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# ANALYSIS OF CYTOKININS BY IMMUNOASSAY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF *IN VITRO* CULTIVATED *DIANTHUS CARYOPHYLLUS*

***Todor Genkov*[1], Ivailo Ivanov[2], Iordanka Ivanova[1]***

*Department of Plant Physiology[1], Laboratory of Bioorganic Synthesis[2], Faculty of Biology, Sofia University, 8 Dragan Tzankov Blvd., 1421 Sofia, Bulgaria*

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**Summary**. A method was used for determination of cytokinins zeatin, *iso*-pentenyladenine and dihydrozeatin by direct enzyme immunoassay (ELISA) and HPLC with isocratic programme of elution. The reliability of ELISA was proved by HPLC. In this research we determined the losses of cytokinins during their purification and their endogenous level in carnations, cultivated *in vitro*.

***Key words***: cytokinins, ELISA, HPLC

***Abbreviations***: BSA – bovine serum albumin; DHZ – dihydrozeatin; DHZR – dihydrozeatin riboside; ELISA – enzyme linked immunosorbent assay; HPLC – high performance liquid chromatography; iP – *iso*-pentenyladenine; iPA – *iso*-pentenyladenosine; PBS – phosphate buffered saline; Z - *trans*-zeatin; ZR – *trans*-zeatin riboside

## Introduction

Cytokinins by definition promote cell division in plant tissue culture and are now known to exhibit a wide range of other physiological effects on a variety of plants and plant tissues. Most cytokinins occur as free purine bases, nucleosides or nucleotides and as t-RNA constituents. They can be divided into the following groups: *iso*-pentenyladenine (iP) and derivatives, zeatin (Z) and derivatives, dihydrozeatin (DHZ) and derivatives (Letham and Palni, 1983). The quantification of endogenous cytokinins

* Corresponding author

poses difficulties because they are nearly always trace components in any crude plant extract and are present together with many structurally similar compounds. Thus, the selectivity and sensitivity of the measurement system is extremely important (Horgan, 1988).

In this research we develop a method for the extraction, purification, separation of cytokinins (DHZR, DHZ, ZR, Z, iPA, iP) and their quantitative determination from samples of carnation shoots.

## Materials and Methods

### Plant material

Axial buds of virus-free plants of *Dianthus caryophyllus* (White Sim) were cultivated on solid Murashige and Skoog's medium which contained 0.8% agar. pH was adjusted to 5.7 before autoclaving. Cultures were grown at 24–25 °C for a 16 hour photoperiod, provided by cool-white fluorescent tubes giving approximately 50.4 $\mu mol.m^{-2}.s^{-1}$.

One month after the culture initiation, the shoots were removed and endogenous cytokinin level was assayed.

### Cytokinin extraction and purification

The plant material was ground in liquid nitrogen; samples were extracted with 80% methanol, for 12 h at 4 °C in the dark. The homogenate was filtered and methanol fractions of the extracts were passed through a $C_{18}$ Sep-Pak cartridge (Waters Ass., Milford, MA). The eluates were evaporated in vacuum, at 40 °C to leave an aqueous phase, diluted with 9 volumes of 40 mM ammonium acetate (pH 6.5). 4 ml of diethylaminoethyl (DEAE) cellulose equilibrated in 40 mM ammonium acetate (pH 6.5) were added and the mixture was shaken for 10 min (Banowetz, 1992). After centrifugation to remove the DEAE cellulose, the supernatant was passed through a $C_{18}$ Sep-Pak cartridge. The cartridge was then washed with 0.1 M acetic acid and Z/ZR; DHZ/DHZR were eluted with 6 ml 25% methanol in 0.1 M acetic acid (v/v); iPA/iP were eluted with 10 ml 60% methanol. After concentration to 0.5 ml, cytokinins were quantified by ELISA, using polyclonal antibodies. The same purification protocol was used for HPLC separation of cytokinins in extracts.

### Preparation of immunogen conjugate and enzyme tracer

Cytokinin-BSA conjugates were synthesised by the method of Weiler (1980; 1981) and modifications of Eberle et al. (1986). DHZR, ZR or iPA were coupled, via periodate-oxidized *vic*-hydroxy groups of the ribose, to free amino groups of BSA or alkaline phosphatase (3 000 $units.mg^{-1}$), type VII-S from Sigma (St. Louis, USA).

### Immunization protocol

Each rabbit (12–16 weeks old) was injected subcutaneously and intramuscularly with 1 mg of dialysed and lyophilised conjugate, dissolved in 1 ml 5 mM PBS (pH 7.2) and an equal volume of Freund's complete adjuvant (multiple sites) on days 0, 7, 14, 21, 28, 42, 56, 70, 84 and 102. The blood was collected and IgG fraction was precipitated with 40% saturated ammonium sulphate, dialysed, lyophilised, and stored at -20ºC.

### ELISA

ELISA was performed with microtitration plates coated with 200 µl of 10 $\mu g.ml^{-1}$ solution of antibodies against iPA, ZR, DHZR, respectively, in 50 mM $NaHCO_3$, pH 9.6, and incubated for 16 hours at 4 °C. After washing, the wells were incubated for an additional period of 30 min at room-temperature with 250 µl of a 0.5% solution of BSA in PBS. A competitive technique was used for determining the unknown quantities of cytokinins (Weiler et al., 1981). The wells were filled with 150 µl of either cytokinin standard (0.1–100 pmol dilution range) or extract. After one hour incubation at 37°C were added 50 µl of a preparation from the respective cytokinin bound to alkaline phosphatase. The plate was incubated for 1 h at 37 °C and washed twice. The phosphatase activity was measured by adding 200 µl of a *p*-nitrophenylphosphate solution (1 $mg.ml^{-1}$ 50 mM $NaHCO_3$, pH 9.6) to each well and incubated for 1 h at 37 °C. The reaction was stopped by adding 50 µl 2 N NaOH and the absorbency of each well was measured at 405 nm.

### HPLC separation and quantification of cytokinins

The analyses were performed on a Waters HPLC system, pump 510, tunable absorbance detector 484, data module 745. The reverse phase column was $C_2/C_{18}$ (4.6×200 mm; 5 µm; MinoRPC$^{TM}$S 5/20 – Pharmacia, Sweden), using isocratic eluation programme of 30% or 40% methanol for Z/ZR, DHZ/DHZR and iPA/iP respectively, the flow rate was 1.2 $ml.min^{-1}$, the temperature 30 °C and the detection at 270 nm. Peaks were identified from their retention times by comparison with standards.

## Results and Discussion

### Purification of cytokinins

To determine the losses of cytokinins in the purification procedure, we used standards with quality determined on each step of this procedure (Fig. 1). At the end of the purification the losses varied from 15 to 61% for the different cytokinins (Table 1).

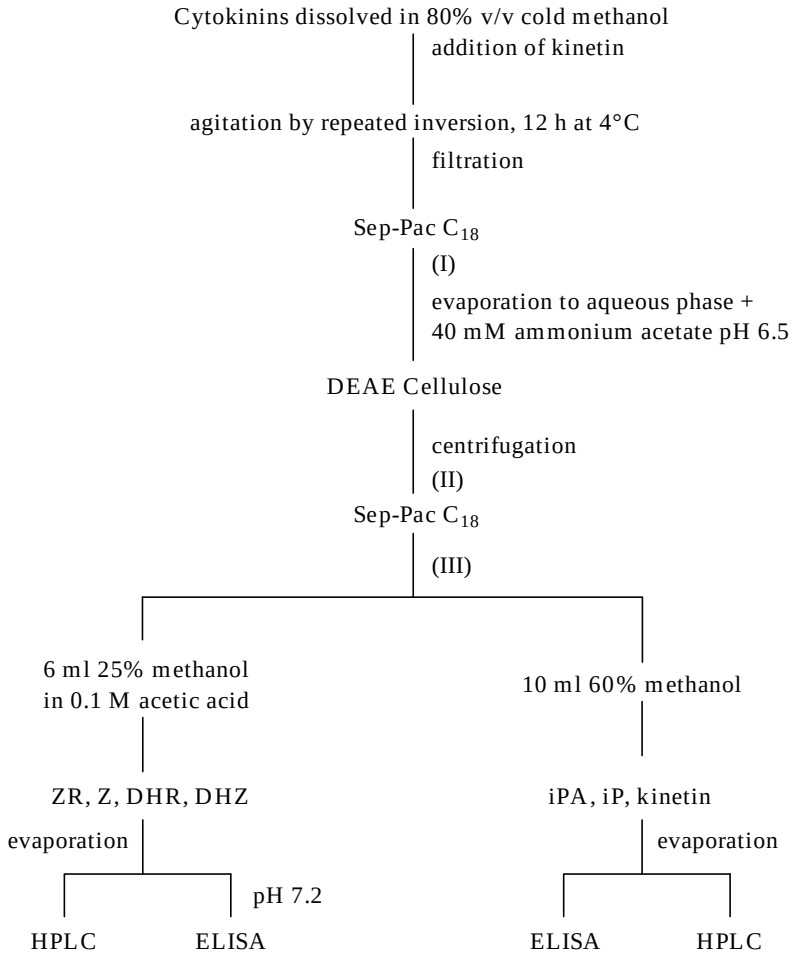


**Fig. 1**. Flow chart showing analysis of cytokinins. The numbers I to III indicate the points where aliquots were taken to measure

They were higher for corresponding ribosides as compared to free bases. The major were the ones after DEAE cellulose (step II) and when cytokinins remained on $C_{18}$ Sep-Pak after washing and eluting with methanol of 25% and 60% (step III). The values were similar to those stated by other authors (Hansen et al., 1984; Guinn and Brummet, 1990; Fernandez et al., 1995).

For determining the losses during purification of the extract was used kinetin, whose retention time was different from those of the analysed cytokinins. Its losses were similar to the losses of *iso*-pentenyl cytokinins and the latter were not taken into account.

For assessment of the losses during the different steps of purification most often as an internal standard was used $^{3}$H-iPA or $^{3}$H-iP, added before homogenization of the sample (Dermastia and Ranvikar, 1996) or all steps of the purification were tested in advance (Bollmark et al., 1988). As an internal standard was also used [$^{14}$C]-BA for the re-evaluation of the natural cytokinins in comparison to the losses (Fernandez et al., 1995; Kataeva et al., 1995). Often more than one internal standard was used (Scott and Horgan, 1984), but this is not a common practice, probably, due to the high price of the analysis.

**Table 1**. Losses of cytokinins (percentage) after each step of the purification procedure, determined by HPLC

| Cytokinins | Step* | | |
|---|---|---|---|
| | $C_{18}$ (I) | DEAE (II) | $C_{18}$ (III) |
| ZR | 7.6 | 41 | 59 |
| Z | 4 | 13 | 56 |
| DHZR | 7 | 28 | 61 |
| DHZ | 4 | 9 | 33 |
| iPA | 4 | 8 | 19 |
| iP | 5 | 6 | 15 |
| kinetin | 6 | 10 | 22 |

* The roman numbers correspond to those of Fig. 1

## HPLC separation

The gradient elution is most often used for HPLC analyses of cytokinins (Fernandez et al., 1995). An alternative approach for cytokinin separation is the more economical isocratic eluation programme, used by some authors (Soedjima et al., 1992). These authors use as a mobile phase a 44% aqueous methanol. We found that with the reverse phase column we used, iPA and iP were separated very well by this eluent not only from each other but from the other cytokinins as well (due to their higher hydrophobity), while ZR, Z, DHZR and DHZ were practically not separated from each other under these conditions. The decrease in the amount of methanol to 38% led to separation of ZR, DHZ, iPA and iP, as the retention times of iPA and iP increased considerably (29.8, respectively, 37.8 min; Table 2). These conditions are convinient for separation of *iso*-pentenyl cytokinins but not for the other cytokinins. Because of that we separated the *iso*-pentenyl cytokinins in advance with $C_{18}$ Sep-Pak before HPLC and determined them under the above conditions. In order to determine proper conditions for separation of ZR/Z and DZR/DZ, we studied the influence of the methanol concentration in the mobile phase (from 38% to 28%). The best separation was achieved at a

**Table 2**. HPLC-retention times (min) of cytokinins at some mobile phases

| Cytokinins | Aqueous methanol | | |
|---|---|---|---|
| | 38% | 30% | 40% |
| ZR | 6.5 | 10.7 | – |
| Z | 7.3 | 11.4 | – |
| DHZR | 7.3 | 12.7 | – |
| DHZ | 8.5 | 13.8 | – |
| iPA | 29.8 | >90 | 13.4 |
| iP | 37.2 | >90 | 17.1 |
| kinetin | – | – | 6.5 |

methanol concentration in the phase of 30%, as its further decrease to 28% led to a dramatic deterioration of the separation. The temperature variation from 25 °C to 40 °C did not affect the separation but only the retention times.

Using acetonitrile in concentration from 12% to 8%, we found that a proper separation was achieved at the lowest concentration of the organic solvent but at the expense of a considerable increase of the retention times (about three times).

## ELISA

Specificity of the antibodies was determined by cross-reactivity studies. Table 3 shows the cross-reactivities of different structural analogues, expressed as a percentage of cross-reactivity on a molarity base (Weiler and Zenk, 1976). This detailed cross-reaction study revealed a selectivity of the three antibodies. The antibodies showed only

**Table 3**. Cross-reactivity (%) of three polyclonal antibodies to related compounds

| Compounds | anti-ZR | anti-iPA | anti-DHZR |
|---|---|---|---|
| *trans*-zeatin riboside | 100 | 3 | 5 |
| *trans*-zeatin | 85 | 1 | 4 |
| 2-*iso*-pentenyladenosine | 1.6 | 100 | 4.5 |
| 2-*iso*-pentenyladenine | 0.9 | 64 | 3 |
| dihydrozeatin-riboside | 2 | 2.5 | 100 |
| DL dihydrozeatin | 1.4 | 1 | 86 |
| adenosine | >0.03 | >0.03 | >0.03 |
| adenine | >0.03 | >0.03 | >0.03 |
| benzyladenine | 1.5 | 4 | 1.5 |
| kinetin | 0.2 | 0.6 | 0.9 |

Note: Cross-reactivities were measured by an enzyme immunoassay in which the concentration of a cross-reactant which inhibited the binding of antigen by 50% was determined

a minimal specificity for the ribose moiety, i. g. they poorly distinguish the base from the corresponding riboside. This has been pointed out by other authors and is due to the fact that these antibodies are polyclonal (Barthe and Stewart, 1985). Data are reported on a higher specificity of polyclonal antibodies for the ribose moiety (Ivanova M. et al., 1994; Redig et al., 1996).

The ZR antibodies were highly reactive with ZR and Z. They had a weak cross-reactivity with iPA, iP from 0.9 to 1.6%, and higher – with DHZR and DHZ, from 1.4 to 2%.

The iPA antibodies were highly reactive with iPA and iP. Weak was the cross-reactivity with ZR, Z, DHZR and DHZ, from 1 to 3%.

The DHZR antibodies were highly reactive with DHZR and DHZ. They cross-reacted almost equally with ZR, Z, iPA and iP from 3 to 5%.

Antibodies against the three cytokinins showed a low cross-reactivity with adenine and adenosine (lower 0.03%).

With BA, antibodies against ZR and DHZR reacted weakly to 1.5%, whereas the cross-reactivity of iPA antibodies was higher to 4%. The three antibodies showed a low cross-reactivity from 0.2 to 0.3% against kinetin.

Typical standard curves were obtained with the three different antibody preparations (Fig. 2). The curves could be linearized over the entire measuring range by logit transformation of B/Bo values.

The ZR standard curve provided a measuring range that extended from 0.1 to 60 pmol of ZR, and from 0.5 to 90 pmol, respectively, for Z. The iPA and DHZR standard curves had a measuring range from 0.5 to 50 and, respectively, 60 pmol. For the corresponding bases the method was valid from 1 to 100 pmol for iP and from 1 to 90 pmol for DHZ.

To test the reliability of the assay, the quantitative data obtained with ELISA were compared with data from an independent physico-chemical method – HPLC (Table 4). We observed an increase of the cytokinin values after ELISA assay. This is observed by many other authors (Hansen et al., 1984; Ivanova A. et al., 1994; Redig et al., 1996). The reason for this is, probably, the cross-reactivity of the antibodies with structural compounds, found in the plant extract. In the determination of cytokinins by ELISA the highest was the content of DHZR/DHZ, followed by that of ZR/Z and the lowest one was the content of iPA/iP. After HPLC separation we observed that among the zeatin cytokinins the riboside form was predominant and among the dihydrozeatin ones – the free base. Among the *iso*-pentenyl cytokinins the corresponding riboside was predominant.

As a rule, some authors analyse the cytokinin amount only by ELISA (Bollmark et al., 1988) or HPLC (Lennox et al., 1993; Kovac and Zel, 1994). In other cases the analysis is carried out by ELISA, and the validity is proved by HPLC (Bangerth, 1994) or by a combination of the two methods (Hansen et al., 1984; Ivanova A. et al., 1994; Bollmark et al., 1995; Feito et al., 1995). In the latter variant the HPLC-step is used only for cytokinin separation and their quantitative determination

**Table 4**. Comparison of the cytokinin concentrations in carnation shoots determined after purification by ELISA and HPLC

| | ELISA (pmol eq.g $DW^{-1}$) | HPLC (pmol.g $DW^{-1}$) |
|---|---|---|
| ZR | 960 | 589 |
| Z | | 325 |
| DHZR | 1350 | 45 |
| DHZ | | 1290 |
| iPA | 530 | 310 |
| iP | | 165 |

Note: Purification losses of cytokinins are presented in Table 1 and are not included in the above values

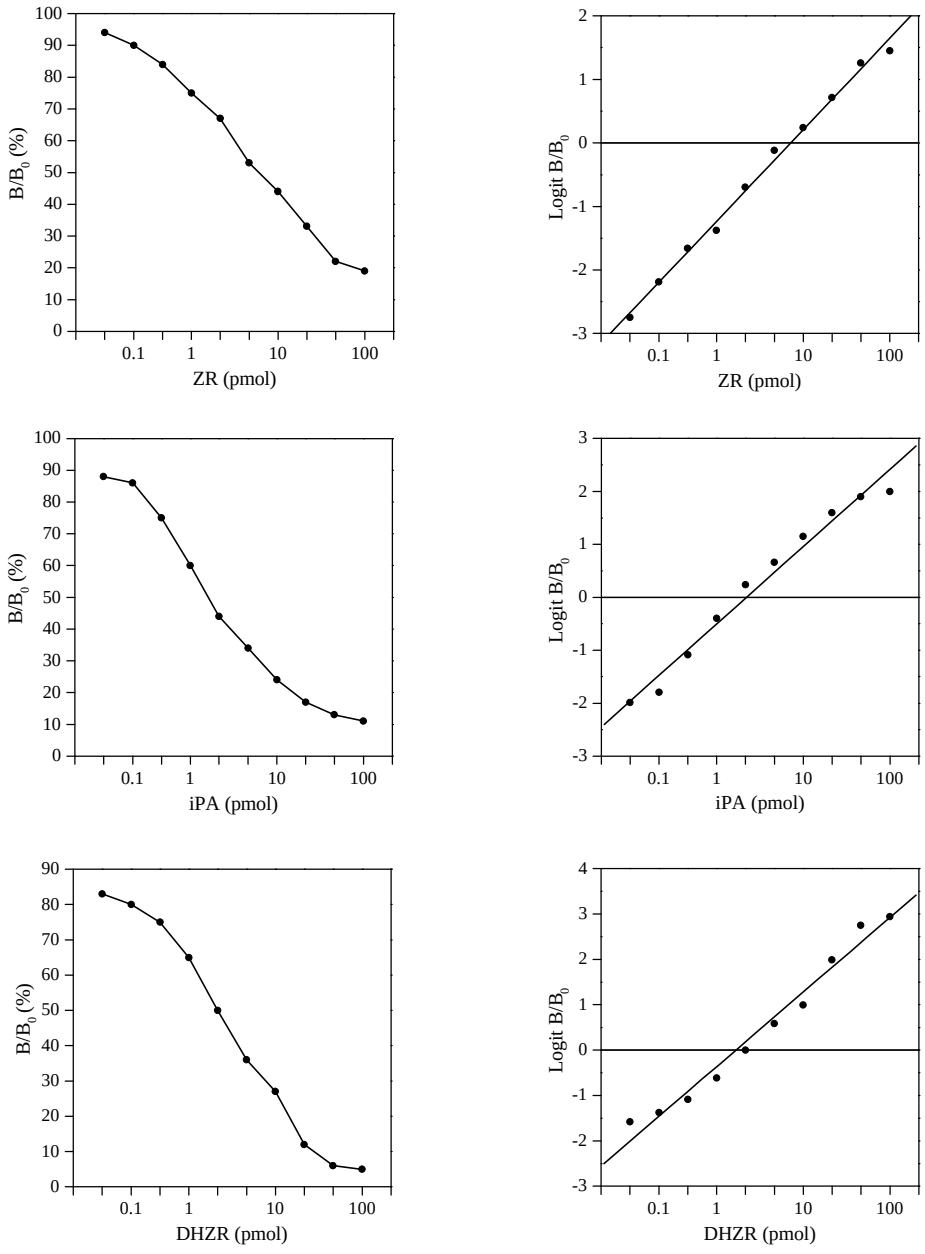


**Fig. 2**. Typical standard curves for antibody ELISA obtained with ZR, iPA and DHZR as standards. B – binding of tracer to antibody in the presence of cytokinins, $B_0$ – binding of tracer in the absence of cytokinin, both values corrected for unspecific binding, logit $B/B_0 = \ln[(B/B_0)/(100-B/B_0)]$

by ELISA, because the amount of cytokinins in the tissues is below the limit of HPLC UV-detection (Fernandez et al., 1995).

The ELISA-method we developed allows determination of natural cytokinins as equivalents. Our data from the HPLC prove the reliability of ELISA with the antibodies which we obtained against ZR, iPA, DHZR and this method is reliable in a determined detection range. For determination of the amount of the corresponding ribosides or bases it is necessary to separate the cytokinins and to analyse them by HPLC.

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