J Plant Growth Regul (2007) 26:1–14
DOI: 10.1007/s00344-006-0045-2

J P G R
Journal of Plant Growth Regulation

NOVEL TECHNIQUES

New Techniques for the Estimation of Naturally Occurring Brassinosteroids

Jana Swaczynová,[1] Ondřej Novák,[1] Eva Hauserová,[1] Květoslava Fuksová,[1] Miroslav Šíša,[2] Ladislav Kohout,[2] and Miroslav Strnad[1]*

[1]*Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany ASCR, Šlechtitelů 11, 783 71 Olomouc, Czech Republic;* [2]*Department of Steroid Chemistry, Institute of Organic Chemistry and Biochemistry ASCR, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic*

Abstract

We have developed enzyme-linked immunosorbent assays (ELISAs) for measuring 24-epicastasterone and related brassinolide analogs, with detection ranges of 0.005 to 50 pmoles. Polyclonal antibodies used in these assays were raised against 24-epicastasterone carboxymethyloxime-bovine serum albumin conjugates and were found to have high specificity for 24-epibrassinosteroids. Natural brassinosteroids (BRs), such as brassinolide and 24-epibrassinolide, exhibited relatively high cross-reactivities with the generated antibodies, whereas other BR analogs with β-oriented hydroxyl groups at C-2, C-3, C-22, and C23 lacked immunoreactivity. Through the use of internal standardization, dilution assays, recovery of authentic [^{3}H]24-epicastasterone, and immunohistograms, the ELISAs have been shown to be applicable for estimating 24-epibrassinosteroid levels in crude plant extracts. To analyze brassinosteroids in tissues from young bean (*Phaseolus vulgaris* L., cv. Pinto), *Daucus carota* ssp.*sativus* plants and *Arabidopsis thaliana* L. Heynh. seedlings, and rape (*Brassica napus* L.) pollen, the extracts were fractionated by high performance liquid chromatography (HPLC) and the resulting fractions were analyzed by the ELISA method. Immunohistogram ELISA analysis of HPLC fractions indicated that major peaks of immunoreactivity co-chromatographed with the labeled and unlabeled 24-epibrassinolide. A highly sensitive electrospray ionization mass spectrometry (MS) technique (LOD: 50 fmol) was also developed and the results obtained by the HPLC-ELISA and HPLC-MS approaches were compared.

Key words: Antibodies; *Arabidopsis*; Brassinosteroids; Enzyme immunoassay; HPLC-MS; Rape pollen

Introduction

Brassinosteroids (BRs) are polyhydroxylated steroid plant hormones that are essential for both the growth and the development of plants (Zullo and

Received: 2 May 2006; accepted: 3 August 2006; Online publication: 16 February 2007
*Corresponding author; e-mail: strnad@aix.upol.cz

others 2003). They induce cell elongation and cell division, increase DNA and RNA polymerase activity, interact synergistically with auxins, stimulate ethylene production, and increase tolerance to stress caused by temperature, water, or salinity (Sasse 1999; Khripach 1999; Sakurai 1999). Many different techniques have been developed for the estimation of BRs in plant tissues. The most widely used are bioassays and gas chromatography-mass spectrometry. There are also some reports describing the development of antibodies against BRs. Horgen and co-workers prepared monoclonal antibodies against a synthetic analogue of brassinolide, but this analogue was non-covalently bound to a carrier protein (Horgen and others 1984). The resulting antibodies showed high cross reactivity (35%–48%) to major plant sterols such as sitosterol, ergosterol and stigmasterol, and therefore were not useful for brassinosteroid analyses. Yokota and others (1990) have reported the synthesis and use of a hapten–protein conjugate prepared from the carboxymethyloxime of castasterone, which exposes the A-ring diol and the side-chain for recognition. This conjugate induced the production of a very useful antiserum that had high affinity for a range of naturally occurring brassinosteroids, especially those having a 6-keto group or a lactone in the B ring (that is, castasterone or brassinolide types). Schlagnhaufer and others (1991) described the preparation of a hemisuccinate of an unnatural brassinolide analog (2α,3α,22β,23β-tetrahydroxy-24β-methyl-B-homo-7-oxa-6-one) and its use to prepare an antiserum in mice. However, they provided no information on the structure of the hemisuccinate, and the limited cross-reactivity data they presented indicated that it had only relatively low affinity for natural brassinolide and its analogs. Therefore the success of this approach is difficult to assess at the present time.

High performance liquid chromatography (HPLC) is one of the most frequently used analytical methods for the separation and analysis of BRs (Gamoh and Takatsuto 1994). Brassinosteroids have no suitable chromophore for detection, and hence they are often derivatized with pre-labeling reagents to make them responsive to ultraviolet (UV), fluorometric, or electrochemical detectors. Several boronic acid reagents that react with the vicinal diol groups of BRs have been used as pre-labeling reagents, including naphthaleneboronic acid for UV detection (Gamoh and others 1988); 9-phenanthreneboronic acid (Gamoh and others 1989b), 1-cyanoisoindole-2-m-phenylboronic acid (Gamoh and Takatsuto 1989a), and (dansylamino)phenylboronic acid (Gamoh and others 1990a) for fluorimetric detection; and ferrocenboronic acid for electrochemical detection (Gamoh and others 1990b). The reported detection limits were dependent on the derivative used, ranging from 25 to 100 pg per injection. Of these reagents, (dansylamino)phenylboronic acid seems to be the most useful because the derivatives it yields can be detected at longer wavelengths (excitation 345 nm/emission 515 nm) than other boronates, and hence they are subject to the least interference by matrix contaminants. The derivatized BRs are effectively separated by HPLC using octadecyl silica gel (ODS) columns with acetonitrile–water mixtures as the mobile phase.

Gamoh and others (1992) developed an HPLC method based on a combination of pre-column labeling and post-column fluorescence detection that could be used to separate and detect 24-epimers of brassinolide and castasterone. Related reversed-phase HPLC methods have also been successfully applied to analyze BRs in pollen samples from broad bean, corn, sunflower, buckwheat, and orange (Takatsuto 1991; Gamoh and others 1994; Motegi and others 1994).

Microanalytical liquid chromatography/mass spectroscopy (LC–MS) methods for analyzing BRs as their boronates have also been developed using either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). The APCI-based method of LC–MS was used to analyze naphthalenoboronate derivatives of BRs, and optimum results were obtained by using a reversed-phase HPLC acetonitrile-water gradient to elute the analytes from a C18 column (Gamoh and others 1996). Typical ions observed in the positive-ion spectra of the naphthalenoboronates were a pseudo-molecular ion $[M+H]^+$ and a fragment ion, $[M+H-H_2O]^+$. The parent ion was the most abundant for BL fragmentation, whereas the $[M+H-H_2O]^+$ ion was the most abundant in castasterone, teasterone, and typhasterol mass spectra. Full-scan mass spectra were readily obtained from 400 ng of free BRs, whereas the limit of detection in the selected ion monitoring (SIM) mode was around 2 ng. The most effective mobile phase for naphthalenoboronates was found to be a mixture of acetonitrile and water (9:1) containing 0.5% HCOOH. Svatoš and others (2004) described a highly sensitive and selective LC-(ESI)-MS method for analyzing BRs in plant extracts, involving the use of a microbore C18 column (1.0 mm) and chemical derivatization of free BRs to dansyl-3-aminophenylboronates, with a much lower limit of detection (LOD) than previous analytical methods. The limit of detection in SIM mode for derivatized BRs was 125 attomole. The practical utility of the

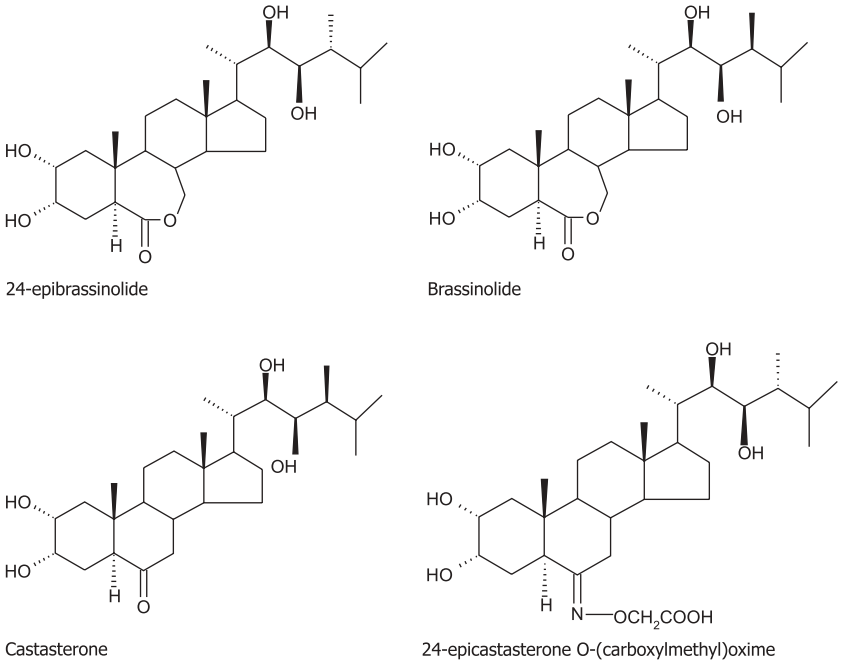

Figure 1. Structure of brassinolide, castasterone, 24-epibrassinolide, and 24-epicastasterone O-(carboxylmethyl)oxime.

method was illustrated by demonstrating the transformation of a deuterium-labeled precursor of castasterone to brassinolide in *Arabidopsis thaliana* plants (Svatoš and others 2004).

Here, we describe the development of polyclonal antibodies against the brassinosteroid, 24-epicastasterone. Antisera against this substance were produced by immunizing rabbits with 24-epicastasterone carboxymethyloxime (24-epiCS-CMO) (Figure 1) conjugated to bovine serum albumin (BSA). The conjugates were prepared using a mixed anhydride procedure, and the antibodies obtained were tested by enzyme-linked immunosorbent assay (ELISA) using a 24-epiCS-CMO-peroxidase conjugate. We report the usefulness of the ELISA with the polyclonal antibodies raised against 24-epicastasterone for the analysis of endogenous BRs in different plant materials. Furthermore, we also developed a sensitive electrospray HPLC/MS assay for the direct analysis of BRs and compared the two approaches for estimating levels of BRs in different plant tissues.

Materials and methods

Chemicals and Reagents

24-Epicastasterone (24-epiCS) and 24-epibrassinolide (24-epiBL) were synthesized according to our improved methods (Kohout 1994; Šíša 2005). The synthesis of 24-epicastasterone-O-(carboxymethyl)oxime (24-epiCS-CMO) has also been described previously (Swaczynová and others 2006). Other unlabeled brassinosteroids were obtained from Olchemim Ltd. (Olomouc, Czech Republic) or CIDtech Research Inc. (Cambridge, Ontario, Canada). [$^2H_6$]Brassinolide and [$^2H_6$]castasterone were generous gifts from Dr. B. Schneider (Jena, Germany). Horseradish-peroxidase for enzyme immunoassay (2500 U · mg^{-1}), bovine serum albumin, tributylamine, isobutylchloroformate, dimethylformamide, dimethylsulfoxide, *N*-hydroxysuccinimide, *N*,*N*′-dicyclohexylcarbodiimide, picrylsulfonic acid, 3,3′,5,5′-tetramethylbenzidine, and DEAE-Sephadex were from Sigma (St. Louis, MO, USA). C18-reversed phase columns and cartridges were from Waters (Prague, Czech Republic), Strata X reversed-phase columns (33 μm, surface modified styrene divinylbenzene) were from Phenomenex (Torrance, CA, USA), and ELISA plates were from Nunc A/S (Roskilde, Denmark). All other chemicals were obtained from Lachner (Neratovice, Czech Republic).

Preparation of [^{3}H]epicastasterone

[^{3}H]Epicastasterone (specific activity 50 TBq · mol^{-1}) containing tritium in the α-position to the keto group was obtained by a base-catalyzed isotopic exchange reaction in a dioxane solution containing [^{3}H]H_2O and [^{3}H]methanol (specific activity of reaction mixture 65 GBq). A solution for tritium labeling was prepared by stirring dioxane (0.1 ml), methanol (0.02 ml), and platinum dioxide (7 mg) for 1 h in a 15% [^{3}H] atmosphere (5 ml) at 0.075 MPa. The labeling procedure was started by

lyophilization of a 1.8 M methanolic solution of KOH (20 µl) in a reaction bulb, to which a solution containing 1.5 mg epicastasterone in dry dioxane (0.1 ml) was added, followed by lyophilization of the tritiated solution to the reaction bulb. The mixture was stirred for 2 h under an atmosphere of argon. After lyophilization, the product was dissolved in an absolute toluene:ethyl acetate mixture (1:1, v/v), separated from the remaining KOH by centrifugation, and lyophilized, after which the labile radioactivity was removed by repeated cycles of solubilization in *tert*-butanol:H_2O (9:1, v/v) and lyophilization. The crude product (200 MBq) obtained had 94% radiochemical purity, as determined by thin-layer chromatography (TLC) analysis on a Merck silica gel ($CHCl_3$: CH_3CN: acetone, 45:45:10, v/v/v, $R_f = 0.20$), and was purified by preparative TLC chromatography under the same conditions. The total yield of [^{3}H]epicastasterone was 110 MBq with radiochemical purity higher than 99%.

Plant Material

Rape (*Brassica napus* L.) pollen collected from the field and 10-day-old *Phaseolus vulgaris* L., cv. Pinto and *Arabidopsis thaliana* L. Heynh. (accession Col-0) plants were used for brassinosteroid analysis. The bean seedlings were grown in pots containing perlite and a 1/10 dilution of Hoagland's solution in a light-controlled cultivation room (25°–27°C, light 48 $W \cdot m^{-2}$, light–dark period 16/8 h). *Arabidopsis* plants were grown in 250-ml Erlenmeyer flasks containing 50 ml of 50% Murashige-Skoog basal growth medium, 3% (w/v) sucrose, pH 5.6 (20 seeds per bottle). The flasks were agitated and kept at 23°C, with an 18-h photoperiod. After 10 days, the plants were harvested, immediately frozen in liquid nitrogen, and stored at –70°C until extraction and purification. Fresh *Daucus carota* plants were bought in an open market in Olomouc, Czech Republic, on June 2006 and extracted the same day.

Immunological Reagents

24-Epicastasterone-O-(carboxymethyl)oxime (24-epiCS-CMO; Figure 1) was conjugated to BSA using a modification of the mixed anhydride procedure described by Erlanger (1967). Briefly, 24-epiCS-CMO was dissolved in dimethylsulfoxide and dioxane and treated with tributylamine and isobutyl chloroformate for 1 h at 4°C (final volume 350 µl, 24-epiCS-CMO/tributylamine/isobutyl chloroformate, 1/2/1.5, v/v/v). The activated hapten solution was carefully added to BSA dissolved in 5 mM phosphate buffer (15 mg/ml, pH 7.4), and the mixture was gently stirred for 12 h at 4°C. The conjugate was dialyzed against PBS (pH 7.4) for 5 days. The molar ratio of 24-epiCS-CMO:BSA (15:1) was determined by the trinitrobenzene sulfonate method (Satake 1960). The 24-epiCS-CMO–horseradish peroxidase conjugate used as the labeled antigen in competitive ELISA was prepared with carbodiimide (O'Sullivan 1979; Gross 1968). 24-EpiCS-CMO (2.46 µmol) was dissolved in 200 µl dimethylformamide and mixed with equal amounts of *N*-hydroxysuccinimide and *N,N'*-dicyclohexylcarbodiimide. The mixture was stirred for 12 h at room temperature. The activated ester was added dropwise to a solution of horseradish peroxidase (3.4 mg in 300 µl 0.13 M $NaHCO_3$) and stirred for 3 h at room temperature. The mixture was then dialyzed against phosphate buffer (pH 7.4) for 3 days. The immunization schedule and purification of antibodies are described in detail elsewhere (Strnad and others 1997). Antibodies for ELISA were purified by either ammonium sulfate precipitation or by protein A affinity chromatography (Harlow and Lane 1988).

Enzyme-linked Immunosorbent Assay (ELISA)

Individual wells of microtiter plates (Nunc-Maxisorp) were first coated with 150 µl of anti-24-epiCS-CMO polyclonal antibody (0.66 µg/ml, 50 mM $NaHCO_3$, pH 9.6), incubated overnight at 37°C for binding, and then washed and blocked twice with 200 µl of 1% PBS-TWEEN 20 buffer per well for 15 min. After decanting, the wells were filled, in the following sequence, with 100 µl of PBS (50 mM Na_2HPO_4/ NaH_2PO_4 buffer, 0.15 M NaCl, pH 7.2), 50 µl of standard in phosphate buffered saline (PBS), and 50 µl of tracer solution (0.1%, w/v, BSA in PBS buffer), after which the plates were incubated at 37°C for 2 h. Unbound conjugate was removed by rinsing the plates three times with PBS containing 1% (w/v) TWEEN 20. The plates were then filled with 150 µl of a solution of 3,3',5,5'-tetramethylbenzidine (TMB) to act as a substrate (1 mg TMB, 500 µl CH_3COONa, 10 µl 16%, w/v, H_2O_2/10 ml H_2O). The reaction was stopped after 15 min of incubation at 25°C by adding 50 µl 2 M H_2SO_4, and the final absorbance was measured at 450 nm (Titertek Multiscan® PLUS). Sigmoid curves for standards and cross-reacting compounds were linearized by the log-logit transformation: $\text{logit } B/B_0 = \ln\{(B/B_0)/(100–B/B_0)\}$. The resulting data were processed by the ImmunoRustregAnalyser computer program (Laboratory of Growth Regulators, Olomouc, Czech Republic).

Extraction and Purification of Brassinosteroids

A previously described method was used for the extraction and purification of the endogenous brassinosteroids (Yokota and others 1984, 1997; Nomura and others 1997). Freeze-dried plant tissues were ground to a fine powder under liquid nitrogen and extracted twice in ice-cold 80% (v/v) methanol for 2 h (10 ml/g FW). About 1400 Bq (80,000 dpm) of [^{3}H]24-epicastasterone (for HPLC-ELISA) or 10 pmol of [$^2H_6$]brassinolide and [$^2H_6$]castasterone (for HPLC-MS) was added to the extracts to measure recovery during purification, to facilitate the location of natural BRs on the basis of their co-elution with authentic internal standards, and to estimate exact levels of brassinosteroids in plant samples. After centrifugation, supernatants were recovered and purified by extraction on C18 column cartridges (0.5 g, Waters). The samples were diluted to 20 ml in 40 mM ammonium acetate buffer (pH 6.5) and passed through two columns in tandem: a DEAE Sephadex A-25 (Sigma, USA; 1 ml/g FW) coupled to a Strata X reversed-phase column (33 μm, Phenomenex, Torrance, CA, USA). After the samples were loaded, columns were washed with 15 ml of 40 mM CH_3COONH_4 (pH 6.5). Brassinosteroids retained on the Strata X were eluted with 3 ml 100% methanol, and the eluates were dried under vacuum and stored at −20°C until further analysis. Before use, the Strata X sorbent was activated with 10 ml 70% (v/v) EtOH, 5 mL H_2O, and 5 ml 40 mM CH_3COONH_4 (pH 6.5).

Equipment

The HPLC-MS experiments were carried out in an Alliance 2695 Separations Module (Waters, Milford, MA, USA) linked to a Waters PDA 996 photodiode and array detector and a ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, UK). Data were processed by Masslynx software (Data Handling System for Windows, version 4, Micromass, Altrincham, UK).

The HPLC system described above was linked in series to a PDA 2996 (Waters) and Frac-100 fraction collector (Pharmacia, Uppsala, Sweden) for the fractionation of samples prior to ELISA.

HPLC-MS Conditions

Samples were dissolved in 100% methanol, filtered through a micro-filter (PTFE, 4 mm, 0.45 μm, Waters), and 5 μl (25% of total sample volume before filtration) of each sample was injected onto a 150 mm × 2.1 mm, 5 μm Symmetry C18 reversed-phase column (Waters). The column thermostat was set at 30°C and the mobile phase was the following binary gradient of solvent A (methanol) and solvent B (5 mM formic acid) at a flow rate of 0.25 ml/min: 0 min, 70% A; 0–12 min, 75% A, 12–16 min, 100% A. The column was equilibrated to initial conditions for 10 min. Using post-column splitting (1:1), effluent was simultaneously introduced into the DAD (scanning range 210–400 nm; with 1.2 nm resolution) and electrospray source (source temperature 100°C, capillary voltage + 3.2 kV, cone voltage +20 kV, desolvation temperature 250°C). Nitrogen was used as both the desolvation gas (550 l/h) and the cone gas (50 l/h). The detector parameters were set to a span size of 0.5 m/z and an interchannel delay of 0.02 s. Quantification was performed by SIM of molecular $[M+Na]^+$. The dwell time of each SIM channel was calculated to obtain 16 scan points per peak and individual mass.

HPLC-ELISA Analyses

Samples were dissolved in 100% methanol and 5 μl (25% of the total sample volume) was separated on an analytical reversed-phased column (150 mm × 2.1 mm, 5 μm particles; Symmetry C18, Waters) as described above for HPLC-MS. The separation was combined with testing of HPLC-fractionated extracts by ELISA specific for 24-epiCS. For the ELISA analysis, fractions of 0.5 min were collected, evaporated to dryness *in vacuo*, and re-dissolved in 15 μl methanol and 185 μl PBS buffer. Fifty-microliter aliquots were investigated in duplicate by scintillation counting and ELISA. The content of individual brassinosteroids in the appropriate immunoreactive fractions was assessed using a series of different ELISA analyses including dilution and internal standardization (Weiler 1982; Badenoch-Jones and others 1984). The brassinosteroid values obtained by the ELISA of the fraction(s) were calculated from the 24-epiCS and 24-epiBL calibration curves and corrected by the appropriate recovery values to obtain estimates of BR levels in plant tissues (expressed as 24-epiCS or 24-epiBL equivalents, respectively).

Results and Discussion

Assay Characteristics

All immunized rabbits produced antisera to the 24-epiCS-CMO-BSA conjugate, but serum titers

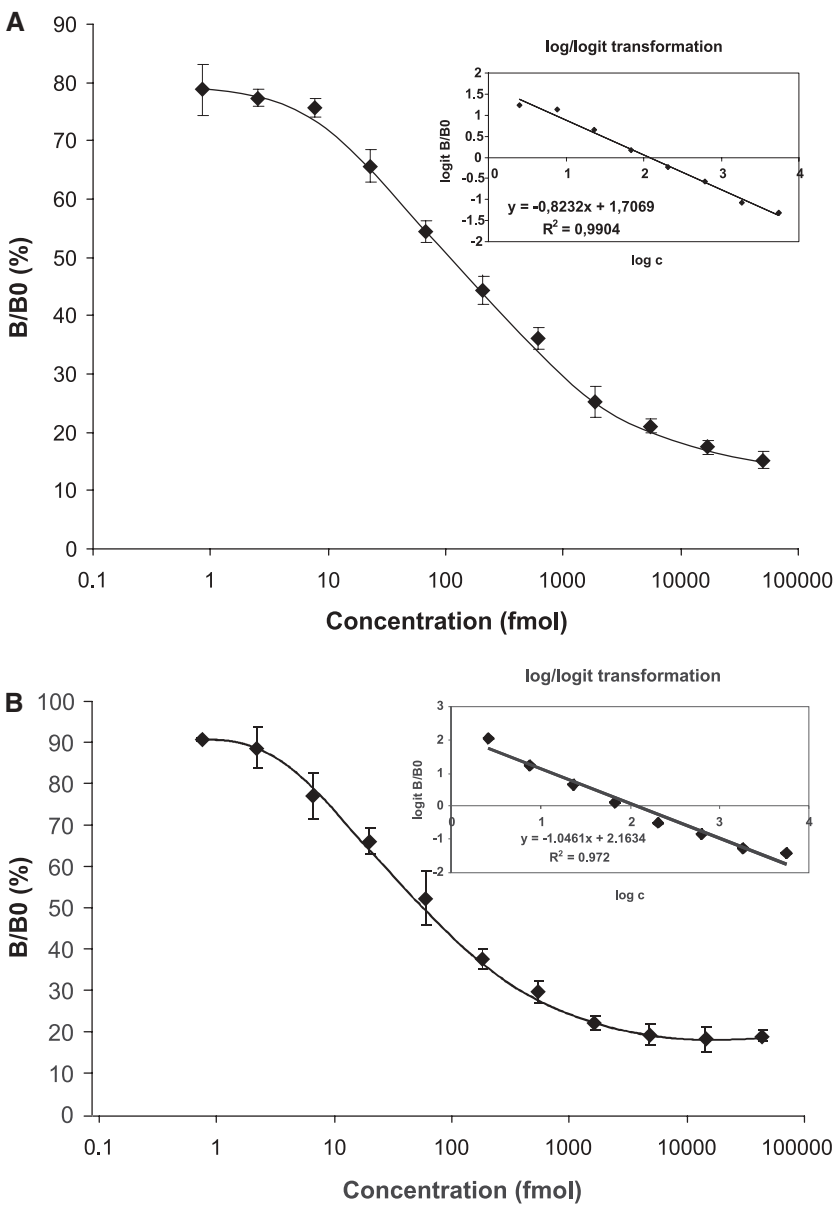

Figure 2. Typical standard curves obtained for 24-epibrassinolide (**A**) and 24-epicaststerone (**B**) ELISAs and linearized logit/log plots of the same data (inset). Bars indicate standard deviations of duplicates ($n = 20$); B and Bo represent binding of peroxidase tracer in the presence and absence of 24-epiCS or 24-epiBL.

differed considerably, reflecting variations in the reactions of individual animals. Antibody CMO 2, specific for 24-epiCS, was selected and routinely used for brassinosteroid analysis because of its high selectivity. Mean standard curves and the corresponding log/logit plots obtained with this antibody are shown in Figure 2. The inset shows the linearized curve ($r = –0.97$), providing a measuring range that was significant at $p = 0.001$ over the range $1.1.10^{-7}$–$1.5.10^{-10}$ and $1.1.10^{-7}$–5.10^{-11} mol.l^{-1} for 24-epiCS and 24-epiBL, respectively. Unspecific binding (in the presence of an excess of 24-epiCS, 100 pmol) amounted to less than 5.5% of the total. The average coefficient of variation of standard B/Bo values within the measuring range was less than 3.5%, and between-assay variation was around 9%. The assay parameters compare favorably with those of other published BR immunoassays (Horgen and others 1984; Schlagnhaufer and others 1991; Yokota and others 1990).

Like gibberellins, brassinosteroids present a challenging problem for the production of applicable antibodies because they possess few unique structural features. The majority of natural metabolites consist of structures containing a 2,3-diol grouping, a vicinal *cis*-diol functionality along with alkyl groups of varying length and a degree of unsaturation in the side-chain, and a 6-carbonyl functionality, either as a ketone or a lactone. In addition, a

Table 1. Molar Cross-reactivities of Various Brassinosteroids with Antibodies Raised Against 24-epicastasterone

Brassinosteroids	Cross-reactivity (%)	Brassinosteroids	Cross-reactivity (%)
24-Epicastasterone	100	24-Epibrassinolide	24.3
Castasterone	0.27	25-Hydroxybrassinolide	0.08
25-Hydroxycastasterone	0.11	22S,23S-Epibrassinolide	0.2
28-Homocastasterone	0.11	22S,23S–Homobrassinolide	0.3
22S,23S-Homocastasterone	0.1	Cholesterol	0.05
22S,23S-Epicastasterone	0.4	Stigmasterol	<0.01
Brassinolide	1.3	β-Sitosterol	<0.01
28-Homobrassinolide	1.0	Ergosterol	<0.01

Data presented are expressed as percentage ratios of the molar concentrations of 24-epicastasterone and the cross-reacting competitor giving 50% binding.

number of structurally closely related steroids may occur in tissues at much higher levels, for example, sitosterol, cholesterol, or ergosterol. Therefore, a range of structurally or physiologically very similar compounds had to be tested for cross-reactivity with the antibody to prove the applicability of the assay for the determination of BR in plant extracts. The specificity of the antibodies was therefore determined by detailed cross-reactivity studies, and the results are given in Table 1. The compounds were tested for antibody binding over a range of 0.01 to 500 pmol per assay. Our newly generated antibody is clearly highly specific for 24-epiCS (relative reactivity: 100%). No cross-reactivity was found toward any of the non-brassinosteroid plant sterols like cholesterol, stigmasterol, ergosterol, β-sitosterol, and related compounds, even when tested in amounts up to 500 pmol per assay. Other natural brassinosteroids, such as castasterone, 28-homocastasterone, 25-hydroxycastasterone, and 25-hydroxybrassinolide showed only marginal cross-reactivity (<0.3%), and all SS-brassinosteroids behaved in a similar fashion. In addition to 24-epicastasterone, the antibodies also cross-reacted with 24-epibrassinolide (24.3%) and brassinolide (1.3%), and thus they are useful for the determination of these phytohormones in plant tissues. The more flexible conformational behavior of the 24R side chain in 24-epiBL compared with BL, as determined by nuclear magnetic resonance (NMR) and modeling studies (Drosihn and others 2001; Stoldt and others 1997), is a critical property decreasing its biological activity (brassinosteroids with a 24S-methyl or -ethyl group have a tenfold higher bioactivity than corresponding hormones with a 24R-alkyl function). The 24R side-chain is also a decisive group lowering the reactivity of BL with the 24-epiCS antibodies. 28-Homobrassinolide, which has an ethyl group in the side-chain at C24, reacted so poorly that its interference in the determination of 24-epiCS or 24-epiBL, even if present in higher amounts than these BRs in extracts, can be regarded as negligible. The slopes of the log/logit transformation of all brassinolide analogs were similar to the standard curve of 24-epicastasterone.

Three groups have reportedly developed antibodies against BRs, but none of them have described antibodies against 24-epiCS-CMO (Horgen and others 1984; Schlagnhaufer and others 1991; Yokota and others 1990). In contrast, our antibodies show the highest specificity toward 24-epicastasterone and 24-epibrassinolide. These findings are in accordance with the different positions used for attaching the BRs to the immunogenic carrier, and they agree with earlier observations that antigenic structural features located near the coupling site are not usually as well discriminated by the elicited antibodies as features remote from the coupling site (Weiler 1982; Strnad and others 1992a, b).

Validation of the Direct ELISAs

The apparent endogenous BR content obtained by comparing proportions (percentages) of immunotracer that bound in the presence of a standard (the B/Bo value) was plotted against BR concentration. This procedure makes it possible to detect inactivation of the antibody or the binding of compounds other than 24-epiCS in the samples, which could lead to spurious estimates of BR contents. In principle, the exogenous 24-epiCS curve should be a straight line with a unit slope and a *y*-intercept equal to the amount of BR originally present in the sample. The presence of interfering compounds that adversely affect accuracy will be indicated by a change in the slope of this curve. The analyses should also show additivity, that is, doubling the amount of extract should

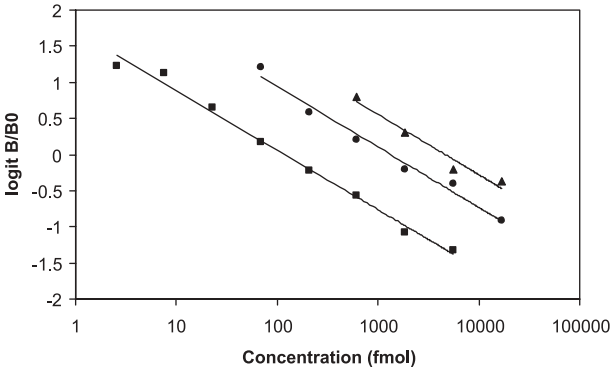

Figure 3. Validation of the ELISA data for 24-epibrassinolide. Logit transformation of enzyme-linked immunosorbent assay (ELISA) standard curves (■) and dilution curves of crude extract (▲) and extract spiked with varying amounts of unlabeled standard added to a fixed amount (50 µl) of crude extract (●). Logit $B/B_0 = \ln[(B/B_0)/(100\text{-}B/B_0)]$.

give double the reading in the immunoassay. These validation protocols have been described previously by Pengelly and Meins (1977) and further elaborated by Pengelly (1986) and Jones (1987) in a discussion of potential sources of errors in plant hormone immunoanalyses.

Details of such a validation for one of the samples are shown in Figure 3. Initial experiments showed that dilution curves for the crude *Arabidopsis* extracts did not always parallel the standard curve. However, partial purification of the samples by Strata X chromatography resulted in parallel standard and sample dilution curves, suggesting that the samples did not contain substances that interfered with the assays. When activity was detected in HPLC fractions that were assayed at more than one dilution, the dilution curves were also parallel to the standard curve. Furthermore, the recoveries of internal standard spiked into the crude and HPLC-purified extracts were found always to yield satisfactory parallel lines (see Figure 3). The cross-reactivity and dilution analysis data suggest that the assay allows the reliable estimation of 24-epiCS and/or 24-epiBL levels in crude extracts. This approach may have useful applications in the control of exogenously applied 24-epiBL in field trials, because this brassinosteroid is used to improve crop production in many different countries (Kripach and others 1999).

Accurate quantification of brassinosteroids in plant extracts was performed by HPLC–ELISA analysis of partially purified extracts in conjunction with recoveries of internal tritium-labeled standard. Recovery of [^{3}H]24-epiCS between 32% and 38% was obtained. The classical purification protocol based on the C18-DEAE-Strata X procedure was also standardized by deuterium-labeled BRs. In HPLC-MS analysis, recoveries of 25%–32% were achieved, values similar to those obtained for [^{3}H]24-epiCS and other plant hormones in this type of purification procedure (Novák and others 2003). The comparatively poor recoveries were probably due to the relatively complex C18-DEAE-Strata X purification procedure applied to this series of samples. Thus, the accurate determination of endogenous BRs presented above enabled purification by C18-DEAE-Strata X chromatography and the quantitation by HPLC-ELISA/MS to be validated by an internal standardization procedure. The addition of labeled derivatives to the extracts further facilitated the detection of natural BRs, giving better resolution of products that eluted close together, as well as a measure of the percentage recovery of different BRs throughout the purification procedure. Ideally, recovery markers are required for each hormone metabolite that is being measured (Prinsen and others 1998; Novák and others 2003). However, in many cases only a few internal standards have been used, often added later during the extraction process, or just before quantitative analyses (Prinsen and others 1997, 1998; Van Rhijn and others 2001). Clearly, our methodology presented here could be improved by internal standardization of each of the measured BRs.

LC-ESI-MS

Brassinosteroids yielded quasi-molecular stable ions of $[M+Na]^+$ in electrospray-positive mode (ESI+ for brassinolide 503.24; see Figure 4) as the most abundant ions in the mass spectrum. This phenomenon is most likely due to the strong and stable attachment of sodium to the ketone and hydroxyl groups of steroids (Ma and Kim 1997). In positive ion mode, *m/z* 427.26, 445.29, 463.28, 481.28, 519.28 ions corresponding to, respectively, $[M\text{-}3H_2O+H]^+$, $[M\text{-}2H_2O+H]^+$, $[M\text{-}H_2O+H]^+$, $[M+H]^+$, and $[M+K]^+$ were also detected. Under the conditions tested, however, strong and specific $[M+Na]^+$ ions were mainly observed that have not previously been used in BR analysis, because only $[M+H]^+$ were employed in previous investigations (Gamoh and others 1996; Svatoš and others 2004). Competition between sodium adduct formation and protonation also appears to occur during the electrospray ionization process of other steroids (Ma and Kim 1997; Díaz-Cruz and other 2003). Because the ESI sensitivity improved threefold when 5 mM was used instead of 50 mM formic acid, we attempted to eliminate protonation altogether by use

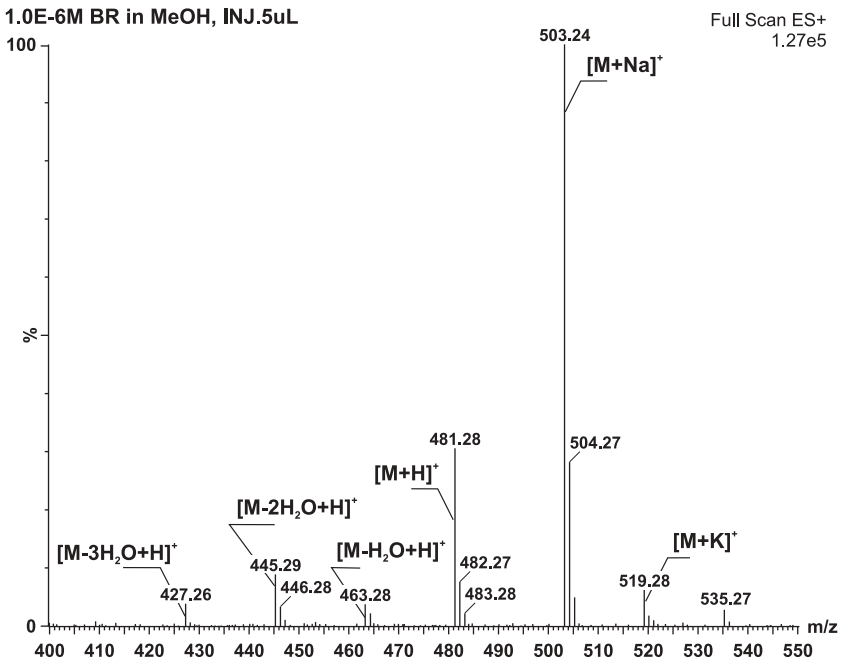

Figure 4. Isotopic cluster pattern of brassinolide obtained in a full-scan electrospray ionization (ESI) mass spectrum.

of a formic acid-free CH_3OH/H_2O mobile phase. Elimination of the proton source required extensive washing of the LS-MS system with water, where formic acid or ammonium acetate had been used previously. The effectiveness of the washing process could be followed by monitoring the decreased abundance of protonated methanol (*m/z* 33). After extensive washing, almost no electrospray current was observed (fluctuating between 0 and 0.05 mA), and the background signal was very low. The sodium ion (*m/z* 23) is usually the most abundant ion observed under these conditions. The brassinosteroid spectra obtained contained primarily the natriated molecules $[M+Na]^+$, as shown in Figure 4, thereby considerably improving detection limits during SIM analysis (Table 2). Comparable results were obtained for all BRs in ESI+ mode. Monitoring stable $[M+Na]^+$ ions in ESI mode also provided the best LC/MS sensitivity for hydroxylsteroids related to either progesterone/testosterone or ketosteroids related to the estradiol group (Ma and Kim 1997). We further investigated the effects of various parameters (for example, desolvation temperature, capillary and cone voltage) on the sensitivity of mass spectrometric detection, and the optimal values found were used for the LC-ESI-MS method (Table 2).

Quantification was performed using the standard isotope-dilution method. Final concentrations were calculated from the areas of the *m/z* $[M+Na]^+$ peak for labeled and authentic brassinosteroids in the SIM chromatograms. Chromatograms of one ion are not conclusive, and therefore two other diagnostic ions $[M-2H_2O+H]^+$and $[M+H]^+$ were used to measure the endogenous brassinosteroid levels. We were, however, able to get exact values for brassinolide in pollen rape extracts alone. The $[M-2H_2O+H]^+$and $[M+H]^+$ ions were usually of approximately 30% (BL-like structures) and 10% (CS-like structures) ion intensity compared to appropriate $[M+Na]^+$ ions. For this reason, the limits of detection (LOD) of these ions were 5–10 times lower than that presented for $[M+Na]^+$ in Table 2 (for comparison see ions for BL in Figure 4). Under the LC separation conditions, the limits of detection (LOD), retention time stability, linear ranges, and expression of linearity obtained for the standard compounds are given in Table 2. The LOD (defined as a signal to noise ratio of 3) of all the brassinosteroids measured was 50–75 fmol. The data shown were obtained by analyzing each analyte at nine different concentrations, ranging between 0.01 and 100 pmol per injection. After log-transformation, a linear regression function adequately described the ratios between concentration and integrated area units of the corresponding peak signals within the concentration range. The results clearly show that the method gives good linear correlation, with correlation coefficients ranging from 0.9990 to 0.9993. The linear ranges found for the brassinosteroids (0.1–50 pmol) are also in good agreement with previously published data (Gamoh and others 1996; Konstantinova and others 2001) and also with the results obtained for other plant and animal steroids (Ma and Kim 1997; Kuronen and others 1999; Van Aerden and others 1998; Shimada and

Table 2. The Dynamic Range, Detection Limit, Retention Time, Dwell Time, and Expression of Linearity (linear regression and correlation coefficients) for the Estimation of Various Brassinosteroids by LC–ESI–MS

Compound	Retention time (min)	m/z	Detection limit (fmol)	Dynamic range (pmol)	Dwell time (s)	Equation of linear regression	ρ^2
Brassinolide	9.13 ± 0.02	503.24	75	0.1–50	1.29	y = 1.01 ± (0.01)*x* + 0.14 ± (0.02)	0.9995
24-Epibrassinolide	8.40 ± 0.04	503.24	50	0.1–50	1.29	*y* = 0.99 ± (0.02)*x* + 0.16 ± (0.03)	0.9993
28-Homobrassinolide	12.28 ± 0.02	517.27	50	0.1–50	0.85	*y* = 1.06 ± (0.11)*x* + 0.20 ± (0.03)	0.9996
Castasterone	12.65 ± 0.03	487.28	50	0.1–50	0.85	*y* = 1.02 ± (0.01)*x* + 0.37 ± (0.03)	0.9993
24-Epicastasterone	11.81 ± 0.03	487.28	75	0.1–50	0.85	*y* = 1.02 ± (0.01)*x* + 0.04 ± (0.02)	0.9991
28-Homocastasterone	16.19 ± 0.02	501.29	50	0.1–50	2.55	*y* = 0.98 ± (0.07)*x* + 0.25 ± (0.07)	0.9991

Table 3. Endogenous Brassinosteroid (BR) Levels in Different Plant Materials (pmol/g FW)

	Brassinolide	24-Epibrassinolide		
Plant material	HPLC/MS	HPLC/ELISA	HPLC/MS	HPLC/ELISA
Phaseolus vulgaris	0.98 ± 0.21	ND	<0.72	<8.8
Arabidopsis thaliana	2.59 ± 0.15	ND	7.56 ± 1.65	9.5 ± 2.57
Rape pollen	211.5 ± 38.86	ND	<0.72	<8.8
Daucus carota	1.34 ± 0.14	ND	1.55 ± 0.56	<8.8
	Castasterone	24-Epicastasterone		
	HPLC/MS	HPLC/ELISA	HPLC/MS	HPLC/ELISA
Phaseolus vulgaris	2.08 ± 0.32	ND	<1.08	<2.2
Arabidopsis thaliana	1.21 ± 0.07	ND	<1.08	<2.2
Rape pollen	26.18 ± 0.74	ND	<1.08	<2.2
Daucus carota	0.68 ± 0.06	ND	1.37 ± 0.77	2.56 ± 2.05

Two triplicate series of the same samples were extracted with 80% (v/v) methanol and purified using a C18 column, a coupled DEAE Sephadex and Strata X chromatography system. Brassinosteroids were analyzed either by LC–ESI–MS or by 24-epicastasterone-specific LC–ELISA of 0.5 min fractions collected from the chromatography steps. The values have been corrected for the recovery of internal standards of [$^2H_6$]- or [^{3}H]-BR added during extraction.
ND: not detected.

others 2001; Schlüsener and others 2005). The LC–MS method described by Svatoš and others (2004), which is based on the chemical derivatization of free BRs to dansyl-3-aminophenylboronates is, however, more sensitive, giving a LOD in SIM mode of around 125 attomole. Nevertheless, the LOD of 100 pg/ml reported for samples obtained from native plant extracts was significantly higher and in the range of the method described here in which only extracts from 25% of the initial amount of fresh plant material (1 g) were injected (see results for *P. vulgaris* in Table 3).

Immunodetection and Quantification of BRs in Plant Extracts

To further validate the methods for brassinosteroid analysis in plant samples the LC–ESI–MS-based approach was introduced and the results obtained compared with the LC–ELISA data. The method achieving selectivity in the presence of other signals was based on co-chromatography of SIM and immunoactivity traces with signals of authentic labeled standards. This was reached by separating all cross-reactive compounds using HPLC on the Symmetry C18 column (Figure 5).

The applicability of the HPLC–ELISA procedure for detecting BRs in the young tissues was demonstrated using extracts from *A. thaliana* seedlings and *D. carota* plants. Assays of HPLC-purified extracts from *A. thaliana* using the ELISA for 24-epiCS detected only the cross-reactive compound co-eluting with the authentic and labeled 24-epibrassinolide standard (Figure 5). The specificity of the antibodies may have allowed more derivatives to be detected and quantified, but no other cross-reacting compounds were present in the immunohistograms. The level of 24-epiBL determined from three duplicate estimates was found to be 9.5 ± 2.57 pmol.g^{-1} FW in *A. thaliana*. We established that the

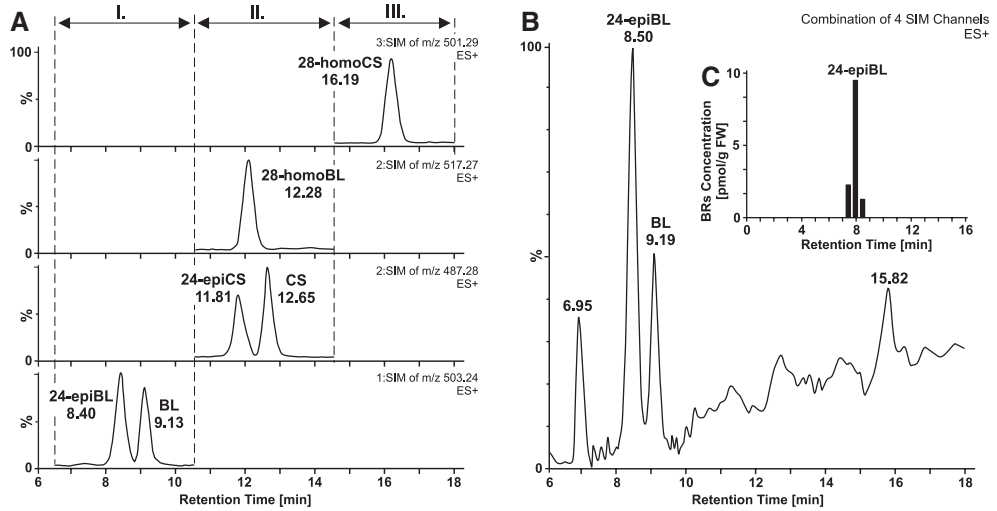

Figure 5. Reversed-phase high performance liquid chromatography (HPLC) separation of brassinosteroid standards (**A**), distribution of immunoreactivity in LC fractions (each of 0.5 min) (**C**) and results for analysis (**B**) of natural brassinosteroids in purified extracts of *A. thaliana* seedlings. The data in **B** were derived by combining the results from 4 SIM channels (m/z 487.28, 501.29, 503.24, 517.27). The immunoreactive region corresponds to the 24-epibrassinolide standard. For chromatography conditions, see *Materials and Methods*. Brassinolide (BL), Castasterone (CS), 24-epibrassinolide (24-epiBL), 24-epicastasterone (24-epiCS), 28-homobrassinolide (28-homoBL), 28-homocastasterone (28-homoCS). I, II,III - retention windows of SIM channels.

absolute levels of 24-epiBL determined by the two methods were very similar (Table 3), although the ELISA values tended to be a little higher than the LC-ESI-MS values. To date, 24-epiBL has been detected primarily in seeds (Schmidt and others 1997; Kauschmann and others 1997); for example, *A. thaliana* seeds have been found to be very rich in 24-epiBL, with a concentration of 220 ng.kg^{-1} FW (Schmidt and others 1997).

The most immunoreactive substance, 24-epiCS, has been identified in *Daucus carota* ssp. *sativus* seeds (Adam and others 1996; Schmidt and others 1998). The levels of 24-epiCS in this plant material as determined by LC–ELISA and LC–ESI–MS were 2.56 ± 2.05 and 1.37 ± 0.77 pmol · g^{-1} FW, respectively (Table 3). The occurrence of the 24-epiCS has been described in several other plant materials, including (*inter alia*) sugar beet seeds at 54 pmol · g^{-1} (25 µg.kg^{-1}) (Schmidt and others 1994), *Ornithopus sativus* (L.) seeds (Schmidt and others 1993) and panicles of *Rheum rhababarum* (L.) (Schmidt and others 1995) at 10.7 pmol · g^{-1} (5 µg · kg^{-1}), and green algae *Hydrodictyon reticulatum* (L.) at 0.65 pmol · g^{-1} FW (0.3 µg · kg^{-1}) (Yokota and others 1987). The endogenous concentrations of 24-epiCS in our samples are one order of magnitude lower than those reported for green algae, whereas valid comparisons for carrot cannot be made because no quantitative data are available in the literature. It should be noted here that the amounts of 24-epiCS in green plant tissues are usually much lower than those in seeds (Fujioka 1999).

The first BR to be detected, brassinolide, was isolated as just a few crystals (4 mg) from 40 kg of rape pollen (Grove and others 1979), and it occurs in many plant species, if not all (see Appendix in Kripach and others 1999). It was also identified in the plants selected here. However, because this substance is only very slightly immunoreactive with our antibody (CR = 1.3%), it was probably not detected by HPLC–ELISA, even though it was present in rape pollen at concentrations of 211.5 ± 38.86 pmol · g^{-1} FW according to HPLC–MS. Brassinolide levels obtained by calculation from the area of the *m/z* $[M+Na]^+$ peak closely agreed with that measured using diagnostic ions $[M-2H_2O+H]^+$and $[M+H]^+$ and reached 197.2 ± 41.7 and 219.4 ± 28.41 pmol · g^{-1} FW, respectively. Unfortunately, the background level of the immunosignal in the HPLC-fractionated extracts was much higher than in any of the other HPLC–ELISA assayed samples, and this led to a much lower LOD per g FW with these samples. The published brassinolide concentrations, including for example, rape pollen (208 pmol · g^{-1} FW; Grove and others 1979), *Vicia faba* pollen (376.5 pmol · g^{-1} FW; Gamoh and others 1989b) and seeds (395 pmol · g^{-1}FW; Park and others 1987), *Arabidopsis* whole plant (83 fmol · g^{-1} FW) and pea shoots (58 foml · g^{-1} FW;

Nomura and others 2001), correlate very closely with the levels presented in Table 3. The results also clearly show that the immunoassay system used here is less sensitive than the electrospray HPLC–MS method for analyses of samples from native plant tissues (Table 3). However, as mentioned above, our ELISA assay may be useful for testing extracts from field crops sprayed with synthetic 24-epiBL.

Finally, we also report here the identification and quantification of castasterone in *D. carota, P. vulgaris* and *A. thaliana* seedlings, as well as in the rape pollen. Whereas castasterone represents the most widely distributed BR, its levels vary between different tissues taken from different plants. Some reported concentrations include 288 pmol · g^{-1} FW in bean pollen, 45 pmol · g^{-1} FW in sunflower pollen (Gamoh 1994), 5.2 pmol · g^{-1} FW in galls of chestnut (Yokota and others 1982) and 774 fmol · g^{-1} FW (360 ng.kg^{-1}) in *A. thaliana* seeds (Schmidt and others 1997). Our data are within a range similar to these published levels (see Table 3).

To conclude, the above-mentioned methods were found to be applicable for the estimation of endogenous BR levels in different plant tissues. As a result of the application of the different principles of enzyme immunoassay and mass spectrometry, together with the use of two different systems of internal standardization, the identity of quantified derivatives was well verified. There is room for further improvement of the analytical methods, and for reduction of their LODs by using capillary LC systems coupled to a nano-ESI source. Further improvements should be achievable through the use of ultra performance liquid chromatography (UPLC) and may be enhanced even further by implementation of brassinosteroid-specific immunoaffinity chromatography in the analysis of natural plant samples. Techniques based on these promising strategies are under development.

ACKNOWLEDGMENTS

The authors thank Jarmila Balonová for excellent technical assistance. We would also like to thank Sees-editing Ltd. (www.sees-editing Ltd.) for the excellent editing of this manuscript. This work was supported by a grant from the Czech Ministry of Education (No. MSM 6198959216, LCO 6034, Z406605061).

REFERENCES

Adam G, Porzel A, Schnidt J, Schneider B, Voight B. 1996. New developments in brassinosteroid research. In: Rahman A. editor. Studies in Natural Product Chemistry. Amsterdam, The Netherlands: Elsevier Science. pp 495–549.

Badenoch-Jones J, Letham DS, Parker CW, Rolf BG. 1984. Quantification of cytokinins in biological samples using antibodies against zeatin riboside. Plant Physiol 75:1117–1125.

Díaz-Cruz S, Lopez de Alda M, Lopez R, Barcelo D. 2003. Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS). J Mass Spectrom 38:917–923.

Drosihn S, Porzel A, Brandt W. 2001. Determination of preferred conformations of brassinosteroids by means of NMR investigations and Boltzmann stastistical analysis of simulated annealing calculations. J Mol Model 7:34–42.

Erlanger BF. 1967. The preparation of steroid-protein conjugates to elicit antihormonal antibodies. Methods Immunol Immunochem 1:144–150.

Fujioka S. 1999. Natural occurence of brassinosteroids in the plant kingdom. In: Sakurai A, Yokota T, Clouse SD, editors. Brassinosteroids: Steroidal Plant Hormones. Tokyo, Japan: Springer Verlag. pp 21–45.

Gamoh K, Takatsuto S. 1989a. A new boronic acid derivative as a highly sensitive fluorescence derivatization reagent for brassinosteroids in liquid chromatography. Anal Chim Acta 222:201–204.

Gamoh K, Takatsuto S. 1994. Liquid chromatography assay of brassinosteroids in plants. J Chromatogr A 658:17–25.

Gamoh K, Kitsuwa T, Takatsuto S, and others. 1988. Determination of trace brassinosteroids by HPLC. Anal Sci 4:533–535.

Gamoh K, Omote K, Okamoto N, and others. 1989b. HPLC of brassinosteroids in plants with derivatization using 9-phenanthreneboronic acid. J Chromatogr 469:424–428.

Gamoh K, Okamoto N, Takatsuto S, and others. 1990a. Determination of traces of natural brassinosteroids as a dansylaminophenylboronates by LC fluorometric detection. Anal Chim Acta 228:101–105.

Gamoh K, Sawamoto H, Takatsuto S, and others. 1990b. Ferrocene boronic acid as a derivatization reagent for the determination of brassinosteroids by HPLC with electrochemical detection. J Chromatogr 515:227–231.

Gamoh K, Takatsuto S, Ikekawa N. 1992. Effective separation of C-24-epimeric brassinosteroids by LC. Anal Chim Acta 256:319–322.

Gamoh K, Yamaguchi I, Takatsuto S. 1994. Rapid and selective sample preparation for the chromatographic determination of brassinosteroids from plant material using solid-phase extraction method. Anal Sci 10:913–917.

Gamoh K, Abe H, Shimada K, and others. 1996. LC/MS with atmospheric pressure chemical ionization of free brassinosteoids. Rapid Commun Mass Spectrom 10:903–906.

Gross H, Bilk L. 1968. Zur reaktion von *N*- hydroxysuccinimide mit dicyklohexylcarbodiimid. Tetrahedron 24:6935–6939.

Grove MD, Spencer FG, Rohwedder WK, Mandava NB, and others. 1979. Brassinolide, a plant growth promoting steroid isolated from *Brassica napus* pollen. Nature 281:216–217.

Harlow E, Lane D. 1988. Antibodies—A Laboratory Manual. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory. 726 pp.

Horgen PA, Nakagawa CH, Irvin RT. 1984. Production of monoclonal antibodies to steroidal plant growth regulator. Can Biochem Cell Biol 62:715–721.

Jones HG. 1987. Correction for nonspecific interference in competitive immunoassays. Physiol Plant 70:146–154.

Kauschmann A, Jessop A, Koncz C, Altmann T. 1997. Molecular/genetic analysis of brassinosteroid synthesis and action. Proc Plant Growth Regul Soc Am 24:95–96.

Khripach VA, Zhabinski V, de Groot AE. 1999. A New Class of Plant Hormones. San Diego, CA, USA: Academic Press.456 pp.

Kohout L. 1994. New method of preparation of brassinosteroids. Coll Czech Chem Commun 59:457–460.

Konstantinova VO, Antonchic AP, Zhabinskii VN, Khripach V, Schneider B. 2001. Analysis of underivatized brassinosteroids by HPLC/APCI-MS. Occurrence of 3-epibrassinolide in *Arabidopsis thaliana*.. Collect Czech Chem Commun 66:1729–1734.

Kuronen P, Vaananen T, Pehu E. 1999. Reversed-phase liquid chromatographic separation and simultaneous profiling of steroidal glycoalkaloids and their aglycones. J Chromatogr A 25:25–35.

Ma Y-Ch, Kim H-Y. 1997. Determination of steroids by LC/MS. J Am Soc Mass Spectrom 8:1010–1020.

Motegi C, Takatsuto S, Gamoh K. 1994. Identification of brassinolide and castasterone in the pollen of orange (*Citrus sinensis*) by HPLC. J Chromatogr A 658:27–30.

Nomura T, Nakayama M, Reid JB. 1997. Blockage of brassinosteroid biosynthesis and sensitivity causes dwarfism in garden pea. Plant Physiol 113:31–37.

Nomura T, Sato T, Bishop JG, and others. 2001. Accumulation of 6-deoxocathasterone and 6-deoxocastasterone in *Arabidopsis*, pea and tomato is suggestive of common rate-limiting steps in brassinosteroid biosynthesis. Phytochemistry 57:171–178.

Novák O, Tarkowski P, Tarkowská D, Lenobel R, Doležal K, Strnad M. 2003. Quantitative analysis of cytokinins in plants by LC–MS. Anal Chim Acta 480:207–218.

O'Sullivan MJ. 1979. Enzyme immunoassay: a review. Anal Biochem 210:145–154.

Park KH, Yokota T, Sakurai A, Takahashi N. 1987. Occurrence of castasterone, brassinolide and methyl 4-chloroindole 3-acetate in immature *Vicia faba* seeds. Agric Biol Chem 54:3081–3086.

Pengelly WJ. 1986. Validation of radioimmunoassay for IAA using gas-chromatography-selected ion monitoring-mass-spectrometry. In: Bopp M, editor. Plant Growth Substances. Heidelberg, Germany: Springer-Verlag. pp 35–43.

Pengelly WJ, Meins F. 1977. The relationship of indole-3-acetic acid content and growth of crown-gall tumor tissues of tobacco in culture. Planta 136:173–180.

Prinsen E, Van Dongen W, Esmans E, Van Onckelen H. 1997. HPLC linked electrospray tandem mass spectrometry: a rapid and reliable method to analyse indole-3-acetic acid metabolism in bacteria. J Mass Spectrom 32:12–22.

Prinsen E, Van Dongen W, Esmans E, Van Onckelen H. 1998. Micro and capillary liquid chromatography tandem mass spectrometry: a new dimension in phytohormone research. J Chromatogr A 826:25–37.

Sakurai A. 1999. Brassinosteroid biosynthesis. Plant Physiol Biochem 37:351–361.

Sasse J. 1999. Physiological actions of brassinosteroids. In: Sakurai A, Yokota T, Clouse SD, editors. Brassinosteroids: Steroidal Plant Hormones. Tokyo, Japan: Springer-Verlag. pp 219–262.

Satake K. 1960. The spectrophotometric determination of amine, amino acid an peptide with 2,4,6-trinitrobenzene 1-sulfonic acid. J Biochem 47:654–660.

Schlagnhaufer CD, Arteca RN. Phillips AT. 1991. Induction of anti-brassinosteroid antibodies. J Plant Physiol 38:404–410.

Schlüsener MP, Bester K. 2005. Determination of steroid hormones, hormone conjugates and macrolide antibiotics in influents and effluents of sewage treatment plants utilising high-performance liquid chromatography/tandem mass spectrometry with electrospray and atmospheric pressure chemical ionisation. Rapid Commun Mass Spectrom 19:3269–78.

Schmidt J, Spengler B, Yokota T, Adam G. 1993. The co-occurrence of 24-epicastasterone and castasterone in seeds of *Ornithopus sativus*. Phytochemistry 32:1614–1615.

Schmidt J, Kuhnt C, Adam G. 1994. Brassinosteroids and sterols from seeds of *Beta vulgaris*. Phytochemistry 36:175–177.

Schmidt J, Himmelreich U, Adam G. 1995. Brassinosteroids, sterols and lup-20(29)-en-2α,3β,28-triol from *Rheum rhababarum*. Phytochemistry 40:527–531.

Schmidt J, Altmann T, Adam G. 1997. Brassinosteroids from seeds of *Arabidopsis thaliana*.. Phytochemistry 45:1325–1327.

Schmidt J, Porzel A, Adam G. 1998. Brassinosteroids and a pregnane glucoside from *Daucus carote*. Phytochem Anal 9:14–22.

Shimada K, Mitamura K, Higashi T. 2001. Gas chromatography and high performance liquid chromatography of natural steroids. J Chromatogr A 935:141–172.

Stoldt M, Porzel A, Adam G, Brandt W. 1997. Side chain conformation of the growth-promoting phytohormones brassinolide and 24-epibrassinolide. Magn Reson Chem 35:629–636.

Strnad M, Hanus J, Vanek T, Kaminek M, Ballantine JA, Fussell B, Hanke DE. 1997. Meta-topolin, a highly active aromatic cytokinin from poplar leaves (*Populus* × *canadensis* Moench, cv *Robusta*). Phytochemistry 45:213–218.

Strnad M, Vereš K, Hanuš J, Siglerová V. 1992a. Immunological methods for quantification and identification of cytokinins. In: Kamínek M, Mok DWS, Zažímalová E, editors. Physiology and Biochemistry of Cytokinins in Plants. The Hague, The Netherlands: SPB Academic Publishers. pp 437–446.

Strnad M, Peters W. Beck E, Kamínek M. 1992b. Immunodetection and identification of N^6-(o-hydroxybenzylamino)purine as a naturally occurring cytokinin in *Populus* × *canadensis* Moench cv *Robusta* leaves. Plant Physiol 99:74–80.

Svatoš A, Antonchick A, Schneider B. 2004. Determination of brassinosteroids in the sub-femtomolar range using dansyl-3-aminophenylboronate derivatization and electrospray mass spectrometry. Rapid Commun Mass Spectrom 18:816–821.

Swaczynová J, Šíša M. Hniličková J, Kohout L, Strnad M. 2006. Synthesis, biological, immunological and anticancer properties of a new brassinosteroid ligand. Polish J Chem 80:629–635.

Šíša M. 2005. New Brassinosteroids Analogues, PhD. Thesis, Charles University, Prague.

Van Aerden C. Debrauwer L, Tabet JC. 1998. Analysis of nucleoside-estrogen adducts by LC-ESI-MS–MS. Analyst 123:2677–2680.

Van Rhijn JA, Heskamp HH, Davelaar E, Jordi W, Leloux MS, and others. 2001. Quantitative determination of glycosylated and aglycon isoprenoid cytokinins at sub-picomolar levels by microcolumn LC combined with electrospray tandem mass spectrometry. J Chromatogr A 929:31–40.

Takatsuto S. 1991. Microanalysis of naturally occuring brassinosteroids. In: Cutler HG, Yokota T, Adam G, editors. Brassinosteroids: Chemistry, Bioactivity, and Applications, ACS Symp Ser 474. Washington, DC, USA: American Chemical Society. pp 1391–1397.

Weiler EW. 1982. An enzyme-immunoassay for *cis*-(+)-abscisic acid. Physiol Plant 54:51–514.

Yokota T, Watanabe S, Ogino Y. 1990. Radioimmunoassay for brassinosteroids and its use for comparative analysis of brassinosteroids in stems and seeds of *Phaseolus vulgaris*.. J Plant Growth Regul 9:151–159.

Yokota T, Arima M, Takahashi N. 1982. Castasterone, a new phytosterol with plant-hormone potency, from chestnut insect gall. Tetrahedron Lett 23:1275–1278.

Yokota T, Kim SK, Fukui Y, Tekematsu T. 1987. Brassinosteroids and sterols from a green alga, *Hydrodictyon reticulatum*: configuration at C-24. Phytochemistry 26:503–506.

Yokota T, Baba J, Koba S. 1984. Purification and separation of eight steroidal plant-growth regulators from *Dolichos lablab* seed. Agric Biol Chem 48:2529–2534.

Yokota T, Nomura T, Nakayama M. 1997. Identification of brassinosteroids that appear to be derived from campesterol and cholesterol in tomato shoots. Plant Cell Physiol 38:1291–1294.

Zullo MAT, Kohout L, de Azevedo MBM. 2003. Some notes on terminology of brassinosteroids. Plant Growth Regul 39:1–11.