Talanta 85 (2011) 151–156

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

New approach to quantitative analysis of benzo[a]pyrene in food supplements by an immunochemical column test

Natalia V. Beloglazova[a,b], Irina Yu. Goryacheva[a,*], Sarah de Saeger[c], Marie Louise Scippo[d], Reinhard Niessner[e], Dietmar Knopp[e]

[a] Saratov State University, Chemistry Institute, Department of Common and Inorganic Chemistry, Astrakhanskaya 83, 410012 Saratov, Russia
[b] M.V. Lomonosov Moscow State University, Department of Chemical Enzymology, Leninskie Hills 1, 119992 Moscow, Russia
[c] Ghent University, Faculty of Pharmaceutical Sciences, Laboratory of Food Analysis, Harelbekestraat 72, Ghent 9000, Belgium
[d] University of Liège, Faculty of Veterinary Medicine, Laboratory of Food Analysis, CART (Centre of Analysis of Residue in Traces), Bld de Colonster 20, 4000 Liège, Belgium
[e] Technische Universität München, Institute of Hydrochemistry and Chemical Balneology, Chair for Analytical Chemistry, Marchioninistrasse 17, D-81377 München, Germany

ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 9 March 2011
Accepted 20 March 2011
Available online 1 April 2011

Keywords:
On-site method
Quantitative test
Benzo[a]pyrene
Screening
Food supplements

ABSTRACT

A quantitative immunochemical rapid test for sensitive determination of benzo[a]pyrene (BAP) as a model analyte was developed making use of a handheld reader for results evaluation. The covalent immobilization of antibodies to different Sepharose gels, i.e., CNBr-activated Sepharose 4B and CNBr-activated Sepharose 4 Fast Flow was compared with adsorption to a polyethylene support. The lowest limits of detection (LOD) were 4 ng L^{-1} and 40 ng L^{-1}, respectively, using optimized assay conditions. The developed test was applied to food supplements (garlic, black radish and maca), including a pre-treatment procedure. LOD of 9 ng kg^{-1} and linear range of 13–80 ng kg^{-1} were obtained. Results of BAP determination in naturally contaminated samples were confirmed by high-performance liquid chromatography coupled to fluorescence detection and a good correlation was achieved. We suggest that the developed test format can be used to quantitative detection of the low molecular weight analytes, such as mycotoxins, pesticides, other pollutants in food and environmental samples.

1. Introduction

Many of the man-made chemical compounds which are released into the environment are toxic to surrounding biota or humans via the food chain. Therefore, the concern over increasing sample load and rising costs motivate the search for rapid, preferably without any time-consuming sample concentration step and prior sample pre-treatment, low-cost, simple, and reliable tests that could also be automated or carried out on-site (field-portable assays). General features of immunoassays first of all their specificity, sensitivity and speed of analysis led to the development of numerous environmentally and food-related assays and applications over the last 40 years.

Polycyclic aromatic hydrocarbons (PAHs) are formed by incomplete combustion or pyrolysis of organic carbon-containing materials and during various industrial processes. According to various sources, they have been determined in the atmosphere, in terrestrial and aquatic ecosystems, also in remote areas, and in dietary products, i.e. they are considered ubiquitious in the environment. PAHs can be found in food products also as a consequence of certain industrial processing method, such as smoking, heating (grilling, roasting) drying, and addition of smoke flavoring products (produced from smoke condensates), which are utilized to improve organoleptic characteristics. Recent reports have drawn attention to PAH contamination of toys and possible contribution to the increased incidence of cancer in children. Due to the carcinogenic and mutagenic nature of many PAHs, there has been increased concern in their detection and monitoring in recent years. In the EC Directive 98/38/EC concerning the quality of water intended for human consumption (drinking water directive) a limit value of 10 ng/L (10 ppt) was set for benzo[a]pyrene (BAP) which is the lowest of all limit values set for individual chemical parameters in this directive. BAP was the first PAH to be identified as a carcinogen and it has often been used as a marker for PAH contamination in general. During the last decade, several immunoassays using different formats have been reported for PAHs.

Nowadays one of the trends in food and environmental analysis is the development of test formats, which allow a rapid answer about presence or absence of a contaminant in a sample. If possible, such tests should be applicable on-site to screen many samples in a short time. Most popular are immunologically based tests as they are characterized by high sensitivity and specificity [1–4].

The lateral-flow immunoassay (LFIA) format, also known as immunological dipstick or immunochromatographic assay, is by far the most common, because it can be manufactured very

* Corresponding author.
E-mail addresses: goryachevaiy@mail.ru, goryachevaiy@info.sgu.ru (I.Yu. Goryacheva).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.03.052

cost-efficiently and can be used by untrained personnel. This non-instrumental technique combines the chromatographic principle and immunochemical recognition of analyte [5]. The first labels for LFIA were enzymes [6]. Recently, colloidal particles, such as carbon [7–9], selenium [10] and more common gold [11] were found optimal. Nowadays, the latter is very popular as it is characterized by chemical stability, inertness and simplicity of use and preparation [12]. Plasmon-resonance scattering was used for nanoparticle-based tests for semi-quantitative or quantitative evaluation of analyte concentrations. At present, the LFIAs are widely used for qualitative testing in clinical diagnostics [13] and food safety control [14–16] where the analytes are present in relatively high concentrations. This limitation is mainly caused by rather low signal intensity and poor quantitative discrimination of the colour-formation reaction based on label accumulation. In addition, it is difficult to use LFIAs for screening of samples with complicated coloured matrices without special pretreatment procedure.

Another principle was used for the gel-based column immunoassay technique, which allows to combine preconcentration by an immunoaffinity support and visible detection of the analyte by an enzymatic reporter. To our experience, the sensitivity can be tuned over a larger range compared to LFIA and interfering (coloured) compounds can be easily removed. This method was used for detection of different kinds of mycotoxins [17,18] and PAHs [19,20]. In the mentioned studies the method was primarily applied for a qualitative screening, i.e., to sort out samples with concentrations above a set limit value. Recently, we reported on the quantitative detection of 2,4,6-trinitrotoluene by scanning the gels and digital processing of the colour data with Adobe Photoshop CS3 program [21]. However, the obtained results were not entirely satisfying, i.e., several parameters like room lighting, scanning and image processing affected the test evaluation heavily. We herein report the development of a quantitative immunochemical column test for the polyaromatic hydrocarbon compound BAP in food supplements using a commercially available handheld reader.

2. Experimental

2.1. Reagents and materials

BAP, pyrene (PYR), microzide (2-chloroacetamide) and Tween 20 were purchased from Sigma Chemical Co. 1-Benzo[a]pyrenebutyric acid (BAP-BA) was obtained from the Institute of PAH Research (Greifenberg, Germany). Preparation and characterization of monoclonal mouse anti-BAP antibody 22F12 was described [1]. Rabbit anti-mouse immunoglobulin (Ig) G was purchased from Dako (Glostrup, Denmark). CNBr-activated Sepharose 4B (S4B) and CNBr-activated Sepharose 4 Fast Flow (S4FF) were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Plastic tubes (Bond Elut reservoir, 1 mL and 3 mL) and polyethylene frits (1/4 in. and 3/8 in. diameter) were supplied by Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium). Abicap columns and polyethylene filters were purchased from Senova GmbH (Jena, Germany). BAP–horseradish peroxidase (HRP) conjugate (BAP-BA–HRP) was synthesised as described [19]. The substrate chromogenic solution used for the HRP-enzyme was Colorburst™ Blue TMB/Peroxide (ALerCHEK, Inc., USA). All other chemicals and solvents were of analytical grade; doubly distilled water was used throughout.

Phosphate-buffered saline (PBS) 0.01 M, pH 7.4, was used as an assay buffer for the gel-based immunoassay. It was also used to prepare the washing solution (PBS with 0.05% Tween, v/v) and the blocking buffer (1% casein in PBS, w/v) for the gel-based assay, and also for the blocking buffer (1% casein in PBS, w/v) and the washing buffer (0.01 M PBS with 0.2% BSA w/v, 0.05% w/v microzide) (PBS–BSA) for abicap colums. PBS with 0.05% Tween (v/v) was used also for conjugate dilution in column gel-based immunoassay with quantitative result interpretation and polyethylene filter-based immunoassay. According to the Senova protocol, carbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 9.5; 0.05% microzide) was used for activation of filters and as coupling buffer for antibody adsorption on the filters. The substrate buffer was composed of 0.1 M sodium acetate, 0.1 M citric acid and 0.05% microzide.

Stock solution of BAP (1 mg mL^{-1}) was prepared in methanol and stored at −20 °C. Working solutions were prepared by dilution of standard solution in methanol/water (10:90, v/v) to give the range of 1–1000 ng L^{-1}. As a read-out system the portable abicap photometer (Senova) was used.

Food supplements (garlic, black radish, maca) were purchased from different manufacturers. Kind of supplements and name of manufacturers are presented in Table 1.

Table 1
Benzo[a]pyrene determination in food supplements by the quantitative immunochemical column test and HPLC–FLD.

Name and producer of samples	Concentration BAP, μg kg^{-1}	
	Column immunoassay	HPLC–FLD
Garlic		
BioActive Garlic (Pharma Nord)	0.7	0.5
Garlic (IPPA)	2	1.7
Garlic (Dieti Natura)	19	15
Garlic (Laboratoires Fenioulx SPRL)	0.3	<0.2
Garlic (Nature Made)	0.3	0.3
Garlic (Mannavita BVBA)	0.2	<0.2
Black radish		
Black radish (Dieti Natura)	0.6	0.5
Black radish (Laboratoires Fenioulx SPRL)	23	19
Black radish (Ephyto)	16	12
Maca		
Maca (Dieti Natura)	0.2	<0.2
Maca (Decola Vivadis SA)	0.3	0.2
Maca 500MG (AOV)	0.6	0.5
Active Maca (Mattisson Healthcare)	0.2	<0.2
Maca (Laboratoires Fenioulx SPRL)	0.2	<0.2
Maca 500 (Biodynamics BVBA)	0.4	0.2

2.2. Preparation of the gels

Anti-BAP antibodies were immobilized onto S4B and S4FF gels in two ways: (a) binding by rabbit anti-mouse IgG antibodies which were covalently immobilized prior to that and (b) direct covalent binding.

(1) *Preparation of rabbit anti-mouse IgG antibodies immobilized gels.* Shortly, 0.5 g of freeze-dried S4B or S4FF were washed on a sintered glass filter using 100 mL of 1 mM HCl. 740 μL of rabbit anti-mouse immunoglobulins (2.7 mg mL^{-1}) and 300 μL of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) were mixed with the gel on a shaker for 2 h at room temperature (RT). The gel was washed with 5 mL of coupling buffer to remove the excess of antibody. After this the unlinked active groups were blocked with 7 mL of glycine buffer (0.2 M glycine, 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0) for 2 h at RT. The gel was washed with 3 cycles of alternating pH. Each cycle consisted of a wash with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl (10 mL) followed by a wash with coupling buffer (10 mL). The prepared gel was suspended in PBS (1:3, v/v) and stored at 4 °C. These gels are designated as Rab-S4B and Rab-S4FF.

(2) *Preparation of anti-BAP antibodies immobilized gels.* Instead of rabbit *anti*-mouse immunoglobulins 740 μL of mouse anti-BAP antibody (1 mg mL^{-1}) was used. All other steps were performed

as described above. Accordingly, these gels are designated as Mou-S4B and Mou-S4FF.

Blocked gel. The gels with blocked active groups were prepared by the same procedures as described above except for addition of antibody reagent. These gels are designated as BLO-S4B and BLO-S4FF.

For quantitative results evaluation with the Senova reader the nonspecific interactions should be reduced as much as possible. Therefore, an additional blocking step with PBS, containing 1% casein was included in the preparation procedure of all gels.

2.3. Preparation of gel-based test column

For the preparation of test layers the Mou-S4B and Mou-S4FF gels were mixed with blocked gels BLO-S4B and BLO-S4FF, respectively, at appropriate ratios in a 3 mL plastic tube with bottom frit. This dilution of the primary antibody-coupled gels was necessary to optimize the quantity of specific antibody binding sites and thus to obtain high assay sensitivity. 250-μL aliquot of the mixture was placed on the bottom frit of an empty 1-mL Bond Elut cartridge and then covered with a second frit, constituting the test layer.

For the preparation of assay gels on the base of Rab-S4B and Rab-S4FF, 0.2 mL of Rab-S4B or Rab-S4FF was mixed with 1 mL of blocked gel BLO-S4B and BLO-S4FF, respectively, in a 3 mL tube with a bottom polyethylene frit. PBS excess was removed under gravitation. Then 100 μL of specific anti-BAP antibodies in selected concentrations were added and carefully mixed. This solution was flowed through using a plunger and the gel was washed two times with 3 mL of PBS afterwards. Then 3 mL of PBS was added and the assay gel was mixed. For preparation of the single-use test columns the assay gel (250 μL) was placed on the bottom frit of an empty 1-mL Bond Elut cartridge and then covered with a second frit. The prepared columns were kept at 4 °C.

2.4. Gel-based immunoassay procedure

For tests with visual detection, the dilutions Mou-S4B/BLO-S4B ($1{:}10^9$, v/v) and Mou-S4FF/BLO-S4FF ($1{:}10^6$, v/v) were found optimal. With Rab-S4B and Rab-S4FF gels the quantity of coupled BAP-specific antibodies was varied and a concentration of 0.1 ng mL^{-1} was found optimal for both gels.

For the assay procedure 1 mL of the BAP standard solution prepared in methanol/water (10:90, v/v) or the same volume of food supplement extract was passed through the test tube. With Mou-S4B and Rab-S4B assay gels, after the sample extract passed the column, a washing step with 4 mL of PBS–Tween (0.05%) followed. Then, 50 μL of BAP-BA–HRP conjugate solution (dilution 1:500, corresponds to 2.0×10^{-6} M) was applied and assay gel incubated for 3 min. Excess of conjugate was removed with 7 mL PBS–Tween (0.05%, v/v) and followed by the addition of the chromogenic substrate (50 μL). After 20 s incubation, the substrate solution was removed by the plunger. Visual detection was done within 5 min after chromogenic substrate application. The cut-off level was defined as lowest analyte concentration, which is characterized with no colour development at a fixed detection time.

The procedures were similar with Mou-S4FF and Rab-S4FF diluted gels, except that 12 mL of washing buffer was used instead of 7 mL to remove the excess of conjugate. This was done for the leveling of the non-specific interaction for both support materials.

The tests with quantitative evaluation (readout of the optical density) were performed almost the same way, however, only Mou-S4B diluted gel was used owing to its outstanding characteristics. In detail, Mou-S4B/BLO-S4B at a ratio of $1{:}10^9$ (v/v) and a dilution of 1:300 (corresponds to 3.5×10^{-6} M) of BAP-BA–HRP conjugate was used. The latter was diluted in PBS–Tween (0.05%, v/v) for leveling non-specific interactions. Further, a 20 min substrate incubation time was used in order to obtain the colour intensity required for readout by the handheld optical reader.

2.5. Polyethylene filter-based immunoassay procedure

The operation with the polyethylene filter support was adapted from the supplier's protocol. After degassing of the filters by placing them in ethanol and using 15 min ultrasonication they were inserted into the columns. Following, activation was done by passing through 750 μL of ethanol/water (50:50, v/v), 750 μL of water and the same volume of carbonate buffer. The activation procedure was performed twice.

Next, the antibody was adsorbed by the addition of 750 μL of anti-BAP antibody in carbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 9.5; 0.05% microzide) (dilution of antibody stock solution $1{:}10^5$, corresponding to about 0.9×10^{-8} mg of primary antibody per polyethylene filter) onto the column and incubation for 20 min at RT. Free sites on the filter surface were blocked with 750 μL of blocking solution (PBS containing 1% casein) during 20 min and followed by a final washing step with 750 μL of PBS with 0.2% BSA and 0.05% microzide.

BAP standard solution or sample (750 μL) was placed into the column, incubated during 6 min, and then passed through the column very slowly (1 drop per second). The column was washed with 750 μL of PBS–BSA. Next, 750 μL of BAP-BA–HRP (dilution 1:300 in PBS–Tween (0.05%, v/v), corresponds to 3.5×10^{-6} M) was added, incubated during 3 min and passed through the column very slowly. After another washing step with 750 μL of PBS–BSA, 500 μL of substrate buffer were passed to adjust the pH before the chromogenic substrate was added. After 20 min substrate incubation time the signal was evaluated by the handheld reader.

2.6. Sample pretreatment

Before analysis of food supplement samples by the developed column-based immunoassays an extraction step was required. The procedure as described [22] was used. Briefly, 5 g of food supplement sample was mixed with 25 mL of a 2 M KOH solution prepared in ethanol/water (90:10, v/v). The mixture was heated during 30 min under stirring, then 20 mL of water and 20 mL of cyclohexane was added, and the mixture was further heated during 10 min. The cyclohexane fraction was collected. The procedure was repeated three times by using only 15 mL of cyclohexane for second and third cycles. The pooled cyclohexane fractions were evaporated to dryness and the residue was reconstituted with 5 mL of methanol/water mixture (10:90, v/v).

For reference, BAP was determined by HPLC–FLD as described [22]. Sample pretreatment procedure was similar as described above, with the exception that the organic phase was dried with anhydrous sodium sulphate and filtered before evaporation. The residue was reconstituted with 500 μL of cyclohexane and passed through a silica SPE column (Bond Elut Si, 500 mg, 3 mL). BAP was eluted with 7 mL of cyclohexane, the solvent again evaporated and residue redissolved in acetonitrile. 5 μL of extract or standard were injected on the HPLC C18 column and using for separation a gradient of water/acetonitrile/methanol as mobile phase [23].

3. Results and discussion

3.1. Choice of optimal solid support material for the gel-based immunoassay

To investigate the influence of Sepharose structure on the solid support properties two different materials, i.e., S4B and S4FF, were compared. The latter is a highly cross-linked agarose with lower

coupling capacity (according to supplier's information, 25–60 and 13–26 mg of α-chymotrypsinogen/mL drained medium, for S4B and S4FF, respectively).

After having performed BAP determination it became clear that the two supports, disregards their great structural similarity, revealed different results (Table 2). The S4FF gel, under the used experimental conditions, was characterized by high non-specific interaction. To make it clear, the non-specific interaction in this method appears as colour development of the blocked gel (no antibody immobilized) which was used as a reference in a separate column. For leveling of this interaction an increased volume of buffer was used in the washing step (12 mL instead of 7 mL in the cases of S4B gels). However, as a disadvantage, the more intense washing runs the risk of eluting some amount of antibody bound BAP-BA–HRP conjugate, i.e., leads to weakened colour of the assay gel. For compensation, the antibody density of the gel could be increased but in parallel the cut-off level would be corrupted. Concluding, S4B was preferred and was used in the following experiments.

In parallel, two ways of antibody immobilization on the gel, i.e., direct covalent coupling of the primary *anti*-BAP antibody and coupling of the primary antibody via the previously immobilized secondary rabbit *anti*-mouse IgG antibody, were investigated. No or only small difference regard the obtained assay sensitivity was observed (Table 2). Therefore, it became obvious that both immobilization strategies are applicable without prejudice. Because of saving reagent costs, the direct coupling method was used in further experiments.

3.2. Optimization of filter-based immunoassay

In addition, the non-covalent coupling of primary antibody to the abicap columns from Senova company was checked. The column is a plastic tube, which houses a 3-dimensional filter made of sintered polyethylene material for the immobilization of proteins, e.g. antibodies. Thereby, the principle of coupling of the antibodies to the support differs from gel-based columns, i.e., antibodies are immobilized by simple adsorption. An important advantage of the abicap columns is the opportunity to get quantitative results by using the available handheld photometer. This makes the results independent on subjective visual inspection by the experimenter. The read-out system allows to solve the problem of subjectivity of result interpretation, and to transform qualitative visual results into precise information. For quantitative measurement the conjugate concentration was increased to gain more intensive colour for the photometric detection of the analytical signal.

The first step in filter-based immunoassay development was to ascertain the optimal immunoreagents concentrations and detection time. A 1:300 dilution of BAP-BA–HRP conjugate (corresponds to 3.5×10^{-6} M) in PBS–Tween (0.05%, v/v) for non-specific interaction leveling and use of about 0.9×10^{-8} mg of primary antibody per polyethylene filter were chosen as optimal. Lower amounts of antibody gave weak colour development and made interpretation of results more complicated. The obtained cut-off level for BAP determination of 40 ng L^{-1} was, however, significantly higher than those which were obtained with the different gel-based assays (Table 2).

In addition, it was indispensable to perform an optimal blocking, as the photometric measurement was done in comparison with a blank (no antibodies immobilized) column. 0.01 M PBS, containing different concentration of casein and BSA were used for the frit surface blocking, and PBS containing 1% casein was revealed as the best blocking solution. The optimal blocking time was 20 min and was in agreement with Senova protocol. Also, composition of washing buffer was varied. The standard washing buffer recommended by the company is 0.15 M PBS (pH 7.3), containing 0.05% microzide; 0.1% BSA and 0.05% Tween 20. Unfortunately, in the present investigation, the addition of the surfactant led to a decrease in signal intensity. Therefore, 0.01 M PBS containing different concentration of BSA was tested. The addition of 0.2% BSA was found optimal. Microzide (0.05%) was added to the washing solution as a preservative.

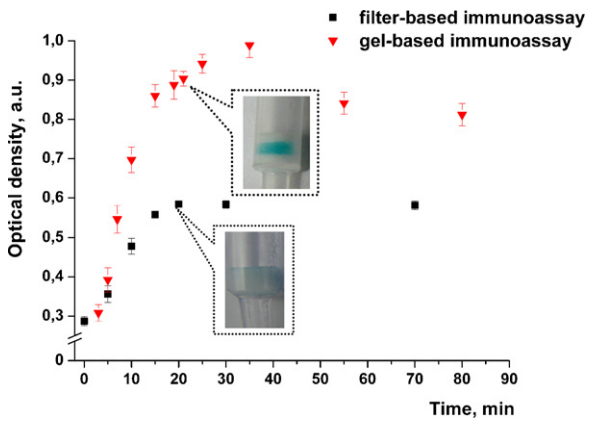
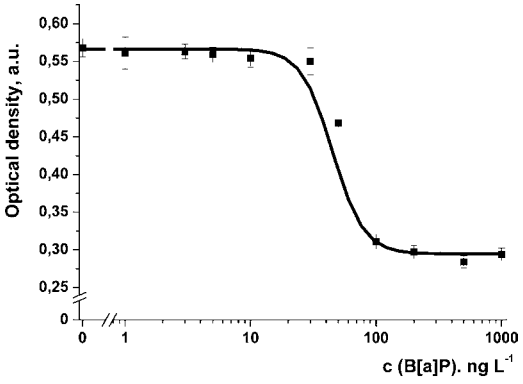


Fig. 1. Kinetic of colour development for filter-based and gel-based column tests ($n = 4$).

One of the main steps of the assay development was to assess the optimal time of colour formation. The incubation time was varied from 5 to 70 min. Significant change of colour intensity was obtained during the first 10 min, but it did not reach the maximum value in this time period. After 20 min the maximum optical density value and the lowest cut-off level was reached (Fig. 1). Using quantitative detection, the calibration curve obtained with the filter-based assay is shown in Fig. 2. It becomes obvious that the assay sensitivity was not high enough for the determination of the analyte at the required detection level. Additional efforts are necessary to decrease the LOD by about one order of magnitude.

3.3. Quantitative gel-based immunoassay

Because the filter-based immunoassay did not provide us the required sensitivity we investigated the applicability of the available abicap photometer for read-out of the gel-based column tests. As the Bond Elut plastic tubes were not transparent for the used radiation, the gel-based assay was performed in plastic tubes from Senova company. Because photometric read-out requires saturated colour of the assay gel in the case of blank sample analysis, the conjugate concentration should be increased compared to visual



Detection limit, ng L^{-1}	30
IC 50, ng L^{-1}	58
Linear range, ng L^{-1}	35-80

Fig. 2. Calibration curve for quantitative filter-based tests ($n = 3$).

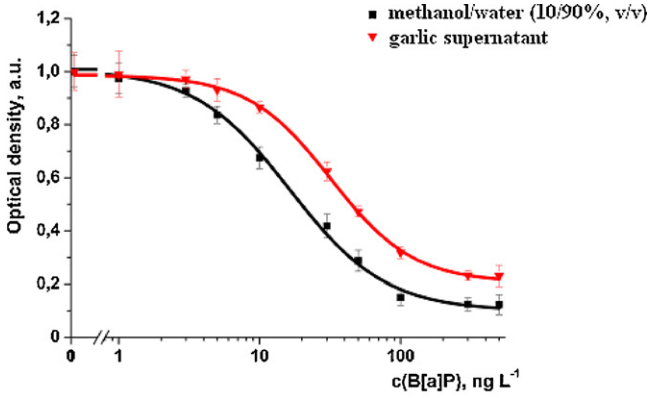
Table 2
Comparison of solid supports for antibody coupling.

	Sepharose 4B gel		Sepharose 4 Fast Flow gel		Polyethylene frit
	Coupled with primary antibody	Coupled with secondary antibody	Coupled with primary antibody	Coupled with secondary antibody	Coupled with primary antibody
Nonspecific interaction	–[a]	–	+[b]	+	–
Number of analytical steps	5	5	6[c]	6[c]	6[d]
Sensitivity[e], ng L^{-1}	4	4	20	10	40

[a] Absence of non-specific interaction (no colour development within 6 min).
[b] Presence of non-specific interaction (colour development in less than 6 min after chromogenic substrate application).
[c] Including additional washing step.
[d] Including activation step.
[e] Visual detection.

detection. Concluding, the optimization of conjugate concentration for the quantitative gel-based immunoassay was very important. A variety of assay gels, which were prepared with different mixtures of anti-BAP antibody loaded gels and blocked gels was tested in the range from $1{:}10^3$ to $1{:}10^{10}$. The optimal mixture was $1{:}10^9$. Additional dilution of the antibody loaded gel did not drop down the cut-off level further. Also, different BAP-BA–HRP conjugate dilutions, i.e., 1:100, 1:300 and 1:500, were compared. The 1:100 diluted conjugate gave a very high non-specific interaction, and the colour intensity of assay gel did not show a significant difference on whether a blank or spiked sample was run through. On the opposite, the 1:500 diluted conjugate gave a too low optical density value. Thus, the BAP-BA–HRP dilution of 1:300 (corresponding to 3.5×10^{-6} M) was chosen as optimal, i.e., the blue coloured assay gel appeared in the case of a blank. For minimization of non-specific interaction an additional blocking step (1 h incubation with PBS containing 1% casein (w/v)) was included in the procedure of gel preparation and PBS–Tween (0.05%, v/v) was used for conjugate dilution. Following, the detection time was defined. As demonstrated in Fig. 1, a visual difference appeared already after 6 min but the signal was already very low. Therefore, the optimal detection time was established at 20 min (same as in the case of the filter-based immunoassay). Concluding, the total analysis time was about 30 min, which included about 6 min for the different analytical steps and 20 min for colour development.

The calibration curve for BAP determination was built and analytical performance evaluated (Fig. 3). A detection limit of 4 ng L^{-1} of sample for BAP was calculated which allows determination of the analyte at the decision level in foodstuff and drinking water.



	Methanol/water mixture 10/90 (v/v)	Garlic supernatant
Detection limit, ng L^{-1}	4	9
IC 50, ng L^{-1}	16	32
Linear range, ng L^{-1}	5 - 51	13 - 80

Fig. 3. Calibration curves for quantitative gel-based tests ($n = 3$).

3.4. Determination of benzo[a]pyrene in food supplements

A set of food supplements, comprising very complicated matrices, were analyzed using special sample pretreatment as described recently [19]. As a difference, additional purification step with silica SPE column, which was required for HPLC analysis, could be eliminated, as described in Section 2.6. (Sample pretreatment). This was possible, because column test allows to combine clean-up and analyte concentration procedures in one step. BAP was extracted with cyclohexane and, after this solvent was evaporated, the residue was reconstituted with methanol/water (10:90, v/v), passed through the test column and analyzed with quantitative detection. For correction of matrix effects a calibration curve was built using standards prepared in blank garlic sample (absence of BAP was confirmed by HPLC) extract (Fig. 3). The calculated LOD values were 4 ng L^{-1} for standard solution and 9 ng L^{-1} for supernatant extract (corresponds to 9 ng kg^{-1}). This calibration curve was also used for estimation of BAP concentration in black radish and maca samples. The results which were obtained for naturally contaminated garlic, black radish and maca are presented in Table 1. If it was necessary, extracts were diluted with methanol/water (10:90, v/v). The comparison of the quantitative immunochemical column test outcomes and related results from confirmation method (HPLC–FLD) revealed a good agreement. Some overestimation of BAP concentration with the immunochemical test could be a result of cross-reacting polyaromatic compounds which were present in these samples.

4. Conclusions

For the first time an immunochemical gel-based column test with quantitative detection was developed and applied for the low molecular weight analyte benzo[a]pyrene (BAP). A commercially available handheld photometer was used for analytical signal measurement and, therefore, to get rid of subjectivity of result interpretation. Three different principles of antibody immobilization were compared, i.e., direct covalent binding of primary mouse anti-BAP antibodies to Sepharose gels, coupling via covalently on Sepharose immobilized secondary rabbit–anti mouse IgG antibodies, and adsorption onto sintered polyethylene frits. The direct covalent binding was found to be optimal using the Sepharose 4B gel based column immunoassay. Screening of BAP in different kinds of food supplements with the developed test revealed results which were very comparable to an SPE–HPLC–FLD method.

Acknowledgments

The research was supported by German Academic Exchange Service (DAAD), German Ministry of Education and Research (BMBF, project 02WU0969) and Russian Foundation of Basic Research (RFBR, project 10-03-91168-GFEN). We further acknowledge SENOVA GmbH (Jena, Germany) for making available the handheld reader.

References

[1] D. Matschulat, A. Deng, R. Niessner, D. Knopp, Analyst 130 (2005) 1078–1086.
[2] J.C. Chuang, J.M. Van Emon, Y.-L. Chou, N. Junod, J.K. Finegold, N.K. Wilson, Anal. Chim. Acta 486 (2003) 31–39.
[3] A. Meimaridou, W. Haasnoot, L. Noteboom, D. Mintzas, J. Pulkrabova, J. Hajslová, M.W.F. Nielen, Anal. Chim. Acta 672 (2010) 9–14.
[4] S. Boujday, S. Nasri, M. Salmain, C.-M. Pradier, Biosens. Bioelectron. 26 (2010) 1750–1754.
[5] G.A. Posthuma-Trumpie, J. Korf, A. van Amerongen, Anal. Bioanal. Chem. 393 (2009) 569–582.
[6] M.G. Pappas, R. Hajkowski, W.T. Hockmeyer, J. Immunol. Methods 64 (1983) 205–214.
[7] M. Blažková, P. Rauch, L. Fukal, Biosens. Bioelectron. 25 (2010) 2122–2128.
[8] M. Blažková, B. Mičková-Holubová, P. Rauch, L. Fukal, Biosens. Bioelectron. 25 (2009) 753–758.
[9] M. Lonnberg, J. Carlsson, Anal. Biochem. 293 (2001) 224–231.
[10] S.C. Lou, C. Patel, S.F. Ching, J. Gordon, Clin. Chem. 39 (1993) 619–624.
[11] S. Rong-Hwa, T. Shiao-Shek, C. Der-Jiang, H. Yao-Wen, Food Chem. 118 (2010) 462–466.
[12] P. Ghosh, G. Han, M. De, C.K. Kim, V.M. Rotello, Adv. Drug Deliv. Rev. 60 (2008) 1307–1315.
[13] A. Warsinke, Anal. Bioanal. Chem. 393 (2009) 1393–1405.
[14] K. Li, L. Liu, C. Xu, X.G. Chu, Anal. Sci. 23 (2007) 1281–1284.
[15] X. Wang, K. Li, D. Shi, X. Jin, N. Xiong, F. Peng, D. Peng, D. Bi, J. Chromatogr. B 847 (2007) 289–295.
[16] H. Watanabe, A. Satake, Y. Kido, A. Tsuji, Anal. Chim. Acta 472 (2002) 45–53.
[17] I.Y. Goryacheva, S. De Saeger, M. Lobeau, B. Delmulle, S.A. Eremin, I. Barna-Vetró, C. Van Peteghem, Anal. Chim. Acta 590 (2007) 118–124.
[18] E.Y. Basova, I.Y. Goryacheva, T.Y. Rusanova, N.A. Burmistrova, R. Dietrich, E. Martlbauer, C. Detavernier, C. Van Peteghem, S. De Saeger, Anal. Bioanal. Chem. 397 (2010) 55–62.
[19] N.V. Beloglazova, I.Y. Goryacheva, D.A. Mikhirev, S. De Saeger, R. Niessner, D. Knopp, Anal. Sci. 24 (2008) 1613–1617.
[20] I.Y. Goryacheva, N.V. Beloglazova, D.A. Mikhirev, R. Niessner, D. Knopp, Talanta 75 (2008) 517–522.
[21] E.Y. Basova, I.Y. Goryacheva, D.A. Mikhirev, T.Y. Rusanova, N.A. Burmistrova, B. Kerkaert, T. Cucu, S. De Saeger, B. De Meulenauer, Anal. Methods 1 (2009) 170–176.
[22] C. Brasseur, F. Brose, A. Pirlot, C. Douny, G. Eppe, G. Maghuin-Rogister, M.L. Scippo, Accredit. Qual. Assur. 12 (2007) 535–542.
[23] S. Danyi, F. Brose, C. Brasseur, Y.-J. Schneider, Y. Larondelle, L. Pussemier, J. Robbens, S. De Saeger, G. Maghuin-Rogister, M.L. Scippo, Anal. Chim. Acta 633 (2009) 293–299.