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Identification and Quantification of Several Mammalian Steroid Hormones in Plants by UPLC-MS/MS

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Abstract We have developed an effective method for the isolation, identification, and quantification of several mammalian steroid hormones and their metabolites in different plant tissues. The purification protocol was based on solid-phase extraction (SPE) combined with immunoaffinity chromatography (IAC) using immobilized generic polyclonal anti-Δ^4-3-keto-steroid antibodies covalently bound to Affi-Gel 10 sorbent. The antibodies were characterized by means of enzyme-linked immunosorbent assay (ELISA). The detection limit of the ELISA was 6.0×10^{-10} mol L^{-1} and cross-reactivity with most Δ^4-3-keto-steroids was very high as predicted (68–122%). The IAC allowed fast, single-step purification of different plant extracts prior to analysis by ultra-performance liquid chromatography-electrospray tandem mass spectrometry [UPLC-ESI(+)-MS/MS]. In multiple-reaction-monitoring (MRM) mode, the detection limit of the method for most of the steroids analyzed was close to 10 fmol and the response was linear up to 50 pmol injected. The analytical accuracy was validated using tobacco leaf samples spiked with known amounts of authentic and deuterium-labeled standards. The newly developed method was capable of detecting and quantifying at least 12 specified steroid compounds in plant extracts. In the analyzed extracts from three plant species, that is, common foxglove (*Digitalis purpurea* L.), tobacco (*Nicotiana tabacum* L.), and elecampane inula (*Inula helenium* L.), four endogenous steroids were detected, identified, and quantified. Progesterone was found in all three plants at concentrations comparable to those reported in previous studies. Three other steroids, androstendione, 17α-hydroxyprogesterone, and 16-dehydroprogesterone, were identified for the first time in plant extracts. 17α-Hydroxyprogesterone and 16-dehydroprogesterone occurred at significant concentrations in *D. purpurea*, whereas androstendione was found in *N. tabacum* and *I. helenium* but not in *D. purpurea*.

Keywords Ultra-performance liquid chromatography (UPLC) · Tandem mass spectrometry (MS/MS) · Immunoaffinity purification · Steroids · Plant extracts · *Digitalis purpurea* · *Nicotiana tabacum* · *Inula helenium*

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Introduction

Steroid compounds play vital functions in biological systems. In addition to an essential role in maintaining the integrity of biomembranes, steroids typically serve as regulatory molecules in a wide range of physiologic processes. In mammals, steroid hormones are classified into five families based on their structure and biological roles (Fig. 1). These families are: male sex hormones, androgens; female sex hormones, estrogens; mineralocorticoids and glucocorticoids, which are essential for the regulation of homeostasis in the body; and progestins, with progesterone as the principal representative, the most important functions of which are associated with the onset and maintenance of pregnancy. The naturally occurring steroids include not only animal hormones with a variety of

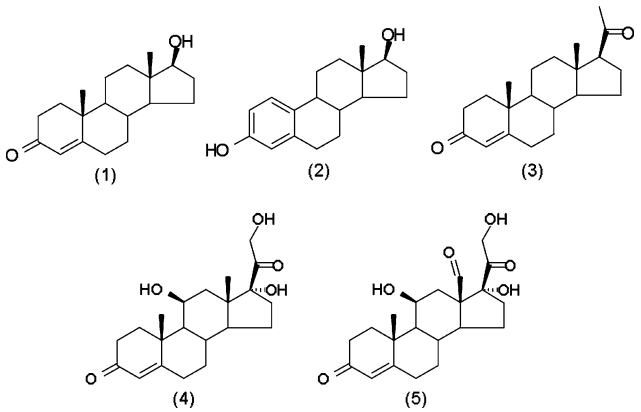

Fig. 1 Structures of steroid compounds representing basic classes of mammalian steroid hormones: 1, testosterone (androgens), 2, estradiol (estrogens), 3, progesterone (progestins), 4, cortisol (glucocorticoids), 5, aldosterone (mineralocorticoids)

molecular structures and functions but also a range of steroids that are regarded as typical plant compounds. These include the brassinosteriod group of plant growth regulators, cucurbitacines, withanolides, cardenolides or steroidal saponins, and alkaloids.

First reports of the occurrence of mammalian sex hormones in plant extracts appeared in articles by Dohrn and others (1926) and Skarzynski (1933). Bonner and Axtman (1937) reported stimulation of the *in vitro* growth of isolated pea embryos by estrone. Estrone was later isolated from the seeds of date palm (*Phoenix dactylifera* L.; Bennett and others 1966) and progesterone was identified in apple seeds (*Mallus* sp.) by Gawienowski and Gibbs (1968). Pioneering studies of steroid metabolism in *Digitalis* sp. following the application of radio-labeled compounds to leaves were undertaken by Bennett and colleagues (Bennett and Heftmann 1965; Sauer and others 1967; Bennett and others 1968). However, although much research on the occurrence and physiologic functions of steroids in plants has been undertaken (see Geuns 1978; Janeczko and Skoczowski 2005), our understanding of the mechanism of action and metabolism of steroid hormones in plants remains relatively sparse. Estrogen receptor-like proteins, localized in the nuclei, were recently isolated from various plant organs of *Solanum glaucophyllum* Desf. (Milanesi and others 2001). Membrane Steroid Binding Protein 1 (MSBP1), which plays a part in growth regulation and has a very high affinity to progesterone, was identified and characterized in *Arabidopsis thaliana* (L.) Heynh. (Yang and others 2005). These authors also identified a soluble Steroid Binding Protein (SBP) that lacks a hydrophobic membrane anchor sequence and seems to be plant-specific. Homologous genes for these proteins as well as genes for similar binding proteins from rice (*Oryza sativa* L.) have been cloned and their abundant expression described (Iino and others 2007). Several enzymes participating in steroid metabolism have now been isolated and characterized from plant sources, primarily from woolly foxglove (*Digitalis lanata* Ehrh.; Seidel and others 1990; Wendroth and Seitz 1990; Lindemann and Luckner 1997). These enzymes exhibited highly similar catalytic properties to those involved in mammalian steroid metabolism but it seems that they evolved independently of the analogous mammalian enzymes. An exception is the enzyme 5α-reductase, which seems to be encoded by the descendants of the same ancestor gene in both animals and plants (Li and others 1997; Rosati and others 2005).

Studying mammalian-type steroids in plants requires powerful analytical tools able to detect and identify trace amounts of these compounds in plant samples. Most early studies utilized radio immunoassay (RIA), thin-layer chromatography (TLC), and gas chromatography (GC) (reviewed by Janeczko and Skoczowski 2005). However, unequivocal identification of detected compounds using these methods presents difficulties such as cross-reactions of antibodies with structurally related substances (RIA) or coelution of interfering compounds together with the analytes respectively. These disadvantages have been largely resolved by the introduction of mass spectrometry (MS) into plant steroid research (Young and others 1977). Saden-Krehula and others (1991) used TLC-MS to identify progesterone in the steroid fraction of extracts from *Vitex agnus-castus* L. Iino and others (2007) used GC-MS to identify and quantify progesterone in several plant species [*Arabidopsis thaliana* (L.) Heynh., *Pisum sativum* L., *Oryza sativa* L.]. High-performance liquid chromatography (HPLC) in combination with MS is routinely used for analysis of natural steroids but to our knowledge has not yet been used for the analysis of steroids in plant extracts. Recently introduced ultra-performance liquid chromatography (UPLC) combined with MS enables very sensitive analysis of minute concentrations of several groups of compounds involved in plant physiologic processes (Li and others 2006; Gruz and others 2008; Novak and others 2008). UPLC is a modern separation technology, using a chromatography column containing 1.7 μm bridged ethylsiloxane/silica hybrid particles, that provides higher peak capacity and greater resolution and sensitivity in comparison to HPLC (Zhao and others 2006). Storbeck and others (2008) developed a method for the analysis of seven adrenal steroid metabolites from heterologous expression media (the yeast *Pichia pastoris*) using UPLC-coupled atmospheric pressure chemical ionization mass spectrometry (UPLC–APCI–MS). In this study, we have introduced a very sensitive, rapid, and versatile method for the simultaneous analysis of 12 mammalian steroids in plant extracts from *Nicotiana tabacum*, *Digitalis purpurea*, and *Inula helenium* by ultra-performance liquid chromatography-

electrospray tandem mass spectrometry in a positive mode [UPLC–ESI(+)–MS/MS]. Tandem mass spectrometry affords the precise identification of compounds of interest. Moreover, the sensitivity of the assay is significantly enhanced by including an immunoaffinity chromatography (IAC) purification step (Gaskell and Brownsey 1983; Ferguson and others 2001; Hradecka and others 2007) using generic anti-Δ^4-3-keto-steroid antibodies.

Materials and Methods

Plant Material

Tobacco plants (*Nicotiana tabacum* L.) were planted under standardized conditions at 23°C and a 16/8-h photoperiod. After 8 weeks of growth the leaves were harvested and immediately plunged into liquid nitrogen. The leaves were lyophilized and stored at −80°C until extraction. Common foxglove plants (*Digitalis purpurea* L.) and elecampane inula plants (*Inula helenium* L.) were grown under field conditions at the Crop Research Institute, Department of Vegetables and Special Crops, Olomouc, Czech Republic. Leaves were harvested from 4-month-old vegetative plants. Leaves were immediately frozen in liquid nitrogen, lyophilized, and stored at −80°C until required.

Sample Preparation

Plant samples (2.5 g) were homogenized using a ball mixer mill (Retsch MM301, Retsch GmbH, Haan, Germany) with 4 mL of 80% MeOH at room temperature. The grinding jar was rinsed with 3 mL of 80% MeOH; both volumes were combined and 100 pmol of internal standards (IS) in methanolic solution were added to every sample. The following IS were used: [16,16,17-$^2H_3$]-testosterone (Sigma-Aldrich, St. Louis, MO, USA); [17,21,21-$^2H_3$]-pregnenolone (Laboratory of Radioisotopes, Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic); and [1,2,4,5,6,7-$^2H_6$]-5α-pregnan-3,20-dione and [3,4-$^{13}C_2$]-progesterone (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). Extraction was for 60 min on a shaker at 4°C and afterward the mixture was centrifuged (20 min, 29,200 rcf, 4°C) using an Avanti®30 centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The first purification step was carried out through Strata-X 200-mg/3-ml cartridges (Phenomenex, Inc., Torrance, CA, USA) conditioned by 3 mL of MeOH and equilibrated by 3 mL of H_2O. After sample application the column was washed with 3 mL of H_2O and the bound fraction was eluted by 3 mL of MeOH cooled to −20°C to prevent the degradation of compounds to be analyzed. The eluent was evaporated to dryness with a vacuum centrifugal evaporator (Jouan RC 1010, Thermo Scientific, Inc., Waltham, MA, USA), resuspended in 1 mL of 7.5% MeOH in phosphate-buffered saline (PBS) (50 mM NaH_2PO_4, 15 mM NaCl, pH 7.2), and passed through an immunoaffinity column.

Immunoaffinity Purification

The polyclonal antibodies used in this study were raised against 4-androsten-3-one-17-carboxymethyloxime (CMO; Pouzar and Cerny 1994) linked to bovine serum albumin (BSA) at position C17. This hapten was conjugated to the carrier protein by the carbodiimide method (Gross and Bilk 1968). Three rabbits were immunized following the immunization schedule described by Strnad and others (1997). The antisera were purified by affinity chromatography on protein G and characterized by means of enzyme-linked immunosorbent assay (ELISA). The 4-androsten-3-one-17-CMO-peroxidase conjugate used as labeled antigen in the competitive ELISA was also prepared by the carbodiimide method (Gross and Bilk 1968). From the three antibody strains obtained, the antibody designated T1 showed the highest cross-reactivity with Δ^4-3-keto-steroids and thus was used for the preparation of immunoaffinity gels (Affi-Gell 10, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The capacity of the immunoaffinity columns was tested in triplicate using a mixture of steroid standards at concentrations from 0.5 to 250 pmol that were applied to immunoaffinity columns loaded with various volumes of immunoaffinity gel (250–600 μL). Every lot of standards was repeatedly (3×) applied to a column and then incubated with the gel for 30 min before eluting in 3 mL of MeOH (−20°C). Cooled MeOH was used as an elution solution in IAC to decrease the danger of antibody denaturation. Eluates were evaporated to dryness. Immunoaffinity columns were regenerated by rinsing with PBS, H_2O, MeOH, H2O, and PBS (3 mL each). NaN_3 (0.05% in PBS) served as a preservation medium. The optimized volume (500 μL) of gel was used for purification of the plant extracts according to the schedule described above.

Instrumental

Immunoaffinity purified samples were dissolved in 20 μL of MeOH and 3 μL of each sample was injected onto a reversed-phase column (BEH C18, 2.1 × 50 mm, 1.7 μm, Waters, Corp., Milford, MA, USA). Compounds were separated by an Acquity UPLC™ System (Waters), which included a binary solvent manager, sample manager, and 2996 PDA detector. The binary solvent gradient was processed as follows: initial proportion of solvent A (100% MeOH) to solvent B (10 mM formic acid) was 40:60; 2 min, 40:60; 10 min, 50:50; 15 min, 65:35; 17 min, 80:20; flow-rate was 0.3 mL min^{-1} and column

temperature was 40°C. At the end of the elution the column was washed with 100% A (1 min) and equilibrated to initial conditions for 2 min. Retention times for the analyzed compounds ranged from 2.6 to 16.3 min. The effluent was introduced into the tandem mass spectrometer Micromass Quattro *micro*™ API (Waters MS Technologies, Manchester, UK). Steroids were detected using multiple-reaction monitoring (MRM) of the transition of $[M + H]^+$ ion to the appropriate product ion. The second transition of $[M + H]^+$ ion to another product ion was used to confirm endogenous occurrence and to calculate the MRM ratio. Conditions for the MRM experiment were optimized as follows: capillary voltage, 1.0 kV; source/desolvation gas temperature, 100/350°C; cone/desolvation gas flows, 2/550 L h^{-1}; LM/HM resolution, 12.5; ion energy 1, 0.3 V; ion energy 2, 1.5 V; entrance, exit, and multiplier voltages, 2.0 V, 2.0 V, and 650 eV, respectively. Argon at a pressure of 4.5×10^{-3} mbar was used as the collision gas. Other settings such as dwell times, cone voltages, and collision energies were optimized for every diagnostic transition.

A hybrid Q-Tof *micro*™ mass spectrometer (Waters MS Technologies) was used for the high-resolution identification and confirmation of steroid compounds in plant extracts. Aliquots of 20 µL were taken for analysis. The measurement was performed using HPLC analysis on an Alliance separation module 2965 and equipped with a photodiode array detector 2996 (Waters) using a reversed-phase column Symmetry C18 (150 mm × 2.1 mm i.d., 5 µm; Waters) and a post-column splitting of 1:1. Following injection, analytes were eluted with a 35-min binary linear gradient (0 min, 50% B; 10–25 min, 70% B; 25–35 min, 100% B; flow rate of 0.25 mL/min; column temperature of 30°C) of 5 mM formic acid and methanol (B). Electrospray ionization in the positive ion mode was performed using the following parameters: source block/desolvation temperature, 100°C/350°C; capillary/cone voltage, 2500/30 V; and spray/cone gas flow (N_2), 50/500 L/h. In the full-scan mode, data were acquired in the mass range *m/z* 50–1000, with a cycle time of 33 ms, a scan time of 2.0 s, and collision energy of 4 V. For the exact mass determination experiments, a lock spray was used for external calibration with a mixture of 0.1 M NaOH/10% formic acid (v/v) and acetonitrile (1:1:8 by volume) as a reference. Accurate masses were calculated and used for the determination of the elementary composition of the analytes with a fidelity of 5 ppm.

Calibration Curves

Calibration curves were constructed from triplicate analyses of steroid standards by plotting the calculated response area of the analyte to IS ratio against the defined concentration of each unlabeled standard 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0/10 pmol, where IS concentration is the known concentration of the added IS. The amount of each steroid present in the plant extracts was determined by comparing the peak area of the endogenous steroid to that of the corresponding stable isotope-labeled steroid. QuanLynx™ software (Waters) was used to calculate the calibration curves, their corresponding correlation coefficients, and the limits of detection (LOD; signal-to-noise ratio of minimum 3) for all the steroid compounds analyzed.

Results and Discussion

Sample Preparation

The extraction procedure was optimized to obtain the greatest possible yield of steroid compounds from each plant sample. A ball mixer mill provided effective and time-saving homogenization of the samples. Polar extraction solvents were used because nonpolar solvents could lead to unwanted contamination of the extract by interfering hydrophobic compounds. Various modifications to the extraction solvent were tested, including the use of 80% MeOH (Yokota and others 1984) and $CHCl_3$-MeOH (1:2) (Skliar and others 2000). As 80% MeOH was found to be the most effective (data not shown), this was chosen as the extraction solvent. Attempts were made initially to remove coextracted plant pigments from the extracts by partitioning them twice against n-hexane before further analysis (Schmidt and Steinhart 2002). However, this approach led to a significant decrease of the recoveries of the steroid standards (data not shown) and was consequently abandoned. The purification of steroids from biological materials is usually carried out on C18-bonded silica or on polymeric sorbents. Following Vulliet and others (2007), we used Strata-X (200 mg) SPE cartridges with a high surface polymeric reversed phase. Using this extraction procedure, recoveries in the region of 50% were obtained. These recoveries indicate a considerable influence of the plant material on purification efficiency. Unfortunately, contamination from plant pigments and/or hydrophobic compounds was still very high, making it difficult to analyze trace amounts of steroids reproducibly. To overcome this problem an immunoaffinity purification step was included in the protocol.

Immunoaffinity Purification

Because steroid compounds may occur in complex plant materials in minute concentrations and because the presence of hydrophobic compounds in extracts may interfere with their determination, an immunoaffinity purification

step was deemed necessary. Analytical approaches utilizing broad-spectrum generic antibodies to extract whole groups of structurally related compounds from plants have been described previously (for example, Novak and others 2003; Hauserova and others 2005). The specificity of antibodies depends greatly on the position at which the hapten is linked to the protein carrier (Bosch and others 1974). To produce generic antibodies able to bind the group of steroid compounds of interest in the present work, rabbits were immunized with 4-androsten-3-one-17-CMO-BSA conjugate. Analytical characteristics of the recovered antisera were determined by ELISA. It was believed that the antibodies obtained would show high cross-reactivities with steroid compounds containing a 3-one group conjugated to a 4-ene group and varying structurally only in the moiety on C17 (see Table 1). These steroids, namely, testosterone, progesterone, androstendione, and 20α-hydroxyprogesterone, were really bound with very similar affinity; 16-dehydroprogesterone also showed high cross-reactivity. Pregnenolone, with a 3β-ol group and a double bond in the C5 position, attained a cross-reactivity of over 23% in contrast to 5α-pregnan-3,20-dione where there was stronger decrease of cross-reactivity (6.89%), probably caused by substitution by hydrogen in position 5α. Cross-reactivity of 16α-hydroxyprogesterone also exceeded 20% in contrast to 17α-hydroxyprogesterone (7.47%) where the –OH substitution on C17 seemed to interfere with the antigen-binding site. An essential characteristic of broad-spectrum generic antibodies is that they should have low specificity to the preferred group of analyzed compounds. The specificity of the antibodies prepared in the work described here was similar to or lower than that of analogous antibodies previously described (Bosch and others 1974; Fantl and Wang 1983). Nevertheless, from the performed cross-reactivity tests it is obvious that variations in the basic Δ^4-3-keto- structure led to a significant reduction in binding to the antibody (Table 1). This fact contributes to minimizing the number of the stereoisomeric compounds that could interfere in the identification of the analyzed steroids. As the obtained antibodies possessed the required sensitivity in the ELISA test (Table 2) and high affinity for several Δ^4-3-keto-steroids, they were deemed suitable for use in the preparation of immunoaffinity gels for the purification and preconcentration of steroids in different plant extracts. The high affinity of the antibodies for 12 steroids (cross-reactivities ranging from 6.89% for 5α-pregnan-3,20-dione to 122% for androstendione) enabled these to be routinely analyzed in plant samples. The use of such IAC columns greatly improved the sensitivity of subsequent UPLC–ESI(+)–MS/MS.

Table 1 Molar cross-reactivities of various steroids with anti-4-androsten-3-one-17-CMO antibodies T1

Compound		Cross-reactivity (%)
Testosterone	4-Androsten-17β-ol-3-one	100.00
Androstendione	4-Androsten-3,17-dione	122.00
19-Nortestosterone	4-Estren-17β-ol-3one	10.70
19-Hydroxyandrostendione	4-Androsten-19-ol-3,17-dione	0.57
Progesterone	4-Pregnen-3,20-dione	112.00
11α-Hydroxyprogesterone	4-Pregnen-11α-ol-3,20-dione	1.55
17α-Hydroxyprogesterone	4-Pregnen-17α-ol-3,20-dione	7.47
16α-Hydroxyprogesterone	4-Pregnen-16α-ol-3,20-dione	20.80
16-Dehydroprogesterone	4,16-Pregnadien-3,20-dione	118.00
20α-Hydroxyprogesterone	4-Pregnen-20α-ol-3-one	65.80
Cortisol	4-Pregnen-11β,17α,21- triol-3,20-dione	0.81
Cortexolone	4-Pregnen-17α,21-diol-3,20-dione	8.28
Corticosterone	4-Pregnen-11β,21-diol-3,20-dione	5.63
Aldosterone	4-Pregnen-11β,21-diol-3,20-dioxo-18-al	12.80
DHEA	5-Androsten-3β-ol-17-one	0.02
Pregnenolone	5-Pregnen-3β-ol-20-one	23.80
17α-Hydroxypregnenolone	5-Pregnen-3β,17α-diol-20-one	0.27
Cholesterol	5-Cholesten-3β-ol	<0.01
Etiocholanolone	5β-Androstan-3α-ol-17-one	1.16
Androsterone	5α-Androstan-3α-ol-17-one	0.87
Dihydroprogesterone	5α-Pregnan-3,20-dione	6.89
Estrone	1,3,5(10)-Estratriene-3-ol-17-one	0.08
Estradiol	1,3,5(10)-Estratriene-3,17β-diol	<0.01

Table 2 Characteristics of polyclonal anti-4-androsten-3-one-17-CMO antibodies T1 in ELISA

Concentration of antibody (μg mL^{-1})	0.47
Limit of detection (mol L^{-1})	6.0×10^{-10}
Midrange B/B_0 (50% binding) (mol L^{-1})	4.6×10^{-9}
Range of measurement (mol L^{-1})	4.8×10^{-8} to 6.0×10^{-10}
Nonspecific binding (%)	2.93
Intra-assay variability (%)	0.76
Interassay variability (%)	2.17

Optimization of UPLC–ESI(+)–MS/MS

Effective separation of steroid compounds of similar hydrophobicity and molar mass was achieved by using ultra-performance liquid chromatography (UPLC). A mixture of 12 unlabeled and 4 labeled internal steroid standards was separated on a short reversed-phase column (2.1 × 50 mm). Various mobile phase modifiers were tested during the validation phase of the LC separation. In general, the LODs of some steroid analytes were decreased by millimolar additions of formic or acetic acid. Ma and Kim (1997) compared atmospheric pressure ionizations (APCI and ESI) for the analysis of several steroids. The APCI mode was less sensitive depending on steroid proton affinities, which can be controlled by choice of a mobile phase. The ESI mode provided stable $[M + Na]^+$ ions when methanol-water were used as a mobile phase, but their occurrence can be considerably reduced by acid modifiers. Therefore, we used methanol-10 mM formic acid as a mobile phase to obtain a stable baseline chromatographic separation and optimal ionization conditions (see Table 3), providing $[M + H]^+$ ions.

The total run time of the procedure, including equilibration, was 20 min, representing a considerable reduction in run time compared to the HPLC method previously developed in our laboratory (45 min, data not shown). The separation of 12 steroid compounds by UPLC is shown in Fig. 2. The system had high reproducibility during the chromatographic separations (variation of retention time ranged between 0.05 and 0.55% RSD). This allowed the chromatographic run to be split into seven retention windows (1.0–4.0, 4.0–7.1, 7.1–8.5, 8.5–10.0, 10.0–12.0, 12.0–14.5, and 14.5–17.00 min), which increased the sensitivity of the subsequent ESI(+)–MS/MS measurements. Consistent with the elution gradient used, the most polar steroid (aldosterone) was eluted in the first retention window, whereas pregnenolone and 5α-pregnan-3,20-dione were eluted in the last, seventh window. For steroid compounds with identical molecular masses and similar MRM transitions, *m/z* 347 (corticosterone/cortexolone) and *m/z* 317 (20α-hydroxyprogesterone /pregnenolone/5α-pregnan-3,20-dione), respectively, baseline separation was achieved. Structural isomers 16α- and 17α-hydroxyprogesterone were also separated with high efficiency. This fact further increases the credibility of the subsequent MS/MS identification. Importantly, successful separation of 16-dehydroprogesterone, progesterone, and 20α-hydroxyprogesterone was also achieved (Fig. 2). The isotopic peaks (M + 2) of

Table 3 Optimized product ion scanning, quantitation and confirmation transitions, cone voltage (CV), collision energy (CE), and dwell time (DT) parameters of the triple quadrupole mass spectrometer (electrospray interface in positive ion mode) for each of the analyzed steroids

	CV (V)	Quantitation MRM transitions	CE (eV)	Confirmation MRM transitions	CE (eV)	DT (s)
Aldosterone	25	361 > 343	17	361 > 315	20	0.500
Corticosterone	25	347 > 329	15	347 > 121	20	0.090
Cortexolone	27	347 > 97	21	347 > 109	25	
17α-Hydroxyprogesterone	27	331 > 97	20	331 > 109	22	
Androstendione	28	287 > 97	18	287 > 109	21	0.500
Testosterone	28	289 > 97	19	289 > 109	22	0.200
$^2H_3$-testosterone	28	292 > 97	19	292 > 109	22	
16α-Hydroxyprogesterone	27	331 > 97	21	331 > 109	24	0.500
16-Dehydroprogesterone	26	313 > 109	22	313 > 97	19	0.055
Progesterone	27	315 > 97	19	315 > 109	22	
$^{13}C_2$-progesterone	26	317 > 99	19	317 > 111	22	
20α-Hydroxyprogesterone	27	317 > 97	19	317 > 109	22	
Pregnenolone	22	299 > 281	15	317 > 281	15	0.055
$^2H_3$-pregnenolone	22	302 > 284	16	320 > 284	16	
5α-Pregnan-3,20-dione	22	317 > 281	15	317 > 299	15	
$^2H_6$-5α-pregnan-3,20-dione	22	323 > 287	16	323 > 305	16	

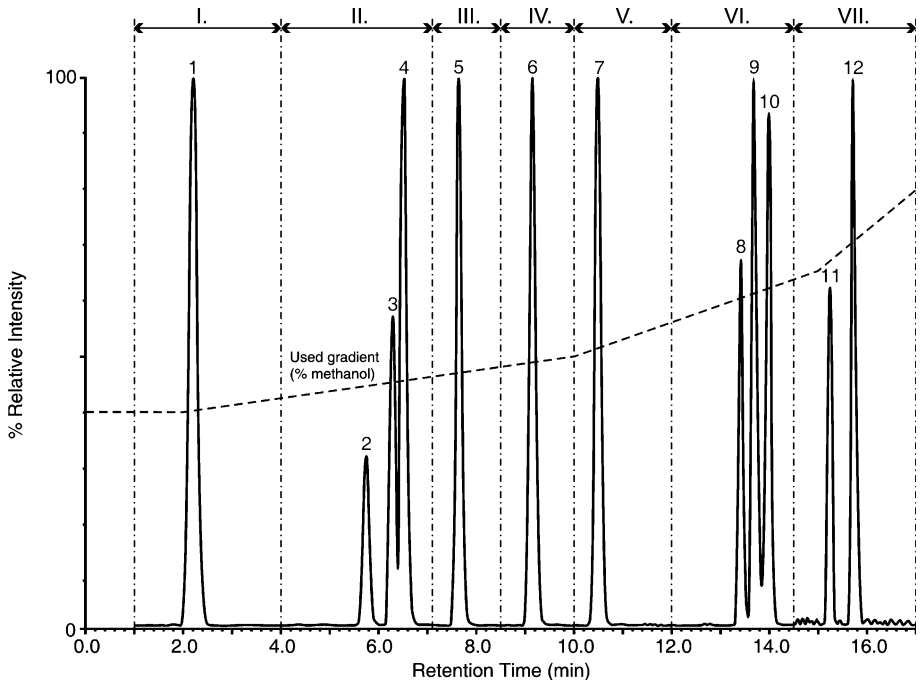

Fig. 2 Combined UPLC-ESI(+)-MS/MS chromatogram of a mixture of the following 12 steroid standards: Aldosterone (1), Corticosterone (2), Cortexolone (3), 17α-Hydroxyprogesterone (4), Androstendione (5), Testosterone (6), 16α-Hydroxyprogesterone (7), 16-Dehydroprogesterone (8), Progesterone (9), 20α-Hydroxyprogesterone (10), Pregnenolone (11), 5α-Pregnan-3,20-dione (12). For illustration, the gradient used (% of methanol) is also shown. I.–VII. Represent splitting of the chromatographic run into seven retention windows. For chromatography conditions, see Materials and Methods

molecular ions of 16-dehydroprogesterone (*m/z* 313) and progesterone (*m/z* 315) are isobars of progesterone and 20α-hydroxyprogesterone (*m/z* 317), respectively. They have the same product ions and increase the apparent concentration of the latter steroids without sufficient separation.

The MRM transitions from $[M + H]^+$ ion to appropriate product ion (Table 3) allowed precise quantification of the analyzed compounds. To determine other characteristic diagnostic MRM transitions, 100 pmol of each steroid standard was injected separately onto the column and the precursor and product ions were identified. The $[M + H]^+$ molecular ions of underivatized steroids were observed with electrospray ionization in positive mode. Only pregnenolone exhibited a high abundance of protonated $[M\text{-}H_2O + H]^+$ molecules due to the loss of water, while the intensity of $[M + H]^+$ ions was only 50%. The second MRM transition was also measured for each analyte to enable steroid conformation to be determined and the MRM ratio to be calculated. The MRM ratio can be used as another criterion by which an analyte may be distinguished from interfering substances—a useful feature of the MS method (Kushnir and others 2006a). Electrospray capillary and cone voltages (CV) were adjusted to generate the precursor ions in ESI + mode. The collision energy (CE) was optimized for dissociation of the molecular ions into the product ions of each steroid. The CV varied from 22 to 28 V, whereas the CE was between 15 and 25 eV. In accordance with changes in the character of the elution bands (number of transitions, width and/or height of peaks) different dwell times were used to find the best detection parameters (16 data points across the peak). The data are summarized in Table 3.

Validation of the Method

After the separation and MS/MS conditions had been optimized, the method was validated as follows. After log-transformation, linear calibration curves with correlation coefficients greater than 0.9985 were obtained over the concentration range 0.010–50 pmol for most steroids (Table 4). The results clearly show a good agreement with previously published results obtained for other plant and animal steroids (Ma and Kim 1997; Van Aerden and others 1998; Kuronen and others 1999; Shimada and others 2001; Schlüsener and others 2005; Storbeck and others 2008). Data on method sensitivity are also given in Table 4. The limits of detection were determined for each steroid and based on three times the signal-to-noise ratios. The determined LODs were mostly in the range of 2.5–10 fmol injected; higher values were found for pregnenolone (0.25 pmol) and 5α-pregnan-3, 20-dione (0.5 pmol). In addition to peak retention time, the MRM ratios were also determined for steroid confirmation (Table 4). The use of the MRM ratio as an identification criterion is possible when the relative transition intensity corresponds to that of the calibration standard from either calibration solutions or from spiked samples at comparable concentrations (Kushnir and others 2006b; Hernando and others 2007). For LC–MS techniques, recommended tolerance ranges from 20 to 50% RSD. Our data, calculated for standards and spiked samples, showed ranges of 2–12% RSD and 4–15% RSD,

Table 4 Limit of detection, dynamic range, expression of linearity (correlation coefficients, R^2), recovery, and analytical accuracy for the estimation of various steroids by UPLC–ESI(+)–MS/MS

	LOD[a] (fmol)	Dynamic range (pmol)	R^2	MRM ratio	Recovery[b] (%)	Analytical accuracy[b] (%)
Aldosterone	5.0	0.010–50	0.9994	2.41 ± 0.15	7.4 ± 0.4	73.0 ± 0.7
Corticosterone	5.0	0.010–50	0.9986	2.74 ± 0.21	14.7 ± 1.3	93.1 ± 3.0
Cortexolone	10.0	0.025–50	0.9992	1.06 ± 0.06	15.6 ± 0.9	88.9 ± 2.0
17α-Hydroxyprogesterone	10.0	0.025–50	0.9991	1.27 ± 0.03	18.3 ± 1.3	102.1 ± 3.1
Androstendione	2.5	0.005–50	0.9989	1.50 ± 0.03	21.9 ± 1.2	109.3 ± 1.2
Testosterone	5.0	0.010–50	0.9990	1.18 ± 0.06	22.5 ± 1.4	114.7 ± 4.6
16α-Hydroxyprogesterone	10.0	0.025–50	0.9992	1.08 ± 0.09	23.9 ± 2.5	116.1 ± 7.4
16-Dehydroprogesterone	10.0	0.025–50	0.9990	1.09 ± 0.07	25.2 ± 1.5	102.2 ± 6.0
Progesterone	5.0	0.010–50	0.9993	1.16 ± 0.09	19.5 ± 1.3	117.3 ± 5.7
20α-Hydroxyprogesterone	10.0	0.025–50	0.9989	1.04 ± 0.06	34.7 ± 2.4	109.9 ± 8.5
Pregnenolone	500.0	1.000–50	0.9985	2.44 ± 0.25	38.2 ± 9.3	101.4 ± 10.8
5α-Pregnan-3,20-dione	250.0	0.500–50	0.9988	1.02 ± 0.11	10.1 ± 1.5	95.4 ± 9.9

[a] Signal-to-noise ratio was set to 3:1

[b] Mean value of three independent measurements ± standard deviation

respectively. This indicates a minimal matrix effect during MS determination and optimal system performance. Each analyte was scanned to confirm its retention time and optimized characteristic MRM transitions. In addition, the determination of the elementary composition of the steroids detected in plant extracts was performed by Q-TOF MS.

Recovery during extraction and purification was determined by replicate analysis ($n = 3$) of nonspiked samples and samples spiked with defined amounts of analyzed steroid standards. Recoveries were calculated as a percentage of starting concentration of 10 pmol and 100 pmol of steroid standards added to the sample prior to the extraction process. Recoveries achieved using the new method ranged from 10 to 40%. The strong hydrophobic character of the analyzed steroids made it difficult to separate them from plant pigments and other hydrophobic compounds, which are responsible for the undesirable matrix effect. Because SPE purification had been found insufficient, the use of an immunoaffinity purification step was necessary. The involvement of IAC demands dissolving the evaporated plant sample in the polar PBS solution which leads to the particular losses of steroids from the analyzed extracts and thus lowers the recovery of the assay. Nevertheless, the use of IAC significantly increased the selectivity and sensitivity of high-throughput UPLC-MS/MS analysis as well as reduced the necessity for frequent system clean-up (Novák and others 2008). In this way the effects of the sample matrix were reduced resulting in an improved signal strength.

The analytical accuracy of the steroid estimation was checked by the addition of stable-isotope-labeled standards during extraction. The standard isotope dilution analysis provides quantitative results with high accuracy and precision. Analysis of the spiked samples showed good agreement between actual and expected values (Table 4).

Table 5 Steroid levels in *Nicotiana tabacum* spiked with testosterone and progesterone

	Steroid levels[a]	
	Testosterone	Progesterone
Nonspiked	n.d.	55.46 ± 3.89
Spiked with 10 pmol	11.59 ± 2.10	65.88 ± 3.79
Spiked with 100 pmol	117.87 ± 6.96	161.00 ± 8.98

n.d. not detected

[a] Fresh weight (pmol/g), mean ± SD

Table 6 Steroid content quantified in *N. tabacum*, *D. purpurea*, and *I. helenium* (pmol/g FW)

	Steroid content[a]		
	Nicotiana tabacum	*Digitalis purpurea*	*Inula helenium*
Androstendione	7.69 ± 2.13	n.d.	11.18 ± 2.98
17α-Hydroxyprogesterone	n.d.	173.53 ± 20.70	n.d.
16-Dehydroxyprogesterone	n.d.	28.94 ± 3.99	n.d.
Progesterone	55.46 ± 3.89	58.92 ± 5.77	2.10 ± 0.62

n.d. not detected

[a] Values are mean ± SD

The accuracy of the developed method varied by up to ±20% of the true amount present in the sample which is sufficiently accurate for the determination of trace components in complex plant materials (van Rhijn and others 2001). The validity of the steroid measurements is also summarized in Table 5. Steroid contents analyzed in tobacco leaf extracts with the addition of labeled testosterone or progesterone were very close to the expected values following spiking with 10 and 100 pmol of each compound. Analysis of tobacco extracts without the

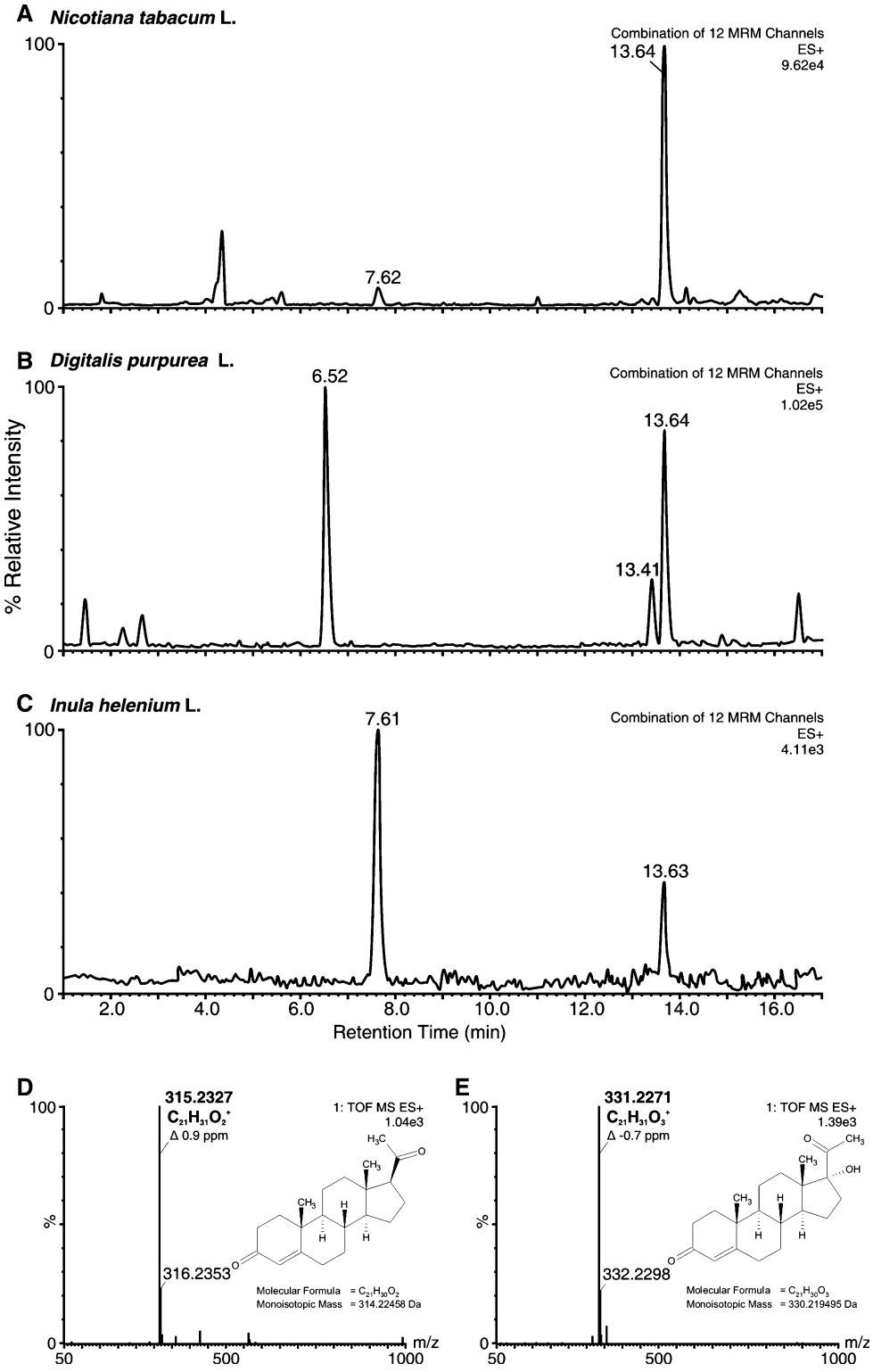

Fig. 3 UPLC-MS/MS chromatograms and spectra of natural steroids (retention time: 17α-hydroxyprogesterone, 6.5 min; androstendione, 7.6 min; 16-dehydroprogesterone, 13.4 min; progesterone, 13.6 min) in purified extracts of *N. tabacum* (**a**), *D. purpurea* (**b**) and *I. helenium* (**c**). The data were derived by a combination of the results from 12 quantitation MRM channels, see Table 3. Examples of MS spectra of detected steroids—progesterone (**d**) and 17α-hydroxyprogesterone (**e**) from extracts of *D. purpurea*

addition of labeled steroid standards was used for identification of analyzed compounds. The validation tests carried out on the method confirmed its suitability for the trace analysis of mammalian-type steroids in plant material.

Steroid Analysis in Plant Extracts

The newly developed method described above was used to investigate the occurrence and amounts of steroids present in three model plants: tobacco (*Nicotiana tabacum*), common foxglove (*Digitalis purpurea*), and elecampane inula (*Inula helenium*) (Table 6, Fig. 3). The presence of steroids was confirmed by Q-TOF MS which provided highly accurate mass determination (Fig. 3d, e). The presence of mammalian steroids in *Digitalis* sp. had been reported previously (Sauer and others 1967; Bennett and others 1968). Progesterone is regarded as an intermediate in the cardenolide biosynthesis pathway in cardenolide-producing plants such as *Digitalis* sp. (Caspi and Lewis 1967; Lindemann and Luckner 1997). In our study, progesterone was actually detected in *Digitalis* extract at a concentration of about 59 pmol g^{-1} FW, at a similar concentration (55 pmol g^{-1} FW) in *N. tabaccum*, and at a much lower concentration (2.1 pmol g^{-1} FW) in *I. helenium* (Table 6). When compared to results of Iino and others (2007), the concentrations we measured were approximately onefold lower than those that Iino and coworkers detected in shoots of *A. thaliana* (508 pmol g^{-1} FW), twofold lower than in *Oryza sativa* shoots (4897 pmol g^{-1} FW), but very close to the amounts measured in leaves of *Lycopersicon esculentum* (79.5 pmol g^{-1} FW).

In addition to the presumed progesterone, our extracts from *D. purpurea* also contained two other steroid compounds, 16-dehydroprogesterone (29 pmol g^{-1} FW) and 17α-hydroxyprogesterone (at the surprisingly high concentration of around 173 pmol g^{-1} FW; Table 6). In mammalian metabolism, 17α-hydroxyprogesterone is an important intermediate in the biosynthesis of the glucocorticoid cortexolone (11-deoxycortisol) and the weak androgen androstendione. Nevertheless, these compounds have not been detected in extracts from *D. purpurea* and the role of 17α-hydroxyprogesterone in *Digitalis* metabolism remains unknown. Elecampane is a traditionally used medicinal plant known to contain sesquiterpene compounds (Seaman 1982), but as far as we know the occurrence of steroids in this species has not previously been reported. The extracts of elecampane were found to contain both progesterone (2 pmol g^{-1} FW) and androstendione (11 pmol g^{-1} FW). These steroids were also found in *N.tabaccum* (progesterone at 55 pmol g^{-1} FW and androstendione at 7.7 pmol g^{-1} FW). As with *I. helenium,* the presence of steroids in tobacco has also been uncertain, although conversion of pregnenolone into progesterone by sterile cell suspension culture of *N. tabacum* was reported by Graves and Smith (1967). 16-Dehydroprogesterone and 17α-hydroxyprogesterone were not detected in extracts from either *N. tabaccum* or *I. helenium.*

Although mammalian steroid hormones undoubtedly occur in higher plants, their physiologic roles still remain uncertain despite the fact that a number of studies have demonstrated that both vegetative and reproductive development of plants may be affected by exogenous application of steroids (Janeczko and Skoczowski 2005). The detection of progesterone in all the species analyzed here is not surprising as it has been previously reported from a wide range of plant taxa (Gawienowski and Gibbs 1968; Janeczko and Skoczowski 2005; Iino and others 2007). Furthermore, the occurrence of putative progesterone-binding proteins in *Arabidopsis* and rice (Yang and others 2005; Iino and others 2007) points to a physiologic role. There has been no report of reliable detection of the other steroids found, namely, androstendione, 17α-hydroxyprogesterone, and 16-dehydroprogesterone.

In conclusion, we have developed and rigorously validated an immunoaffinity chromatography combined with ultra-performance liquid chromatography–electrospray tandem mass spectrometry method for the detection and accurate quantitative measurement of 12 steroids in natural plant samples. Using this new method, four steroids were identified and quantified in three model plants. The approach we have developed represents a substantial improvement in the analysis of naturally occurring steroids and provides a useful procedure for studying the occurrence, metabolism, and roles of mammalian steroid hormones in plants.

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