AgCl. The LOD for sucrose with and without co-immobilised MUT was 3 and 300 pM (10 μl inj.), respectively. A similar system was used by Kiba et al. [99] for the reversed-phase separation and detection of sucrose and glucose in foods. They used post-column IMERs containing invertase and pyranose oxidase with chemiluminescence detection of H_2O_2. The LOD for sucrose and glucose was 0.2 and 0.3 μM (50 μl inj.), respectively.

Kakemoto et al. [100] separated glucose and lactose by reversed-phase CLC and then detected the analytes with a post-column co-immobilised enzyme reactor containing GOD and β-galactosidase. Lactose and glucose elute from the CLC-column and lactose is then converted by β-galactosidase to β-D-glucose and D-galactose. The produced β-D-glucose is then converted by GOD to H_2O_2 which is detected amperometrically at +750 mV vs. Ag/AgCl. Lactose, glucose, ascorbic acid and L-cysteine could be detected in various milk products with an LOD for lactose and glucose of 10 and 5 ng, respectively.

Anion-exchange CLC combined with a pyranose-based biosensor was used for the detection of 1,5-anhydroglucitol (1-deoxyglucose) in urine [96]. The enzyme was immobilised in a chitosan membrane which was cast onto a Pt

Table 3
Biospecific detection of saccharides using either IMER(s) or biosensor configurations

Saccharides	Biosensing configuration	Biochemical transducer	Physical transducer	Ref.
Lactose/glucose in milk	IMER	β-galactosidase/ glucose oxidase	EC/H_2O_2	[90]
Glucose in carbonated water,sake,soy sauce, wine	IMER	glucose oxidase	EC/H_2O_2	[276]
L-Lactate, pyruvate	IMER biosensor	lactate oxidase/ lactate dehydrogenase	EC/peroxidase/ H_2O_2	[108]
Glucose/maltose in foods	IMER	glucoamylase/ glucose oxidase	EC/H_2O_2	[277]
Glucose in urine and fruits	IMER	glucose oxidase	EC/H_2O_2	[278]
Glucose/sucrose in pear juice	IMER	invertase/ mutarotase/ glucose oxidase	EC/H_2O_2	[27]
Galactose/lactose in soft cheese	IMER	galactose oxidase	EC/H_2O_2	[27]
Maltooligo-saccharides in beer	IMER	amyloglucosidase /glucose oxidase	EC/H_2O_2	[27]
Sucrose/glucose	IMER	invertase/ pyranose oxidase	chemilumines-cence	[89]
Oligosaccharides in wort and beer	IMER	amylogucosidas/ glucose dehydrogenase/ mutarotase	UV/NADH	[45]
Mono- and disaccharides	IMERS	hexose oxidase/ lucose oxidase	EC/H_2O_2	[47]
Maltodextrins	IMER	glucoamylase/ glucose oxidase	EC/H_2O_2	[46]
Maltooligo-saccharides	IMER	oligosaccharide dehydrogenase	EC	[39]
Stachyose,raffinose, sucrose,fructose	IMER	invertase/ fructose dehydrogenase		[279]
β-Glucan oligosaccharides ≤DP30	IMERS	cellulase/glucose oxidase	EC/H_2O_2	[44]

(Continued on p. 206)

Table 3 (*continued*)

Saccharides	Biosensing configuration	Biochemical transducer	Physical transducer	Ref.
Alditols	IMER	sorbitol dehydrogenase	Fluorescence/ NADH	[88]
D-Fructose, sucrose, L-sorbose	IMER	sorbitol dehydrogenase	Fluorescence/ NADH	[86]
Glucose, 1-deoxyglucose in serum	IMER	pyranose oxidase	chemilumines-cence/H_2O_2	[278]
Stachyose, raffinose, melibiose, galactose	IMER	galactose oxidase peroxidase	horeseradish peroxidase/H_2O_2 fluoresence	[279]
Glucose, cellodextrines	biosensor	cellobiose oxidase glucose oxidase	EC/H_2O_2	[18]
1,5-Anhydroglucitol (1-deoxyglucose) myo-inositol	biosensor	pyranose oxidase	EC/H_2O_2	[85]

electrode for detection of H_2O_2 at +700 mV vs. Ag/AgCl. The LOD of human urine 1,5-anhydroglucitol was 0.1 mg/l and the measurable range was 0.1–60 mg/l (150 μl inj.), which covers the range of concentrations most likely to be encountered. The method was compared with the GC–MS technique and showed good correlation.

4.2.2. Oligo- and polysaccharides

For the determination of oligosaccharides and polysaccharides, hydrolytic enzymes are usually combined with oxidoreductases. These enzymes hydrolyse the oligo- and/or polysaccharides to their monomeric constituents which then can be detected by a suitable oxidoreductase.

At this laboratory we have been working for several years with flow systems for starch analysis and have recognised some very important aspects. The main problem with polysaccharides is that the physical and chemical properties are usually very complex, e.g., a homogeneous starch molecule does not exist. The single common feature that all starch molecules share in nature is that the main structural building block is the α-D-glucose unit with the molecular formula $C_6H_{10}O_5$. If starch can be totally broken down to its glucose constituents, a good quantitative measure of starch is achieved. Only the enzymatic method of degradation leads to the production of solely glucose. Hydrolysis with acid is very prone to error because not only starch but also other polysaccharides, e.g. cellulose, are hydrolysed. Additionally, prolonged treatment with acid can lead to the production of isomaltose and thus give a false glucose content [101]. Alkaline treatment of starch may lead to a mixture of several monosaccharides because the glucose first formed in the depolymerisation may isomerise under extreme pHs and elevated temperatures [102].

To be able to quantify the total glucose content in starch or the total monomer content of any polysaccharide and thereby the concentration of the polysaccharide itself, it is most essential that 100% degradation of the polysaccharide to its monomer is achieved. Since an electrode surface offers only a restricted area for attachment of the enzyme(s), thus resulting in a low conversion, enzyme electrodes are not, in

this instance, applicable [103,104]. Several papers from our group have been presented where two starch hydrolysing IMERs, containing α-amylase and amyloglucosidase (AMG), were used in conjunction with FI and CLC for the total breakdown of starch-related molecules to glucose [1,105–107]. α-Amylase is an endo-enzyme, hydrolysing starch randomly to maltose, maltooligosaccharides and α-limit dextrins, while AMG is an exo-enzyme hydrolysing from the non-reducing ends producing glucose. The produced glucose can then be oxidised by a co-immobilised GDH/MUT or GOD/MUT reactor as described in section 3.1. As not only the total glucose content of starch is of interest but also the molecular mass distribution and the amylose/amylopectin ratio, the introduction of an IMER, containing starch hydrolysing enzyme(s) as a post-column reactor in conjunction with gel-permeation chromatography (GPC), has been proposed in several papers [106,107]. Since, the various molecular mass fractions and the amylose and amylopectin of starch are expected to have different RI factors and absorptivities in UV, quantitation of the chromatographic peaks is very difficult. A post-column detection system capable of totally hydrolysing all the various fractions of separated native starch, followed by a selective detection system for the glucose formed, should thus be able to solve this problem. Continuous efforts have been made to improve the efficiencies of the two starch-hydrolysing IMERs so that their size and thereby their contribution to band-broadening in post-column detection for GPC could be decreased [108,109]. In a recent work, Emnéus and Gorton [108] studied twelve different supports for the immobilisation of AMG (Table 4). Five of these were controlled porous glass (CPG) supports with various pore sizes ranging from 117 to 729 Å to study the effect of enzyme loading and the ability of the large substrates to reach the immobilised enzyme inside the pores. The other supports included spherical ceramic silica (Micropil A) and alumina (Micropil C) based materials, neutral uncoated unispher-PBD alumina (Biotage), Lichrosphere, Kromasil, and hydroxyapatite, most of them with restricted use as enzyme supports. All reactors were analysed for kinetic parameters using soluble starch (Zulkowsky), oligosaccharides, and maltose as substrates. The substrates were chosen to study the combined effects of both enzyme loading and enzyme availability. Starch and oligosaccharides with a DP (degree of polymerisation) >10 have the highest turn-over rates with AMG, whereas maltose has the lowest. The reactor volumes necessary to obtain a 99% conversion of soluble starch (Zulkowsky) to glucose calculated with Eq. (2) is included in Table 4. When comparing the kinetic parameters and the reactor volumes necessary for 99% conversion for the five pore sizes of CPG, it can be seen that the best pore size was 170 Å. Here, the amount of immobilised AMG is high, and the pores are large enough for both substrate and enzyme to enter, which is verified by high K_{ps}^{app} and V_{max}^{app} and the small reactor volume necessary to obtain 99% conversion. With a pore size of 117 Å, the amount of AMG immobilised is low in spite of the large surface area available, indicating that AMG is too large to enter and use this area. However, when studying the other supports, the Micropil A support was very much superior (Table 4). Similar work was also done for α-amylase [109].

Ortega [46] developed a cation-exchange separation system for the detection of maltooligosaccharides (DP 1–11) in wort and beer, using two post-column IMERs with AMG and GDH coupled in series. The final enzymatic product NADH was detected by UV absorbance (Fig. 10). The LODs for DP2, DP4 and DP7 were 25, 15 and 25 $\mu g\ ml^{-1}$ (50 μl inj.), respectively. Mögele et al. [27] developed a similar system using a co-immobilised AMG and GOD for the detection of maltose and maltooligosaccharides in corn syrup and Pils beer. The enzymatically produced H_2O_2 was detected amperometrically at +600 mV vs. Ag/AgCl with an LOD for maltose of 1 p*M* (10 μl inj.).

Maes et al. [44] separated and detected linear α-(1–4)- [43] and β-(1–3)-linked glucooligomers in maltodextrins and hydrolysate of laminarin with a co-immobilised AMG or cellulase reactor and a GOD reactor coupled in series after a

Table 4
Physical and kinetic parameters for the hydrolysis of maltose, oligosaccharides, and soluble starch by amyloglucosidase immobilised on various solid supports

Reactor no., support	Pore size (Å)	Surface area ($m^2 g^{-1}$)	mg AMG/ 100 mg^{-1} support	K_{ps}^{app} (min^{-1})	V_{max}^{app} (mM min^{-1})	K_M^{app} (mM)	99% conv. reactor vol. (μl)
Soluble starch							
1, CPG	117	210	16.4	2.64	108.0	40.8	523
2, CPG	170	150	26.5	4.98	193.8	38.9	277
3, CPG	230	110	20.3	4.38	175.8	40.2	315
4, CPG	500	50	17.1	2.22	98.4	44.4	622
5, CPG	729	36	13.1	1.32	57.6	43.7	1047
6, Micropil A	300	100–150	24.3	10.32	401.4	38.9	134
7, Micropil C	130	100	11.0	1.68	74.4	44.4	822
8, Biotage	250	50	14.4	3.72	142.2	38.3	371
9, Lichrosphere	1000	5	14.3	1.80	76.2	42.3	768
10, Kromasil	100	340	11.0	0.42	20.4	47.9	3289
Maltooligosaccharide							
1, CPG	117	210	16.4	2.22	93.0	41.7	622
2, CPG	170	150	26.5	6.13	261.0	42.6	225
3, CPG	230	110	20.3	3.90.	151.2	38.7	354
4, CPG	500	50	17.1	2.28	99.6	43.8	606
5, CPG	729	36	13.1	1.08	49.8	46.2	1279
6, Micropil A	300	100–150	24.3	8.16	329.4	40.4	169
7, Micropil C	130	100	11.0	4.92	180.6	36.7	281
8, Biotage	250	50	14.4	6.72	242.4	36.1	206
9, Lichrosphere	1000	5	14.3	3.12	127.2	40.7	443
10, Kromasil	100	340	11.0	0.72	33.6	46.8	1919
Maltose							
1, CPG	117	210	16.4	0.46	21.6	46.9	1495
2, CPG	170	150	26.5	0.78	35.4	45.1	886
3, CPG	230	110	20.3	0.57	26.4	46.3	1212
4, CPG	500	50	17.1	0.30	15.0	47.8	2303
5, CPG	729	36	13.1	0.18	9.0	48.6	3838
6, Microphil A	300	100–150	24.3	1.08	46.8	43.3	640
7, Microphil C	130	100	11.0	0.30	14.4	47.7	2303
8, Biotage	250	50	14.4	0.42	19.8	47.1	1645
9, Lichrosphere	1000	5	14.3	0.21	10.2	48.2	1289
10, Kromasil	100	340	11.0	0.06	3.0	49.1	11513

Reproduced with permission from Ref. [108].

separation column. The resulting H_2O_2 was detected amperometrically at +750 mV vs. SCE (Figs. 11, 12). Detection limits were very low, in the ng range for the various oligosaccharides with linear calibration graphs over more then 3 orders of magnitude. The standard deviation, σ, of the retention times for DP 2 to DP 40 was found to be lower than 2% ($n = 5$).

Nordling et al. [18] developed a combined electrode, where the signal could be recorded simultaneously for up to four independent biosensors symmetrically mounted in a flow cell. In this work two biosensors were mounted in the flow cell and used for the detection of glucose and soluble cellodextrines after CLC separation. The biosensors used contained GOD or cellobiose oxidase immobilised through co-polymerisation with an osmium redoxpolymer pOs-

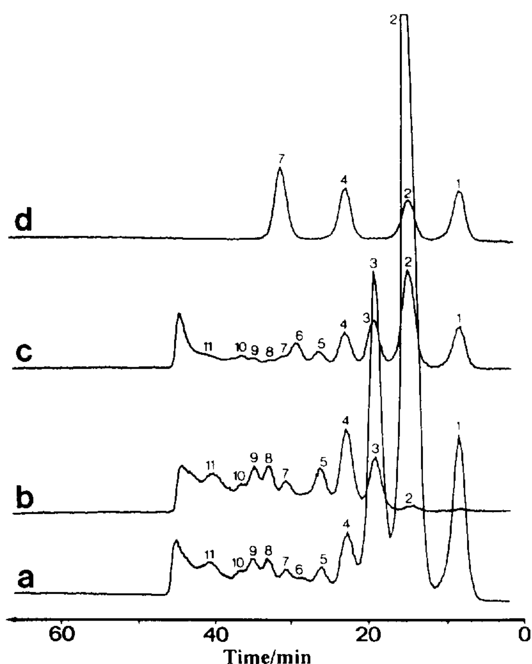


Fig. 10. Chromatograms obtained with two cation-exchange resin columns coupled in series using post-column immobilised amyloglucosidase and glucose dehydrogenase reactors of (a) wort diluted 25-fold, (b) beer containing 4.5% alcohol diluted 20-fold, (c) beer containing 1% alcohol diluted 20-fold, (d) standards (DP1, DP2, DP4, DP7). Final detection of NADH with UV. The peak numbers refer to the DP number. Reproduced with permission from Ref. [46].

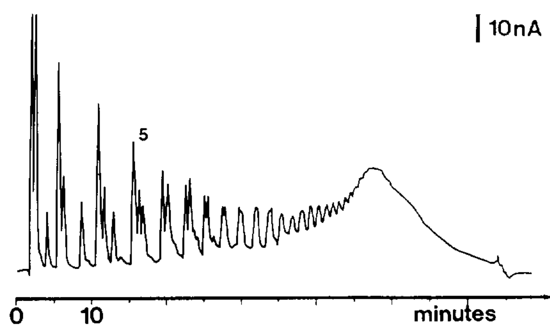


Fig. 11. Gradient separation of β-(1–3)-linked glucooligomers in hydrolysed laminarin using a post-column co-immobilised cellulase and glucose oxidase IMER; 5 indicates the peak corresponding to a degree of polymerisation (DP) of 5. Reproduced with permission from Ref. [44].

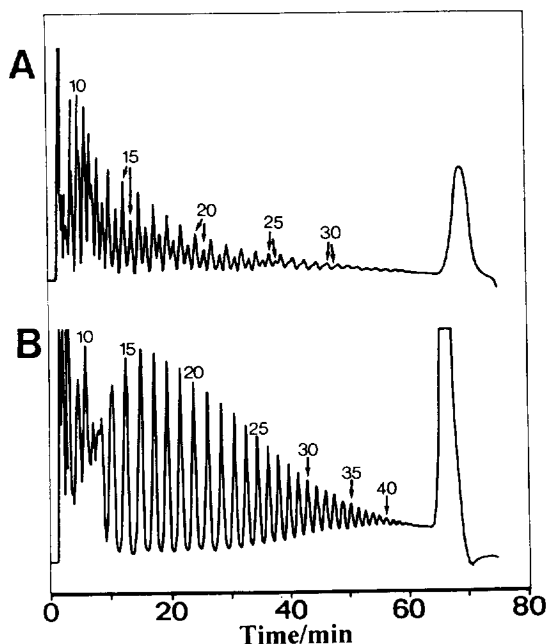


Fig. 12. Gradient separations of (A) maltodextrins and (B) reduced maltodextrins obtained on a Bio-Sil reversed-phase C_{18} column with a post-column co-immobilised amuloglucosidase and glucose oxidase IMER. Peak numbers indicate the DP number. Reproduced with permission from Ref. [43].

EA on a glassy carbon electrode set at +500 mV vs. Ag/AgCl. The current resulted from the shunting of electrons from the redox enzymes via the osmium complex to the electrode in the absence of oxygen and was proportional to the rate of substrate conversion.

4.3. Post-column detection of acetylcholine and choline

Acetylcholine (ACh) plays a significant role as neurotransmitter of cholinergic neurons present in the central nervous system. Choline (Ch) serves as precursor and metabolite of brain membrane lipids and acetylcholine. ACh also plays an important role in Alzheimer's disease, which is characterised by a decrease in the number of cholinergic neurons. The importance of these compounds in clinical research explains the increasing activity in the development of new

highly sensitive methods for their continuous determination.

Conventional methods for assaying ACh include bioassay, radioenzymatic assay and gas chromatography–mass spectrometry (GC–MS). Since 1983, a number of CLC methods for ACh and Ch have been described based on chromatographic separation of both compounds and on their post-column enzymatic derivatisation and electrochemical detection [110–120].

Usually the enzymes acetylcholine esterase (AChE) and choline oxidase (ChO) are immobilised in an IMER(s), following a separation column. ACh and Ch are chromatographically separated and then eluted into the IMER where ACh is hydrolysed by AChE to Ch which then is oxidised by ChO under the production of H_2O_2. The H_2O_2 can then be detected amperometrically or photometrically as described in section 3.1.2.1 and 3.1.3. A number of papers describe interference problems encountered in this field by using electrochemical oxidation [121,122] and some of the solutions to these problems are presented below.

Rícny et al. [123] separated ACh, Ch, and their analogues on polymeric ion-pairing reversed-phase CLC-columns (TESSEK HEMA C_{18} and TESSEK EDMA-DVB) at pH 8.9–9.1 with SDS (sodium dodecyl sulfate) as ion-pairing reagent. The separated fractions were passed into a co-immobilised enzyme reactor containing AChE, ChO, and peroxidase (POD). First ACh is hydrolysed by AChE to acetate and Ch. Ch is then oxidised by ChO to betain and H_2O_2. A highly fluorescent compound is then produced by the reaction of H_2O_2 and 3-(hydroxyphenyl)propionic acid (HPPA) catalysed by POD. With the internal standard, acetylethyl choline, the separation and detection took 8 min and without the internal standard it took 4–5 min with a flow-rate of 1 ml/min. The LODs for Ch and ACh were 1 and 3 pmol (20 μl inj.), respectively. This method was used to detect ACh and Ch in rat brain tissue. The solvent front and other interferences generally associated with electrochemical detection are absent in this system, due to the selective fluorescence detection with POD.

Flentge et al. [124], developed a similar CLC–IMER system as described above. However, in their paper the enzymes were physically immobilised in a sandwich-type enzyme reactor in a minimal dead space between two cellulose membranes, thus retaining maximal enzyme activity. The IMER was placed after an Aminex A-9 Biorad separation column. The enzymatic product H_2O_2 was then detected at a Pt electrode set at +250 mV vs. Ag/AgCl. Interference free electrochemical oxidation was achieved by incorporating a preoxidator, which is an electrochemical detector cell with a large oxidising surface (50 mm^2), a spacer of 50 μm between a glassy carbon and carbon/Teflon electrode, and a potential set at +750 mV. The LODs for ACh and Ch were 15 and 10 pmol (0.5 μl inj.), respectively. This detection system using only immobilised ChO, was employed successfully in conjunction with a microdialysis probe placed stereotaxically in rat brain to measure the increase in Ch after cardiac arrest due to intracardial injection of $MgCl_2$ in the rats.

Greaney [125] developed a post-column CLC–IMER system with the same enzymes as above. However, they combined this CLC method with microdialysis in vivo and a unique analytical flow cell featuring a solid-state palladium reference electrode and two different designs of Pt working electrodes with a resulting applied potential of +300 mV, leading to reduction of interferences. An LOD of less than 20 fmol ACh (10 μl inj.) was achieved in this system.

4.4. Post-column detection of other analytes

Steroid hormones are synthesised from esters of cholesterol in the adrenal gland, gonads and placenta. After secretion, the steroids are transported into the circulation of the target tissues. Lam and Malikin [126] have extensively worked on the development of enzyme-based detection systems for CLC and their application to the clinical analysis of biological fluids for the determination of hydroxysteroids [126]. They used various types and sources of dehydrogenases, and depending on the type of IMER used in the post-column mode, the detection could be made

specific for hydroxy groups at various positions on the steroid molecule. Specificity for α- and β-conformations could also be obtained. The hydroxysteroid substrates were oxidised to the ketosteroid product by the enzyme hydroxysteroid dehydrogenase in the presence of the co-factor NAD^+. Enzymatically produced NADH was then detected by fluorescence detection. Optimisation of the various reactors was made by activity measurements of the enzymes in the IMERs. Many of the hydroxysteroids were converted with close to 100% efficiency, which is especially advantageous in real applications since the sensitivity will be equal for most metabolites present in the sample. Another great advantage of these enzyme-based detection units is the compatibility with up to 50% organic solvent in the mobile phase without significant influence on the IMER activity [127]. This resistance towards organic solvents makes reversed-phase separations fully applicable. For more apolar steroids, where a higher level of e.g. methanol is needed, dilution by the introduction of a make-up reagent stream of buffer is necessary. Fig. 13 gives a good example of separations of (a) 3α-hydroxysteroids in IMER-hydrolysed urine, (b) the background without the IMER and (c) a reference standard mixture.

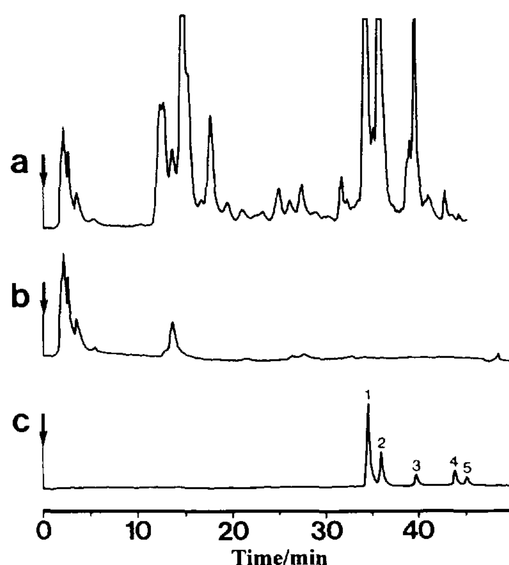


Fig. 13. Chromatograms of a reversed-phase separation of 3α-hydroxysteroids found in the enzyme catalysed urine of (a) a male subject, (b) the background without the IMER, and (c) a reference standard mixture. Peaks: 1 = ethiocholanolone, 2 = androsterone, 3 = $5\alpha PR3\alpha,17\alpha 20\beta$triol, 4 = $5\alpha PR3\alpha,20\alpha$diol, 5 = $5\alpha PR3\alpha,20\beta$diol. Reproduced with permission from Ref. [126].

Similar work was performed by Ikegawa et al. [128] who determined conjugated bile acids in human urine by using a post-column 3α-hydroxysteroid dehydrogenase reactor with chemiluminescence detection of the generated NADH.

Takeuchi and co-workers [129,103] used an aldehyde dehydrogenase IMER for the determination of aliphatic- and furan-aldehydes. Both ion-exchange and reversed-phase separations were used with fluorescence detection of the enzymatically produced NADH. Results were shown for both a standard reference mixture and whisky where the acetaldehyde and furfural present could be determined.

Acetyl-CoA is the acetyl donor in the biosynthesis of acetylcholine, acetyl-L-carnitine and N-acetylserotonin. Yamamoto et al. [131] developed a CLC system for the determination of Acetyl-CoA and other acyl-CoA esters. Phosphotransacetylase was immobilised in an IMER following a reversed-phase ion-pair separation using tetra-n-butylammonium phosphate (TBAP) as ion-pairing reagent; 2 mM TBAP and 25% methanol in the eluent resulted in a 65% retained enzyme activity. CoA was enzymatically liberated by immobilised phosphotransacetylase and was subsequently reacted with Ellman's reagent (DTNB) forming a coloured compound which was detected spectrophotometrically at 412 nm.

The identification and determination of acylcarnitines in urine is used to diagnose secondary carnitine deficiency caused by various inborn errors and acquired disorders of fatty acid and amino acid oxidation. Matsumoto et al. [132] immobilised acylcarnitine hydrolase, carnitine dehydrogenase and diaphorase in three individual IMERs for the post-column detection of acylcarnitines in human urine samples. A number of different acylcarnitines were separated by reversed-phase chromatography and then eluted into the three IMERs, the first of which con-

tained acylcarnitine hydrolase, which hydrolyses the acyl-group of these compounds under the production of L-carnitine. The produced L-carnitine was then oxidised in the presence of β-NAD^+ and carnitine dehydrogenase in the second IMER whereby β-NADH was produced. β-NADH was then reacted with resazurin in the presence of diaphorase in the third IMER to give the highly fluorescent rezorufin, proportional to the amount of the individual acylcarnitines in the urine sample. The LOD for the various acylcarnitines was <1 μmol/l (100 μl inj.).

Kurth et al. [133] developed a post-column CLC system for the reversed-phase separation and detection of H_2O_2 and hydrophilic organic peroxides by using immobilised HRP. The eluent from the chromatographic separation was mixed with a second flow stream containing *p*-hydroxyphenyl acetic acid which was entered into the HRP reactor and the resulting fluorescence intensities were recorded. The LOD for H_2O_2 was $5 \cdot 10^{-8}$ *M* (20 μl inj.). However, due to peak-tailing of the organic peroxide homologues their LOD was approximately ten times higher.

Analysis of free fatty acids (FFA) has become important in biochemical and clinical research. Kawasaki et al. [134] developed a post-column CLC system incorporating immobilised acyl-CoA synthetase (ACS) and acyl-CoA oxidase. FFAs were separated by reversed-phase chromatography. The separated eluting fractions were mixed with a reagent, containing CoA, ATP and Mg^{2+}, and entered into the ACS reactor under the production of acyl-CoA and AMP. In the presence of O_2 and immobilised acyl-CoA oxidase the produced acyl-CoA was then oxidised forming H_2O_2. The enzymatically produced H_2O_2 was then chemiluminimetrically detected by mixing with a second reagent containing luminol and microperoxidase, resulting in emission of light. A calibration graph for *n*-capric acid was linear between 0.4 and 2.0 μmol (10 μl inj.).

The level of urinary polyamines has been shown to be elevated in patients with cancer and consequently much attention has been paid to them as tumour markers. Watanabe et al. [135] developed a reversed-phase post-column system using immobilised polyamine oxidase for putrescine, spermidine, and spermine using electrochemical detection of the enzymatically produced H_2O_2. A similar system was later developed by Watanabe [136] for the detection of spermidine and spermine. Here, the author tried a polyamine oxidase from a different source than above, i.e. from *Aspergillus terreus*. The sensitivity of the latter system was ca. 10 times higher than that of the former, probably due to the origin of the polyamine oxidase. However, the latter system was not able to detect putrescine, since the polyamine oxidase from *A. terreus* had no activity for this analyte.

Yamato et al. [137] developed an CLC–IMER system for the determination of oxalate in plasma and urine. Oxalate was oxidised by an oxalate oxidase IMER, resulting in the production of H_2O_2 which was detected amperometrically at a Pt electrode set at +500 mV vs. Ag/AgCl. Chromatograms with and without IMER were compared showing that oxalate could be selectively determined in plasma and urine. The LOD for oxalate was 10 nmol/ml (100 μl inj.).

Ortega et al. [138], compared UV detection with tyrosinase-based bioselective detection using an IMER or an amperometric biosensor as post-column detection units for the separation of some phenolic drugs. The phenolic drugs were separated with reversed-phase chromatography using a mobile phase containing only 5% acetonitrile. Tyrosinase is a water producing oxidase that oxidises phenols to quinones in two enzymatic steps. Enzymatically produced quinones were amperometrically reduced at a solid graphite electrode set at −50 mV vs. Ag/AgCl. Compared with UV detection, the combination of enzymatic catalysis and the low applied potential resulted in a more selective detection of phenolic drugs in spiked serum samples.

Similar work as above was performed using two different biosensor configurations based on tyrosinase as detection devices for a CLC separation of environmentally important phenols in waste water samples [139]. The latter paper stresses several factors that influence the per-

formance of a tyrosinase biosensor when immobilised on a solid graphite electrode and in a carbon-paste electrode.

Tissue electrodes with tyrosinase were used for the determination of phenolic compounds in CLC by Connor et al. [90]. However, the sensitivity was found to be low, which was probably due to the necessary diffusion of substrates into the cells before conversion. Nevertheless, with this biosensor similar resolution and chromatographic performances were found compared to normal CLC–UV systems.

Heitzer et al. [140] developed a whole-cell biosensor based on a genetically engineered bioluminescent catabolic reporter bacterium for the continuous on-line monitoring of naphthalene and salicylate bioavailability and microbial catabolic activity potentials in waste streams.

Separation and detection of a number of enzymes and their activities based on post-column derivatisation have been reported. Early work in this area was done by Schlabach and co-workers [141,142]. A recent review covering the area was presented by Lambeth and Muhonen [143].

Luque de Castro and Fernandez-Romero [144] developed an "open–closed" CLC system with post-column derivatisation for kinetic measurement of three isoenzymes of creatine kinase in serum samples. This system can be described as a stop-flow system where the sample is injected into the post-column "open" to the flow of the mobile phase, then the reactor is "closed" to the mobile phase flow by valve-switching and the sample is allowed to react in the post-column reactor until switched "open" again and transported to the detector.

Macholán and Hlavatá [145] developed a method for the determination of arginase during its purification with affinity chromatography. The arginase was continuously monitored with a potentiometric biosensor based on immobilised urease.

The monitoring of plasmin and its precursor plasminogen is of great importance for diagnostic purposes because of their relation to various disorders occuring in blodd vessels [146]. Abe et al. [147] developed a post-column CLC–IMER system using immobilised urokinase for the monitoring of human plasminogen species which was fractionated by affinity chromatography.

4.5. Pre-column systems

The uridine diphosphate-glucuronosyltransferases (UDPGT), present in rat liver microsomes are a family of membrane-bound enzymes concentrated in the lipid bilayer of the endoplasmatic reticulum of cells in the intestine, kidney, and other tissues. They are involved in conjugation of the glucuronic acid moiety of uridine 5′-diphosphate-glucuronic acid with different compounds including drugs, phenolic compounds, steroid hormones, and bilirubin. Alebic-Kolbah and Weiner [148] used rat liver microsomes non-covalently immobilised on an artificial membrane (IAM), which was used in the pre-column mode with CLC to study the glucuronidation of 4-nitrophenol (4-NP) and 4-methylumbelliferone (4Me7OHC) and to study the enzymatic parameters associated with this process.

Conjugation of phenols with D-glucuronic acid and sulphate ions is a common metabolic pathway of benzene in humans and is considered part of the metabolic products of excretion in urine. Since 30% of the retained benzene appears as phenol in vivo, urinary phenol is part of the biological exposure index (BEI) for benzene. Jen and Tsai [149] developed a pre-column reversed-phase CLC system for the determination of glucoronide/sulphate metabolites of benzene using immobilised β-glucuronidase. After dilution of the urine it was injected into the IMER–CLC system, where the conjugates present were hydrolysed by the β-glucuronidase to phenol. The produced phenol was then separated from other urinary components and detected with fluorescence detection. The LOD for urinary phenol was 50 μg/l.

Adenosine phosphates and inorganic ions were simultaneous determined by immobilised adenosine triphosphatase (ATPase) or alkaline phosphatase (ALP) reactors placed in the pre-column mode to an ion-exchange separation column

[150]. A lipase IMER was used in the pre-column mode of a chiral column to determine the enatioselectivity of the enzyme [151].

A pre-column GPC system was developed to investigate the efficiency of the hydrolysis of different starches by incorporating two IMERs with immobilised α-amylase and amyloglucosidase in front of a GPC column [106,107]. Fig. 14 shows gel-permeation chromatograms of glycogen (a), two different potato starches (b and c) and a glucose reference (d) with and without starch hydrolysing IMERs. It can be seen that when only the α-amylase IMER is used, essentially most of the large polymer fractions have been broken down (i- > ii). However, when

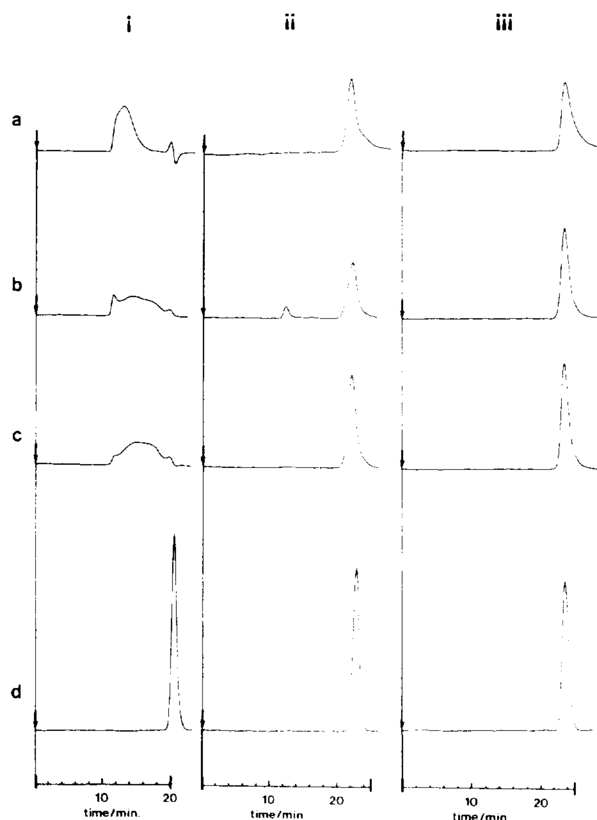


Fig. 14. GPC chromatograms using (i) no IMER, (ii) α-amylase IMER, (iii) α-amylase and amyloglucosidase IMERs in the pre-column mode. (a) Glycogen, (b) native warm water soluble potato starch, (c) native cold water soluble potato starch and (d) glucose as reference. Reproduced with permission from Ref. [107].

comparing the retention times of the three starches with glucose, the former have slightly shorter retention times, indicating, as expected, that total hydrolysis to glucose was not reached only with α-amylse. In (iii), it is clearly seen that all large polymeric fractions have disappeared and that all eluting peaks have the same retention times as the glucose reference when the α-amylse and AMG IMERs were used sequentially. The size of these reactors could be drastically reduced by optimising the pore size and the kind of support used (see section 4.2.2 and Table 4 above).

The possibility to use IMERs in the pre-column mode has great potential for studying the structure and degree of branching of large polysaccharides. The determination of the degree of branching of different polysaccharides is currently performed by debranching with enzymes in batch. Stefansson and Novotny [152] obtained oligosaccharide maps by first debranched α-D-glucans and β-D-glucans using debranching enzymes such as isoamylase, laminarinase, and cellulase enzymes in batch and then analysed the debranched fractions with CZE, yielding high resolution separations. Much efficiency should be gained by using these enzymes in the pre-column immobilised mode. At the moment some of the work at our laboratory is aimed at developing pre-column CLC systems with immobilised isoamylase and/or pullulanase to investigate the degree of branching of different starches.

4.6. Pre- and post-column systems for amino acids, peptides and proteins

4.6.1. L- and D-Amino acids

L-Amino acid oxidase (L-AAOX) and D-amino acid oxidase (D-AAOX) can be utilised for the steroselective determination of L- and D-amino acids. The idea of using L-AAOX and D-AAOX for stereospecific determination was proposed already 1988 by Jansen and Frei [153], however, without showing that the enzymes were strictly stereospecific. The first work utilising amperometric biosensors with immobilised L-AAOX and D-AAOX in a bi-amperometric flow-through cell of a thin layer configuration using a

platinum electrode for stereoselective determination of L- and D-amino acids was made by Yao and Wasa [17]. A reversed-phase separation system was used where a number of L- and D-amino acids were separated without the addition of an organic additive to the mobile phase. The column outlet was split and introduced to the two enzyme electrodes. Marko-Varga and co-workers [28,37,154] showed the strict stereoselectivity of the L-AAOX and D-AAOX to L- and D-amino acids when the enzymes were immobilised onto solid supports contained in IMERs in a parallel configuration in CLC and FI. The system was coupled to an ion-exchange separation system (CLC) and applied to serum samples. Silica based C_{18} phases were deliberately avoided as stationary phases since the stability of these phases was found to be inadequate when purely aqueous mobile phases were used. Polymer based anion-exchange chromatographic supports on the other hand were found to be highly stable although somewhat lower resolution and longer separations were obtained [37].

Amperometric biosensors for L- and D-amino acids were also developed using carbon paste as the electrode material [155,156]. The enzyme in close conjunction with the electrochemical transduction promotes fast overall kinetics. Different additives were mixed into the carbon-paste electrode material in order to stabilise the immobilised enzyme and to promote electron transfer. It was found by Kacaniclic et al. [155] and Johansson et al. [156] that the molecular structure of the additives used in the electrode material as well as the immobilisation method had great impact on both the operational stability and sensitivity of the biosensor. An interesting characteristic was found when L-AAOX was immobilised in an organic phase such as carbon paste, namely that the selectivity of the enzyme changed. This effect was clearly seen when the selectivity of the L-AAOX was compared between soluble, immobilised on solid support and immobilised in carbon paste. As seen in Table 2 only eight L-amino acids respond with the enzyme in solution out of the 20 selected and 13 when immobilised on a CPG support used in an IMER. However, all 20 amino acids could be measured when the carbon-paste electrode was used. Out of these, cystein was directly electrochemically oxidised at the operational potential used (−50 mV vs. Ag/AgCl). This change in selectivity can probably be ascribed to some conformational changes of the enzyme in the hydrophobic environment of the graphite/oil mixture. The same shift in selectivity using CPE was observed using a D-amino acid oxidase biosensor [157] (see Table 2). Although not all 20 D-amino acids could be determined as in the case of L-amino acids, there was still a large improvement of the group specificity compared with the soluble D-AAOX [158].

Recently, Galensa [23] showed that an L-glutamic acid oxidase was successfully used in an IMER configuration when coupled in the post-column mode to CLC. The enzyme from *Streptomyces sp.* enabled the determination of important amino acids in wine which could not be detected by non-specific oxidases.

4.6.2. Peptide and protein mapping

Peptide mapping is one of the most powerful and successful tools available for the study of peptides and proteins. This is usually done by chemical and enzymatic hydrolysis at elevated temperatures into smaller fragments. These fractions are often analysed by some type of chromatographic separation and detection where these results are the basis for the peptide map. Peptide maps can further be used as unique fingerprints and can be very important tools in order to qualitatively identify different proteins and peptides.

Peptide and protein determinations have successfully been performed by utilising different types of peptidases. CLC in combination with mass spectrometry (MS) has been used since the early 80's in combination with enzyme catalysis. At this early stage the enzymes were used in solution [159–162] and many of the problems concerning the elimination of the enzymes from the solution have lead to the logical use of immobilised enzymes contained in IMERs.

Fast atom bombardment, field desorption and thermospray are the most commonly used ionisation interfaces used with MS in combination with

IMER–CLC systems for peptide and protein identification. In one case the enzymatic catalysis was performed directly on the probe tip for MS identification of peptides using fast atom bombardment ionisation [163]. Hydrolytic cleavage by endopeptidase was used to identify peptides derived from recombinant proteins. This technique was applied when MS alone did not give sufficient information and in cases where the mass range of the instrument was limited to 2000 Da. IMERs have also been found to help increase the detectability of proteolytic peptide fragments, which were found to co-elute in CLC separations. The hydrolysed digested protein was determined by the use of continuous-flow fast atom bombardment MS with selective-ion monitoring [164].

Tryptic digestion is one of the most common ways of catalysing the hydrolysis of proteins, due to the quantitative and specific nature of the catalytic performance of the enzyme. Trypsin hydrolyses from the C-terminal side of lysine and arginine residues. Other commonly used endopeptidase and exopeptidase enzymes used for immobilisation are carboxypeptidase Y, carboxypeptidase A, carboxypeptidase B, aminopeptidase M, chymotrypsin, thermolysine and V8 protease [164–170].

In these systems the enzymes were usually covalently coupled to the CPG support via glutaraldehyde. Considerable stabilisation of the IMERs was obtained by reduction of the resulting imine bonds $-N=CH-$ to $-NH-CH_2$ by the use of sodium cyanoborohydride [165]. Surprisingly, many of the papers describe slurry packing techniques similar to those commonly used in traditional packing of CLC stationary phases. Enzymes immobilised on the supports were packed at pressures between 3000–4000 psi with retained stability of the enzyme activity. The particle sizes varied between 10 and 100 μm and the inner diameter of the IMERs were typically around 2 mm. The IMERs were preferably coupled on-line to CLC separation and MS detection. To be able to use optimal CLC conditions enzymes are needed which are compatible with organic additives in the mobile phase. These enzymes were found to be strongly dependent on the choice and percentage of organic solvent in the mobile phase. Voyksner et al. [166] found that 25% (v/v) in water did not appreciably affect enzyme activities while chromatographic efficiency was retained.

Stachowiak et al. [167] showed the efficiency of coupling an IMER at various positions in a chromatographic system. The first IMER was used to catalyse the hydrolysis of peptide into primary fragments. Next, CLC separation was performed of these fragments and subsequently a second IMER digested each fragment as it was eluting from the analytical column. Thermospray MS was used to detect the resulting fragments. Protein sequencing with this technique was made for basic pancreatic trypsin inhibitor.

Various types of endopeptidases such as chymotrypsin, thermolysine, trypsin and V8 protease and exopeptidases such as carboxypeptidase A, B and Y were used in the post-column mode with CLC and thermospray MS for the identification of synthetic endorphins. In the thermospray spectrum found for these β-endorphins $[M+2H]^{2+}$, $[M+H+Na^+]$ and weak $[M+3H]^{3+}$ ions were typically found and the LOD was around 800 fmoles.

Chui and Wainer [168] showed the potential of IMERs using artificial membranes as the solid support. The enzyme was not covalently bound but trapped in hydrophobic cavities within the membrane matrix. This pre-column CLC system could be used to measure both the increase and decrease in enzyme activity by the injection of known enzyme substrates and enzyme inhibitors, respectively.

Kim et al. [170] used immobilised carboxypeptidase Y and trypsin IMERs in combination with CLC coupled to a thermospray mass spectrometer for on-line peptide sequencing. Cobb and Novotny [171] developed IMERs in combination with a microcolumn CLC system and a CZE system for small sample volumes with a detectability in the pico-, and femtomole range, respectively. Trypsin was immobilised on an agarose gel, which catalysed the hydrolysis of tryptic digest samples of β-casein, as well as characterised phosphoprotein with a molecular mass of 23 982 Da. After pre-column derivatisa-

tion, separations could be made by using micro CLC or CZE, and detection with UV at 215 nm. Peptide maps from phosphorylated and dephosphorylated forms of β-casein could efficiently be determined. Even a single amino acid modification in the β-casein structure could be identified with these techniques. Fig. 15 illustrates the separation of a peptide hydrolysate with both micro CLC and CZE. Note that the numbers of the peaks in Fig. 15A,B do not correspond to the same fragments.

In a recent work, Perron and Pagé [172] used CZE separation in combination with an enzyme assay for the development of 11 different synthetic MTX prodrugs. Carboxypeptidase A hydrolysed methotrexate-α-peptides resulting in the release of free methotrexate, which could be followed with this new CZE approach used within the cancer research field. Simultaneous separation and detection of the enzyme activities for glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase was shown with a LOD in the order of $5 \cdot 10^{-16}$ mol.

Similar to the work of Nashabeh and El-Rassi [173], enzyme-catalysed reactions for peptide mapping were recently performed by immobilisation of trypsin, pepsin or carboxypeptidase Y to the inner wall of a fused-silica capillary by avidin–biotin coupling [174]. These microreactors were integrated in the pre-column mode with a CZE separation capillary via an open fluid junction for the digestion and analysis of insulin and heavily glycosylated α_1-acid glycoprotein.

5. Antibodies/antigens as analytical tools

When mammals are invaded by infectious organisms or their toxic products, the immune defence system is triggered through the lymphatic production of specific receptor proteins called immunoglobulins or antibodies (Ab). The compounds which trigger the immune response systems are referred to as antigens (Ag). Antigens with a molecular mass less than 1000 Da are called haptens and they do not trigger an immune response unless bound to a carrier protein, e.g. ovalbumine, bovine serum albumine (BSA) and KLH.

A typical immunoresponse results in the production of polyclonal antibodies (antiserum) with different specificity and affinity for antigenic determinants (epitopes) on the antigen. Antibodies with the desired properties can then be selectively purified from the resulting antisera by affinity purification. However, the result still is a rather heterogeneous antibody preparation which often show broad cross-reactivity. Mono-

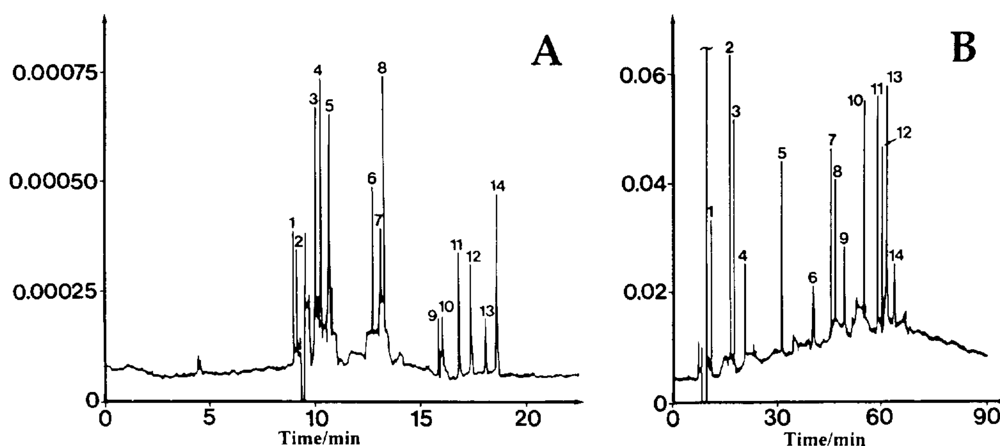
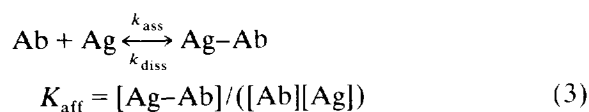


Fig. 15. Separations of peptide hydrolysate using (A) micro CLC and (B) CZE. The numbers corresponds to retention times of peaks obtained in the two systems. The peak numbers in the two chromatograms do not correspond to the same analytes. Reproduced with permission from Ref. [171].

clonal antibodies can be obtained by fusing the antibody producing cells with myeloma (tumour) cells in vitro to form cell lines that will grow in culture, producing antibodies. One such hybridoma cell with the desired property is selected and cultivated under the production of identical more selective monoclonal antibodies.

The binding between antigen and an antibody is very selective and extremely strong, effectuated by multiple, reversible non-covalent bonds. This is the property that makes them very useful as analytical reagents. However, the suitability of a particular antibody preparation in an immunoassay is governed by the affinity with which it binds the corresponding antigen or hapten, and this affinity must be evaluated separately before an immunoassay protocol is established. The affinity between an antigen and an antibody can be expressed according to reaction (3):



$$\mathrm{Ab} + \mathrm{Ag} \underset{k_{\mathrm{diss}}}{\overset{k_{\mathrm{ass}}}{\longleftrightarrow}} \mathrm{Ag{-}Ab}$$

$$K_{\mathrm{aff}} = [\mathrm{Ag{-}Ab}]/([\mathrm{Ab}][\mathrm{Ag}]) \qquad (3)$$

where the equilibrium constant for the reaction is named the affinity constant (K_{aff}), also defined as the ratio of the association rate (k_{ass}) and the dissociation rate (k_{diss}) of the antibody–antigen complex (Ag–Ab).

Immunoassays are usually referred to as quantitative analytical techniques used to detect and measure analytes through antigen–antibody reactions. In a broader sense it is also a qualitative method to characterise the immunological properties of the analytes.

Direct measurement of the antigen–antibody reaction can be performed by using immunoprecipitation techniques where the immunocomplex is spectrophotometrically detected through change in turbidity [175]. However, in most immunoassays, the quantitation is done by detection of a marker or label molecule. Different types of labels, e.g. radioactive isotopes [radioimmunoassay (RIA)), enzymes (enzyme-immunoassay (EIA)], or fluorescent compounds (fluoroimmunoassay), are coupled to the antigen or the antibody to visualise the primary binding reaction. The sensitivity of the assay is essentially governed by the detectability of the label and the affinity of the antibody for the antigen. The description of different types of immunoassays is beyond the scope of this paper and the reader is asked to address reviews and books available on the subject [175–179].

6. Antibody/antigen-based detection systems

More and more research is being devoted towards the automation of immunoassays through the use of continuous-flow systems. As for enzyme-based systems most of the work has been performed in the FI mode creating what usually is referred to as flow injection immuno assays (FIIA). These methods are inherently easier to automate than using microtiter plates or tubes and can lead to rapid and sensitive detection.

In the simplest form, homogeneous FIIA, no separation of bound and unbound label is required. Samples and reagents are mixed by merging two flow streams or by the injection of the sample into the flowing stream containing reagents. Usually, a labelled antibody present in the flow stream give rise to a standing signal which changes when the antigen–antibody complex is formed due to e.g. quenching of a fluorescent label or inhibition of an enzyme label by the binding reaction. Examples of some homogeneous FIIAs are given in Refs. [175,178].

In heterogeneous FIIA systems, separation of the bound and unbound label is required. The antibody or the antigen is usually immobilised to some solid support contained in a immunoreactor which is inserted into a flow system. Samples and reagent are injected into the flow system and antigen or antibodies are allowed to react in the immunoreactor. The removal of unreacted reagents is continuously done by the flow stream. The detection of bound reagent in the reactor takes place either directly in the reactor or downstream from the reactor depending on the configuration chosen. Due to efficient mass transfer and the very high reagent concentration in a immunoreactor, heterogeneous FIIA offers extremely accelerated binding kinetics. These systems are thus more straightfor-

ward, sensitive, and less influenced by interferences than homogeneous systems.

The procedures and available chemistries for immobilisation of a label to an antibody/antigen or the immobilisation of an antibody/antigen to a solid support are essentially the same as those applied to enzymes, which makes the knowledge available in both these fields relevant for the inexperienced scientist [177,180,181]. Care must be taken that the active immuno binding site for either antigen or antibody is not involved in the immobilisation to a support or a label. If the antigen of interest is a hapten, care must be taken in choosing a different immobilisation chemistry than that used for the conjugation of the hapten to its carrier protein for immunisation. The immunoresponse will result in polyclonal antibodies specific for the antigen, carrier protein and sometimes for the coupling group between hapten and carrier protein which later might cause "unspecific" binding problems. Also, steric hindrance of the immobilised antibody for the specific antigen or vice versa can prevent a successful binding reaction, which often necessitates the use of a spacer between the support and the species to be immobilised. A very recent paper discusses the role of antibody density effects on antibody–antigen binding efficiency [182].

Cross-reactivity is a problem often encountered in immunoassays where the goal is to obtain high selectivity towards one single analyte. This occurs because the antibodies fail to discriminate between structurally similar molecules. This problem in conventional immunoassays can be turned into a positive aspect when coupling immunotechniques with chromatography. In this case antibody cross-reactivity can be favourably used for the selective and sensitive detection of a separation of structurally related compounds or the selective capture (enrichment) of structurally related compounds of interest.

6.1. Pre-column systems

CLC–immunoassay (CLC–IA) methods can be devided into two groups. Those where the immunoreaction precedes or is performed in the CLC column (pre-column) and those where the immunoreaction is performed after the CLC column (post-column). Most of the work has been performed in the pre-column mode where the antibodies or antigens were immobilised to simultaneously capture and enrich the analytes, and at the same time remove interferences from the sample matrix [35]. The analytes are then desorbed and eluted into a conventional HPLC column, adding an additional discriminating step. Usually the effluents are detected by conventional detection techniques such as UV-absorbance which limits the sensitivity [183,184]. Many of these pre-column immuno techniques are related to conventional solid-phase extraction (SPE) techniques, only differing in that the interactions of the analyte with the immunoreactor are probably much more specific.

Chromatographic immunoassays were introduced by Mosbach et al. in 1978 [185]. Since then, a vast number of publications have appeared dealing with modifications of this technique in order to improve among other things the sensitivity.

Immunosorbent subtraction is a pre-column technique for identifying antigens at high concentration. An immunoreactor, containing immobilised anti-human growth hormone antibodies, was used in the pre-column mode with gel-permeation chromatography to subtract structural variants of human growth hormone from the sample [186]. The presence of antigens was detected by comparing chromatographic runs with and without the immunoreactor coupled to the system. Frontal immunoaffinity chromatography for differentiating between protein structure variants using polyclonal antibodies was developed by Xu and Regnier [187].

Riggin et al. [188] developed a non-competitive immunoassay for antibodies to human growth hormone (IgG) by pre-incubation of the IgG with excess fluorescent labelled human growth hormone (hGH). This was followed by injection of the mixture and entrapment of all antibody bound antigens on a protein G column and elution of excess labelled antigens. The fluorescent labelled antigen–antibody complex was then desorbed, eluted and detected by fluorescence detection with an LOD of 250 ng/ml.

An on-line immunoassay for the steroid drug budesonide was developed for bioanalytical purposes in the pharmaceutical industry by Kronkvist et al. [189]. The sample, containing the analyte, was incubated off-line with antibody and alkaline phosphatase-labelled analyte. The incubate was then injected onto the flow system where the antibodies bound to a protein G column. The eluting enzyme labelled analyte was then reacted with *p*-aminophenyl phosphate and the product *p*-aminophenol was detected amperometrically at a glassy carbon electrode.

Nilsson et al. [42] and Cassidy et al. [190] developed so called chromatographic sequential addition immunoassay systems. This technique is based on the sequential injection of antibodies, sample antigens and then labelled antigens to an immunocolumn with immobilised antibodies. The excess unbound labelled antigens are then eluted and detected or the bound labelled antigens are desorbed, eluted and detected, the response being proportional to the amount of bound antigen in the sample. This technique depends on the time the reagents are in contact with each other in the immunoreactor and is governed by the flow-rate through the reactor. Similar work was performed by Nilsson et al. [42]. and the theoretical basis for this method was investigated by Hage et al. [191].

Hage et al. [192] developed an automated assay for parathyroid hormone, parathyrin (PTH). PTH from plasma was pre-incubated with excess acridinium ester-labelled antibodies. The mixture was injected into an immunoreactor containing immobilised antibodies against PTH. The retained PTH and associated labelled antibodies were then detected by a chemiluminiscent post-column reaction. A similar system was developed [193] for thyroxine, but in this instance amplification of the post-column reaction was obtained by using enzyme-labelled antibodies. Tyroxine and excess peroxidase (POD) labelled anti-thyroxine monoclonal antibodies were pre-incubated before injection onto a cation-exchange CLC column which separated the antigen–antibody complex from the unbound antibodies. The effluent was then mixed with a second flow containing H_2O_2 and 3-(*p*-hydroxyphenyl)propionic acid (HPPA) which catalysed by POD results in a fluorogenic compound. The amount of fluorescence produced by POD-labelled bound and unbound antibodies was then detected by a fluorescence detector.

A capillary electrophoretic (CE) enzyme immunoassay technique was developed for the measurement of triazine herbicides [194]. The assay was based on competitive binding between the target analyte and enzyme labelled analyte. The substrate *p*-aminophenol phosphate was then introduced in the CE and the enzyme label alkaline phosphatase catalysed its conversion to the product *p*-aminophenol. The product was measured amperometrically with an LOD of 50 mg/l.

6.2. Post-column systems

So far very few immunoassay systems have been used on-line as post-column detection units for CLC separation of several analytes of interest. Below, a presentation of the few existing on-line systems is presented as well as some systems using automated semi on-line systems. Additionally, a presentation will be made concerning FIIA systems that should be suitable in conjunction with CLC.

Several papers report on non-competitive flow systems using immunoreactors with immobilised antigen or antibodies for the determination of the antigen or antibody, respectively [195,196]. These systems work in the following fashion: The sample analyte is pre-incubated or premixed in a mixing coil with excess labelled antibodies. The large excess of antibody will result in the binding of the majority of sample analytes to labelled antibodies. The mixture is then transported into the immunoreactor containing immobilised antigen, where the large excess labelled unbound antibodies will be trapped whereas the antibody-bound analytes will pass right through. The label associated to the antibody bound to the analyte is then monitored by an appropriate detector. This non-competitive method is exemplified in Fig. 16 for digoxin as model analyte using fluorescein-labelled anti-digoxigenin Fab fragments.

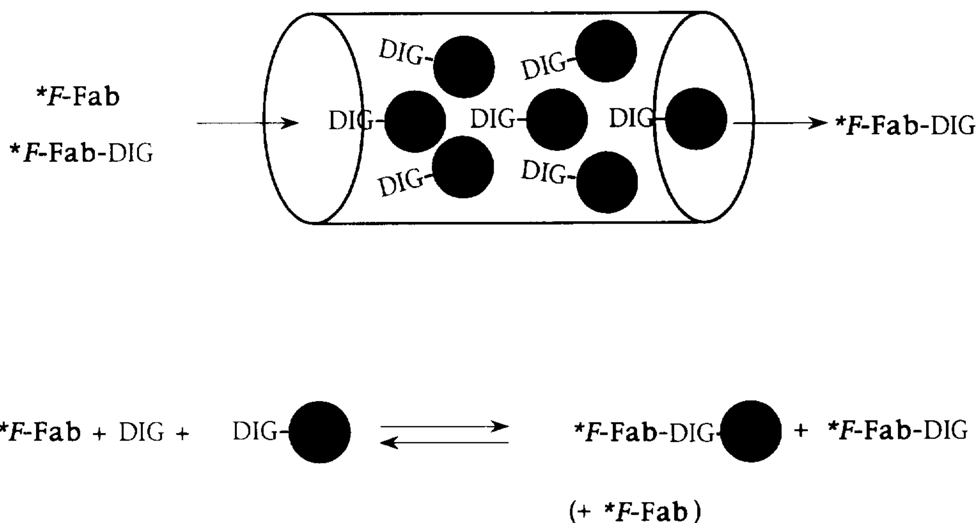


Fig. 16. Schematic illustration of a non-competitive immuno reaction using an immobilised digoxin reactor. F^*-Fab represents fluroscein labelled anti-digoxigenin Fab fragments. DIG represents digoxin and DIG-● represents digoxin immobilised to a solid support.

These systems seem to be suitable as post-column detection devices for CLC and the first true CLC–immunodetection system was developed by Irth et al. [34]. They developed a heterogeneous non-competitive CLC–immunoreactor system for the separation and detection of digoxin and its metabolites by using an antigen immunoreactor containing immobilised digoxin and fluorescence detection of fluorescein-labelled Fab fragments bound to the digoxin and metabolites (see also Fig. 16). The LOD was approximately 200 fmol digoxin (injection volume 200 μl). Fig. 17 depicts the separation and detection of digoxin and its hydrolysis products. Another paper from the same group describes the development of an on-line system incorporating sample clean-up/trace enrichment using a restricted access SPE column, chromatographic separation and the bioselective immuno detection described above [197]. Fig. 18 illustrates a chromatogram for the detection of digoxin and its metabolites in serum from a patient after oral administration of 1 mg digoxin. The peaks represented are (1) digoxigenin monodigitoxose, (2) digoxigenin didigitoxose and (3) digoxin.

Amplification of the signal for digoxin can be

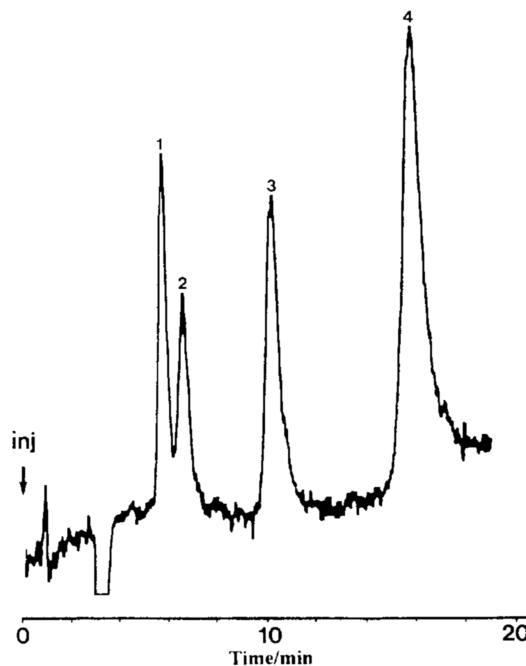


Fig. 17. CLC separation and non-competitive immuno detection of digoxin and its hydrolysed metabolites (10^{-8} M hydrolysed digoxin). Peaks: 1 = digoxigenin monodigitoxose, 2 = digoxigenin didigitoxose, 3 = digoxin and 4 = digoxigenin. Reproduced with permission from Ref. [34].

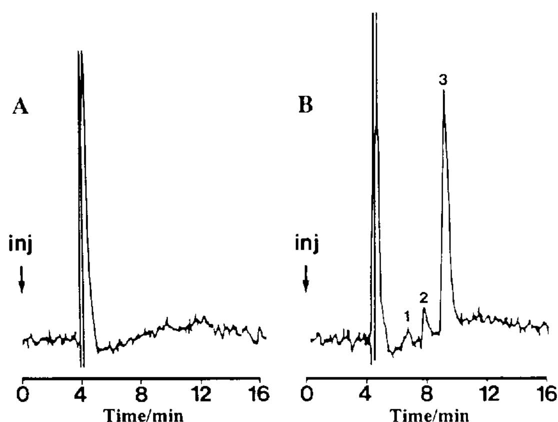


Fig. 18. Illustration of chromatograms for the detection of digoxin and its metabolites of serum from a patient after oral administration of 1 mg digoxin. (A) Serum blank and (B) digoxin metabolites present in serum. Peaks: 1 = digoxigenin monodigitoxose, 2 = digoxigenin didigitoxose and 3 = digoxin. Reproduced with permission from Ref. [197].

obtained by using peroxidase (POD) or alkaline phosphatase-labelled Fab fragments instead of the fluorescein-labelled Fab fragments used above [198]. The POD label was detected by the addition of a reagent flow containing HPPA and H_2O_2, resulting in the production of many fluorescent product molecules. Fig. 19 shows the FI system used where the amplification results from the fact that one single enzyme label gives rise to many fluorescent molecules. How large the amplification will be is dependent on how long the enzyme label is allowed to react with the substrate which is governed by, e.g., the length of reaction coil 2 or the flow-rate used in

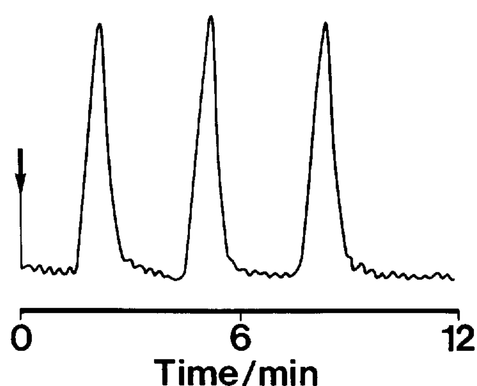


Fig. 20. Triplicate recordings of 1 pmol digoxin into the FIIA system shown in Fig. 19.

the system. Fig. 20 shows three injections of a 1 pmol digoxin solution into the FI system. The LOD at this stage for an unoptimised system was 10 fmol (100 μl inj.). Three different flows are necessary in this system since a CLC column is intended to be inserted right after the injector.

Kusterbeck et al. [199] developed FIIA systems based on displacement of labelled antigens bound to an immobilised antibody support. Antibodies directed against the 2,4-dinitrophenol (DNP) moiety of DNP derivatives were immobilised in an immunoreactor. The immobilised antibodies with free antigen binding sites in the reactor were then saturated with fluorescein-labelled antigen. DNP derivatives were then injected into the column, displacing the fluorescein-labelled antigens, thus resulting in a fluorescence signal downstream at the detector. The

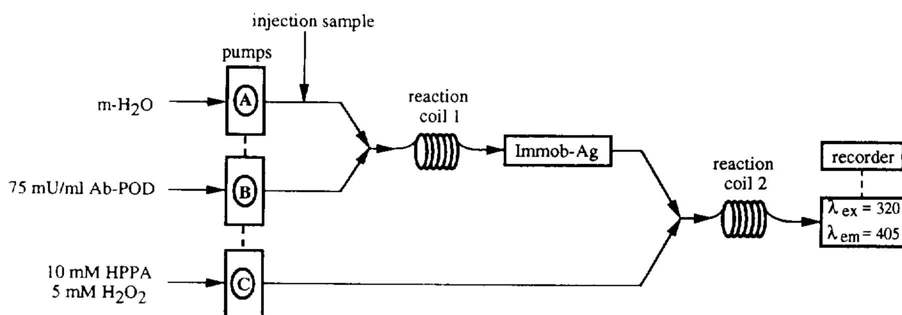


Fig. 19. FIIA non-competitive system for detection of digoxin using an immobilised digoxin immuno reactor and HRP labelled anti-digoxigenein Fab fragments.

immunoreactor could be used for several assays before resaturation with fluorescein labelled antibodies. The assay took 3 min with a LOD of $2.5 \cdot 10^{-7}$ M (200 μl inj.) for DNP-lysine. This system also seems suitable as a post-column detection system for CLC. A column would first separate the DNP derivatives which then would be transported into the immunoreactor, followed by displacement of fluorescent antigens and then fluorescence detection [200].

The possibility to use a restricted-access phase for the separation of free labelled antigen from labelled antigen/antibody complex was exemplified by utilising the difference in molecular mass between avidin and fluorescein-labelled biotin [201]. Biotin was eluted from a CLC column and then mixed and reacted with a flow containing excess unlabelled avidin. A third flow with excess fluorescein-labelled biotin was added to react the unreacted unlabelled avidin. The separation was performed by the entrapment of the smaller labelled biotin molecules in the inner cavities of the restricted-access phase, whereas the larger avidin/fluorescein labelled biotin complex passes right through the restricted access column. In this system, a decrease in signal represents an increase in the biotin concentration.

Imprinted polymers as affinity gels for antibody mimicking in CZE might be a new way to open up possibilities for other future immuno-based detection systems [202].

7. Interfacing of biospecific recognition with biomedical-, biotechnological- and environmental applications

7.1. *Bioselective membranes for biosensors*

Membrane deposition on electrode surfaces can have a large positive impact on the selectivity of biosensor performance if properly designed. Membranes casted directly on or formed through electropolymerisation of electroactive monomers on electrode surfaces have been used for different purposes in conjunction with enzyme-based amperometric biosensors. An increased stability has been observed for enzymes immobilised on/in membrane covered electrodes. These membranes can also act as size or charge exclusion barriers to prevent possible interfering compounds in the sample matrix from reaching the true electrode surface and also prevent enzymes from leaking out from bulk modified electrode materials. A membrane can also act as an additional diffusion barrier for the enzyme substrates to reach the electrode surface. Depending on the membrane thickness, the noticeable effect will be that the linear part of the calibration curve will be shifted towards higher substrate concentrations and a decrease in sensitivity, due to an increase in the apparent Michaelis–Menten constant (K_M^{app}). This may be advantageous in biosensor systems when using enzymes with low K_M values, as the linear part of the calibration curve can be extended.

A series of different membranes were studied in order to increase the operational stability and to extend the linear range of the calibration curve of biosensors. Five different types of electropolymerised membranes were investigated; poly-*o*-phenylene diamine, polyphenol, polypyrrole, polyaniline, and a co-polymer obtained from *m*-phenylene diamine and resorcinol. A polymer, denoted Eastman AQ, containing negatively charged groups covalently bound within the polymeric backbone is commercialised in an aqueous soluble form by Eastman Chemical. This kind of polymer is very well suited as an ion-exchange membrane cast directly on an enzyme-covered electrode [203]. The polymer–water solution will stay in a homogeneous state at room temperature, but once dried it is no longer water soluble unless heated to 95°C. The pH of the polymer–water solution is between 5 and 6 and causes no serious effects with respect to stability and selectivity when applied on an electrode surface containing a number of oxidases, dehydrogenases, and peroxidases [203].

Improved selectivity for analyte determinations was found when a number of possible electroactive interfering compounds and well-known solutes in biological fluids and biotechnological samples were tested. For all electrodes,

including uncovered electrodes, virtually all the initial response remains after exposure to these compounds. Eastman AQ was able to withstand the influence of high cell concentrations from microorganisms such as *Saccharomyces cereviseae*. This was not the case for the other five membranes described above. Biosensors used in biotechnological processes with high levels of cells present in the fermentor were used with a combination of two membranes, poly-*o*-phenylene diamine and Eastman AQ. The combined discriminating effect of these two membranes gave highly stable and precise operation of an alcohol biosensor [203].

7.2. Plasma and urine

Extensive sample handling and sample preparation of plasma and urine in the analysis of low levels of analytes can be avoided through the discriminating nature of an enzyme. However, the proteins, peptides and other interfering compounds must be non-interactive with the electrode surface and other parts of the chromatographic system. Therefore, increasing attention has been devoted to the use of biocompatible membranes [203] (see section 7.1). We used protective membranes cast on electrode surfaces in order to be able to increase the operational stability of the biosensor when applied to the analysis of biological samples.

Ethanol is the most common toxic substance involved in legal cases. Alcohol determination in blood samples is of primary importance in both forensic, medical and clinical toxicology.

An enzyme-based amperometric alcohol biosensor was developed to be used as a detection unit for CLC. This biosensor was based on co-immobilised AOD and HRP in a carbon-paste electrode. The selectivity of the biosensor was found to vary when three different alcohol oxidase enzyme preparations from *Candida boidini* and *Pichia pastoris* were tested [204] (see Table 1). High sensitivity could be obtained for a number of alcohols, organic acids and aldehydes. Optimisation regarding the sensitivity and selectivity of the three alcohol oxidase co-immobilised biosensors was performed. The selectivity and stability of the biosensor was retained by working at an applied potential of −50 mV vs. Ag/AgCl, the optimal operational potential for electrochemical measurements, and by the casting of a bioselective membrane, a mixture of *o*-phenylenediamine and Eastman AQ, on the electrode surface [203].

Separate studies were made to ensure the stability of a CLC separation of methanol and ethanol in a purely aqueous mobile phase at pH 8, the optimal pH for the biosensor performance. It was found that the use of silica-based C_{18} (Nucleosil) material gave good resolution of methanol and ethanol. However, after incorporation of the biosensor, it was found that not only did the separation factor and the plate numbers decrease, but also a decrease in stability of the biosensor was observed. Probably, this was due to hydrolysis of the silica phase at pH 8.0, resulting in adsorption of the released silanol groups on the electrode surface, leading to electrode fouling. A polymer-based PLRP-S separation column was therefore used throughout the experiments and was found to be more stable and almost as efficient in terms of resolution. This analytical column gave optimum operation when flushed with acetonitrile–water (30:70) overnight each third day.

A fast and reliable biosensor-based liquid chromatographic separation system was optimised using the alcohol oxidase from *Pichia pastoris* for the determination of ethanol and methanol in human urine and plasma samples.

Plasma samples were spiked with varying levels of methanol and ethanol and thereafter diluted 10, 50, and 100 times prior to separation. All recovery values were close to 100% (98–108%), except for 50-times diluted 0.25 m*M* alcohol with a recovery of 119%. No significant difference in the recovery of ethanol or methanol was seen for 10 repetitive separations of plasma. The protecting layer on the electrode and its biocompatibility were found to be of utmost importance, since a developed sample handling step along with the fast CLC separation could not eliminate all interfering compounds. This was found in comparison with the use of disposable solid-phase extraction cartridges (Sep-Pak,

C_{18}), where the recovery for both methanol and ethanol in spiked plasma samples was only 50–65%. The low recoveries could not be explained, but these were consistent throughout the experiments. By eliminating the SPE clean-up step, an increase in sensitivity by a factor of ca. 2 could be obtained.

Stable and reliable determinations of ethanol and methanol in plasma and urine could be performed with only a simple dilution and centrifugation step prior to injection onto the liquid chromatographic system. An analysis time of 4 min was required for the assay, with a sample throughput of 13 samples per hour. This means that regardless of analyte and matrix concentration, the bioselective CLC detection system showed good qualitative and quantitative data [204].

Typical chromatograms are shown in Fig. 21 where the separation of a mixture of methanol and ethanol in plasma and urine from a pregnant woman is shown, which can be compared with a separation of a standard mixture (same concentrations as in plasma and urine, methanol 0.5 m*M* and ethanol 1 m*M*). Plasma blanks (Fig. 21e) gave only a minor front peak which did not interfere with the two eluting alcohol peaks. The urine samples taken from a pregnant women were found to behave somewhat different from

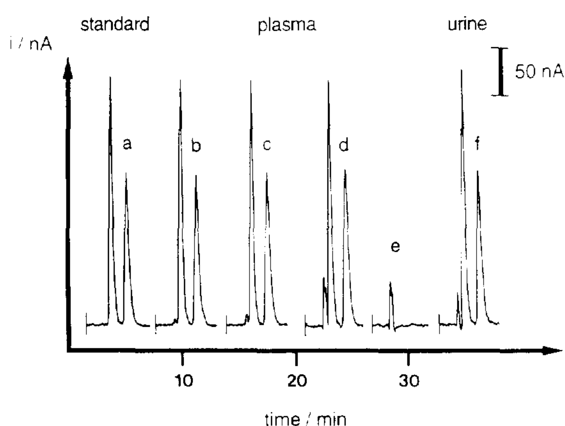


Fig. 21. CLC–biosensor determination of methanol and ethanol in urine and plasma. (a,b) Standard separations of 0.5 m*M* methanol and 1 m*M* ethanol, (c,d) spiked plasma, (e) plasma blank, (f) spiked urine.

the plasma samples. The blank injection of 10-times diluted urine (not shown) was found to be similar to that of the plasma blank without any interference from matrix compounds present in the urine. A somewhat higher spreading of recovery values was found for the spiked urine samples. Most values were close to 100%, but surprisingly, high values were found for samples with larger dilution factors, which varied between 105 to 130%.

7.3. Fermentation processes

In recent years different types of on-line sampling systems have been developed for bio-process monitoring [205–207]. By following processes in an on-line mode, feed-back control of the process itself is possible in order to obtain maximum production yield. The area of bio-technology and the potential of utilising rapid and accurate biosensing detection principles is currently engaging scientists world-wide.

Biotechnological products where enzymes and antibodies are utilised for monitoring purposes, are focused on two categories of products. Production of larger biomolecules such as enzymes and antibodies, and small molecules such as amino acids, other organic acids, antibiotics and ethanol. Another area of interest involves biotechnologically produced pharmaceuticals where the production process can be genetically engineered with very efficient process yields. No doubt this will in the future become a rapidly growing application area. The progress in genetic engineering and molecular cell biology will also play a key role for the success in the field of biotechnology.

Biotechnological processes involve a biological system surrounded by chemical and physical environments. Substrates being consumed by the microorganisms, products being formed, several intermediate and stable metabolites are usually found in the fermentation broth. The intermediate compounds and products are usually formed while the substrates are consumed. In order to calculate and follow the mass balance variations, detailed on-line monitoring of these compounds is of vital importance. This is true for

all processes where feed-back control can be used efficiently for well-known fermentations, or give insight into the state and metabolism of microorganisms in processes which are to be studied and optimised.

In process control biosensors and bioreactors have been used to a larger extent than in other areas. The type of analytical flow systems used is determined by a number of factors, e.g. substrate-, intermediate-, and product concentrations present in the broth, and the complexity of the substrates used. Matrix complexity can in most cases be the determining factor for the complexity of the flow system which is required for accurate monitoring.

7.3.1. Sampling

There are two different ways to continuously sample from a bioreactor if manual sampling methods are not included. The sampling unit can be mounted inside the fermentor or placed outside [208,209]. Several systems of both kinds have been reported during the last ten years. In situ sampling units often consists of a stainless steel carrier serving as a support for a porous membrane. The filtrate is then withdrawn by means of a peristaltic pump and transported for further analysis. It requires a well-stirred position in the bioreactor to avoid or reduce membrane clogging.

In all systems where the sampling unit is placed outside the fermentor, the fermentation broth must be pumped out of the fermentor to the sampling unit and after filtration back again [210]. This increases the risk of contamination. Also, the circulation itself may affect the morphology of the micro-organisms due to the high linear velocities obtained in the tubings. It is possible to change the membrane during a fermentation. However, in many processes the aseptic conditions offered by these instruments is sufficient. This type of sampling unit exists today in several different designs and a number of them are commercially available. The basic principle of such a unit is that the liquid transport of sample across the membrane is to be enhanced [211].

The sampling step is performed by various filtration techniques (as described below) and dialysis [212]. Ultrafiltration, hollow fiber filtration, dialysis using traditional dialysers, microdialysis with a probe approach, or by the use of coaxial catheters are commonly used sampling approaches for withdrawing samples from the process [213–215].

These techniques are to a varying extent effective in removing not only high molecular mass compounds like cells, proteins and macromolecules, but also in some cases excluding groups of compounds. The filtrate "purity" is an important factor which will have a strong influence on the complexity of the system that needs to be developed. A highly efficient membrane filtration technique allows the use of FI or CLC flow configurations where the stability of the method becomes satisfactory. Matrix compounds should to a larger extent be kept out of the analytical flow lines since the often broad spectrum of these groups of compounds has a tendency to adsorb to all exposed surfaces in the system.

In situations where a rather simple fermentation is being performed, an FI system is most often sufficient for accurate measurements, whereas for more complex processes, a liquid chromatographic step needs to be introduced. The chromatographic step may comprise a sample handling technique such as a simple solid-phase extraction step where small disposable or re-usable columns are used. These techniques are used for the separation and elimination of matrix components present in the broth that otherwise would ruin the detectability of the product(s) formed during the process. An analytical column is introduced when a more efficient separation from interfering compounds, or when multi-analyte detection is needed. Sample clean-up often combines sampling and solid-phase extraction techniques to obtain a fairly "clean" fraction of the sample that can be determined without the risk of contamination, clogging of the flow system, or other unwanted effects that ruin even the most carefully optimised and efficient analytical flow system.

The use of various wastes from industry is one type of technical substrate that will give rise to

complications in analysis if not properly handled in the various parts of the system [216–219]. If the bioselective detection is operating sufficiently, and one or several analytes are to be determined, the need of a CLC column may be eliminated.

7.3.2. Biospecific recognition in fermentations

Enzyme-based detection systems are by far the most used bioselective detection techniques in combination with FI for process monitoring. Some papers have appeared in the literature where antibody-based units are developed for this purpose [220]. Fermentation processes are mainly controlled by coupling the analysis to FI with a bioreactor [7]. Lately, enzymatic multichannel FI-systems have become very popular due to the possibility of multianalyte determinations. This technique offers very often analytical results similar to those obtained by CLC separations. IMERs in parallel flow lines have been used in e.g. monitoring mammalian cell fermentations [16]. On-line dialysis was used with IMERs comprising dehydrogenases for the determination of glucose, lactate, glutamine and ammonium. The system allowed the changing of set-points of various operating parameters in order to obtain maximum production of monoclonal antibodies. This system was based on the original work of Spohn et al. [221].

Schügerl et al. [222] investigated both CLC chromatographic separation techniques and FI with IMERs for production processes with recombinant *Echerichia coli*. Schrader et al. [223] developed an IMER-based FI system comprising a cross-flow filtration sampling unit to remove yeast, proteins and other colloidal solubilised substances, followed by a sample preparation step and an alcohol dehydrogenase IMER. The necessary NAD^+ cofactor was added in a reagent stream after the sample preparation step.

On-line monitoring of penicillin V during penicillin fermentations was made by the use of β-lactamase immobilised on both a pH electrode, and in an IMER configuration [224]. Hereby, both the concentration of penicillin and penicilloic acid could be measured.

Shu and co-workers [225,226] recently described the use of co-immobilised L-lactate dehydrogenase and L-alanine aminotransferase in a detection system for on-line monitoring of fermentation processes for the production of D-lactic acid. A culture of *Lactobacillus delbrueckii* was used in a fully automated sequential injection analysis system which had a parallel flow configuration comprising the IMERs. Enzymatically produced NADH was measured by UV detection. The L-alanine aminotransferase was used to eliminate the produced pyruvate by D-lactate dehydrogenase thereby efficiently pushing the reaction of D-lactate to the product side. In this way, the catalytic performance of the overall detectability of D-lactic acid could be improved. A further adjustment of the enzymatic system was the use of D-lactate dehydrogenase in a carbon-paste biosensor configuration. The electrode material was chemically modified with the necessary co-factor NAD^+, a mediator, and an *o*-phenylenediamine containing polymer for D-lactic acid analysis [226]. The reaction sequence in this case was favoured by the use of the mediator which is involved in the electrocatalytic oxidation of NADH. By the use of this biosensor configuration, addition of expensive NAD^+ cofactor could be eliminated since it is no longer added as a soluble additive in the eluent.

Post-column detection for enzyme activity measurements has interesting potential utilising continuous flow procedures in downstream processes. Post-column derivatisation assays were developed for qualitative and quantitative determination of lipase from culture supernatants of *Staphylococcus carnosus* [227]. This technique was also used for the determination of other dehydrogenases and oxidases [228–230].

The production of L-lysine, commonly used as a supplement in animal feed and for special human dietary nutrition, applied a similar post-column detection principle as above [229]. A preparative CLC separation was made using an ion-exchange resin (column volume 7 l) to which an on-line enzyme activity assay was coupled for purity measurements of L-lysine.

Since the oil crisis in the mid 70's alternative energy sources have been of great importance. Technical substrates such as waste waters from

industry have a potential in the search for the total utility of organic matters. Waste waters from pulp and paper industry are an example, containing high levels of sugars. Poly-, oligo- and monosaccharides present in these wastes can efficiently be used as the carbon source in ethanol fermentations.

Ethanol produced in bioprocesses has been considered as a renewable energy source suitable for use 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. In Sweden, fuel ethanol produced from lignocellulosic material has been suggested as a possible energy source, which has been successfully employed for 32 buses in Stockholm.

A fully automated system was developed, using a robotic sample handling instrument (Millilab, Waters), a CLC column and bioreactors, for the monitoring of monosaccharides in an ethanol fermentation process. Since the broth samples were highly complex a multi-phase clean-up of the sample had to be made in order to detect the sugars. Glucose, xylose, galactose, arabinose and mannose were separated and detected by the use of glucose dehydrogenase, mutarotase and galactose dehydrogenase co-immobilised in post-column IMERs, using NAD^+ as the cofactor in the reagent stream. The resulting product NADH could be followed by using a chemically modified electrode where NADH was selectively detected at 0 mV vs. SCE, or by UV at 340 nm [217,218].

Fig. 22a illustrates the separation and detection of a direct crude injection of theses sugars at the end of a fermentation using a conventional RI detector. It is impossible to make qualitative and quantitative determinations due to the background matrix. By introducing solid-phase extraction before the separation, the chromatogram is more suitable for qualitative evaluations, but quantification is still difficult using RI detection (Fig. 22b). However, by the use of the enzyme-based detection both xylose and arabinose are readily determined (Fig. 22c). These samples contain the highest levels of alcohols at the end of the fermentation, resulting in a more complex broth matrix due to solubilisation of apolar phenolic break-down products from lignin.

In another work the products formed in an ethanol fermentation were monitored on-line with a sampling unit (Waters Filter/Acquisition Module) based on tangential flow filtration, in conjunction with CLC [219]. The performance of the various membrane types and pore sizes and their influence on the sampling were investigated and optimised. The influence of feed-rate on the flow-rate of the filtrate for model solutions as well as real fermentation substrates and broths was determined. These optimisations were found to be key factors for the stability of the system. Membrane recovery values for carbohydrates and ethanol in substrates and broths varying in complexity were found to be around 90%.

The total set-up is depicted in Fig. 23 which involves a sample withdrawing step involving three switching valves that were operated automatically. Interfering compounds were trapped efficiently on the mixed anion/cation-exchange column positioned in valve 2. The biosensor comprised a reagentless enzyme electrode based on co-immobilised alcohol oxidase and horse radish peroxidase was used as the working electrode in an amperometric flow-through cell connected to a CLC system for the selective analysis of methanol and ethanol and other aliphatic alcohols [156]. The enzymes were covalently immobilised in carbon paste in the presence of polyethylenimine. Electrodes prepared from the enzyme-modified carbon paste were optimised regarding their sensitivity and selectivity. Different membranes were cast or electropolymerised directly on the surface of the electrode to increase the long term stability of the biosensor. The compatibility with the reversed-phase chromatographic system was outlined. A PLRP-S polymer-based separation column was used with phosphate buffer as the mobile phase. The selectivity of the enzyme electrode was also determined by injecting some easily oxidisable and possibly interfering species normally present in biological samples. The ethanol biosensor was positioned on-line and the detection of a sample is shown in Fig. 24. The two resulting chromatograms are from an early and a late phase of the

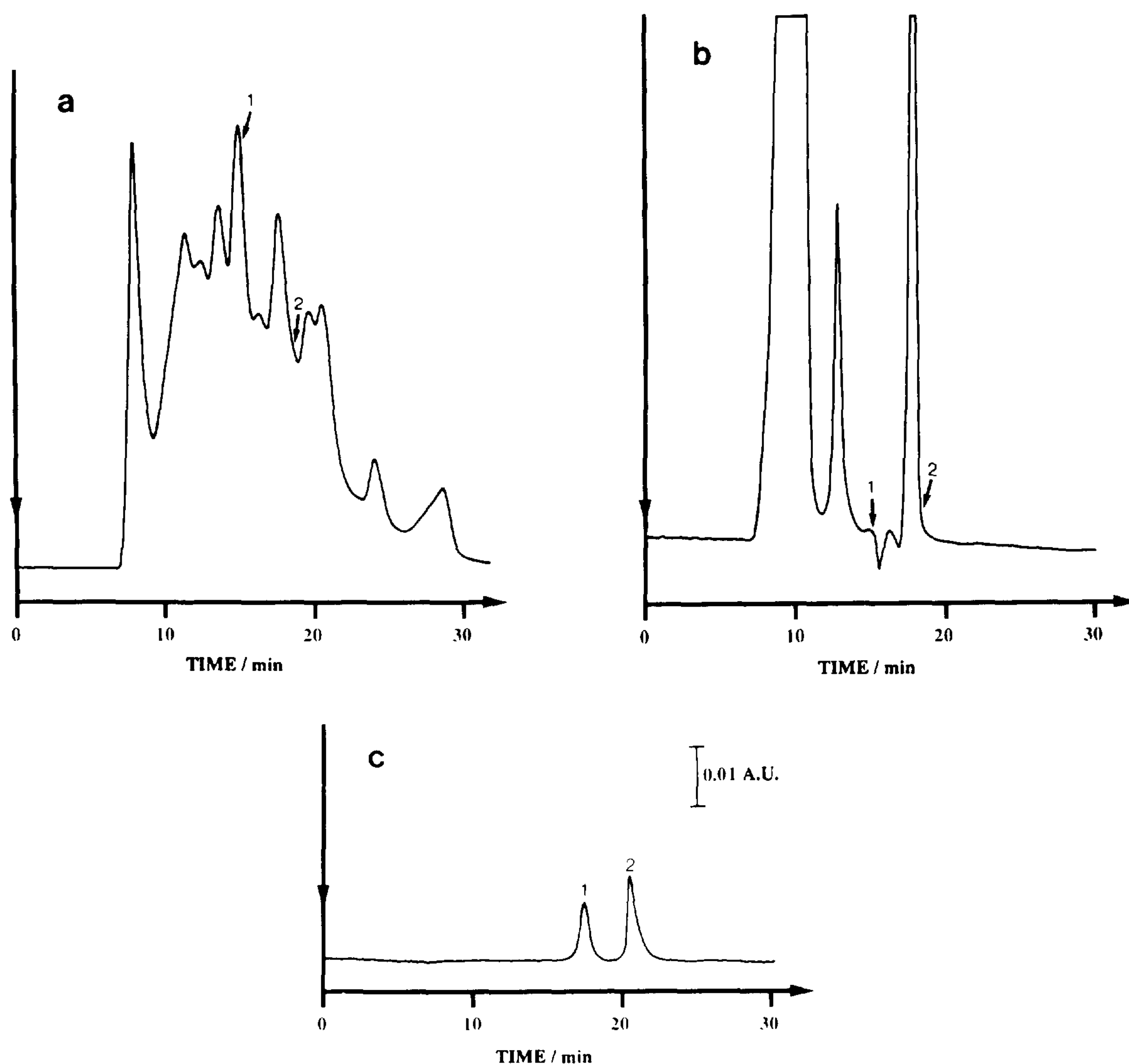


Fig. 22. Chromatographic recordings of a 100-fold diluted fermentation broth (sampled after 32 h). (a) Direct crude injection, RI-detection, (b) as in (a) but with solid-phase extraction, and (c) as in (b) using IMER detection. Peaks: 1 = 13 nmol xylose; 2 = 20 nmol arabinose. Reproduced with permission from Ref. [217].

fermentation. The two first peaks in the chromatograms are front peaks whereas the third reflects the ethanol present in the sample. The same set-up was also used for the separation of sugars present in the fermentation broth.

There is still a need to develop systems which would operate with a more simple instrumental set-up for these fermentations. Microdialysis was therefor used as the sampling technique for on-line bioprocess monitoring in conjunction with CLC and biosensor detection. Applications to a penicillin broth and an ethanol fermentation were studied. Typical recovery values of carbohydrates were found to be close to 100% even after exposure of the microdialysis probe in the process for about 30 h. The microdialysis probe was mounted directly into the fermentor with the probe membrane immersed into the solution. A schematic drawing of the probe is shown in Fig. 25 in the instrumental set-up. The perfusion

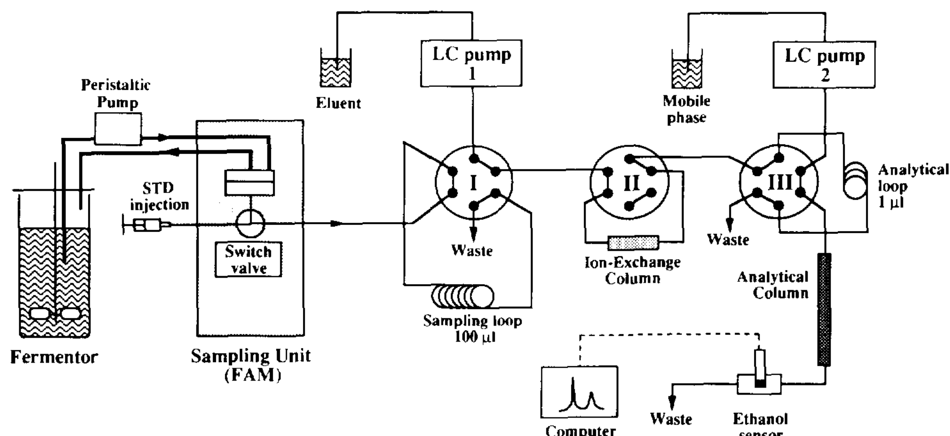


Fig. 23. Chromatographic system for on-line monitoring of ethanol fermentation processes. Reproduced with permission from Ref. [28].

eluent is passed through the capillary in the probe down to the tip from where it is transported upwards through a laser-drilled hole in the capillary as shown in Fig. 25. The perfusion liquid is transported upwards on the inner side of the dialysis tubing, now also containing representative concentrations of solute components from the broth that were able to diffuse through the pores of the membrane. Once it reaches the T-connection on the top of the probe it is transported and trapped in the sampling loop (loop 1 in Fig. 25). The flow-rate of the perfusion eluent through the dialysis unit is controlled by the syringe pump. This flow-rate is one of the important parameters determining the dialysis factor. Other factors are the length of the probe, the chemical structure of the membrane material used, and the thickness of membrane. A very slight over-pressure on the inside of the membrane results in a minimal net flux of perfusion liquid passing out to the fermentation broth, which is assumed to help prevent clogging of the membrane. The dialysed sample is next transported to the six-port injection valve and thereafter introduced into the chromatographic column. Ongoing work demonstrates that the physical properties and the chemical structure of the membranes used have a strong influence on the dialysis factors in various biological matrices [231].

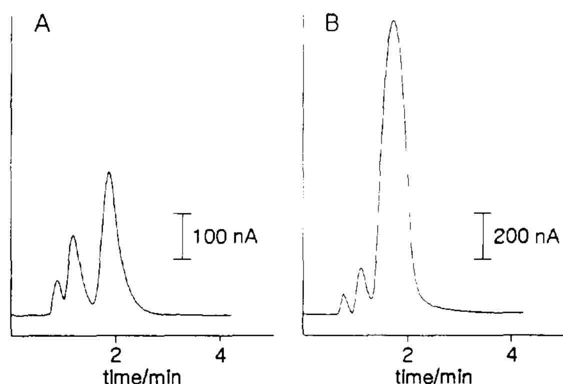


Fig. 24. Separations of a fermentation broth using the ethanol analysis set-up in Fig. 23. (A) Resulting chromatogram after 1 h, (B) after 23 h.

In Fig. 26, the difference in selectivity of this system compared with conventional RI detection is illustrated for an ethanol fermentation using *Saccharomyces cerevisiae*. Many peaks appear in the conventional system while only a single one appears when the enzyme-based detection system is used. Fig. 27 clearly demonstrates the

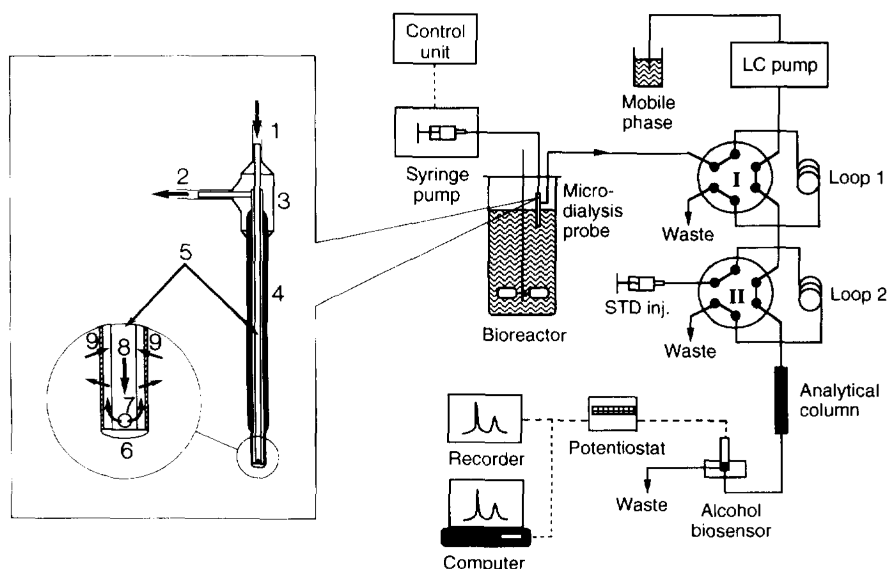


Fig. 25. Integrated sampling and CLC set-up for the determination of alcohols in biotechnological processes. Magnification of the microdialysis probe tip illustrates (1) inlet flow to capillary, (2) outlet flow to valve I, (3) T-piece, (4) outer cannula, (5) inner cannula, (6) probe tip, (7) laser drilled hole in capillary, (8) perfusion liquid pumped down the inner cannula, and (9) dialysate membrane.

efficiency of the system where the increasing level of ethanol could be followed with time. Additionally, pilot-scale ethanol fermentations were made with oxygen feedback control using lignucellulose hydrolysate as a technical substrate with *Escherichia coli* [232].

There are also examples where CLC has been used in combination with immuno detection in biotechnological fermentations. Schmid's group has shown examples where affinity chromatography was coupled to a FI system monitoring the formation of mouse IgG during cultivation of hybridoma cells [233].

Nilsson et al. [42,220] showed recently that post-column ELISA immunodetection can be utilised for the determination of α-amylase. The described method could also be used for the determination of IgG, human serum albumin, lysozyme and N-acetyltryptophan by a GPC separation. Unattended monitoring of the process could be performed by using a sequential competitive binding assay. The ELISA was coupled to ion-exchange separations of the proteins and determinations could be made each 6th min.

7.4. Surface and waste waters

Monitoring the environment for the presence of compounds that may be damaging to human health and nature is fundamental in regulation and law enforcement to protect our environment. Although classical analytical techniques are being improved, these methods are usually expensive and time-consuming. Rogers and Lin [234] described the general environmental problems, cost of classical techniques and possible advantages of using biospecific detection for environmental monitoring. They give numerous examples of what could be done, e.g. which biological component could be used for certain groups of target compounds. Another interesting review was presented by R. Niessner [235] who discussed the strengths and weaknesses of immu-

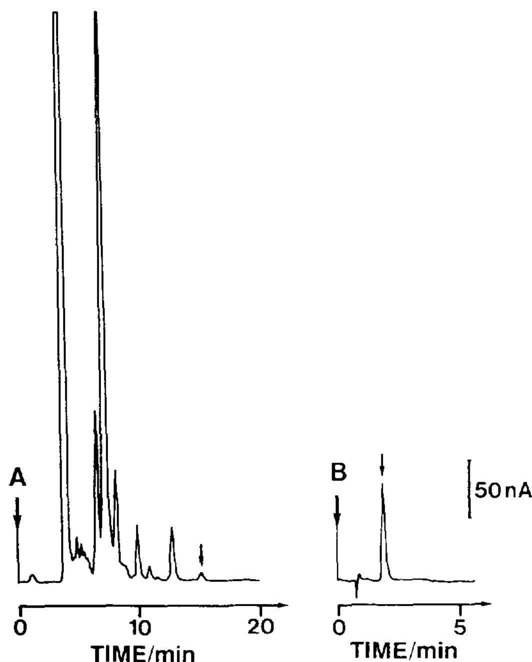


Fig. 26. Chromatograms obtained when injecting the microdialysate into two different chromatographic systems. (A) Cation-exchange separation using 4 mM sulphuric acid as the mobile phase with RI-detection. (B) Reversed-phase separation (PLRP-S column) using a mobile phase of phosphate buffer (50 mM, pH 7.5) and alcohol biosensor detection. Arrow indicates ethanol peak. Reproduced with permission from Ref. [203].

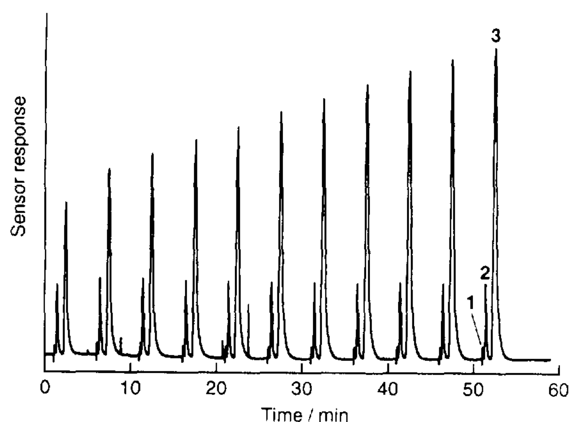


Fig. 27. Eleven sequential chromatograms acquired during the first hour of fermentation. Injection frequency was 12 samples h^{-1}. Peaks 1 and 2 are matrix compounds and peak 3 is ethanol. Reproduced with permission from Ref. [232].

nological techniques in environmental analytical chemistry.

Massive efforts are being made to use bioselective detection systems to decrease the cost of analysis, but also to substantially improve the selectivity as compared with classical techniques. The analytical problems encountered with conventional detection techniques have recently led the European Economic Community (EEC) to initiate a research program, within the environmental program, devoted exclusively to the development of bioselective detection for environmental control [236].

A completely integrated on-line flow system incorporating sample clean-up/trace enrichment, chromatographic fractionation and biosensor detection was developed for the screening for phenols in surface water samples [237] (Fig. 28). The selectivity and sensitivity of this system were obtained through the combination of SPE as the sample pretreatment step and biosensor detection based on the phenol-selective enzyme tyrosinase. In SPE it is usually necessary to use rather high amounts of organic modifier for desorption of enriched analytes from the SPE column, which might be detrimental to the biosensor performance. The main challenge was

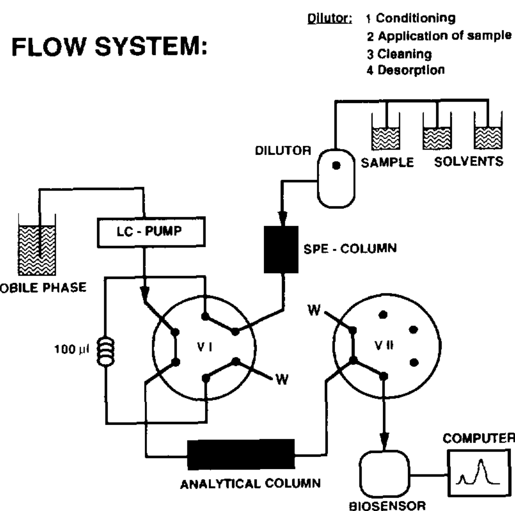


Fig. 28. Fully automated chromatographic system (Gilson Medical Electronics) incorporating robotic sample handling (Aspec XL) with SPE, separation and biosensor detection for the determination of phenols in surface waters.

therefore to solve the interfacing of conventional SPE techniques with biosensor technology so that both techniques perform optimally. This was done by a heart-cut system using a valve-switching technique. The analytes were desorbed with an amount of organic modifier as low as possible and then a heart-cut of the peak was trapped in a 100-μl injection loop. The content of the loop was then injected onto a short fractionation column, the only purpose of which was to separate the organic modifier front from the analyte peak. By the use of valve switching the organic front was thereafter sent to waste and the analyte peak to the biosensor. Two desorption steps for each sample cycle were necessary, since the amount of organic modifier used was too low to desorb the most hydrophobic analyte *p*-cresol. One could argue that higher amounts of organic modifier could be used. However, if the amount is too high the resolution between the analyte peak and the organic front in the fractionation step is lost. Two different biosensor configurations, a solid graphite electrode and a carbon paste-electrode, were tested and the highest sensitivity was obtained with the solid electrode. Seven different spiked surface water samples from the Ebero Delta in Spain were obtained and screened with this system. The three positive samples were identified with signal intensities corresponding to spiked amounts of 1, 10 and 25 μg/l of each catechol, phenol and *p*-cresol. One false positive was also found, but this can be considered less of a problem than a false negative. Seven identical injections of spiked surface waters are depicted in Fig. 29, where the arrows indicate the two different desorption steps. Some problems with repeatability can be seen, which is due to fluctuations in the heart-cut procedure at low analyte concentration.

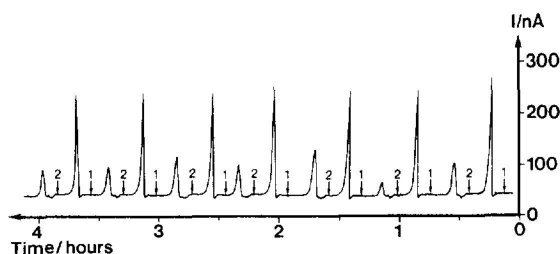


Fig. 29. Seven repetitive separations of phenols using the system described in Fig. 28. with two-step desorption. Peaks: 1 = catechol/phenol peak, 2 = *p*-cresol peak; 1 μg/l of each analyte.

8. New developments and future trends

Several factors influence the kinetics of immobilisation of enzymes, e.g. the mode of immobilisation, enzyme loading properties of the support, and the composition of the reaction medium. Optimisation of these conditions is often time-consuming and many reactions must be carried out. Sampling is critical when studying reactions involving volatile substrates, products or organic solvents since the composition could easily change through the opening and closing of the reaction vials. Wehtje et al. [238] proposed a method to carry out both the reactions and analysis in a fully automated CLC system exemplified by mandelonitrile lyase catalysing condensation of benzaldehyde and HCN and α-chymotrypsin catalysing esterification of N-acetyl phenylalanine with ethanol.

Mixed-culture microbial ecosystems are important in engineered biological waste treatment systems. For many xenobiotic hazardous materials there is only a small number of organisms that possess the necessary genes that code for biodegradation enzymes. In bacteria, these genes are often found on extrachromosomal DNA (plasmids). Stress of different kinds can drastically influence the ability of the critical subpopulation to express this biodegradation activity. For effective environmental control it may be necessary to monitor and control these small subpopulations. A commonly used method is called colony hybridisation and involves the detection of DNA sequences coding for the needed biodegradation trait. However, since this requires culturing of viable cells to form colonies, it is impractical for control applications in many biological waste treatment systems. Moore et al. [239] described how a bioreactor system can be realised for comparably rapid detection of sub-

populations utilising affinity chromatography and DNA hybridisation kinetics. The specific single-stranded DNA is immobilised on a support. DNA is extracted from a sample taken from a biological system and put in single stranded form and injected into the bioreactor. The proposed system should lead to specific genotype analysis in mixed-culture samples within several hours as compared to days or weeks presently required for colony hybridisation.

Millan et al. [240] developed a voltammetric DNA biosensor. Polythymidylic acid of 4000-base average length was immobilised on a stearic acid modified carbon-paste electrode following enzymatic elongation. These DNA-modified biosensors were used to study hybridisation and the results were applied to the selective detection of the cystic fibrosis sequence in an 18-base oligodeoxynucleotide samples.

Eicosanoids and cytokines are reported to play a major role in many inflammatory processes, in cell growth and differentiation and in the development of cancer. They are commonly used as biological markers in many medical areas and are for these reasons important endogenous groups of molecules to determine both in vitro and in vivo. Arachidonic acid (20:4), one of the major lipid constituents of cell membranes, is regulated by its liberation from phospholipids by the action of phospholipases and is the precursor of all eicosanoids. Most mammalian cells possess the capacity to synthesise and release eicosanoids. Eicosanoid synthesis will be determined by the free arachidonic acid levels in the cell by the action of key enzymes forming three major groups of metabolites, e.g. prostaglandins, leukotrienes and thromboxanes.

In order to be able to follow the appearance and disappearance of all arachidonic acid metabolites, bioanalytical methodology has moved towards a combination of chromatographic separation and immunological techniques. Thus, traditional off-line enzyme catalysis in combination with CLC is common, as described by Celardo et al. [241] who used two peptidases for the conversion of leukotriene C4 and leukotriene D4 to form the less potent metabolite leukotriene E4 in blood samples.

Isotope-labelled arachidonic acid has been used extensively to follow the metabolic pathways by the use of CLC and radiodetection [242], GC–MS and immunoaffinity [243] and CLC [242]. These methods have also been compared with CLC–immunodetection methods [244–246]. Radio immunoassays and especially enzyme immunoassays are highly sensitive and selective techniques. However, if they are not combined with chromatographic separation techniques, the total amount of all cross-reacting metabolites will be measured. This is often sufficient information for some research purposes, but both qualitative and quantitative data are in most cases desired.

Biological samples are most often purified by a solid-phase extraction step whereafter the sample is separated by a reversed-phase separation step using methanol or acetonitrile mixed with aqueous buffers. The endogenous metabolites are fractionated by the chromatographic run, evaporated and rediluted into a buffer which is compatible with the immuno assay. These very laborious and time-consuming manual sample handling steps have led to the development of new on-line or semi on-line techniques which can overcome many of the drawbacks described above. Inflammatory cytokines have been determined by CE [247]. Combination of CE and ELISA in a semi on-line configuration has been applied to tissue bound in frozen biopsy specimens. Fractions eluting from the capillary were brought into contact with a pre-wetted membrane onto which the protein peak was collected. An attached electrical motor was used to advance the fraction collector during the electrophoretic run. The different fractions were quantitatively measured using a chemiluminiscence-enhanced immunoassay [248]. With this coupled technique quantitation and validation was performed for interleukin-1, interleukin-6, γ-interferon and tumour necrosis factor α (TNFα). The circular membrane collection technique was first described by other groups [249,250].

The high resolution power of capillary electrophoresis has also had its impact on the developments in biospecific detection. Special attention needs to be given to miniaturisation of detection

units used in CE since the inner diameters of capillary columns range from 10 to 150 μm.

Carbon-paste composite microelectrodes with immobilised glucose oxidase, and 1,1′-dimethylferrocene as the mediator, were positioned inside a 150 μm I.D. polyimide fused-silica capillary column at a depth of 5 mm. Electropherograms were obtained within 2 min for glucose at femtomole levels with an operational potential of 325 mV vs. Ag/AgCl [251].

Other carbohydrate-catalysing enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase, were also analytes of interest and separated with CE. A special post-capillary reactor was used with an on-line mixture of the substrates, glucose-6-phosphate and the cofactor $NADP^+$ [252–254]. This CE post-column micro-enzyme assay could be used for the detection of sub-femtomoles of enzyme.

Separation of enzymes can be combined with simultaneous activity measurements. This is done by introducing the substrate and the cofactor in the capillary while the enzyme plug is eluted down the column. The substrate, which has a higher electrophoretic mobility than the enzyme, moves into the enzyme plug and the product NADPH/NADH is formed which can be detected at 340 nm [255]. This approach can be utilised because of the difference in relative mobilities of the enzyme and substrate/product. Sensitivities down to the attomole level have been reported.

Avila and Whiteside [256] were able to lower this detection limit somewhat and also applied the method for the determination of both glucose-6-phosphate dehydrogenase and yeast alcohol dehydrogenase.

Nashbeh and El-Rassi [173] developed the technique of enzymephoresis using coupled heterogeneous capillary enzyme reactor CZE for the analysis of nucleic acids and their kinetics. The enzymes ribonuclease T, hexokinase and adenosine deaminase were immobilised on the inner side of the capillary. These capillary IMERs coupled in series with CE serve as a peak locator on the electropherogram resulting in improved selectivity and facilitate quantitative determination of different nucleic acids. A resulting electropherogram of the on-line digestion of a secondary structure of transfer ribonucleic acid specific for phenylalanine is shown in Fig. 30 [173].

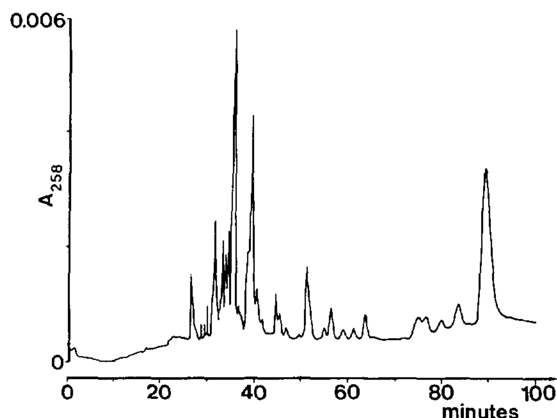


Fig. 30. On-line digestion and mapping of $tRNA^{Phe}$ by tandem RNase capillary IMER–CZE. Reproduced with permission from Ref. [173].

Resulting electropherograms from enzymatically produced products give also kinetic information of the system. Miller and Lytle [257] went even further by immobilising whole cells from Baker's yeast onto a porous chromatographic support (Porous OH-150) which was packed into the beginning of the capillary. The particle structure and the perfusion of analytes through these pores is highly advantageous for high catalytic performance. Peptidases present in the immobilised cells catalyse the β-naphtylamide-aminopeptidase compounds that were introduced sequentially and catalysed by the enzymes present for 1 min. After enzyme catalysis, the products formed were separated by applying a voltage over the column and detection was made by laser-induced fluorescence. Aminopeptidase profiles could be obtained by the immobilisation of 500 cells.

Shimura and Karger [258] developed an affinity-probe CE method. They labelled an Fab fragment of a mouse monoclonal antibody (anti-human growth hormone) with tetrametylrhodamine-iodoacetamide at a hinge region thiol group. The antigen, methionyl recombinant human growth hormone, could be measured

down to detection levels around 5 pM by mixing samples with labelled antibody fragments. The associated complex was subsequently injected and separated by capillary electrophoresis. Special consideration was given to investigate the equilibrium and kinetics of this micro-scale analysis technique.

Affinity CE was developed to study the binding stoichiometry of protein–ligand interactions [259,260]. Application of this technique was used to study the inhibition of carbonic anhydrase from human and bovine erythrocytes, the interaction of a monoclonal antibody to human serum albumin with its antigen HSA, and the binding of streptavidin to biotin derivatives [260]. Bioaffinity studies of receptor–ligand interactions were performed by determining the association- and dissociation constants of a potent immunosuppressant deoxyspergualin and Hsc70, a constitutive or cognate member of the heat shock protein 70 family. The method could also be used for the investigation of the interaction of deoxyspergualin with a synthetic peptide.

Medical challenges have strongly activated the field of analytical chemistry to develop in vitro and in vivo biosensors which are able to follow important biochemical processes.

No doubt the developments in in vivo monitoring where implantable microdialysis probes are used in combination with biosensors will lead to the development of techniques able to measure and follow changes in biological systems. Neuroscience is one example of an area where microdialysis has been used in combination with bioreactors [261]. The development of the microdialysis technique by Ungerstedt made it possible for other groups to combine this technique with CLC separations using bioreactors for the determination of acetylcholine and choline in rat brains [113,261,262]. New developments within the areas of microdialysis and ultrafiltration capillaries used as bioanalytical sample preparation techniques will also be of great importance [263]. Dialysed perfusion solution will not contain blood cells or other macromolecules and can therefore in most cases be directly analysed by chromatographic techniques without any clean-up procedures. Examples of monitored compounds are lactate, choline, glucose, glutamine and glutamate [261].

In vivo pharmacokinetics and metabolism of drugs is another area of great importance and interest for the pharmaceutical industry. Interesting approaches have been described where microdialysis probes and biosensors are combined with electrophysiological techniques in brain research [261].

The search for analytical methods and techniques in biological microenvironments has led to rapid developments within the field of in vivo biosensors. There are several groups that have specialised in implantable biosensors [264,265]. These micro senors are in the range of 0.1 mm in diameter. Csöregi et al. [266] used an implantable glucose sensor by wiring recombinant glucose oxidase with a redox polymer, poly[(vinylimidazol)Os(bipyridine)$_2$Cl$^{+/2+}$], and subsequent crosslinking with poly(ethylene glycol)diglycidyl ether, to form an electron conducting hydrogel [267]. On top of this layer an interference-eliminating membrane layer was deposited consisting of horseradish peroxidase and an outer hydrophilic membrane compatible with the biological micro-environment. Typical current output obtained with this biosensor was 35 nA for a 10 mM glucose solution with a 10–90% response time of around 1 min with a variation in signal response of 5% when operated for 72 h. Several groups are involved in the development of monitoring subcutaneous tissue glucose by combining portable glucose sensors with microdialysis [268,269] or brain glutamate [270]. New types and configurations of microbiosensors have been constructed of carbon fibre with immobilised dehydrogenases with a covalent modification utilising avidine–biotin coupling [271] for the analysis of neurotransmitters, and with immobilised peroxidases used in micro flow systems for the determination of organic peroxides [272]. The development of microarray biosensors enables multi-analyte determinations in multichannel detectors using interdigitated microarrays and independently addressable microarray electrodes [273]. This type of microelec-

trode has been applied to penicillin [274] and NADH analysis [275]. Interdigitated microarray electrodes have also been used in CLC [273].

Another interesting approach is the utilisation of antibody/antigen reactions in an analytical flow system where the immunoreaction is performed directly on the electrode surface with electrochemical techniques. The success of this type of immunosensor lies in the ability of immobilising the antibody on the electrode surface in a stable and sterically correct form for antigen recognition. Sadik et al. [276] developed an immunosensor for the determination of thaumin. The anti-thaumin was immobilised in a poly(pyrrole) polymer film formed on the surface of the electrode. Pulsed amperometric detection was necessary for stability and high sensitivity of the sensor. Other similar voltammetric [277] and optical [278] immunobiosensors have also been reported. Duan and Meyerhoff [279] used self-assembled monolayers of immobilised antibodies on microporous gold electrodes for the determination of human chorionic gonadotropin hCG in whole human blood with a detection limit of 2.5 units/l.

Surface plasmon resonance (SPR) immuno biosensors have been developed during the last decade and there are several companies which have commercialised these biosensors, e.g. the Biacore and Bialight instruments by Pharmacia, Uppsala, Sweden and Biosensor by ASI, Zürich, Switzerland. All types of immuno-interactions can be studied by this technique. Thus valuable kinetic information can be obtained which may be helpful in the application and construction of different immunological techniques [280,281]. Dubs et al. [282] used the Biacore for the mapping the epitopes of tobbaco mosaic virus proteins.

It is expected that developments in the surface chemistry of the nobel-metal surface of sensor chips will lead to improvements in the sensitivity of this type of instrumentation. This would make the technique applicable for sample screening purposes [283].

For improved sensitivity, enzyme-linked and other types of immuno-linked detection principles can probably be interfaced with and incorporated in detection units for use in separation techniques such as CLC and CZE. Developments within the field of genetically engineered monoclonal antibodies and Fab and Fv fragments will have a strong impact on the development of analytical method. Thus we assume that there will be fast and broad developments within the area of on-line immunodetection principles.

9. Abbreviations

ACh	Acetylcholine
AChE	Acetylcholine esterase
AChO	Acetylcholine oxidase
ACS	Acyl-CoA synthetase
ADH	Alcohol dehydrogenase
AOX	Alcohol oxidase
L-AAOX	L-Amino acid oxidase
D-AAOX	D-Amino acid oxidase
ALD	Absolute limit of detection
AMG	Amyloglucosidase
Ag	Antigen
Ab	Antibody
Ch	Choline
CFA	Continuous flow analysis
CZE	Capillary zone electrophoresis
CE	Capillary electrophoresis
CLC	Column liquid chromatography
CPG	Controlled pore glass
DP	Degree of polymerisation
EC	Electrochemical detection
EIA	Enzyme immuno assay
FAD	Flavin adenine dinucleotide
FMN	Flavin mono nucleotide
FET	Field effect transistor
FI	Flow injection
FIIA	Flow injection immuno assay
GC	Gas chromatography
GOD	Glucose oxidase
HRP	Horse radish peroxidase
HPPA	3-(*p*-Hydroxyphenyl)propionic acid
IgG	Immunoglobulin
IA	Immunoassay

IMER	Immobilised enzyme reactor
LACC	Laccase
LOD	Limit of detection
NAD^+/NADH	Nicotinamide adenine dinucleotide (oxidised and reduced form)
Med	Mediator
MS	Mass spectrometry
MUT	Mutarotase
PAD	Pulsed amperometric detection
PEG	Polyethylene glycol
PTH	Parathyrin
POD	Peroxidase
PQQ	Pyrrolo-quinoline quinone
RP	Reversed phase
RI	Refractive index
RIA	Radio immuno assay
SIA	Sequential injection analysis
SFA	Segmented flow analysis
SPE	Solid-phase extraction
SPR	Surface plasmon resonance
TBAP	Tetra-*n*-butylammonium phosphate
TYR	Tyrosinase

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