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Comparison of oriented and random antibody immobilization in immunoaffinity chromatography of cytokinins

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Abstract

Immunosorbents for the plant hormones cytokinins prepared by random antibody immobilization (to Affi-Gel 10) and by oriented approach via oxidized carbohydrate moieties on the Fc region (to Affi-Gel Hz or hydrazide derivative of Perloza MT 200) have been compared. Both approaches yielded immunosorbents with high dynamic capacity (ca. 5–10 nmol ml gel^{-1}). Oriented antibody immobilization did not exhibit crucial effects in the case of low-molecular-mass cytokinins. Antibodies immobilized via a spacer to Affi-Gel 10 have probably enough conformational freedom to enable good accessibility to cytokinins. The sorbents were used in analysis of endogenous cytokinins in maize seeds. In phosphatase treated samples *trans*-zeatin and its riboside were predominant.

Keywords: Immunoaffinity chromatography; *Zea mays*; Immunosorbents; Cytokinins; Plant hormones

1. Introduction

Cytokinins are plant hormones which exhibit various crucial biological activities, as e.g., promotion of cell division, induction of bud formation and development, reduction of apical dominance and delay of senescence. Natural cytokinins are derivatives of adenine substituted at the N^6 position either with isoprenoid or benzyl moiety which may be further modified. Cytokinins exist as free bases, ribosides, nucleotides, glyco-, acetyl- and alanyl-conjugates (e.g., Ref. [1] and references therein).

In plants, cytokinins occur in very low amounts, usually at levels below 30 pmol per g of fresh weight (FW) (e.g., Ref. [2]), together with other much more abundant and often structurally similar substances. Commonly used analytical procedures for cytokinin analysis are based on immunoassays of high-performance liquid chromatography (HPLC) fractions of prepurified plant extracts. However, when a large number of background contaminants is present at high concentrations, the HPLC separation methods, which are effective in fractionation of standards,

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need not be reliable in the case of plant samples [3]. This problem may be overcome by use of immunoaffinity chromatography (IAC) prior to HPLC fractionation. Highly purified cytokinin preparations containing only traces of other UV-absorbing material could be obtained in this way.

Until now immunosorbents for cytokinin analysis have been prepared by random antibody (Ab) attachment using primary amino or carboxyl groups. A number of chromatographic matrices have been used for immobilization of cytokinin antibodies, especially cyanogen bromide (CNBr)-activated cellulose [4–8]. Davis et al. [3] tested CNBr–Sepharose, Fast-Flow Sepharose CL-4B, glycophase silica and Affi-Gel 10. Affi-Gel 10 was used in some other studies (e.g., Ref. [9]).

Antibody binding to solid supports through the ε-amino group of the amino acids results in multiple antibody orientation on the gel surface which may affect accessibility of the antigen binding site. Using binding of antibodies via their carbohydrate moieties located on the Fc region (heavy chain) remote from the antigen binding site Hoffman and O'Shannessy [10] achieved oriented antibody immobilization with excellent steric accessibility of antigen binding site. Oriented immobilization proved to be superior in a number of cases [11,12].

Cytokinin immunosorbents prepared by random and oriented immobilization techniques using two different carriers (Affi-Gel and cellulose) are compared in this paper.

2. Experimental

2.1. Chemicals

Unlabelled cytokinins were obtained from Sigma–Aldrich. Tritium-labelled cytokinins, prepared by alkylation of [2-^{3}H]adenosine with appropriate alkylbromides, were synthesized by Dr. Jan Hanuš, Isotope Laboratory, Institute of Experimental Botany, Prague, Czech Republic. Affi-Gel 10 and Affi-Gel Hz were purchased from Bio-Rad Labs. (Richmond, CA, USA). Bead cellulose Perloza MT 200 (water-swollen, particle size 80–100 μm, bed volume 13.3 ml swollen sorbent g dry matter^{-1}, water content 90%) was obtained from North Bohemian Chemical Works, Lovosice, Czech Republic. Monoclonal antibodies raised against isopentenyladenosine (conjugated to bovine serum albumin via ribose) and nonspecific mouse immunoglobulin G (IgG) were supplied by Dr. Richard Vytášek (96-System, Prague, Czech Republic).

2.2. Oxidation of antibodies

The IgG fraction was isolated from ascite liquid by triple precipitation with 50% ammonium sulphate. The final precipitate was dissolved in 0.1 *M* sodium acetate, pH 5.5 (buffer A). Ammonium sulphate was removed by dialysis against 3×2 l of this buffer at 4°C.

The IgG (concentration approximately 5 mg ml^{-1}) was oxidized by dropwise addition of one tenth volume of fresh 0.1 *M* sodium *m*-periodate (final concentration 10 m*M*). Oxidation was performed in an orbital shaker at 4°C for 20 min in the dark. The reaction was terminated by adding ethyleneglycol to a final concentration 20 m*M* and the mixture was shaken for another 10 min. Unreacted periodate was removed by dialysis at 4°C overnight against the buffer A (2 l) and 0.1 *M* sodium acetate, pH 4.8 containing 0.5 *M* sodium chloride (buffer B, 2×2 l).

2.3. Preparation of cellulose hydrazide

A modified method published by Beneš et al. [13] was used. A 75-g mass of suction-filtered Perloza MT 200 (washed with 0.1 *M* hydrochloric acid and water to neutral pH of eluate) was stirred with 150 ml of acetone for 30 min at laboratory temperature, 75 ml of aqueous acetone was removed after the stirring had been interrupted and the same volume of fresh acetone was added. Stirring was continued for another 30 min, after which another 75 ml of aqueous acetone was removed, 1 ml of 10% sodium hydroxide was added and the stirring continued for 1 h at laboratory temperature. The suspension was cooled to 0°C and 0.51 g of 2,4,6-trichlorotriazine (TCT) was added and allowed to react for 45 min. The product was washed in a column with 300 ml of acetone cooled to 0°C and 300 ml of ice-cold water. The TCT-activated cellulose was stirred with a solution of 0.88 g of adipic acid dihydrazide in 62.5 ml of 0.05 *M* borate buffer, pH 9.0, at room

temperature at constant pH 9.0 for 4 h. The product containing 12.8 μmol of adipic acid dihydrazide ml sorbent^{-1} was finally washed with water.

2.4. Antibody binding to Affi-Gel Hz and cellulose hydrazide

Affi-Gel Hz is cross-linked agarose activated with hydrazide groups. The gel equilibrated in buffer B (settled volume 2 ml) was mixed with solution of oxidized antibodies (ca. 15 mg/3 ml) and coupling proceeded overnight at 4°C on a rotary shaker. The gel suspension was poured into a column (5 ml) and washed with buffer B (10 bed volumes) and with phosphate-buffered saline (PBS, pH 7.4, 10 bed volumes) containing 0.1% sodium azide. The immunosorbent was stored in this buffer at 4°C.

Binding of antibodies to cellulose hydrazide was performed in the same way as to Affi-Gel Hz. Immunoaffinity columns were prepared using monoclonal antibodies raised against isopentenyladenosine. Nonspecific mouse IgG were used for preparation of pre-columns.

2.5. Antibody coupling to Affi-Gel 10

Affi-Gel 10 is a cross-linked agarose gel with *N*-hydroxysuccinimide ester groups, which contains neutral 10-atom spacer arm. It reacts readily with primary amino groups of proteins.

Antibody was dissolved in 0.1 *M* MOPS [3-(*N*-morpholino)propanesulphonic acid, pH 7.5] and dialyzed against this buffer at 4°C overnight. Antibody solution (ca. 20 mg/2 ml) was mixed with Affi-Gel 10 (settled volume 1 ml). The suspension was gently shaken at 4°C for 4 h and after addition of 100 μmol of ethanolamine for additional 1 h. After packing the gel into a column the unbound antibody was washed out with PBS. The immunosorbent was stored in PBS with 0.1% sodium azide at 4°C.

2.6. Determination of the amount of immobilized antibody

The concentration of antibody in solution was determined spectrophotometrically (absorbance at 280 nm) using extinction coefficient 1.4. The quantity of bound protein was calculated as difference in protein content in reaction mixture before and after immobilization. Amino acid analysis in a Durrum D-500 amino acid analyzer was used for precise determination.

2.7. Immunoaffinity chromatography

The carrier with immobilized antibody, either nonspecific (pre-column) or specific (affinity column), was packed into 4-ml polypropylene columns with polytetrafluoroethylene frits. Pre-column (0.5 ml bed volume) was fitted on the top of each affinity column (0.3 ml bed volume) and the column set was washed with 20 ml PBS. Sample (5 ml) was applied on the column set repeatedly three times. The last repetition was applied directly on the affinity column and the flow was stopped for 30 min. After washing with 5 ml PBS the pre-column was removed. The affinity column was washed with 5 ml of cold water and retained cytokinins were eluted with 2 ml of cold methanol (−20°C). After immediate regeneration by washing with 5 ml cold water and 20 ml PBS, the column was ready for another chromatographic run. The pre-column was reactivated by washing with water (5 ml), cold methanol (5 ml), water (5 ml) and PBS (20 ml). Columns and pre-columns were stored at 4°C.

Maximal capacity of immunosorbents was estimated using affinity columns only. Instead of plant sample mixture of cold and tritiated cytokinin standard in 5 ml PBS (radioactivity 30 000 dpm, total concentration 50 nmol) was used. The amount of adsorbed cytokinin was calculated on the basis of radioactivity of methanol eluate.

2.8. HPLC

Cytokinins were separated on Perkin-Elmer 235C chromatograph with diode array detection system (DAD LC 235C) using a LiChrospher 100 column, 250-4, RP-18 (5 μm). Linear gradient was used (A) 40 m*M* triethylammonium acetate buffer, pH 3.9, (B) acetonitrile–methanol (1:1, v/v): 0 min, 81%A+19% B; 16 min, 77% A+23% B; 20 min, 60% A+40% B; 30 min, 57% A+43% B; 32 min, 100% B, flow-rate 0.6 ml min^{-1}. The spectra were re-

corded in the range 240–320 nm. One-minute fractions were collected.

2.9. Cytokinin analysis in maize seeds

Deep-frozen maize seeds were pulverized under liquid nitrogen. Cytokinins were analyzed according to Ref. [14], except the extraction was made with 80% methanol (10 ml per g FW) containing diethyldithiocarbamic acid (40 μg ml^{-1}). [^{3}H]DHZR (30 000 dpm per g FW) was used as an internal standard to calculate the losses during the extraction. The extract was passed through a C_{18} column (1 ml bed volume), evaporated to water phase and dissolved in 40 m*M* ammonium acetate (pH 4.8, 15 ml). After treatment with acid phosphatase (1 mg per g FW, 0.36 U mg^{-1}) and ion-exchange chromatography (DEAE–Sephadex A-25, 2 ml bed volume) cytokinins were retained on the C_{18} column. Elution was made with 80% methanol (5 ml). After evaporation to water phase the samples were diluted with PBS (to 5 ml) and applied to the immunoaffinity column. Retained cytokinins were eluted with cold methanol, evaporated and subjected to HPLC. Fractions corresponding to individual peaks were pooled and cytokinins were quantified by enzyme-linked immunosorbent assay (ELISA) according to Ref. [15].

3. Results

3.1. Antibody characterization

Monoclonal antibodies 16B7 raised against isopentenyladenosine (conjugated with bovine serum albumin) were purified by repetitive precipitation with ammonium sulphate as described in Section 2.2. As the antibody titre in ascites was high (ca. 6 mg ml^{-1}), the IgG fraction was directly used for immobilization. The antibody cross-reactivity was characterized by competitive radioimmunoassay (RIA) by determination of the concentration of cross-reactant which inhibited the binding of the primary antigen by 50% (Table 1). The antibody showed considerable cross-reactivity with cytokinins bearing isoprenoid side chain (67–114%). The higher affinity of cytokinin ribosides in comparison with bases was expected as the ribose formed bridge between hapten and protein when preparing the immunogen. The affinity of cytokinins bearing aromatic side-chains to the antibody was lower, i.e., below 10%, with the exception of benzyladenosine (29.2%). There was no interaction with adenine. The broad cytokinin specificity makes this antibody very suitable for immunoaffinity column construction as it allows to capture different natural isoprenoid cytokinins (with exception of 7- and *O*-glycosyl conjugates).

Table 1
Cross-reactivity of monoclonal antibody with cytokinins and adenine

Cross-reactant	Cross-reactivity (%)
Isopentenyladenine	67.1
Isopentenyladenosine	100.0
trans-Zeatin	114.2
trans-Zeatin riboside	110.0
Dihydrozeatin	81.8
Dihydrozeatin riboside	111.0
Benzyladenine	4.1
Benzyladenosine	29.2
ortho-Topolin	6.0
ortho-Topolin riboside	6.6
meta-Topolin	6.7
meta-Topolin riboside	7.1
para-Topolin	0.1
para-Topolin riboside	0.3
Adenine	0.0

3.2. Column capacities

In the case of immunosorption of small molecules the capture efficiency depends on the density of antibody immobilized on the support. Therefore antibody concentrations recommended by the manufacturer (Bio-Rad Labs.) or higher (comparable with those reported by other authors, e.g., Ref. [3]) were used. All tested methods had a high efficiency of antibody binding (70–97%). Different immunosorbents, i.e., Affi-Gel Hz, hydrazide cellulose and Affi-Gel 10, exhibited similar values of dynamic capacity (i.e., capacity per ml of gel, expressed according to Ref. [3]) for six most frequently analysed cytokinins [*trans*-zeatin (Z), *trans*-zeatin riboside (ZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), isopentenyladenine (iP) and isopentenyladenosine (iPA), Fig. 1]. At high density of

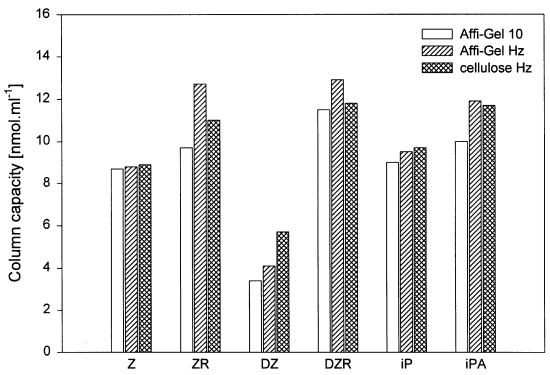

Fig. 1. Dynamic column capacities of different immunosorbents (A) Affi-Gel 10; (B) Affi-Gel Hz; (C) hydrazide cellulose for *trans*-zeatin, *trans*-zeatin riboside, dihydrozeatin, dihydrozeatin riboside, isopentenyladenine and isopentenyladenosine. The column bed volume was 0.3 ml. The bars represent mean value of at least four estimations.

immobilized antibody the differences in capacities paralleled the antibody cross-reactivity towards the individual cytokinins and did not seem to be influenced by the immobilization chemistry.

In spite of the fact that dynamic column capacities of immunosorbents (i.e., capacity per ml of gel) were similar, the specific capacities (i.e., capacity per mg of immobilized antibody) prepared by oriented immobilization methods were rather higher (Table 2). The differences between dynamic and specific capacities were given by the differences in protein loading and to small extent by binding efficiency. In random immobilization method the concentration range reported to be optimal was higher than that for hydrazide carriers. As for hydrazide cellulose high antibody loading was found to have some adverse effects on specific capacity of immunosorbent for ovalbumin [16], two antibody concentrations were tested (''high'' – resulting in immobilized Ab concentration 7.2 mg ml^{-1} of gel and ''low'' – resulting in 4.3 mg ml^{-1}). Specific capacity of both immunosorbents was practically the same. Thus in this case the higher protein concentration used did not limit steric accessibility for cytokinins (Table 2).

3.3. Cytokinin recovery

Even if the immunoaffinity column capacity for a number of cross-reacting cytokinins may be similar for individual isoprenoid cytokinin standards, the recovery of a cross-reactant from a complex mixture may be lower than that of the primary antigen. This may be caused by (1) lower K_a value of antibody for cross-reacting substance in comparison with primary antigen or other cross-reacting substances and/or by (2) substantial differences in concentration of individual substances in the sample. To check the recovery in complex cytokinin mixture, 22 cytokinin standards (in two groups, 100 pmol of each) were applied on Affi-Gel Hz column and analysed by HPLC (Fig. 2a,b). Only 7- and *O*-glucosides were completely unretained. Aromatic cytokinins (e.g., *meta*-topolin) were temporarily retained and then partially removed from the column by repeated buffer washes.

Recovery of six routinely analysed isoprenoid cytokinins (Z, ZR, DHZ, DHZR, iP, iPA) was estimated using 500 pmol cytokinin per 0.3 ml of gel. This amount considerably exceeded the level usually found in plant samples, however, was well below the maximal column capacity. The recovery varied from 88 to 98%.

3.4. Cytokinin analysis in maize seeds

Immunoaffinity columns (with Affi-Gel Hz) were

Table 2
Protein loading of different immunosorbents and their dynamic and specific column capacities

Immunosorbent	Protein loading (mg ml^{-1})	Column capacity (nmol ZR ml^{-1})	Specific capacity (μg ZR mg protein^{-1})
Affi-Gel 10	14.1	9.7	0.24
Affi-Gel Hz	6.6	12.7	0.68
Cellulose Hz (HL[a])	7.2	11.0	0.54
Cellulose Hz (LL[b])	4.3	6.1	0.50

[a] High loading of antibodies.
[b] Low loading of antibodies.

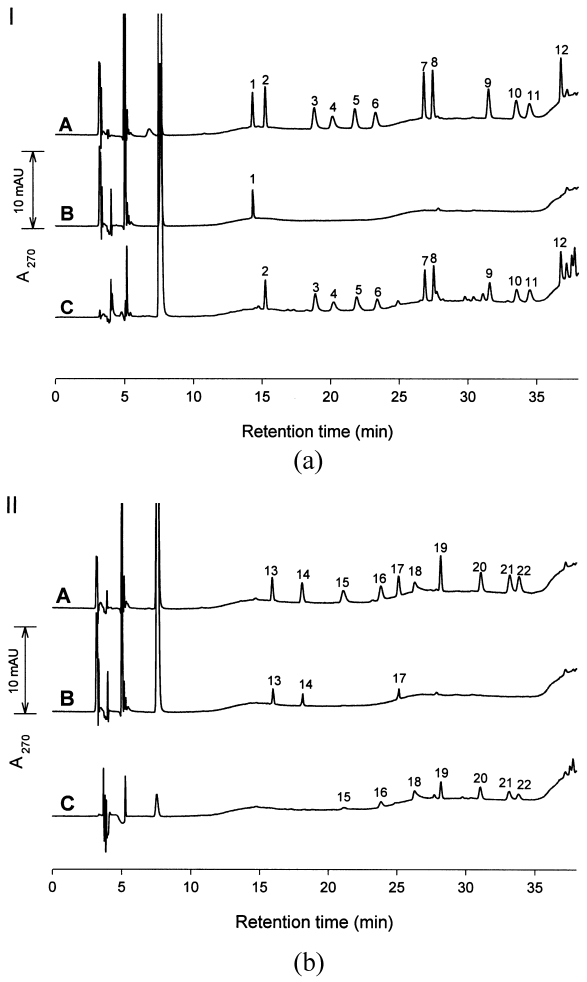

Fig. 2. Recovery of individual cytokinin standards from complex mixture after IAC. (A) HPLC chromatogram of standard mixture before its application to the immunoaffinity column. The injected amount (10 μl) contained 100 pmol each of cytokinin. (B) Sample which passed through the immunosorbent (contains only substances which were unretained). The injected amount (50 μl) corresponds to the concentrated sample which before IAC contained 100 pmol each of cytokinin. (C) Substances eluted from the immunosorbent by methanol. The injected amount (50 μl) corresponds to concentrated methanol eluate after application of 100 pmol each of cytokinin. (a) Mixture I: 1=zeatin-7-glucoside; 2=zeatin-9-glucoside; 3=*trans*-zeatin; 4=dihydrozeatin; 5=*trans*-zeatin riboside; 6=dihydrozeatin riboside; 7=*meta*-topolin; 8=*meta*-topolin riboside; 9=*ortho*-topolin; 10=isopentenyladenosine; 11=isopentenyladenine; 12=isopentyladenine. (b) Mixture II: 13=zeatin-*O*-glucoside; 14=*O*-glucosylzeatin riboside; 15=*cis*-zeatin; 16=*cis*-zeatin riboside; 17=benzyladenine-7-glucoside; 18=isopentenyladenosine monophosphate; 19=isopentenyladenine-9-glucoside; 20=*ortho*-topolin riboside; 21=benzyladenosine; 22=benzyladenine.

used for cytokinin analysis in maize (*Zea mays* L.) seeds at milk maturity. In young seeds with high meristematic activity large number of UV-absorbing substances is present in extract after ion-exchange (DEAE–Sephadex A-25) and reversed-phase (C_{18}) chromatographic steps (Fig. 3). Comparison of HPLC chromatograms before and after IAC (Fig. 3, traces A and B) shows the considerable purification which was achieved by this step.

At milk maturity developmental stage the predominant cytokinin, as determined by ELISA of HPLC fractions of immunoaffinity purified extract, was *trans*-zeatin riboside (306 pmol g FW^{-1}), followed by *trans*-zeatin (66 pmol g FW^{-1}). As the samples were treated with phosphatase, the value for zeatin riboside includes also zeatin nucleotide. Isopentenyladenosine and isopentenyladenine were present only in minute amounts (15 pmol g FW^{-1} and 6 pmol g FW^{-1}, respectively). Only traces of dihydrozeatin and its riboside were found.

The reliability of this method was verified by

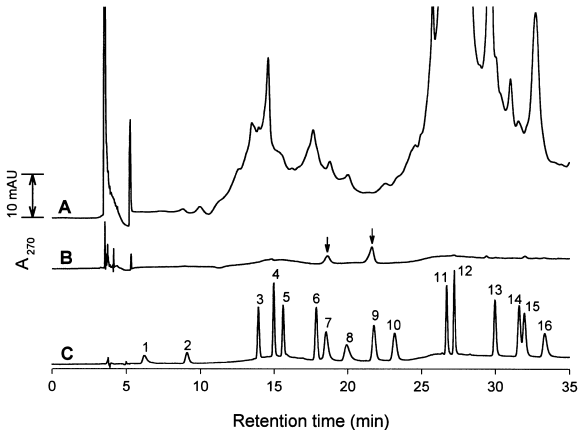

Fig. 3. HPLC chromatogram of the purified extract of maize seeds. (A) Before IAC; (B) after IAC. The injected amount (50 μl) corresponds in both cases to the extract of 250 mg (FW) of maize seeds. (C) HPLC chromatogram of cytokinin standards, adenine and its riboside (1=adenine; 2=adenosine; 3=zeatin-7-glucoside; 4=zeatin-9-glucoside; 5=zeatin-*O*-glucoside; 6=*O*-glucosylzeatin riboside; 7=*trans*-zeatin; 8=dihydrozeatin; 9=*trans*-zeatin riboside; 10=dihydrozeatin riboside; 11=*meta*-topolin; 12=*meta*-topolin riboside; 13=*ortho*-topolin riboside; 14=benzyladenosine; 15=isopentenyladenosine; 16=isopentenyladenine). The injected amount (10 μl) contained 100 pmol each of cytokinin.

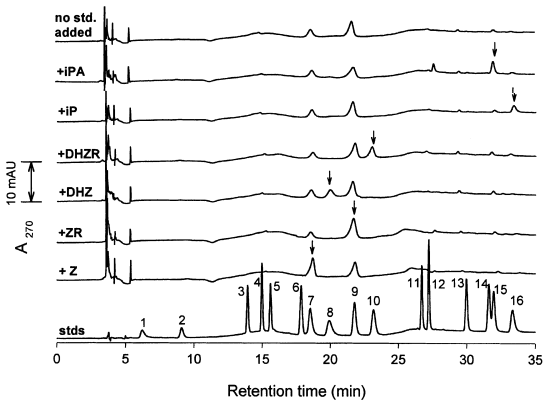

Fig. 4. HPLC chromatograms of IAC-purified extract of maize seeds. Extract was divided into seven equal portions. Individual cytokinin standards were added before IAC (as indicated). The injected amount (50 μl) corresponds in each case to 250 mg (FW) of maize seeds and 50 pmol of individual cytokinin standard. A HPLC chromatogram of cytokinin standards, adenine and adenosine is included at the bottom of the figure (1=adenine; 2=adenosine; 3=zeatin-7-glucoside; 4=zeatin-9-glucoside; 5=zeatin-*O*-glucoside; 6=*O*-glucosyl-zeatin riboside; 7=*trans*-zeatin; 8=dihydrozeatin; 9=*trans*-zeatin riboside; 10=dihydrozeatin riboside; 11=*meta*-topolin; 12=*meta*-topolin riboside; 13=*ortho*-topolin riboside; 14=benzyladenosine; 15=isopentenyladenosine; 16=isopentenyladenine). The injected amount (10 μl) contained 100 pmol of each cytokinin. All HPLC chromatograms are presented in the same absorbance scale (indicated by the bar).

internal standardization. The extract was divided into seven equal portions and to each portion (with exception of the first one) 200 pmol of individual cytokinin was added before IAC. Proportional increase of the corresponding cytokinin was detected by HPLC (Fig. 4) and quantified by ELISA.

4. Discussion

When IAC is used as purification step before final separation and quantitation relatively broad antibody specificity towards the group of closely related substances (e.g., cytokinins) is advantageous. The choice of antibodies does not seem to be restricted to polyclonals, as various monoclonals exhibit considerably differing cross-reactivities [17]. The broad cross-reactivity is probably inherent characteristic of the whole antibody population and does not reflect the effect of small sub-populations of antibodies which would (in case of polyclonals) recognize the individual cross-reactant [3]. Strnad et al. [15] reported wider cross-reactivity in case of antibodies raised against apolar N^6-substituent haptens, i.e., isopentenyl, benzyl and furfuryl groups, in comparison with those raised against zeatin riboside or *m*-topolin riboside. When the cross-reactivity of the antibodies is not sufficiently broad, mixture of different antibodies may be used [9]. In our case the monoclonals 16B7 raised against isopentenyladenosine showed sufficiently wide cross-reactivity to be used as sole antibody in group-specific immunoadsorbent preparation.

For oriented immobilization two hydrazide activated carriers were tested, i.e., widely used Affi-Gel Hz from Bio-Rad Labs. and local carrier bead cellulose Perloza MT 200. Both carriers have excellent binding properties, Perloza being slightly better (binding efficiency ca. 97% in comparison to ca. 86% in case Affi-Gel Hz). Higher mechanical strength of Perloza could not be exploited in IAC as only low flow-rate may be applied to ensure good contact of cytokinins with antibodies.

Oriented immobilization allows to avoid multisite attachment of antibody molecules as well as ensures their good steric accessibility (in optimal concentration range). This method was reported to have great impact on the increase of immunosorbent capacity in case of high-molecular-mass antigens (e.g., Ref. [10]). However, the exceptions from this rule have been also found. Fleminger et al. [18] showed that antibodies against horseradish peroxidase exhibited slightly higher binding activity when immobilized directly to Eupergite C via oxirane groups than after oriented immobilization to hydrazide derivative of the same carrier. It seems that in case of cytokinins, which are rather small molecules, the use of oriented immobilization does not exhibit crucial effects. In their extensive study Davis et al. [3] comparing different carriers for preparation of cytokinin immunosorbents via random immobilization approach found Affi-Gel 10 to be superior. This carrier has a 10 carbon atom spacer arm. It is possible that this spacer allows enough conformational freedom for the antibody to meet the spatial demands of cytokinins.

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