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Determination of aldehydes in food by high-performance liquid chromatography with biosensor coupling and micromembrane suppressors

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Abstract

A high-performance liquid chromatography system for the determination of aldehydes in food was developed incorporating an Cation MicroMembrane Suppressor (CMMS) and enzyme reactors packed with VA-Epoxy on which aldehyde dehydrogenase from bakers yeast and NADH oxidase from *Bacillus licheniformis* were immobilized. The method was based on the principle that the separation efficiency of HPLC is combined with the sensitivity of electrochemical detection and the specificity of enzymes. Main attention was directed to the determination of 5-hydroxymethyl-2-furaldehyde and 2-furaldehyde, the occurence of which is an indication of quality deterioration in several food products. The efficiency of the method has been shown by the analysis of honey, coffee and related beverages, refreshments, sherry, port, dry fruits and breakfast cereals.

Keywords: Food analysis; Biosensors; Detection, LC; Beverages; Aldehydes; Hydroxymethylfuraldehyde; Furaldehyde

1. Introduction

Due to their sensory relevance, aldehydes are widespread in foodstuffs and important products. These aldehydes are mostly minor compounds in foodstuffs, but have attracted increasingly more attention, since consumer protection and quality control have gained increasing influence. The occurrence of certain aldehydes can be an indication of quality deterioration [1–6], temperature overheating [7–9,11,14], microbacterial fermentation [10] and off-flavor [7,11–14]. Furthermore, some aldehydes are considered to be antimutagenic or mutagenic under certain conditions [15–17]. The classical methods for the determination of some aldehydes are based on colorimetric measurements [1,4,5,8,18]. These methods have not been well established, because they are unspecific, time consuming and make use of hazardous or toxic chemicals. Gas chromatographic techniques have also been employed [11,19,20]. In recent years, the most frequently used analytical technique for aldehydes is high-performance liquid chromatography (HPLC) mostly with UV detection after a suitable derivatization step [18,21–24]. Another different possibility for determination arises from the use of biosensors.

Biological components, such as enzymes, are increasingly used in biosensors as tools in analytical chemistry [25–28]. As mentioned in former publications [29,30], a specific, sensitive and rapid analytical technique is given for the determination of aldehydes by the combination of biosensors with HPLC and following electrochemical detection. Commercially available aldehyde dehydrogenase

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(ALDH) and NADH oxidase (NOD) are therefore immobilized and integrated into an HPLC system behind the column. In its function as a redox enzyme, ALDH depends on a co-factor for activity. In this case nicotinamide adenine dinucleotide (β-NAD) is acting as a soluble co-substrate. Under the catalyzation of ALDH the aldehydes are converted to form corresponding acids and NADH:

$$\text{R-CHO} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\textbf{ALDH}} \text{R-COOH} + \mathbf{NADH} + \text{H}^+$$

The resulting NADH can be determined electrochemically at an oxidative potential larger than 400 mV. A problem associated with measuring NADH is that the high operating potentials required allow the possibility of oxidizing other electroactive compounds present in certain samples. For this reason the method involves coupling the sensing enzyme with a second one. The use of NOD enables the detection of hydrogen peroxide at low potentials at which the electroactive compounds do not occur or are at least significantly reduced:

$$\text{NADH} + \text{H}^+ + \text{O}_2 \xrightarrow{\textbf{NOD}} \text{NAD}^+ + \mathbf{H_2O_2}$$

Therefore, a specific determination of aldehydes in complex food matrices is possible without an extensive sample pretreatment like distillation, derivatization or solid-phase extraction.

According to their polarity the aldehydes must be separated by the use of short-chain alkyl reversed-phases like RP-1, RP-2 or RP-8. The dependence of the pH value of the eluent on the maximum response was examined at pH 8.0, the pH optimum of the ALDH [29,30]. As a result, the major limitation for a continuous operation has been the hydrolysis and lack of long-term stability of the silica based stationary phases under these conditions. Furthermore, the hydrolysis byproducts are probably the cause for enzyme degeneration and loss of activity.

The use of a Cation MicroMembrane Suppressor (CMMS) allows a chemical modification of the mobile phase without substantial loss of sensitivity. So far, this has not been possible by the employment of commercially available dynamic or static mixing chambers and an electrochemical detector. The combination ensures long-term stability by using a neutral phosphate buffer as a mobile phase and high sensitivity by working at the required alkaline pH optimum of the enzyme after pH adjustment.

The present paper describes the HPLC–enzyme reactor coupling as an automated technique for the sensitive determination of aldehydes in foodstuffs and the function of the CMMS.

2. Experimental

2.1. Chemicals

The hydroxymethylfuraldehyde (HMF) and furaldehyde (F) solutions of analytical grade were purchased from Sigma, Fluka (Deisenhofen, Germany). As an eluent, a 40 m*M* phosphate buffer of pH 7.0 was used, consisting of potassium dihydrogenphosphate (analytical-reagent grade, Merck, Darmstadt, Germany) and disodium hydrogenphosphate (analytical-reagent grade, Fluka). The buffer additionally included 2 m*M* of the coenzyme β-NAD (Fluka), 1 m*M* Titriplex III (analytical-reagent grade, Merck) and 40 m*M* potassium chloride (analytical-reagent grade, Merck). As CMMS regenerant we used a 100 m*M* potassium hydroxide (KOH) solution or tetrabutylammonium hydroxide (TBAOH) solution, respectively, both of analytical-reagent grade from Fluka. For the regeneration of the aldehyde dehydrogenase we used a 10 m*M* 2-mercaptoethanol solution (Fluka). Acetonitrile of HPLC grade was also obtained from Fluka. Water was purified with an Elgastat UHG II purification system (Elga, Kaarst, Germany).

2.2. Sample preparation

A small amount (4 g) of breakfast cereals and dry fruits were milled in a ball mill MM 2000 from Retsch (Haan, Germany) followed by liquid nitrogen cooling for 3 min (amplitude 100%). The ground sample (1–2 g) was weighed into a 25-ml flask to which 20 ml of the eluent was added. The supernatants were clarified with 2 ml Carrez I and 2 ml Carrez II solutions. The Carrez clarification reagent consisted of a 85 m*M* solution Carrez I (potassium ferrocyanide, Fluka) and of a 250 m*M* solution of Carrez II (zinc sulfate, Fluka). The sample solution

was then diluted to a total volume of 25 ml with eluent. Folded filters having a diameter of 125 mm ($595^{1}/_{2}$ Schleicher & Schuell, Dassel, Germany) were used for the filtration after the Carrez clarification.

Honey, coffee and related beverages were injected after Carrez clarification, while refreshments, sherry and port did not undergo sample treatment. Before injection, all samples were filtered through a 0.2-μm CM (cellulose acetate) filter (CS-Chromatographie Service, Langerwehe, Germany).

2.3. Enzymes and immobilization

The reaction of the aldehydes was obtained by the use of aldehyde dehydrogenase isolated from bakers yeast (ALDH, EC 1.2.1.5, immobilized enzyme amount: 50–100 U). The ALDH was purchased from Boehringer Mannheim (Mannheim, Germany) or Sigma, Fluka. The developing NADH was oxidized to form hydrogen peroxide by NADH-oxidase isolated from *Bacillus licheniformis* (NOD, EC number not clarified, immobilized enzyme amount: 5–8 U). The NOD was also purchased from Sigma, Fluka. VA-Epoxy E 3 (bead size: 15–25 μm) or VA-Epoxy E 12 (bead size: 15–25 μm) obtained from Riedel-de Haën (Seelze, Germany) was used to support enzyme immobilization. A 40-mg amount of the carrier material was therefore suspended together with the respective enzyme in 4 ml phosphate buffer, and the following reaction was carried out under shaking at room temperature. A 1 *M* potassium phosphate buffer (pH 8.0) was used as a coupling solution. After a reaction time of 12 h the suspension was washed with 0.1 *M* potassium phosphate buffer (pH 8.0) and the support with immobilized enzyme was packed into a reactor cartridge (8.0 mm×6.0 mm I.D.). The reactor cartridge was used as a mini-reactor and placed in a special reactor holder which in turn was directly screwed onto the flowcell. Both – the reactor cartridge and the holder – were available from Trace Biotech (Braunschweig, Germany). The enzyme reactors were stored in 0.1 *M* phosphate buffer (pH 8.0) at 4°C.

2.4. Equipment

The chromatographic system is depicted in Fig. 1 and consisted of two Model 2250 HPLC Compact Pumps from Bischoff (Leonberg, Germany) equipped with polyether ether ketone (PEEK) micro pump heads, additional pulse-dampers (ESA, Chelmsford, USA), a Triathlon Autosampler (Bischoff) fitted with a 100-μl injection loop and a Cation MicroMembrane Suppressor CMMS-II (4 mm) or CMMS (2 mm) from Dionex (Idstein, Germany). A solvent degasser unit Degasys DG-1310 from Uniflows (Tokyo, Japan) for degassing the eluent and the regenerant was used. The detection unit consisted of a pulsed electrochemical detector PED 300 from Biometra (Göttingen, Germany) in combination with a wall-jet flowcell with a silane-modified platinum electrode from Trace Biotech. Moreover, a column oven (Techlab, Erkerode, Germany) and column inlet filters (Knauer, Berlin, Germany) were used. A RP-8 Multosphere 120 analytical column (125×4.0 mm I.D., film thickness 3 μm) with guard column (10×4.0 mm I.D.) were purchased from CS-Chromatographie Service. Chromatographic analysis was carried out within the EZChrom Elite Chromatography Data System, Version 2.0 from Scientific Software (San Ramon, USA).

3. Results and discussion

The utilization of enzymes as the main sensing element requires the optimization of different parameters of the HPLC system. Biological components are inherently created to work in an aqueous-based medium and are not very stable in organic solvents or organic–aqueous mobile phases. The stability of the enzymes varies, depending on the enzyme and the operating conditions. Wessels [31] has investigated an extreme decrease in ALDH activity over a few hours by using acetonitrile amounts higher than 10%. When using ALDH/NOD enzyme reactors as detection unit, we found a very high operational stability by using acetonitrile–40 m*M* phosphate buffer (1.5:98.5, v/v) as the mobile phase over several weeks. Isocratical operation was necessary because the used electrochemical detector was not gradient fit. With respect to retention time, band-broadening effects and signal response a flow-rate of 0.5 ml min^{-1} was investigated. Several experiments

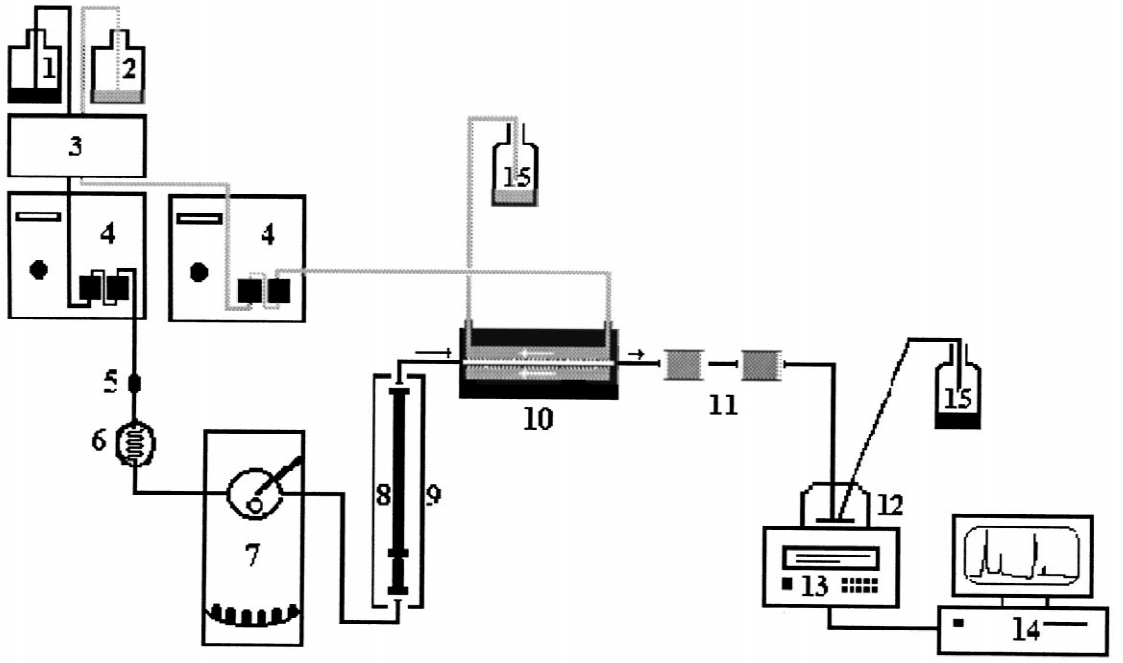

Fig. 1. Scheme of the instrument. 1=Eluent; 2=regenerant; 3=degasser; 4=HPLC pump; 5=inline filter; 6=pulse damper; 7=autosampler; 8=column with guard column; 9=column oven; 10=Cation MicroMembrane Suppressor; 11=enzyme reactors (ALDH/NOD); 12=wall-jet flowcell (with platinum electrode); 13=electrochemical detector; 14=evaluation unit; 15=waste.

using reversed-phase or polymer phases were carried out under these conditions to determine HMF and F. Only short alkyl chain reversed-phases like the RP-8 Multosphere 120 column, e.g., Spherisorb RP-8 (Bischoff) were found to perform fast separations and fulfil the chromatographic requirements. The method was, however, limited by the instability of these silica-based columns with time by using the alkaline phosphate buffer. However, after ~400–500 working hours and opening these columns it was determined that the packing on the front of the column had partly gone – due to hydrolysis of the silica support. Non silica-based phases did not reveal the required separation efficiency. As mentioned [31], the ALDH activity and reaction ratio depended on the pH value. A pH range above or below the pH optimum 8.0 resulted in less than 50% of maximal activity [32]. However, as argued above, for a better operational stability of the column a phosphate buffer with a pH value of $\leq$7.0 was needed and on the other hand for a high sensitivity a pH value of 8.0 was necessary. A pH adjustment using a multitude of commercially available dynamic or static mixing chambers was not suitable without negative consequences on the baseline, huge losses of detection sensitivity or destruction of the preceeding separation. As described below, it can be clearly seen that the use of a Cation MicroMembrane Suppressor in conjunction with HPLC–biosensor coupling is well adapted to ensure optimum performance.

3.1. Cation MicroMembrane Suppressor

Micromembrane suppressors have normally been used as a neutralization and selective desalting unit in combination with ion chromatography and suppressed conductivity detection [33,34]. For the first time we have investigated in this report the potential use of a Cation MicroMembrane Suppressor as a suitable unit for pH adjustment. Anion micromembrane suppressors in combination with enzyme reactors were also used in our working group [35]. Fig. 2 shows the schematic construction of a micromembrane suppressor [33]. The CMMS consists of an eluent compartment and two regenerant compartments separated by anion-exchange membranes. Two

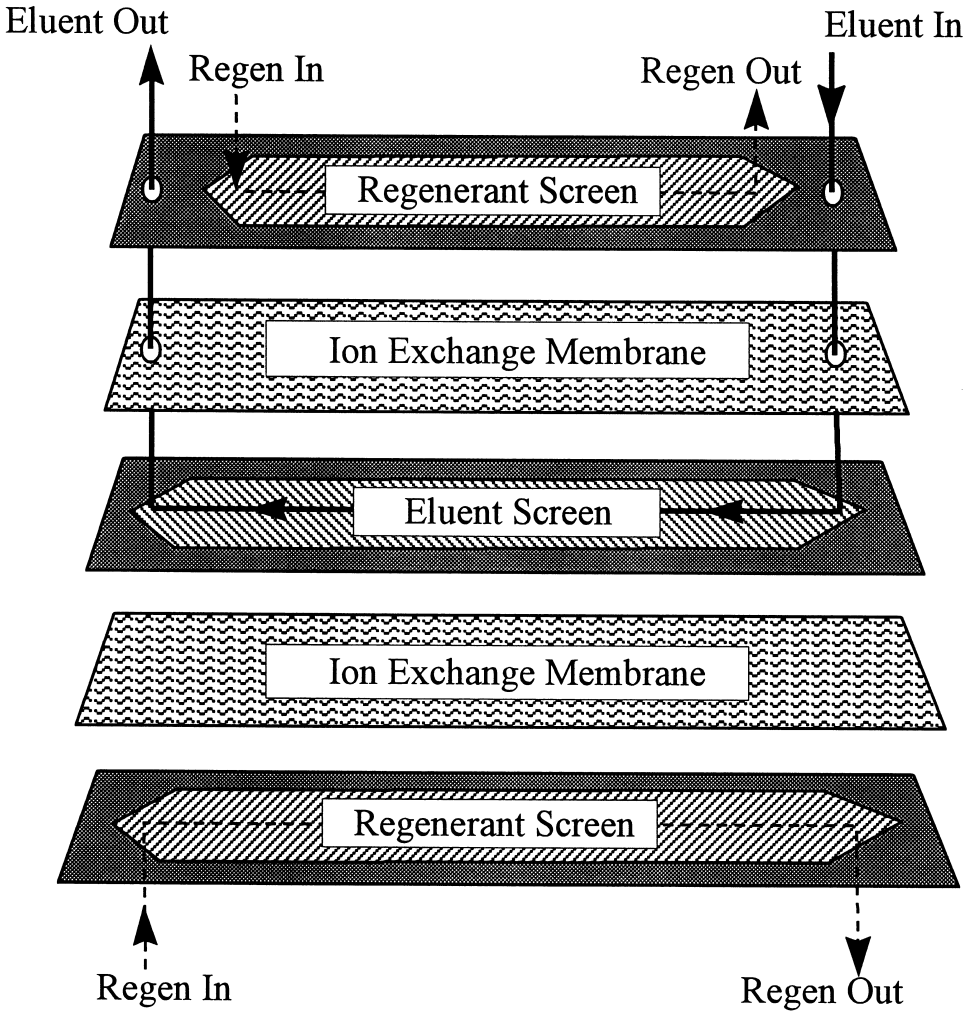

Fig. 2. Schematic construction of a MicroMembrane Suppressor (MMS).

outer regenerant flow channels and an inner eluent flow channel are defined on opposite sides of the membrane. The regenerant is delivered continuously by a HPLC pump in the countercurrent direction of the eluent flow. As an eluent we use the 40 m*M* acetonitrile–phosphate buffer (1.5:98.5, v/v) (pH 7.0) and as regenerants in the chemical suppression mode 100 m*M* KOH and 50 m*M* TBAOH, respectively. The hydroxide ions in the regenerant cross the anion-exchange membranes and combine with the eluent cations, in this case mainly sodium ions. By this reaction the formation of NaOH leads to an alkaline pH value. As demonstrated in Fig. 3 the eluent phosphate anions simultaneously cross the membranes into the regenerant chambers to maintain charge balance.

The influence of dead volume on the analytical system was tested by using CMMS-II 4-mm format and a CMMS 2-mm format in comparison with a system without the suppressor technique. As shown in Fig. 4, both suppressors, the CMMS-II (dead volume 50 μl) and especially the CMMS (dead volume 12.5 μl) cause a minimum of band-broadening effect. Due the reduced eluent flow-rates used on HPLC–biosensor coupling, the equivalent suppression capacity of the 2 mm version was, however

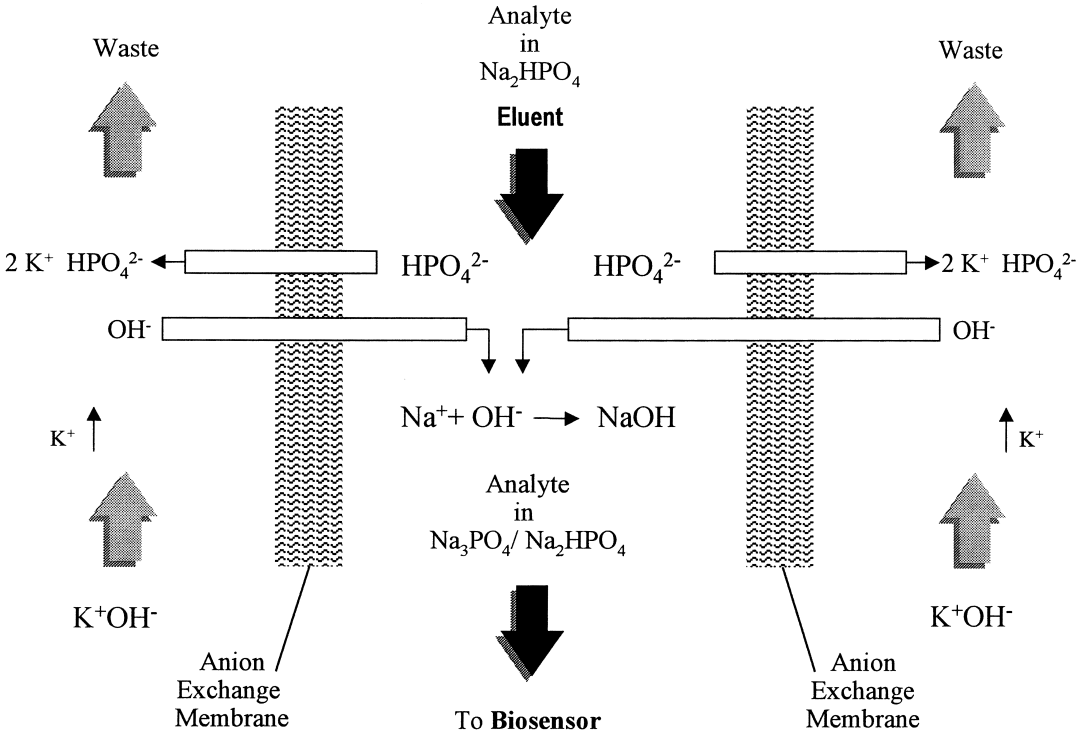

Fig. 3. Chemical suppression with the Cation MicroMembrane Suppressor (CMMS 2 mm).

greater than the suppression capacity achieved with the 4 mm version. The dependence of the suppression capacity on several parameters was investigated by choosing different regenerants and regenerant flow-rates. To ensure a continuous suppression, attention was paid to bubbles and to the fact that the membranes were fully hydrated. In this case – while changing the regenerant – the suppressors had been left for at least 15 min before pumping eluent through the suppressor. Generally, the use of low regenerant flow-rates caused higher molarities for an optimum performance. A pH adjustment was made by using the 100 m*M* KOH solution and permanent pH measurement with a pH meter 761 calimatic equipped with an Ag/AgCl electrode (Knick, Berlin, Germany). It will probably take some time to find the required conditions and to adjust the desired pH value exactly. In contrast, a continuous working without further regulation was possible for several days. It is evident that the suppressor technique is also well adapted for pH adjustment in conjunction with other applications.

3.2. Determination of HMF and F in foodstuffs

A general trend in the development of sophisticated and highly selective detection techniques has been observed over the last years. Especially in food analysis a demand for the determination of progressively smaller amounts of compounds in complex matrices increased. Furthermore, automated systems for the detection of large numbers of samples were required. As described in this study, the use of the ALDH/NOD biosensor enables an increase in the selectivity for the detection step without further chromatographic requirements. Electroactive disturbance compounds which probably eluted at the same retention time as the corresponding substrate could be located by making an enzyme-free measurement. In this case the HPLC–biosensor coupling offers an additional possibility for the examination of results other chromatographic methods do not contain. A further advantage of this method is the possibility of on-line monitoring of both, HMF and F, in a single run with a simple and

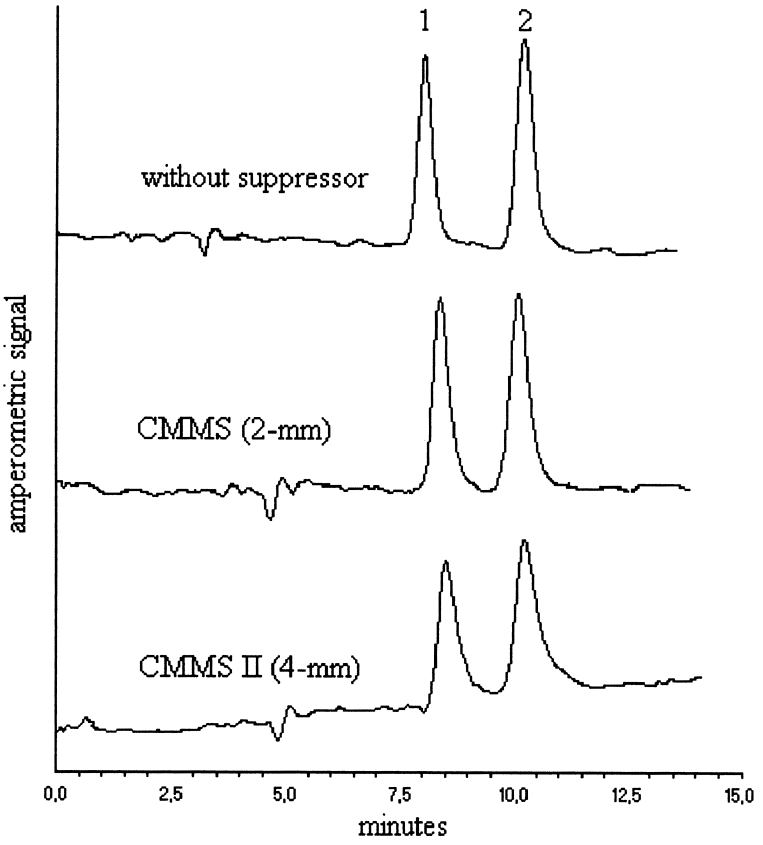

Fig. 4. Chromatographic recording of the separation of HMF (peak 1) and F (peak 2) using the set-up shown in Fig. 1. Conditions: ALDH 100 U, NOD 8 U; eluent: 40 m*M* acetonitrile–phosphate buffer (1.5:98.5) (pH 7.0), flow-rate: 0.50 ml min^{-1}; regenerant: 100 m*M* KOH, flow-rate: 0.23 ml min^{-1}; operational potential: 200 mV; injection volume: 20 μl; temperature: 30°C. Omitting suppressor: same as above; eluent: pH 8.0.

fast separation. Fig. 5 and Fig. 6, for example, show a typical separation obtained on a commercial sample of sherry or instant coffee powder, respectively. Quantification was made by frequently repeated external standard runs. Table 1 shows the results for a determination of HMF and F in some foodstuffs.

3.3. Calibration

The calibration graphs were obtained by employing standard solutions of HMF and F over the range of concentrations from 0.05 to 2 mg/l under optimum experimental conditions as described in the preceding sections. This range represents values typically found in real samples. Coefficients of regression of >0.9995 were obtained for both aldehydes, HMF and F. The detection limits determined by the German Industrial Standards (Deutsche Industrie Norm DIN 32645) [36] are therefore 0.06 mg/l for F and 0.1 mg/l for HMF using a 20 μl injection volume. It is, however, difficult to indicate a general detection limit, since the detection limit can differ depending on the enzyme activity. In comparison with other aldehydes, especially the determination of HMF has to rely on a high enzyme activity by using relatively new ALDH or ALDH regenerated with 2-mercaptoethanol.

3.4. Shelf life, recovery and reproducibility

Shelf life of the enzyme reactors was determined by measuring define standard solutions frequently. It

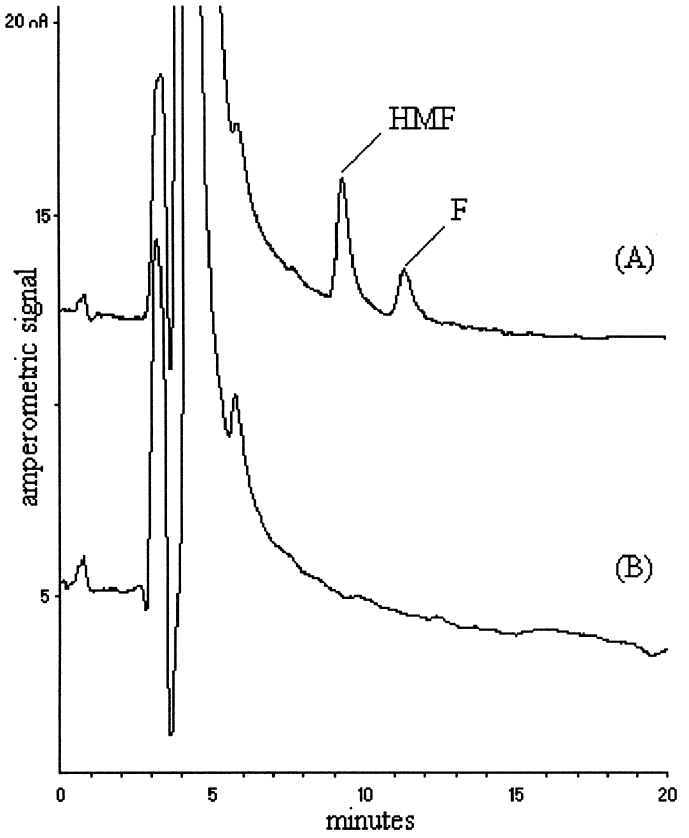

Fig. 5. Determination of HMF and F in sherry. Chromatogram obtained when injecting the sherry (1:10 dilution) into a system (A) with and (B) without using the ALDH/NOD reactor. Amounts: HMF 23.5 mg/l, F 1.9 mg/l. For sample treatment and further details, see Experimental.

was found that the shelf life of the NOD reactor is much longer than that of the ALDH reactor. Sufficient activity was even observed by the NOD reactors after one year, while the ALDH reactors show no sufficient activity after one to two months depending of the analytes.

Recovery was evaluated by adding known amounts of HMF and F to a sample of honey or a solution of instant coffee powder, respectively. It was observed, that the recovery value will differ depending on the structure of the actual analytes. Recoveries for both analytes ranged from 93 to 102% and no significant loss was observed during the clarification step by employing the Carrez solution.

Reproducibility of the biosensor unit was investigated for 24 h continuous sampling of standards and samples with good results showing no drastic decrease by the determination of F. Moreover the determination of HMF was remarkably less precise, because of the strongly depend on high enzyme activity. Reproducibility was evaluated by carrying out the determination seven times on the same standard sample of HMF (2.0 mg/l) and F (2.4 mg/l); each solution was injected twice. The relative standard deviation (RSD) of HMF was 2.0% and of F was 1.1% [37].

4. Conclusions

The described HPLC–biosensor system allows the analysis of aldehydes like HMF and F present in different kind of foodstuffs over a wide range of

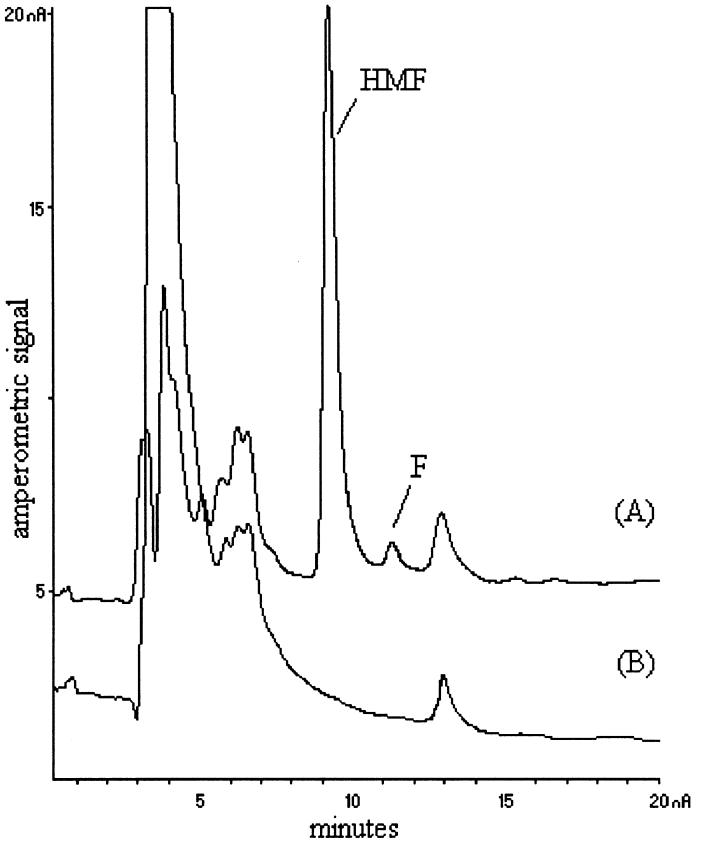

Fig. 6. Determination of HMF and F in instant coffee powder. Chromatogram (A) with the ALDH/NOD reactor, (B) without enzymes. Amounts: HMF 4176.2 mg/kg, F 49.1 mg/kg. For sample treatment and further details, see Experimental.

concentrations. The technique uses enzyme-based biosensors and electrochemical detection as a detection system in combination with Cation MicroMembrane Suppressor and a silica-based chromatographic separation system. The improved use of Cation MicroMembrane Suppressor as a

Table 1
Concentrations of HMF and F found in some commercial foodstuffs (n = number of samples)

Sample	n	HMF (mg/kg)		F (mg/kg)	
		Minimum value	Maximum value	Minimum value	Maximum value
Coffee powder	5	209	606	70	160
Instant coffee powder	8	959	6181	14	95
Honey	15	<0.1	41	*	0.5
Dry fruits	2	114	1196	*	9
Breakfast cereals	2	26	44	*	*
		HMF (mg/l)		F (mg/l)	
Sherry	2	3	24	1	2
Refreshments	4	1.8	2.6	*	*
Fruit juice	3	<0.1	0.7	0.3	5.9

suitable unit for pH adjustment can easily be applied not only in this system but in other as well. More over the described system can be utilized for the separation of other aldehydes using different silica-based phases without loss of operational stability. However, a specific, sensitive and rapid analytical monitoring system has been developed for food analysis.

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