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Enzyme amplification as detection tool in continuous-flow systems II. On-line coupling of liquid chromatography to enzyme-amplified biochemical detection after pre-column derivatization with biotin

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Abstract

Enzyme-amplified biochemical detection (EA-BCD) was used as a post-column detection technique, coupled on-line with high-performance liquid chromatography (HPLC). The enzyme detection system was developed to detect biotin or biotin containing compounds. Biotinylation is widely used to label analytes of interest ranging from small molecules to proteins and DNA. Naphthalene aldehyde and anthracene aldehyde were used as model compounds. Both compounds were biotinylated off-line with biotin aminocaproic hydrazide (BACH). On-column biotinylation was performed by preconcentration of anthracene aldehyde on copper phthalocyanine. After biotinylation, samples were introduced to the HPLC system. Enzyme-labeled streptavidin, which possesses high affinity to biotin, was added post-column to the HPLC effluent. Excess of enzyme-labeled affinity protein was removed by means of an immobilized biotin column. After separation of free and bound fraction, substrate was added, which was converted to a fluorescent product by the enzyme label. Using alkaline phosphatase as an enzyme label, a mass detection limit after on-column preconcentration and biotinylation of 250 fmol was achieved.

Keywords: Enzyme amplification; Derivatization, LC; Detection, LC; Biotin; Alkaline phosphatase

1. Introduction

The demands of analytical techniques are increasing in almost all fields of chemistry. Therefore, modern analytical techniques have to be developed with high sensitivity, high selectivity, high sample throughput and which are able to determine multiple analytes in a sample. Nowadays, one of the most sensitive detection methods involves enzyme amplification. The use of enzymatic detection systems is attractive due to the phenomenon that each enzyme converts a manifold of substrate molecules into product molecules. The amplification step offers the possibility to determine analytes at low concentration level. The advantages of enzyme-amplified biochemical detection (EA-BCD) in continuous-flow systems compared to enzyme assays in microtiterplates are improved reproducibility, ease of automa-

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tion and high sample throughput. Furthermore, EA-BCD in continuous-flow system offers the possibility for on-line coupling to high-performance liquid chromatography (HPLC).

Several EA-BCD systems coupled to flow-injection analysis (FIA) have been described in the last years [1–4]. Detection was based on the interaction of analytes or haptens to enzyme-labeled antibodies. The incubation of analytes with enzyme-labeled antibodies was performed off-line [1–3] as well as on-line [4]. After the incubation, free and analyte-bound fractions were separated, followed by on-line addition of substrate, which can be converted by the enzyme label to a detectable product.

A limitation of enzyme assays in microtiterplates or coupled to FIA is cross-reactivity of other compounds to antibodies, which is characteristic for most biological assays [5]. In batch or FIA assays, it is not possible to distinguish the analytes from other compounds, which have affinity to the antibody used. Therefore, the presence of cross-reactive compounds will lead to erroneous results. Another problem arises when analyzing samples, which contain several analytes of interest. An additional technique, for example HPLC is required to separate the analytes and to avoid cross-reactivity. However, direct coupling of a separation technique to enzyme assays in microtiterplates leads to problems with the reproducibility and is difficult to automate. Therefore, the on-line coupling of HPLC to EA-BCD is preferred.

The overall strategy of the present study, which was introduced previously [6], is the development of an uniform EA-BCD system coupled post-column to HPLC, which responds to compounds which can used to label analytes of interest. Such a labeling compound behaves as a universal probe.

The advantage of EA-BCD that responds to a universal probe is that the development of antibodies for the detection of analytes can be omitted. Furthermore, most antibodies are specific to only a few (cross-reactive) analytes. Analyzing multiple (non-cross-reactive) analytes in a sample needs the preparation of multiple antibodies to the analytes. Preparation, isolation and enzyme labeling of antibodies are laborious and expensive procedures, which can be avoided when a universal probe is applied. We have previously described the development of EA-BCD coupled on-line to FIA [6]. The system was developed to detect biotin-containing compounds and was based on the interaction of biotin with enzyme-labeled streptavidin or anti-biotin Fab fragments. Biotin was chosen as universal probe because the high affinity of biotin for avidin and streptavidin ($K_D = 10^{-15}$ M) [7,8]. Furthermore, biotinylation is widely used as a labeling procedure for analytes [9–11].

One of the most utilized biotinylation procedure is the labeling of proteins and peptides with biotinyl-*N*-hydroxysuccinimidyl ester (BNHS) [12,13]. The succinimidyl ester is reactive to free amino groups, and is therefore linked to the N-terminal of the protein. Furthermore, BNHS is linked to proteins via the ε-amino groups of lysine, resulting in biotinylated proteins with multiple biotin residues. The multiple labeling will enhance the signal due to the fact that several, enzyme-labeled streptavidin molecules will interact with the biotinylated proteins. Numerous examples of biotinylation of proteins with BNHS are given in different reviews [9,10,12,14]. Next to BNHS, biotinyl-*p*-nitrophenyl esters can be used for the biotinylation for proteins and peptides via their free amino groups [12,15]. The interaction between the affinity protein and biotin can be improved by the use of an extended spacer arm between the biotin moiety and the reactive probe [11,13].

Alternatively, biotin hydrazide can be used to label glycoproteins after oxidation of the sugar moieties [12,16,17]. Likewise, this procedure can be applied to oligosaccharides [18] and other carbohydrates [19]. In addition to the procedure described, more specific labels for the biotinylation of proteins are available. Biotinylation of thiols, carboxylic acids, tyrosyl and histidyl amino acid side chains of proteins are reviewed by Bayer and Wilchek [10,12]. However, these biotinylation procedures are not often applied.

Besides protein labeling, another interesting subject is the biotinylation of DNA and RNA. BNHS can be used to label nucleic acids according to the procedures used for the biotinylation of proteins [20,21]. However, a more selective and efficient biotinylation can performed with the use of enzymes [22–24]. Basically, ribonucleotides or deoxyribonucleotides are biotinylated via their nucleobase. Next, the biotinylated nucleotides are added to oligonu-

cleotides via the phosphate group by enzymes. The advantages of enzyme induced biotinylation, compared to chemical derivatization, are the improved selectivity, controllability of the reaction and the increased efficiency the enzyme reaction.

Although most applications concern the biotinylation of biopolymers, biotinylation can be also employed in the labeling of small molecules. Our interest was the development of an analytical method to detect polycyclic aromatic hydrocarbon–DNA adducts (PAH–DNA), PAH–protein adducts or PAH derivatives in biological samples. Nowadays, detection of PAH–DNA adducts at low concentration level is done by ^{32}P labeling [25–27]. A limit of detection (LOD) of 1 adduct/10^8 unmodified nucleotides, corresponding to 0.3 fmol, was achieved by autoradiography at −90°C for one to four days [27]. While providing extremely low detection limits, the ^{32}P method is very laborious and time-consuming and involves radioactive materials. Therefore, the development of a relative fast, non-radiographic analytical method for the detection of PAH metabolites and PAH adducts at low concentration level, to monitor humans exposed to PAHs, is desirable. Although the described system is focused on the analysis of PAH metabolites, other compounds can be analyzed with EA-BCD after biotinylation.

A crucial part of the universal procedure is the removal of excess biotinylation reagent after the labeling procedure. A very selective sample clean-up is based on the interaction of PAHs with copper phthalocyanine. Copper phthalocyanine (CP) forms hydrophobic ''face-to-face'' complexes in aqueous solutions with compounds, which contain three or more aromatic fused rings. These complexes are dissociated by organic solvent [28]. CP, immobilized on silica (CP-silica), was used in the on-line coupling of solid-phase extraction (SPE) to HPLC, for the determination of PAHs in surface water [29]. Furthermore, CP-silica was used for the isolation of PAH metabolites from urine [30] and for the analysis of PAH–DNA adducts from DNA hydrolysate [31]. From these applications, it can be deduced that CP is a very group-specific sorbent, able to preconcentrate PAHs and PAH derivatives, while the (biological) matrix can be removed completely.

The present paper describes the on-line coupling of HPLC to EA-BCD after pre-column derivatization. The EA-BCD system used, was described in a previously paper [6] and can be coupled to HPLC without further adaptation. Naphthalene aldehyde and anthracene aldehyde were used as model compounds. The aldehyde functionality of both compounds can be biotinylated with biotin aminocaproic hydrazide (BACH) in solution. After biotinylation an extensive SPE sample clean-up is necessary to remove excess BACH, which is a crucial part of the procedure. A problem arises when analytes are labeled at low concentration level. The kinetics of reactions becomes slower at low concentration level, which can not be compensated by increasing the concentration of the labeling reagent. Therefore, biotinylation of anthracene aldehyde was also performed on-column to preconcentrate the analyte prior derivatization. On-column derivatization has the advantage over in solution derivatization of improved reaction kinetics, providing higher yields [32,33]. Streptavidin and anti-biotin Fab fragments, both labeled with alkaline phosphatase (AP) were examined for EA-BCD. Monitoring of the enzyme product was done by fluorescence detection.

2. Experimental

2.1. Chemicals

Anthracene aldehyde was obtained from Acros (Geel, Belgium). BACH was purchased from Sigma (St. Louis, MO, USA). Alkaline phosphatase labeled streptavidin (S-AP) was from Pierce (Rockford, IL, USA). Sodium acetate, citric acid, sodium chloride, sodium nitrate and Tween 20 were all purchased from Merck (Darmstadt, Germany). Naphthalene aldehyde and tris-(hydroxymethyl)-aminomethane (Tris) were acquired from Aldrich (Steinheim, Germany). Methanol and diethanolamine (DEA) was from Mallinckrodt Baker (Deventer, The Netherlands). Acetonitrile (HPLC grade) was obtained from Rathburn (Walkerburn, UK). Attophos substrate set and alkaline phosphatase-labeled Fab fragments from monoclonal anti-biotin antibody (Fab-AP) were from Boeringer Mannheim (Mannheim, Germany). All aqueous solutions were prepared with water purified with a Milli-Q system from Millipore (Bedford, MA, USA). All solutions were filtered through a 0.2-μm

cellulose acetate filter (Sartorius, Groningen, The Netherlands) prior to use.

2.2. Pre-column set-up and preconcentration procedure

A coupled-column HPLC system with two pre-columns, Fig. 1, was used for preconcentration of off-line biotinylated anthracene aldehyde as well as for the on-column biotinylation of anthracene aldehyde. The biotinylation procedures are described the next section. The HPLC system consisted of three HPLC pumps. A microgradient pump from Brownlee (Applied Biosystems, Santa Clara, CA, USA) was connected to an Aspec XL autosampler equipped with a 402 dilutor from Gilson (Villiers-Le-Bel, France). Two Rheodyne six-port injection valves (Cotati, CA, USA) were implemented in the autosampler. A CP-silica pre-column (5×4 mm I.D.) was connected to the first injection valve. The pre-column was packed with CP trisulfonic acid modified silica (30 μm). The silica particles were made of restricted access material with a molecular mass cut-off of 15 000 and was a gift from Professor Boos (University of Munich, Munich, Germany).

Sample introduction and flushing of the CP-silica pre-column was performed with the 402 dilutor. Rinsing the CP-silica pre-column with 6 ml acetonitrile–water (10:90, v/v) followed sample loading of off-line biotinylated anthracene aldehyde. After sample clean-up, the first injection valve was switched to the inject position and the biotinylated anthracene aldehyde was desorbed with 1 ml acetonitrile at a flow-rate of 70 μl/min delivered by pump 1. The desorbed analyte was diluted on-line with water added via a mixing union by a spectroflow 400 HPLC pump (Kratos, Rotterdam, The Netherlands) at a flow-rate of 630 μl/min. The sample was re-concentrated on a 5×1 mm I.D. pre-column packed with polygosil C_{18} silica (40–63 μm, Macherey-Nagel, Düren, Germany), which was connected to the second injection valve. The overall preconcentration procedure on the dual pre-column system takes 25 min. Finally, the second valve was switched to the inject position and the C_{18} pre-column was connected on-line with an Alltima C_{18} analytical column (150×1 mm I.D., Alltech, Breda, The Netherlands). The pre-column was desorbed with the LC mobile phase, methanol–water (50 μl/min, 70:30, v/v), which was delivered by a Merck 655-12A pump controlled by a L-5000 controller (Darmstadt, Germany). Desorption of the pre-column was performed in a back-flush direction to avoid peak broadening. During the HPLC analysis, a new sample can be pretreated on the CP-silica pre-column.

The LC column was connected to the EA-BCD

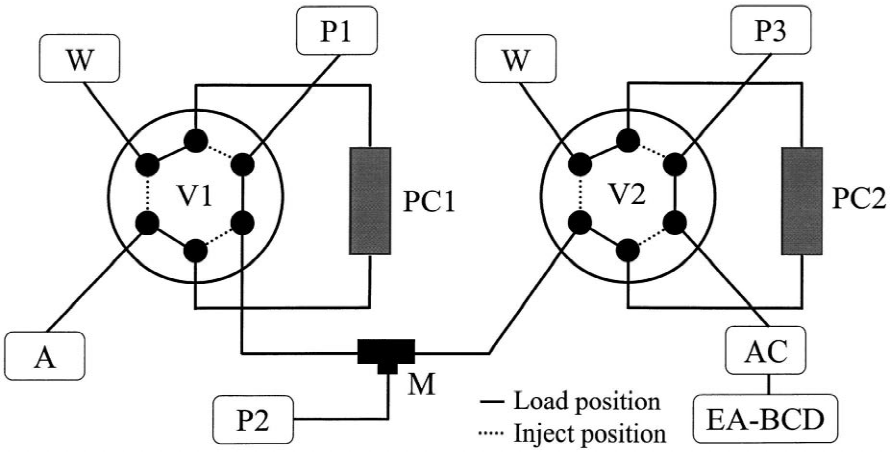

Fig. 1. Schematic representation of the dual-pre-column set-up coupled to liquid chromatography. A=Autosampler and dilutor, V1=first injection valve, V2=second injection valve. PC1=CP-silica pre-column (4×5 mm I.D.), PC2=C_{18} pre-column (1×5 mm I.D.), P1=pump 1, 100% acetonitrile, 70 μl/min. P2=Pump 2, water, 630 μl/min, P3=pump 3, 50 μl/min, methanol–water (70:30, v/v), M=mixing union, AC=analytical column, 150×1 mm I.D., W=waste, EA-BCD=enzyme-amplified biochemical detection, see Fig. 3.

system. A 2×0.8 mm I.D. C_{18} micro guard column (LC Packings, Amsterdam, The Netherlands) was connected to protect the analytical column. For monitoring of the off-line biotinylation reaction the LC column was coupled to a 119 UV–Vis detector from Gilson.

The same set-up was applied for the preconcentration of biotinylated naphthalene aldehyde. However, the CP-silica pre-column was replaced by a 100 μl injection loop. After filling the injection loop with biotinylated naphthalene aldehyde, the first injection valve was switched and the analyte was concentrated on a C_{18} pre-column (5×1 mm I.D.). Analyte transfer from the 100 μl injection loop to the C_{18} pre-column was performed with a spectroflow 400 HPLC pump at a flow-rate of 650 μl/min. On-line dilution of the analytes with water, as described for the dual pre-column set-up was omitted. Next, the C_{18} pre-column was rinsed with 6.5 ml acetonitrile–water (10:90, v/v) to remove excess BACH. Desorption of the C_{18} pre-column and HPLC analysis was performed with 65:35 (v/v) methanol–water at a flow-rate of 50 μl/min.

2.3. Derivatization procedures

During sample handling, naphthalene aldehyde and anthracene aldehyde should be protected from light, because of the instability of the aldehyde functionality. For the biotinylation of naphthalene aldehyde, 100 μl sample in acetonitrile was diluted with 900 μl methanol–100 mmol/l sodium acetate (10:90, v/v, pH 5.6). Next, 0.5 mg BACH was added. The reaction was performed in the dark for at least 1 h under continuous stirring. The biotinylated naphthalene aldehyde was diluted with acetonitrile–water (10:90, v/v) prior to sample introduction.

Anthracene aldehyde was biotinylated off-line as well as on-column. For the off-line procedure, 100 μl anthracene dissolved in acetonitrile was diluted with 900 μl methanol–50 mmol/l citric acid (10:90, v/v, pH 3.0). 0.5 mg BACH was added to the solution and allows reacting for at least 1 h in the dark under continuous stirring. The reaction mixture was further diluted with acetonitrile–water (10:90, v/v) prior to sample introduction. After off-line biotinylation, the products were confirmed by mass spectrometry. Both reactions, resulted in a single reaction product.

The on-column biotinylation was performed directly on a 5×4 mm I.D. CP-silica pre-column. The time schedule of the on-column procedure is given in Table 1. The 402 dilutor was applied for the preconcentration and biotinylation procedure at a flow-rate of 0.5 ml/min (unless otherwise stated). Prior to sample introduction, the CP-silica pre-column was flushed with 1 ml acetonitrile and 1 ml acetonitrile–water (10:90, v/v). Then, 1 ml anthracene aldehyde solution was loaded followed by 1 ml acetonitrile–water (10:90, v/v). Next, the CP-silica pre-column was equilibrated with 1 ml 50 mmol/l citric acid, pH 3.0 containing 10% methanol (biotinylation buffer). Biotinylation was performed by rinsing 2 ml 50 μg/ml BACH in biotinylation buffer at a flow-rate of 0.1 ml/min. After biotinylation, the CP-silica pre-column was flushed with 2 ml of biotinylation

Table 1
Time schedule of the on-column preconcentration and biotinylation procedure

Step (min)	CP-silica pre-column position	C_{18} pre-column position	Action
1 (0)	Load	Inject	Rinse CP-silica with 1 ml acetonitrile and 1 ml acetonitrile–water (10:90, v/v)
2 (5)			Load sample
3 (8)			Rinse CP-silica with 1 ml acetonitrile–water (10:90, v/v) and with 1 ml biotinylation buffer
4 (13)			Rinse CP-silica with 2 ml BACH, 50 μg/ml in biotinylation buffer, flow-rate=0.1 ml/min.
5 (34)			Rinse CP-silica with 2 ml biotinylation buffer and 6 ml acetonitrile–water (10:90, v/v)
6 (52)		Load	Equilibrate C_{18} pre-column
7 (54)	Inject		Desorb CP-silica with 1 ml acetonitrile (70 μl/min) and dilute on-line with water Re-concentrate sample on C_{18} pre-column
8 (69)		Inject	Desorb sample from C_{18} pre-column, followed by HPLC analysis and EA-BCD
9 (70)	Load		Start new procedure during the analysis of the previously biotinylated sample

buffer and 6 ml of acetonitrile–water (10:90, v/v) to remove excess BACH. Finally, the biotinylated anthracene aldehyde was transferred to the analytical column according to the procedure described in previous section. The on-column preconcentration, biotinylation and analyte transfer was fully automated and was performed by the autosampler.

2.4. Enzyme-amplified detection coupled on-line to liquid chromatography

The EA-BCD system which was described in detail previously [6] was used with some minor modifications. Samples were introduced via a 1-μl loop, connected to the second injection valve, or via the pre-column system. To the LC effluent (50 μl/min), S-AP (3.0 U/l) or Fab-AP (7.5 U/l) in carrier buffer was added via a mixing union at a flow-rate of 170 μl/min by a Micro-star K-100 pump (Knauer, Berlin, Germany). The carrier buffer (pH 7.5) consists of 20 mmol/l Tris, 150 mmol/l sodium chloride, 150 mmol/l sodium nitrate and 0.5% (v/w) Tween 20. The incubation of analytes with the affinity proteins was performed in a PTFE-knitted reaction coil with a volume of 351 μl (250 μm I.D.). Excess affinity protein was separated from the bound fraction by means of a 5×2 mm I.D. column, containing immobilized biotin (see Ref. [6] for biotin immobilization procedure). After the separation of the bound and the unbound fraction, substrate was added via a second mixing union at a flow-rate of 90 μl/min by a minipulse 3 peristaltic pump from Gilson. The substrate buffer consists of 10 μ*M* Attophos in 0.1 mol/l DEA, pH 9.9. The enzyme–substrate reaction was performed in a 955 μl PTFE-knitted reaction coil (500 μm I.D.) at 50°C, followed by fluorescence detection. The affinity protein solutions and substrate solutions were prepared freshly every day.

3. Results and discussion

3.1. Design of the analytical system

Biotin containing compounds, eluting from the LC column, are incubated on-line with AP-labeled affinity protein in a reaction coil. The reversed-phase LC effluent contains organic modifier which can disturbed the immuno reaction between biotin and the affinity protein [34,35]. Therefore, a mixing ratio of LC effluent and carrier buffer is chosen, resulting in a maximum organic modifier content of 15% methanol after mixing. This amount of methanol has no influence of the immunoreaction (results not shown). Excess affinity protein is separated from the biotin-bound fraction by means of an immobilized biotin column. In principle, the interaction of biotin with affinity protein must be complete before reaching the immobilized biotin column. Ideally, only biotin-bound affinity protein passes the column, while excess affinity protein is totally trapped by the immobilized biotin. Next substrate is added via a mixing union. Substrate is converted to a fluorescent product by the enzyme label in a second reaction coil. The EA-BCD system was optimized for FIA of BACH [6]. However, the interaction can be influenced by steric hindrance of the biotinylated product.

3.2. Choice of affinity protein

To investigate the response of biotinylated compounds to EA-BCD, both S-AP and Fab-AP were examined. Fab-AP and S-AP were examined with EA-BCD by 1 μl injection of off-line biotinylated anthracene aldehyde diluted to a concentration of $1 \cdot 10^{-7}$ *M*. The chromatograms obtained with both affinity proteins are shown in Fig. 2a and b. The peak area of excess BACH observed when Fab-AP was used as affinity protein was approximately two-times higher compared to the peak area observed with S-AP. The higher response of BACH for Fab-AP was also observed during the optimization of the EA-BCD system [6]. However, the response of biotinylated anthracene aldehyde for Fab-AP was 37% lower than for S-AP. The difference in response for the biotinylated product is probably caused by steric hindrance of the product for both affinity proteins. The affinity of the biotinylated product is more negatively influenced for Fab-AP than for S-AP. Consequently, S-AP was used as affinity protein in further experiments.

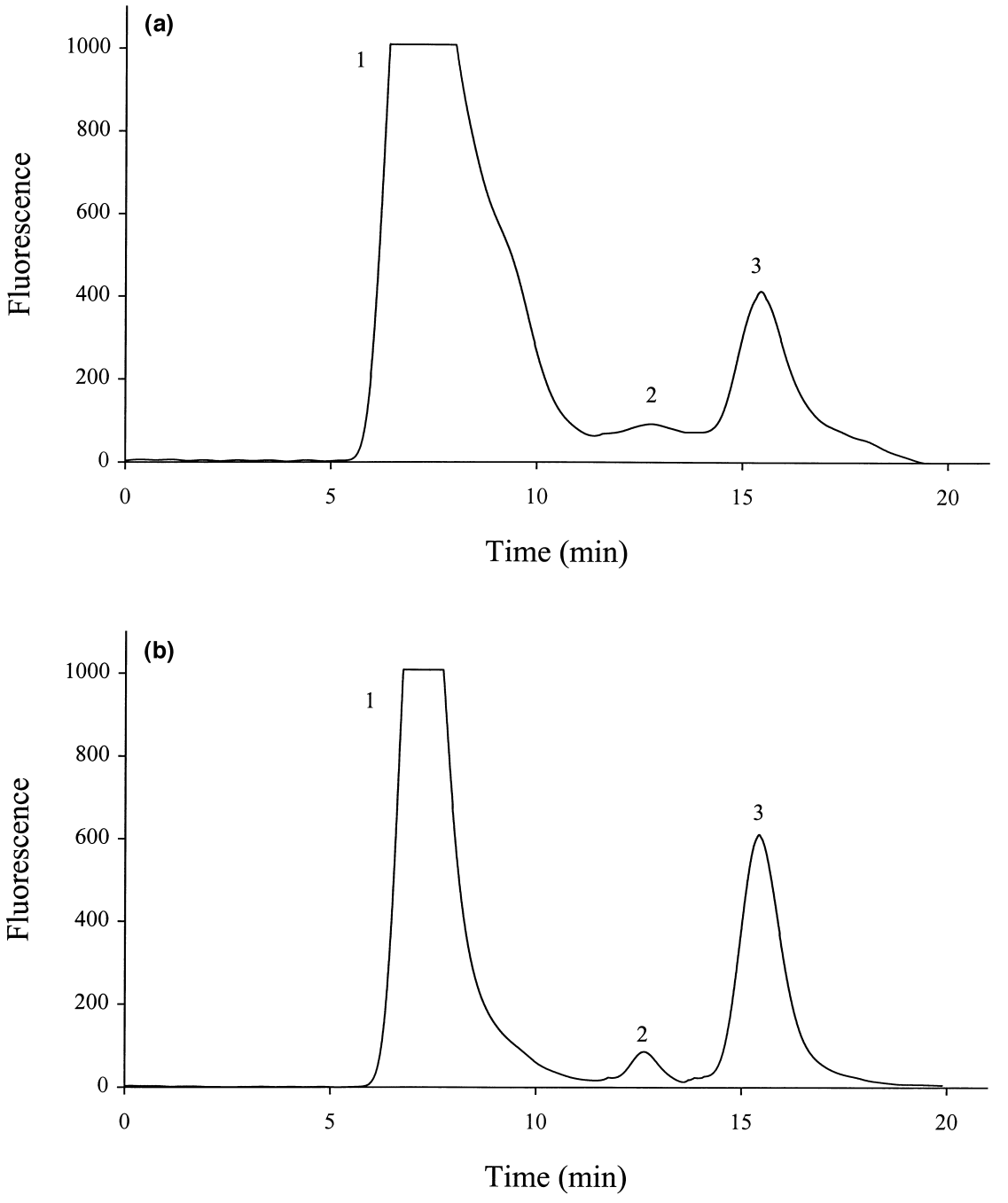

Fig. 2. Comparison of enzyme-labeled affinity proteins by the injection of 100 fmol off-line biotinylated anthracene aldehyde. (a) Fab-AP used as affinity protein. (b) S-AP used as affinity protein. Peaks: 1=excess BACH, 2=impurity, 3=biotinylated anthracene aldehyde. For conditions, see text.

3.3. Off-line derivatization

Naphthalene aldehyde ($1 \cdot 10^{-3}$ *M*) was biotinylated off-line and after biotinylation diluted with acetonitrile–water (10:90, v/v) to a concentration of $1 \cdot 10^{-8}$ mol/l. The diluted sample, which contained $1.3 \cdot 10^{-7}$ mol/l BACH was preconcentrated on a C_{18} pre-column. Next the C_{18} pre-column was switched on-line and the retained analytes were eluted to the analytical column with the LC mobile phase. Fig. 3a represents the chromatogram obtained when 100 μl, $1 \cdot 10^{-8}$ mol/l biotinylated naphthalene aldehyde was preconcentrated on the C_{18} pre-column. It can be concluded that the excess BACH, which elutes unretained from the HPLC column, was almost completely removed. However, the recovery of biotinylated naphthalene aldehyde amounted only to 46%.

Next, $1 \cdot 10^{-6}$ mol/l naphthalene aldehyde was biotinylated according to the described procedure. The reaction was performed overnight to ensure the complete biotinylation of the sample. After the reaction, the solution was diluted with acetonitrile–water (10:90, v/v) to a concentration of $1.3 \cdot 10^{-5}$ mol/l BACH and $1 \cdot 10^{-8}$ mol/l biotinylated naphthalene aldehyde. Fig. 3b shows the chromatogram obtained after preconcentration of 100 μl of the diluted reaction mixture on the C_{18} pre-column. The excess BACH disturbed the analysis of the biotinylated product dramatically even when the reaction solution was diluted 100 times, prior to sample introduction. BACH removal at this high concentration had to be performed with a higher concentration acetonitrile in the rinsing buffer. However, flushing the C_{18} pre-column with a higher concentration acetonitrile will decrease the recovery of biotinylated naphthalene aldehyde even further. The difference of polarity between BACH and biotinylated compound is too small for an efficient sample clean-up. Therefore, a more selective preconcentration is necessary.

To examine the derivatization of anthracene aldehyde with BACH, an off-line biotinylation was performed with 100 μl $1 \cdot 10^{-3}$ mol/l anthracene aldehyde according to the procedure described for naphthalene aldehyde at pH 5.6. Following biotinylation, 1 μl of the sample containing either anthracene aldehyde or the biotinylated product was injected via an injection loop and monitored at 279 nm with UV detection after HPLC. However, even after 4 h of derivatization, no biotinylated product could be detected. Therefore, the off-line biotinylation was repeated in methanol–50 mmol/l citric acid (10:90, v/v) at pH 2.5, 3.5 and 4.5. The yield after 1 h reaction time was monitored with UV detection and is listed in Table 2.

The biotinylated product was well separated from anthracene aldehyde. The reaction was completed after 1 h with a yield of 100% if a pH of 3.5 or lower was used. Consequently, a pH of 3.0 was used for further biotinylation.

Theoretically, compounds attached to the valeric acid side chain of biotin do not influence the interaction of biotin and streptavidin [6–8]. The signal obtained with HPLC–EA-BCD from BACH and biotinylated compounds should therefore be the same. To investigate the response of biotinylated compounds, naphthalene aldehyde and anthracene aldehyde were biotinylated off line. After the biotinylation, the reaction solutions were diluted to $1 \cdot 10^{-7}$ mol/l biotinylated compound and 1 μl was introduced. The response of both compounds was compared to the response obtained when 1 μl $1 \cdot 10^{-7}$ mol/l BACH was injected (see Fig. 4). The response of the biotinylated compounds is somewhat lower than for BACH probably caused by steric hindrance of the analytes. From these results it can be concluded that small biotinylated compounds can detected with approximately the same sensitivity. The influence on the interaction between streptavidin and large biotinylated compounds, such as proteins and oligonucleotides should be investigated in future research.

3.4. Selective preconcentration of biotinylated anthracene aldehyde

The dual pre-column set-up was introduced previously for the determination of PAH–DNA adducts in DNA hydrolysate [31]. This set-up was applied because the affinity of PAH–DNA adducts for CP was higher than the affinity for the analytical column. Desorption of the PAH–DNA adducts from the CP-silica pre-column with the LC mobile phase resulted in a low and irreproducible recovery caused by slow desorption kinetics. Therefore, desorption of

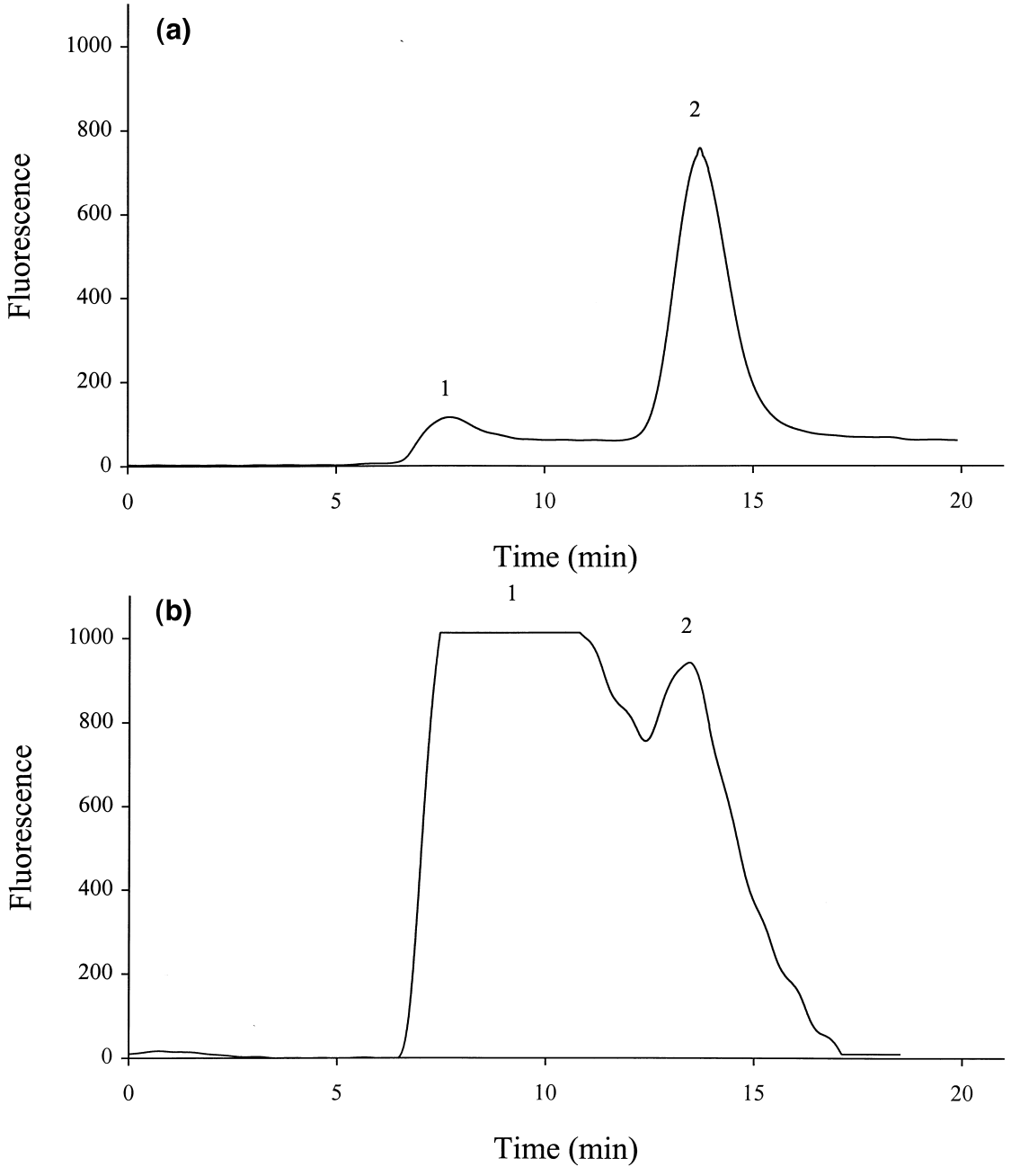

Fig. 3. HPLC–EA-BCD of 100 μl off-line biotinylated naphthalene aldehyde preconcentrated on the C_{18} pre-column. (a) $1 \cdot 10^{-8}$ mol/l biotinylated naphthalene aldehyde with $1.3 \cdot 10^{-7}$ mol/l BACH. (b) $1 \cdot 10^{-8}$ mol/l biotinylated naphthalene aldehyde with $1.3 \cdot 10^{-5}$ mol/l BACH. Peaks: 1=excess BACH, 2=biotinylated naphthalene aldehyde. For conditions, see text.

PAH–DNA adduct was performed with 100% organic modifier, followed by on-line dilution with water. Water was added to the desorption effluent with a ratio of 9:1, resulting in a solution of 10% organic modifier–water. The analytes were re-concentrated on a C_{18} pre-column, which can be de-

Table 2
Yield at different pH after 1 h biotinylation, for conditions, see text

pH	Yield (%)
2.5	98
3.5	99
4.5	60
5.6	0

sorbed with the LC mobile phase. The recovery of PAH–DNA adducts obtained with the dual-pre-column set-up was 95% (RSD 1%, $n=11$) [31]. The same dual pre-column set-up was applied to pre-concentrate biotinylated anthracene aldehyde.

To examine the recovery of the dual pre-column set-up for biotinylated anthracene aldehyde, a sample was biotinylated off-line. After biotinylation the sample was diluted to a final concentration of $1\cdot10^{-7}$ *M* and 1 μl of this solution was injected via an injection loop. Next, the sample was further diluted to a concentration of $1\cdot10^{-9}$ mol/l and 100 μl was loaded on to the CP-silica pre-column connected to the first injection valve. The first injection valve was switched and the biotinylated analyte was desorbed with 100% acetonitrile at a flow-rate of 70 μl/min. Water was added via a mixing union at a flow-rate of 630 μl/min. The resulting solution, containing acetonitrile–water (10:90, v/v), was then re-concentrated on the C_{18} pre-column. Finally, the analytes retained on the C_{18} pre-column were eluted to the analytical column with the LC mobile phase, followed by EA-BCD. A recovery of 95.5% (RSD= 4%, $n=4$) was determined with the dual pre-column set-up.

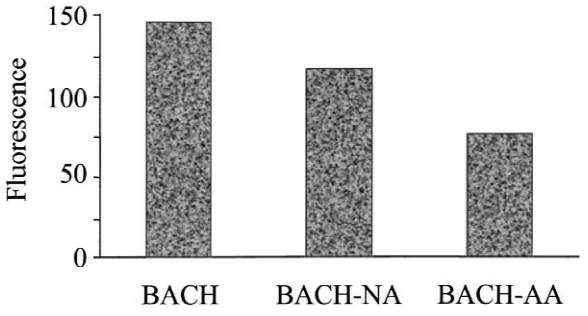

Fig. 4. Response of BACH, biotinylated naphthalene aldehyde (BACH-NA) and biotinylated anthracene aldehyde (BACH-AA) to EA-BCD. For conditions, see text.

The biotinylation reaction at lower concentration levels was performed overnight to ensure maximal biotinylation of the sample. Next, 0.5 ml water was added to the sample and 1.0 ml was introduced to the dual-pre-column system. A minimum detectable concentration (MDC) was determined of $1\cdot10^{-8}$ mol/l biotinylated anthracene aldehyde with HPLC–EA-BCD. The excess BACH ($9\cdot10^{-4}$ mol/l) was completely removed by the dual pre-column set-up, which demonstrate the selectivity of the CP-silica. Introduction of 100 μl $1.3\cdot10^{-5}$ mol/l BACH directly to the C_{18} pre-column, see Fig. 3b, resulted in a large disturbance of the analysis, which can be avoided by using CP-silica.

A major problem of derivatization is the slow reaction kinetics at low concentration level, resulting in low yields. To examine the yield at low concentration, the response was compared to the response obtained after derivatization at high concentration level. After biotinylation of $1\cdot10^{-4}$ mol/l anthracene aldehyde, which was monitored with UV detection and resulted in a 100% yield, the sample was diluted to a concentration of $1\cdot10^{-8}$ mol/l biotinylated anthracene aldehyde and 1 ml was introduced to the dual-pre-column system. Next, $1\cdot10^{-8}$ mol/l anthracene aldehyde was biotinylated overnight, followed by 1 ml sample introduction to the dual pre-column system. In principle, the response of the biotinylation of anthracene aldehyde at high and low concentration level should be the same after dilution. However, the yield of biotinylated anthracene aldehyde, which was labeled at low concentration level was only 9%, compared to the peak area observed with the derivatization at high concentration level. Although the entire amount anthracene aldehyde was biotinylated at high concentration level, the yield at low concentration level was rather insufficient, caused by an incomplete reaction even after 16 h of derivatization.

3.5. On-column derivatization of anthracene aldehyde in combination with selective preconcentration

Because the low yields obtained with the off-line derivatization at low concentrations, the biotinylation procedure was performed directly on the CP-silica pre-column. The advantage of biotinylation directly

on the CP-silica pre-column is the possibility to preconcentrate anthracene aldehyde before derivatization. Furthermore, BACH has no affinity to CP and can be removed easily after biotinylation, whereas the CP-silica will retain the biotinylated analyte. Fig. 5 shows the chromatogram obtained after preconcentration and biotinylation of 1 ml $5 \cdot 10^{-9}$ mol/l anthracene aldehyde. The first peak (1) was the signal obtained from excess BACH, which was eluted unretained from the analytical column. Peaks 2, 3 and 5 were impurities, which were also found in blank analysis. Plasticizers probably cause these signals, although this can not be confirmed by mass spectrometry, due to the low concentration. Peak 4 was the biotinylated anthracene aldehyde. A MDC of $2.5 \cdot 10^{-10}$ mol/l was achieved with EA-BCD when 1 ml anthracene aldehyde was preconcentrated and biotinylated.

The yield of the on-column derivatization was compared with the off-line biotinylation of anthracene aldehyde at a concentration of $1 \cdot 10^{-4}$ mol/l (100% yield). After labeling, the solution was diluted to $2.5 \cdot 10^{-10}$ *M*. A 1-ml volume of this solution was introduced to the dual-pre-column system. Compared to the off-line derivatization, a yield of 11.5% was obtained with the on-column derivatization. Despite the relative low yield, the LOD was 40-times lower with the on-column biotinylation compared to the off-line biotinylation at low concentration level. An explanation for the lower LOD is the preconcentration of the analyte prior the reaction followed by continuous rinsing of biotinylation reagent resulting in improved reaction kinetics [32,33]. Therefore, the on-column biotinylation procedure is preferred according to LOD, speed of analysis and ease of automation. However, removal of excess biotinylation reagent is a crucial step in the on-column procedure.

3.6. Quantitative aspects

Calibration curves were generated in order to demonstrate the feasibility of the on-column preconcentration and biotinylation procedure with the dual-pre-column system followed by EA-BCD. A calibration curve for anthracene aldehyde has obtained with a concentration range from $2.5 \cdot 10^{-10}$ mol/l to $5 \cdot 10^{-8}$ mol/l with a correlation of 0.999

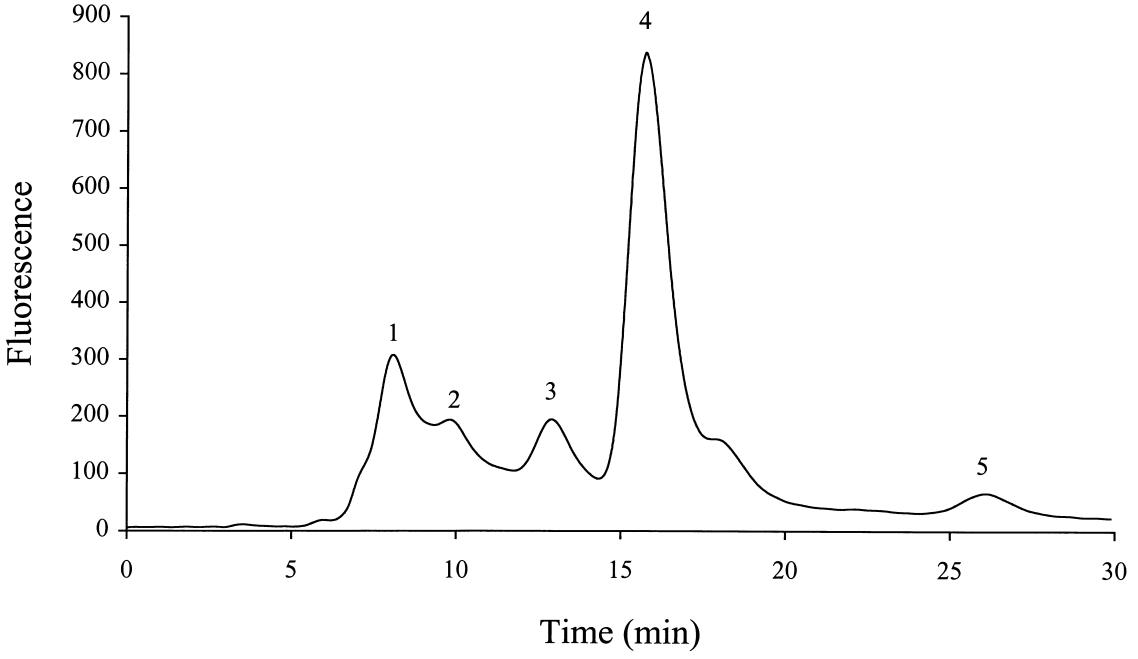

Fig. 5. Chromatogram of on-column biotinylated anthracene aldehyde ($5 \cdot 10^{-9}$ *M*, 1 ml). Peaks: 1=excess BACH, 2, 3, 5=impurities, 4=biotinylated anthracene aldehyde, For conditions, see text.

($n=4$). The calibration curve was characterized by $y=9.895x^9+2.45$ (x=concentration anthracene aldehyde, y=peak area). The relative standard deviation (RSD) was below 6.5% for all data points. A mass detection limit of 250 fmol has been achieved. The LC analysis time was 30 min. However, during the LC analysis, a next sample can be preconcentrated and biotinylated.

4. Conclusions

A fully automated preconcentration and biotinylation procedure coupled on-line to HPLC and post-column EA-BCD has been developed. Alkaline phosphatase-labeled streptavidin was applied as affinity protein, because a higher response for the biotinylated product was obtained, compared to anti-biotin Fab fragments. Analytes were biotinylated off-line as well as on-column. However, a selective sample clean-up after biotinylation is a crucial step. Therefore, CP-silica was used to preconcentrate biotinylated anthracene aldehyde and for removal of excess biotinylation reagent. A drawback of the derivatization is the relatively low yield obtained after off-line or on-column biotinylation. The LOD of the off-line procedure was enhanced by a factor 40 with the on-column procedure. An MDC of $2.5\cdot 10^{-10}$ mol/l anthracene aldehyde (1 ml sample) was accomplished. However, the yield was only 11.5% with the on-column procedure. Therefore, improvement of the biotinylation of analytes, which can lower the LOD, is a challenging task. Future research will be devoted to the biotinylation of PAH–DNA adducts and PAH–protein adducts, followed by HPLC–EA-BCD. Compared to ^{32}P labeling of PAH–DNA adducts followed by autoradiography [27] for one to four days, the analysis time was reduced dramatically. However, the LOD achieved with ^{32}P labeling of PAH–DNA adducts is much lower than for the described HPLC–EA-BCD when anthracene aldehyde was biotinylated. An advantage of HPLC–EA-BCD is the possibility to quantify the biotinylated compounds accurately with a fully automated system.

In addition to PAH adduct analysis, the described system is in principle suitable for detection at low levels of other analytes, if they contain functional groups which can be labeled with biotin derivatives. The HPLC–EA-BCD system can be implemented in different area's of analytical chemistry, for example in environmental and pharmaceutical analysis. However, separation of biotinylated analytes and excess biotinylation reagents is a crucial step. Removal of excess biotinylation reagent can be performed based on difference in polarity, size or ionogenic interactions.

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