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Determination of organic acids, amino acids and saccharides by high-performance liquid chromatography and a postcolumn enzyme reactor with amperometric detection

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ABSTRACT

A technique for the determination of organic acids, amino acids and sugars is described. The compounds of interest are separated by high-performance liquid chromatography (HPLC) and converted on-line by immobilized enzymes. The enzymes employed are covalently bound to a synthetic carrier. Hydrogen peroxide, which is produced in the reaction with oxidases, makes possible the application of an electrochemical detector. This arrangement combines the separation efficiency of HPLC, the substrate specificity of enzymes and the high sensitivity of electrochemical detection. The enzymes act according to known reaction mechanisms, but coupling with HPLC leads to a promising extension in the field of biosensors. The simple pretreatment of the samples (often a dilution step is sufficient) allows a rapid analysis of foodstuffs and biological or clinical extracts. The examples presented demonstrate the very high sensitivity of the method with detection limits in the nano- to picomolar range and a wide field of application.

INTRODUCTION

The demand for rapid but specific and exact methods in analytical chemistry, *e.g.*, in the food industry and the biochemical and environmental fields, has increased substantially recently. High-performance liquid chromatography (HPLC) has been widely applied and the disadvantage of insufficient sensitivity has been overcome by the development of sensitive detectors, *e.g.*, the electrochemical detector. By pre- and postcolumn derivatization it is possible to detect electrochemically inactive substances. One form of selective postcolumn reaction is conversion with immobilized enzyme reactors (IMER) [1–4], based on known enzyme reactions [5–7]. IMERs are commonly used in flow-injection analysis (FIA) [8–11]. Because there is no separation in FIA, the coupling of HPLC with biosensors is advantageous. In the method proposed here, the compounds of interest are separated by HPLC and converted in the IMER and the reaction product is detected amperometrically [12]. For example, this approach has already been employed for the determination of the neurotransmitters choline and acetylcholine [13–16].

The presented one-step determination of organic acids, amino acids and sugars represents a development of the combination of HPLC separation, enzymatic conversion and electrochemical detection. Examples of some successful applications are given.

EXPERIMENTAL

Apparatus

For the separation of acids, two HPLC pumps, an LC-XPD from Pye Unicam (Kassel, Germany) and an Economy 2/E from Techlab (Erkerode, Germany), a Model 3512 degasser from ERC (Alteglofsheim, Germany), Model EP 30 electrochemical de-

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tector from Biometra (Göttingen, Germany) equipped with a platinum cell (working potential 0.6 V *versus* Ag/AgCl, range 50 nA) and a Model 7125 injection valve (Rheodyne, Berkeley, CA, USA) equipped with a 10-μl sample loop. The results were recorded with a Shimadzu (Duisburg, Germany) C-R6A instrument. The following columns were used: Superspher 100, 125 × 4.5 mm I.D., packed with 5-μm ODS (Merck, Darmstadt, Germany); Polyspher OA HY, 300 × 6.5 mm I.D. filled with cation-exchange resin (Merck); and Inertsil ODS 2, 250 × 4.6 mm I.D., packed with 5-μm ODS (VDS Optilab, Berlin, Germany).

The HPLC separation of sugars was carried out with two Beckman (Munich, Germany) pumps, an ERC 3512 degasser, a Biometra EP 30 electrochemical detector and a Model 7125 injection valve (Rheodyne) equipped with a 10-μl sample loop. The results were recorded with a Model 3390 A integrator from Hewlett-Packard (Bad Homburg, Germany). The following columns were used: Supelcosil LC 18, 250 × 4.6 mm I.D., filled with 5-μm ODS (Supelco, Bad Homburg, Germany); LiChrospher, 125 × 4 mm I.D., packed with ODS (Merck); and a mixing column, 60 × 4 mm I.D. filled with glass beads, diameter 40 μm.

Reagents

D,L-Amino acids, ascorbic acid, $Co(NO_3)_2 \cdot 6H_2O$, L-lactic acid, $MgSO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot 2H_2O$, $NaH_2PO_4 \cdot H_2O$, nicotinamide adenine dinucleotide free acid, oxalic acid, phosphoric acid and sugars (fructose, galactose, glucose, lactose, maltose, maltotriose and saccharose) were obtained from Merck, citric acid, succinic acid and tris(hydroxymethyl)aminomethane (Tris) from Serva (Heidelberg, Germany) and lithium-D,L-lactate, lithium-D-lactate and maltooligosaccharides from corn syrup were purchased from Sigma (Deisenhofen, Germany). All reagents were of analytical-reagent grade. Water obtained with a NANOpure-A system (Sybron/Barnstead, Boston, MA, USA) was used to prepare all stations.

Enzymes

The following were obtained from Sigma: L-amino acid oxidase (EC 1.4.3.2) type V from *Crotalus adamanteus* venom, lyophilized, A-3016, 8.5 U [U = amount (units) of enzyme used for immobilization]; D-amino acid oxidase (EC 1.4.3.3) type II from porcine kidney, suspension in $(NH_4)_2SO_4$ solution, A-1789, 73 U; amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger*, suspension in 3.2 *M* $(NH_4)_2SO_4$ solution, pH 6.0, A-3514, 940 U; ascorbate oxidase (EC 1.10.3.3) from *Cucurbita* species, lyophilized, A-0157, 250 U; galactose oxidase (EC 1.1.3.9) from *Dactylium dendroides*, lyophilized, G-3385, 150 U; β-galactosidase (EC 3.2.1.23) from *Escherichia coli*, lyophilized, G-6008, 1000 U; glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* lyophilized, G-6125, 2000 U; invertase (EC 3.2.1.26) from baker's yeast, solid, I-4504, 6000 U; L-lactate oxidase from *Pediococcus* species, lyophilized, L-0638, 25 U; oxalate oxidase (EC 1.2.3.4) from barley seedlings, lyophilized, O-4127, 5 U; mutarotase (EC 5.1.3.3) from porcine kidney, lyophilized, M-9776, 5000 U. L-Lactate dehydrogenase (EC 1.1.1.27) from porcine muscle, solution in glycerin, 127221, 160 U, and D-lactate dehydrogenase (EC 1.1.1.28) from *Lactobacillus leichmannii*, suspension in $(NH_4)_2SO_4$ solution, 106914, 160 U, were commercially available from Boehringer (Mannheim, Germany). Glucose isomerase (EC 5.3.1.5), crystalline 9 U, was a gift from Kalie Chemie (Hannover, Germany).

Immobilization

The enzymes were covalently bound to a polymeric carrier by the Biometra method (Galensa *et al.* [12]). Eupergit C, which is based on poly(methylmethacrylamide), from Röhm Pharma (Weiterstadt, Germany) was used as a polymeric matrix. Bearing oxirane groups, it is capable of binding proteins via their amino, thio and hydroxy groups. The immobilized enzymes are filled in steel cartridges (40 × 2.5 mm I.D.), maximum pressure 150 bar.

Sample preparation

Samples and standards were dissolved in and diluted with water or phosphate buffer (0.1 *M*), sonicated and filtered (0.45-μm membrane filters) if necessary.

RESULTS AND DISCUSSION

Different instrumental arrangements were used for the determination of the particular acids, amino acids and sugars. Fig. 1 shows the simplest way if

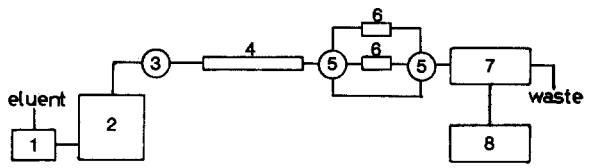

Fig. 1. Schematic diagram of the experimental arrangement without a second eluent. 1 = Degasser; 2 = pump; 3 = injection valve; 4 = HPLC column; 5 = switching valve; 6 = enzyme reactor; 7 = electrochemical detector; 8 = integrator.

the same eluent is needed for the separation and for the enzyme reaction. By the use of switching valves, it is possible to select different enzyme reactors. Interfering electroactive components in the samples can be detected with a reference device, without enzyme. Attention must be paid to the compatibility of the conditions for HPLC separation and enzyme reaction. Enzymes exhibit an optimum pH range for activity, which depends not only on pH but also on ionic strength and type of buffer. The optimum pH can be shifted by the immobilization, *e.g.*, by binding on a multi-charged support [17]. Activity profiles have to be recorded by evaluating the effect of pH on the peak current. If the HPLC separation requires water or an eluent with an unsuitable pH value, the optimum pH is adjusted by feeding a second eluent after the column (see Fig. 2).

To enhance the separation efficiency organic modifiers are sometimes added to the HPLC eluent. Although the covalently immobilized enzymes exhibit an increased stability towards organic solvents compared with the native enzymes [17], the use of such eluents is limited. Among the factors governing the catalytic activity of the IMERs, attention must be paid to the dependence on the presence of a coenzyme or special activators, *e.g.*, inorganic ions [18]. In addition to the investigation of the enzyme parameters, the optimum potential on the working electrode in the flow cell has to be determined. Signal-to-noise ratios and peak current as a function of applied potentials give plots characteristic of the individual electroactive substances. In most instances we used oxidases. The generated hydrogen peroxide was measured at a potential of 0.6 V (*versus* Ag/AgCl/KCl) on a platinum electrode:

$$H_2O_2 \longrightarrow O_2 + 2\,H^+ + 2e^- \qquad (1)$$

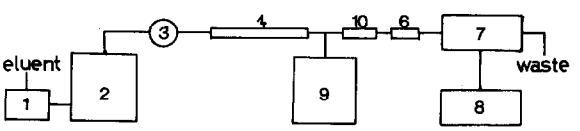

Fig. 2. As Fig. 1, with a second eluent. 9 = Second pump, 10 = mixing column.

Determination of acids

Oxalic acid. Because of the toxicity of free oxalic acid and its salts, determinations in vegetable foodstuffs such as spinach, mangold and rhubarb and in clinical samples are of great interest. Among the various methods for determining oxalic acid [19], the use of enzymes provides a high degree of specificity and sensitivity.

Oxalic acid is converted to H_2O_2 and CO_2 with oxalic acid oxidase (OAD):

$$\text{oxalic acid} + O_2 \xrightarrow{\text{OAD}} CO_2 + H_2O_2 \qquad (2)$$

In contrast to the published FIA method with immobilized OAD and the detection of the liberated hydrogen peroxide by a colour reaction [20,21], no additional reagent is necessary in our method with electrochemical detection. The separation (and also protection of the IMER) takes place by HPLC.

With respect to the optimum pH of the immobilized OAD, a 0.1 *M* citrate or succinate buffer solution (pH 5.0) is used as eluent [21,22]. A linear calibration graph is obtained in the 1–10 ppm range using a 10-μl sample loop (0.1–1 n*M* per injected volume), with a detection limit of 0.63 ppm.

Lactic acid. Lactic acid is important in food technology. It is employed for mild acidification and conservation of sour milk products, sour vegetables, etc. Its determination can serve for fermentation control. The content of lactic acid is also a quality factor indicating the beginning of food degradation. Further, the examination of the lactate blood concentration is an important clinical parameter, *e.g.*, for the diagnosis of lactic acidosis or shock states. Lactic acid can be determined with lactic acid oxidase (LOD), which catalyses the following reaction:

$$\text{lactic acid} + O_2 \xrightarrow{\text{LOD}} \text{pyruvate} + H_2O_2 \qquad (3)$$

Several studies with immobilized LOD have been published, *e.g.*, combined with a Clark oxygen elec-

trode [23], fluorimetric detection [24], differential-pulse polarography [25] or FIA with amperometric detection [26]. In this work, lactic acid is also determined with immobilized LOD, but in combination with HPLC separation and an electrochemical detector. The immobilized LOD has a pH optimum of 7.0–7.5. The eluent is 0.1 *M* phosphate buffer solution (pH 7.3). Under these conditions, the calibration graph is linear from 0.6 to 63 ppm using a 10-µl injection volume (0.07–7 n*M* per injection). The limit of detection is 0.18 ppm. Chromatograms of food samples are presented in Fig. 3.

Lactic acid is an optically active substance that occurs naturally in the dextrorotatory L-(+)-form. The determination of D-(−)-lactic acid as a product of microbial metabolism is of interest, *e.g.*, in the analysis of foodstuffs such as tomato products [27]. LOD only converts the L-enantiomer of lactic acid. For the determination of both forms, we use L- and D-lactic acid dehydrogenase (LDH). L-LDH has also been employed in a FIA system [28]. In our method, nicotinamide adenine dinucleotide (NAD), which is necessary as a coenzyme for the reaction, is added to the eluent (0.1 *M* phosphate buffer, pH 7.3) at a concentration of 1 m*M*.

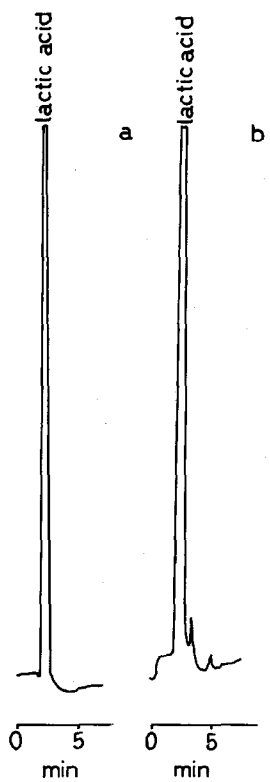

Fig. 3. Chromatography of food samples, diluted in buffer, on a Superspher 100 RP-18e column (125 × 4.5 mm I.D.) with an L-lactate oxidase reactor. Chromatographic conditions: experimental arrangement as in Fig. 1; mobile phase, 0.1 *M* phosphate buffer (pH 7.4); flow-rate, 0.5 ml/min; working potential, 0.6 V; range, 50 nA. (a) Yoghurt, 380 mg/l, L-lactic acid 1.2%; (b) concentrated tomatoes, 1.836 g/l, L-lactic acid 0.5%.

$$\text{lactic acid} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+ \qquad (4)$$

NADH is detected electrochemically at a potential of 0.7 V (in contrast to the usually applied potential of 0.6 V). Separation of the enantiomers is not possible with the tested columns. The determination is achieved by reactor switching as shown in Fig. 1.

Ascorbic acid. Ascorbic acid can be detected by electrochemical oxidation on a platinum electrode at a potential of 0.6 V. Because of the similar retention times on RP-18 columns, the determination of organic acids in real samples is disturbed and ascorbic acid must be eliminated. By the use of ascorbate oxidase (AOD), it is converted into dehydroascorbic acid, which is not electrochemically active. AOD has a pH optimum at pH 5.0–5.5, so the in-line elimination with the immobilized enzyme is only possible during the determination of oxalic acid. AOD and OAD are co-immobilized or two reactors are coupled in series and a buffer solution of pH 5.5 is used as eluent [21]. To determine other analytes, the pH must be changed after the reaction with ascorbic acid oxidase, *e.g.*, for lactic acid from pH 5 to 7.3.

Attempts to shift the pH optimum of AOD by immobilization on another carrier substance are in progress. Alternatively to the destruction, ascorbic acid can be separated chromatographically from oxalic or lactic acid with a suitable chromatographic system. Fig. 4 shows the separation of ascorbic acid, lactic acid and uric acid in blood serum with the IMER system.

Amino acids. L-Amino acids are essential for the biosynthesis of proteins. The optical antipodes, D-amino acids, can be found, *e.g.*, in fermented foods such as dairy products, lactic acid-fermented vegetables and alcoholic beverages [29,30]. The identification of D-amino acids can be evidence of the addi-

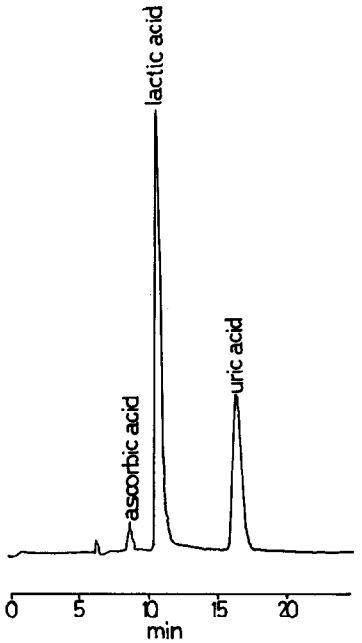

Fig. 4. Separation of ascorbic acid (0.1 μmol/l added), L-lactic acid and uric acid in blood serum, diluted 1:40, on a Polyspher OA HY column (300 × 6.5 mm I.D.) with an L-lactate oxidase reactor. Chromatographic conditions: experimental arrangement as in Fig. 2; mobile phase 1, 0.005 *M* H_3PO_4; mobile phase 2, 0.2 *M* phosphate buffer (pH 7.4); flow-rate, 0.4 ml/min each; working potential, 0.6 V; range, 50 nA.

tion of synthetic, racemic amino acids as adulterants in fruit juices [31]. A method with immobilized L-amino acid oxidase (L-AAOD) as an amperometric enzyme electrode has been proposed [32]. L-AAOD catalyses the oxidative deamination of a number of L-amino acids:

$$\text{amino acid} + H_2O + O_2 \xrightarrow{\text{L-AAOD}} \text{2-oxo acid} + NH_3 + H_2O_2 \qquad (5)$$

The performance of a sensor with L-AAOD has been investigated with the FIA technique. Determination of particular amino acids in one single run is not possible in this way. Other sensors are based on selective oxidases for L-glutamate, L-lysine and L-tyrosine [33]. Recently, a combined FIA–HPLC method was presented, which used immobilized L-AAOD and D-AAOD with amperometric detection [34]. In other studies, racemic resolution of amino acid esters into L-amino acids and D-amino acid esters was achieved using enzyme reactors with immobilized α-chymotrypsin [35] or trypsin [36] as HPLC columns [37]. An HPLC system with immobilized L-AAOD as a postcolumn reactor in combination with chemiluminescence detection has been described [38].

In our work, we also use the optical specificity of immobilized L-AAOD and D-AAOD, but with amperometric detection of the generated hydrogen peroxide. With 0.1 *M* phosphate buffer (pH 7.3), several amino acids, corresponding to the specificity of the oxidase [5,39], were determined after separation on a reversed-phase column (see Fig. 5). The simultaneous determination of both enantiomers with a parallel configuration of two IMERs and a multi-channel flow-through cell should be possible,

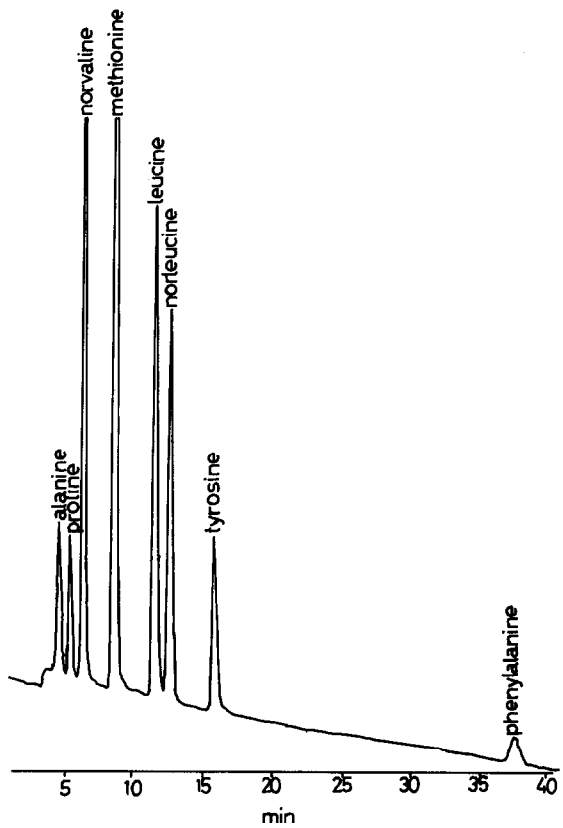

Fig. 5. Chromatography of a standard solution with D,L-amino acids (0.05 m*M*) on an Inertsil ODS-2 column (250 × 4.6 mm I.D.) with a D-amino acid oxidase reactor. Chromatographic conditions: experimental arrangement as in Fig. 1; mobile phase, 0.1 *M* phosphate buffer (pH 7.4); flow-rate, 0.45 ml/min; working potential, 0.6 V; range, 50 nA.

as has been described with a FIA system for sugars, lactate, sulphite and ethanol [40]. Another possibility has been presented for malate and ethanol with a system consisting of two solution lines and a single electrode [41]. A train of two peaks appears in the FIA-trace. To reduce problems in recovery and reproducibility caused by the splitting of the solution line, we prefer alternation of the two amino acid oxidases by means of a switching valve.

Determination of sugars

Another current study is the determination of sugars. Only for glucose and galactose are oxidases available, which produce hydrogen peroxide directly. Galactose oxidase is not very specific, and lactose and raffinose are also suitable substrates.

Other sugars, such as sucrose, maltose and fructose, do not react. Enzymatic hydrolysis of these di- and oligosaccharides and conversion of monosaccharides is necessary before the application of the oxidases [42–44].

Another very sensitive way to determine sugars is high-performance anion-exchange chromatography with pulsed amperometric detection (PAD) [45–50]. However PAD is a universal method, whereas in the method presented the sugars of interest are selectively determined by substrate-specific enzymes.

Glucose. The determination of glucose is accomplished by glucose oxidase (GOD) [7,12]:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{GOD} \text{D-gluconic acid} + H_2O_2 \quad (6)$$

Because GOD is specific only for β-D-glucose, a higher sensitivity can be obtained if GOD is combined with a mutarotase (MUT). MUT catalyses the conversion of α-D-glucose into β-D-glucose [42].

GOD is stable over a wide pH range with maximum activity at pH 5.6. The detection limit in our system is 0.02 ppm (1 p*M* glucose using a 10-μl injection volume).

Sucrose. Sucrose can be hydrolysed in the presence of invertase (IV) to glucose and fructose [42]:

$$\text{sucrose} + H_2O \xrightarrow{IV} \alpha\text{-D-glucose} + \beta\text{-D-fructose} \quad (7)$$

$$\alpha\text{-D-glucose} \xrightarrow{MUT} \beta\text{-D-glucose} \quad (8)$$

Direct conversion of the formed α-D-glucose by

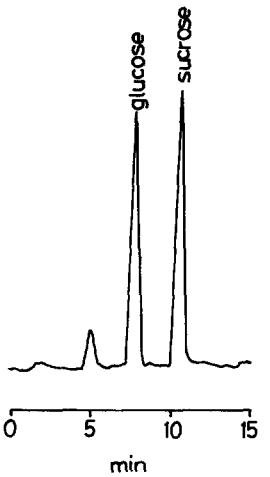

Fig. 6. Separation of glucose and sucrose in a pear juice, diluted 1:40 000, on a Supelcosil LC 18 column (250 × 4.6 mm I.D.) with an invertase, mutarotase and glucose oxidase reactor. Chromatographic conditions: experimental arrangement as in Fig. 2; mobile phase 1, water, flow-rate 0.4 ml/min; mobile phase 2, 0.2 *M* phosphate buffer (pH 6.4), flow-rate 0.3 ml/min; working potential, 0.6 V; range, 50 nA.

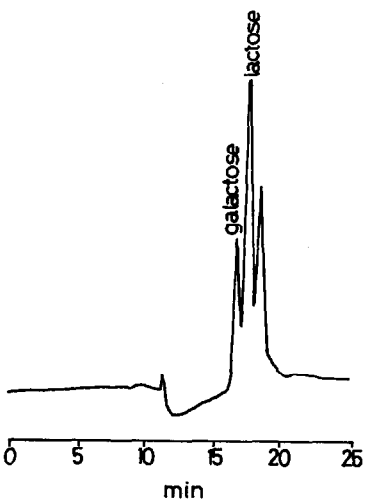

Fig. 7. Separation of galactose and lactose in a soft cheese (130 mg in 100 ml) on a Supelcosil LC 18 column (250 × 4.6 mm I.D.) with a galactose oxidase reactor. Chromatographic conditions: experimental arrangement as in Fig. 2; mobile phase 1, water, flow-rate 0.2 ml/min; mobile phase 2, 0.2 *M* phosphate buffer (pH 6.4), flow-rate 0.25 ml/min; working potential, 0.6 V; range, 50 nA.

GOD to gluconic acid and H_2O_2 (see eqn. 6) is not possible, because GOD is specific for β-D-glucose. For the determination of trace amounts of glucose mutarotase must be used. The detection limit with MUT is 0.1 ppm and without 10 ppm (pH 6.4), corresponding to 3 and 300 p*M* sucrose, respectively, with an injection volume of 10 μl.

Fig. 6 shows the simultaneous determination of glucose and sucrose in a pear juice.

Galactose. The enzymatic conversion is directly possible, because a corresponding oxidase is available [7]. Galactose oxidase (GALOD) reaches maximum activity at pH 5.8 and catalyses the reaction:

$$\text{D-galactose} + O_2 \xrightarrow{\text{GALOD}} \text{D-galactohexodialdose} + H_2O_2 \quad (9)$$

The detection limit is 0.15 ppm (8 p*M* with a 10-μl injection volume).

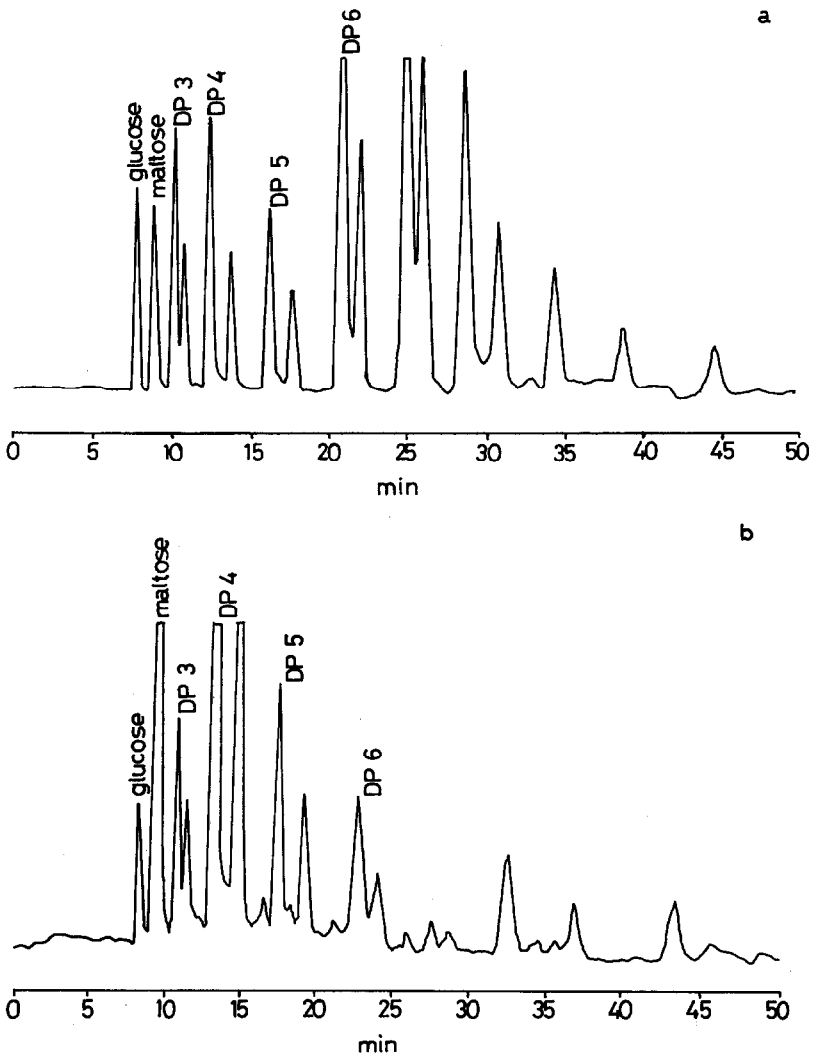

Fig. 8. (a) Chromatography of a standard solution containing 0.06 mg of glucose, 0.08 mg of maltose, 0.14 mg of maltotriose and 3.18 mg of maltooligosaccharides from corn syrup per 100 ml on a Supelcosil LC 18 column (250 × 4.6 mm I.D.) with an amyloglucosidase and glucose oxidase reactor. (b) Separation of glucose, maltose and maltooligosaccharides in a Pils beer, diluted 1:1200, on a Supelcosil LC 18 column (250 × 4.6 mm I.D.) with an amyloglucosidase and glucose oxidase reactor. Chromatographic conditions: experimental arrangement as in Fig. 2; mobile phase 1, water, flow-rate 0.4 ml/min; mobile phase 2, 0.2 *M* phosphate buffer (pH 6.4), flow-rate 0.3 ml/min; working potential, 0.6 V; range, 50 nA.

Lactose. Lactose (β-D-galactose-1→4-D-glucose) can be detected by two different methods.

Hydrolysis of lactose is effected by β-galactosidase, giving galactose and glucose [42]. In a second reaction, glucose can be converted in the presence of GOD (see eqn. 6). The detection limit at pH 7.0 is 0.05 ppm (1.5 p*M* for a 10-μl injection volume).

Likewise, it is possible to determine the galactose with GALOD (see eqn. 9). For the determination using GALOD, because the oxidation with GALOD does not occur at the C-1 position as with GOD, but in the C-6 position [51], enzymatic hydrolysis of lactose to galactose and glucose is not necessary. The detection limit is 5 ppm (150 p*M* for a 10-μl injection).

The separation of galactose and lactose in a soft cheese is shown in Fig. 7.

Maltose. The enzymatic hydrolysis of maltose to glucose can be catalysed by either α-glucosidase or amyloglucosidase (AG). AG hydrolyses α-1,4-glucan linkages in polysaccharides and is therefore not specific for maltose [42]. Nevertheless, AG was most preferred because the sensitivity with AG is much greater and maltooligosaccharides can also be determined (see Fig. 8).

The detection limit is 0.03 ppm (1 p*M* for a 10-μl injection), pH 6.4.

Fructose. Fructose can be converted into glucose with glucose isomerase (GI) [42,43]:

$$\text{fructose} \overset{\text{GI}}{\rightleftharpoons} \text{glucose} \qquad (10)$$

For all of the previous enzymatic sugar reactions, a 0.1 *M* phosphate buffer was taken, whereas the conditions with GI are 5 m*M* Tris buffer (pH 7.5) containing 0.5 m*M* Mg^{2+} and 0.125 m*M* Co^{2+}. To increase the enzyme activity, the GI reactor is heated at 45°C.

Magnesium ions are essential for the glucose isomerase reaction and cobalt ions increase the lifetime of the enzyme, especially when the reactor works at a higher temperature [18]. The magnesium and cobalt ions do not affect the following GOD reactor. Behind the GI reactor, a 0.2 *M* phosphate buffer (pH 6.4) is added by a second pump to the Tris buffer stream (same flow) to change the pH value.

The detection limit is 5 ppm fructose (0.28 n*M* for a 10-μl injection).

CONCLUSION

The method presented offers wide range of applications in the analysis of complex samples. The reagents used are non-toxic and commercially available. The high sensitivity, the specificity of the enzymes and the simple sample pretreatment make the method rapid and inexpensive. The lifetime and stability of the biosensors (often over 1 year) are higher than for FIA techniques. The HPLC separation with reversed-phase or cation-exchange columns allows the simultaneous determination of several substances in various extracts. Additionally, the column protects the enzyme reactor. Currently we are able to determine lactic acid, oxalic acid, ascorbic acid, amino acids and sugars with detection limits in the n*M* to p*M* range. The relative standard deviation in all the cases examined is between 2 and 5%. From the results it can be deduced that the stability of the electrochemical detector is a decisive factor for reproducibility. We are working on further improvements regarding the validity of quantitative results.

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