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RESEARCH ARTICLE

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Small-scale analysis of phytoecdysteroids in seeds by HPLC-DAD-MS for the identification and quantification of specific analogues, dereplication and chemotaxonomy

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Abstract

Introduction: Phytoecdysteroids are analogues of arthropod steroids occurring in plants. They contribute to invertebrate deterrence. A wide diversity of ecdysteroids occurs in phytoecdysteroid-containing plant species, sometimes in high amounts. Ecdysteroids demonstrate potentially useful pharmaceutical actions in mammals.

Objectives: Establish reversed-phase high-performance liquid chromatography with tandem mass spectrometry (RP-HPLC-MS/MS) and RP-HPLC-DAD-MS (diode array detector mass spectrometry) methods for the separation, identification and quantification of ecdysteroids to screen for species containing significant amounts of 20-hydroxyecdysone (20E) and other useful ecdysteroids.

Materials and methods: Micro-extracts of seed samples (ca. 30 mg) in 50% ethanol were subjected to RP-SPE (solid-phase extraction) purification prior to analysis by RP-HPLC-MS/MS and RP-HPLC-DAD-MS.

The method was initially applied to genera (*Amaranthus*, *Centaurea*, *Lychnis*, *Ourisia*, *Serratula*, *Silene* and *Trollius*) where high-accumulating species had been previously encountered. Seeds of 160 randomly selected species, many of which have not previously been assessed, were then analysed. HPLC-MS/MS with a short analysis time initially identifies ecdysteroid-positive extracts and quantifies 20E. The positive extracts (20 ng 20E) are then analysed by HPLC-MS/MS with a longer analysis time to identify and quantify 17 common phytoecdysteroids and, finally, HPLC-DAD-MS (0.1–0.25 μg 20E) is used to obtain UV- and MS-spectra to confirm identifications or as a basis for characterisation of partially identified or novel analogues.

Results: *Lychnis coronaria*, *Silene fimbriata* and *Silene hookeri* ecdysteroids are characterised for the first time and those of *Cucubalus baccifer* and *Ipheion uniflorum* are more extensively characterised.

Conclusions: The procedure provides a rapid/sensitive method for screening small plant samples for the presence, quantification and identification of ecdysteroids. It permits ready dereplication of samples, identifying extracts containing large amounts or novel analogues.

Abbreviations: DAD, diode-array detector; HPLC, high-performance liquid chromatography; MS, mass spectrometry; RIA, radioimmunoassay; RP, reversed-phase; SAR, structure–activity relationship; SPE, solid-phase extraction.

KEYWORDS
20-hydroxy, ecdysone, chromatography, ecdysone, ecdysteroid, mass spectrometry, spectroscopy, steroid profile

1 | INTRODUCTION

Ecdysteroids are the major class of steroid hormones in invertebrates, where they regulate moulting, development and reproduction.[1] The major hormonally-active ecdysteroid in insects is regarded to be 20-hydroxyecdysone (20E, Figure 1), although a total of about 50 analogues have been identified as precursors, intermediates and metabolites, and it is possible that some of these may also have hormonal activity in their own right.[2] Additionally, ecdysteroids occur in a significant proportion of plant species, where they are referred to as phytoecdysteroids, at far higher concentrations (ca. 1000-fold) and with a much greater range of structural diversity than has been found in arthropods (zooecdysteroids).[3] In plants, ecdysteroids are believed to serve to reduce invertebrate predation by acting as feeding deterrents on interaction with taste receptors, or as endocrine disruptors on ingestion bringing about hormonal imbalance involving the mis-regulation of gene expression by intracellular ecdysteroid receptors.[4] Again, the major phytoecdysteroid encountered in plants is 20E, but many others are also found.[3] The levels of ecdysteroids in phytoecdysteroid-accumulating plants depend on the species, the ecotype, the stage of development, the plant part and whether the plant has been subjected to stress or predation. Phytoecdysteroid levels can be extremely high (e.g. 20E at 3.2% of the dry weight of the stem in *Diploclisia glaucescens*[5]), but more typically are in the range of 0.1 to 1% of the dry weight, and it appears that most, if not all, plant species have the genetic capacity to produce ecdysteroids, even if the majority do not normally do so.[6] Phytoecdysteroid profiles can vary from relatively simple, where one or a few analogues predominate to complex cocktails of many analogues. It is now also apparent that ecdysteroids bring about a large range of, essentially beneficial, pharmaceutical effects in mammals,[7] such that there is now a strong and growing interest in using them for medical purposes.[8] For example, the anabolic activity of ecdysteroids which, in contrast to testosterone-like anabolics, does not require associated exercise/training and does not have psychological and physical side-effects, is being exploited for the development of new approaches to the treatment of sarcopenia, Duchenne muscular dystrophy and other muscle-wasting conditions. Up to the present, such trials could only be conducted with 20E, as this is the only pure analogue available commercially in significant amounts.

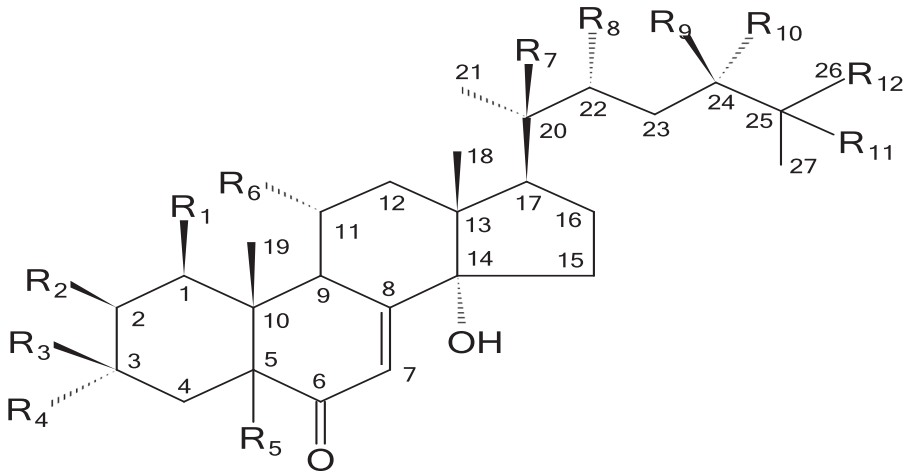
Research on the roles, modes of action and applications of ecdysteroids in invertebrates, plants and mammals all require the availability of adequate amounts of pure ecdysteroids, especially in the case of clinical trials on mammalian species, where kilogram amounts of an analogue are needed. Since the chemical synthesis of ecdysteroids is complicated and proceeds with a low yield,[9] it is not an economically viable strategy for the preparation of large amounts of an ecdysteroid. Semi-synthetic strategies for the conversion of one ecdysteroid analogue (usually 20E) into another may be feasible in a few cases.[10,11] However, without doubt, the generally most applicable strategy to obtain an adequate amount of a specific ecdysteroid analogue is to isolate it from an appropriate plant source. Up to now this approach has been successful for 20E, where commercial, large-scale extraction from plants such as *Cyanotis arachnoidea*, *Pfaffia* spp., *Serratula coronata* and *Leuzea carthamoides* has resulted far greater availability and a reduction in cost from USD2000/g to USD1000/kg over the past two decades. To date, only ca. 2% of plant species have been investigated in any way for their ecdysteroid content.[12,13]

While 20E may continue to remain the most readily accessible and most exploited ecdysteroid, there is a need to identify suitable plant sources from which other ecdysteroid analogues can be readily isolated in high yield and at reasonable cost. Ideally, such plant sources would contain (i) a large amount of the desired ecdysteroid, (ii) few other analogues (which are difficult to separate off by simple/cost-effective purification methods, such as solvent partitions and crystallisation) and (iii) the absence of components in the plant matrix which interfere with the purification. Such analogues are important for structure–activity relationship (SAR) studies on receptors and enzymes in invertebrates and mammals, biosynthetic and metabolic studies in invertebrates, plants and mammals, bioavailability and pharmacokinetic studies in mammals and, ultimately, clinical trials in mammals, including humans. Since the known high-accumulating plant species contain large amounts of ecdysteroids in their seeds, and these are relatively readily accessible and a consistent source of plant material, we have developed a screening procedure, based on micro-extraction of small amounts of commercially available seeds coupled with specific and sensitive identification of ecdysteroids by coupled high-performance liquid chromatography diode array detector mass spectrometry (HPLC-DAD-MS). Here we present the method and discuss its potential use for the characterisation of phytoecdysteroid profiles, for the identification of plant sources of specific ecdysteroid analogues and their wider chemotaxonomical implications.

2 | MATERIAL AND METHODS

2.1 | Source of plant material

Seeds were purchased from Plant World Seeds, Torquay, Devon, UK. Organic solvents were supplied by Carlo Erba, Val-de-Reuil, France.

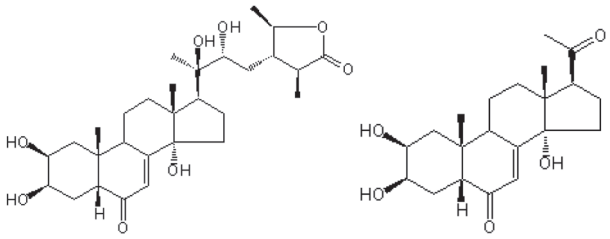


Ecdysteroid	Abbreviation	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
Abutasterone	Abu	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	H-	HO-	HO-	H_3C-
Ajugasterone C	AjuC	H-	HO-	HO-	H-	βH-	HO-	HO-	HO-	H-	H-	H-	H_3C-
Dacryhainansterone	Dacry	H-	HO-	HO-	H-	βH-	$\Delta^{9(11)}$	HO-	HO-	H-	H-	H-	H_3C-
Dacrysterone	Dacryst	H-	HO-	HO	H-	βHO-	H-	HO-	HO-	H_3C-	H-	HO-	H_3C-
3-Dehydroecdysone	3dE	H-	HO-	O=		βH-	H-	H-	HO-	H-	H-	HO-	H_3C-
3-Dehydro-20-hydroxyecdysone	3d20E	H-	HO-	O=		βH-	H-	HO-	HO-	H-	H-	HO-	H_3C-
24(28)-Dehydromakisterone A	24(28)DMakA	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	H_2C=		HO-	H_3C-
2-Deoxyecdysone	2dE	H-	H-	HO-	H-	βH-	H-	H-	HO-	H-	H-	HO-	H_3C-
25-deoxyecdysone	25dE	H-	HO-	HO-	H-	βH-	H-	H-	HO-	H-	H-	H-	H_3C-
2-deoxy-20-hydroxyecdysone	2d20E	H-	H-	HO-	H-	βH-	H-	HO-	HO-	H-	H-	HO-	H_3C-
20,26-dihydroxyecdysone	20,26E	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	H-	H-	HO-	(*R*/*S*) HOH_2C-
Ecdysone	E	H-	HO-	HO-	H-	βH-	H-	H-	HO-	H-	H-	HO-	H_3C-
24-*Epi*-abutasterone	24epiAbu	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	HO-	H-	HO-	H_3C-
24-*Epi*-makisterone A	24epiMakA	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	H-	H_3C-	HO-	H_3C-
20-Hydroxyecdysone	20E	H-	HO-	HO-	H-	βH-	H-	HO	HO-	H-	H-	HO-	H_3C-
(5α-H)20-hydroxyecdysone	5α20E	H-	HO-	HO-	H-	αH-	H-	HO	HO-	H-	H-	HO-	H_3C-
26-Hydroxypolypodine B	26PolB	H-	HO-	HO-	H-	βH-	H-	HO	HO-	H-	H-	HO-	(*R*/*S*) HOH_2C-
Inokosterone	Ino	H-	HO-	HO-	H-	βH-	H-	HO	HO-	H-	H-	H-	(*R*/*S*) HOH_2C-
Integristerone A	IntA	HO-	HO-	HO-	H-	βH-	H-	HO-	HO-	H-	H-	HO-	H_3C-
Makisterone A	MakA	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	H_3C-	H-	HO-	H_3C-
Muristerone A	MurA	H-	HO-	HO-	H-	βHO-	HO-	HO-	HO-	H-	H-	H-	H_3C-
Polypodine B	PolB	H-	HO-	HO-	H-	βHO-	H-	HO	HO-	H-	H-	HO-	H_3C-
Ponasterone A	PonA	H-	HO-	HO-	H-	βH-	H-	HO	HO-	H-	H-	H-	H_3C-
Pterosterone	Pter	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	H-	HO-	H-	H_3C-
Stachysterone C	StachC	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	-CH=C<			H_3C-
Taxisterone	Tax	H-	HO-	HO-	H-	βH-	H-	HO-	H-	H-	H-	HO-	H_3C-
Turkesterone	Turk	H-	HO-	HO-	H-	βH-	HO-	HO-	HO-	H-	H-	HO-	H_3C-
Viticosterone E	VitE	H-	HO-	HO-	H-	βH-	H-	HO-	HO-	H-	H-	H_3COO-	H_3C-

FIGURE 1 Ecdysteroid structures and numbering

2.2 | Extraction

Seeds from a packet (up to 30 mg) were weighed into a weighing boat and then transferred to a Precellys tube, Bertin Technologies, Montigny-le-Bretonneux, France (CK14; 2 mL) containing ca. 60 small porcelain beads. The tubes were firmly sealed and the seeds pulverised in a Savant Fast Prep apparatus, Thermo-Fisher, France (3 × 30 s at speed setting 6.5). Ethanol/water (1:1 *v*/*v*;



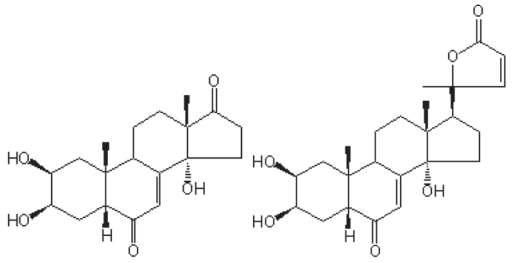


FIGURE 1 (Continued)

1.5 mL) was added to each sample and the tubes were sonicated in a sonication bath for 30 min. The tubes were then heated at 75°C in an oil-bath for 120 min, cooled and then centrifuged in an Eppendorf Centrifuge, Stevenage, UK (30 s at full speed) to pellet solid material.

2.3 | Partial purification by SPE

An aliquot (1 mL) of each seed extract was diluted with 4 mL water and applied to a new, pre-activated C_{18}-Sep Pak cartridge (Waters, Milford, MA, USA) and washed with 5 mL 10% methanol and eluted with 5 mL 100% methanol. The 100% methanol fraction was analysed by HPLC-MS/MS (tandem mass spectrometry) and HPLC-DAD-MS for:

- Quantification of 20E by MS/MS to assess the presence or absence of ecdysteroids in the sample
- Quantification of 20E and 16 other ecdysteroids by HPLC-MS/MS (in triplicate in comparison to calibration curves run before and after the samples and in comparison to older samples of known concentration)
- Determination of chromatographic profiles by HPLC-DAD-MS for the identification of ecdysteroids on the basis of UV- and MS-spectra and retention times

2.4 | Equipment for HPLC-DAD-MS

The Agilent 1260 Infinity LC system consisted of a Model G7167A Injector, a Model G1311B pump, a Model G4212B photodiode-array detector and a Model 6420 Triple Quad Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA). The program used for quantitative and qualitative data analyses was Mass Hunter chromatography software (Version B.07.00).

2.5 | Reference ecdysteroids

Samples of verified ecdysteroids were obtained from the ecdysteroid reference collection of one of us (RL; see www.ecdysone.org for physico-chemical data for these compounds), accurately weighed and dissolved in methanol to give solution of known concentration. The purity of each was determined by reversed-phase (RP)-HPLC with a DAD. Seventeen ecdysteroids, where the purity was > 97%, were used for the preparation of calibration curves (Table 1).

2.6 | Ecdysteroid calibration curves

The HPLC separation of the 17 reference ecdysteroids is shown in Figure 2. Calibration curves (5–5000 ng/mL) were established in triplicate using the HPLC-MS/MS conditions described later for each of the reference ecdysteroids, and the equations, regression coefficients, limit of detection (LOD) and limit of quantification (LOQ) values are summarised in Table 1.

2.7 | Initial quantification of 20E by HPLC-MS/MS

Aliquots (5 μL) of the 100% methanol fraction from the Sep Pak C_{18} purification of each seed extract were mixed with 5 μL water and separated in triplicate on a Fortis C18 2.1 × 50 mm (5 μm particle size) column eluted at 0.3 mL/min with a gradient from 10% to 100%

TABLE 1 Summary of reference ecdysteroid properties and retention times

Compound	MW	Purity (%)	Retention time (min)	MS transition	Calibration equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
Abutasterone	496	99.0	15.8	497.0 → 371.0	$y = 1604x$	.9994	1	4
Ajugasterone C	480	95.8	24.2	481.1 → 445.0	$y = 1753x$	.9996	19	63
Cyasterone	520	97.0	24.9	521.1 → 485.5	$y = 650.0x$	.9994	2	7
Dacryhainansterone	462	99.5	32.4	462.9 → 299.0	$y = 369.4x$	.9998	2	8
2-Deoxyecdysone	448	98.2	35.0	449.0 → 431.0	$y = 11.11x$	.9997	300	1000
25-Deoxyecdysone	448	98.9	40.4	449.0 → 413.1	$y = 760.4x$	.9982	4	13
2-Deoxy-20-hydroxyecdysone	464	99.0	26.7	465.3 → 429.0	$y = 3449x$	.9988	3	9
20,26-Dihydroxyecdysone*	496	99.0	12.1/12.7[aa]	495.2 → 175.0	$y = 75.95x$	.9996	1	2
Ecdysone	464	99.0	26.2	447.0 → 429.0	$y = 1773x$	.9999	38	125
24-*Epi*-Abutasterone	496	99.0	11.7	497.1 → 461.2	$y = 1004x$	.9990	7	23
20-Hydroxyecdysone	480	97.5	18.2	481.1 → 371.2	$y = 1711x$	.9993	2	8
Integristerone A	496	97.2	14.3	497.1 → 386.9	$y = 4.403x$	.9932	200	667
Polypodine B	496	99.0	16.8	497.4 → 351.3	$y = 287.2x$	.9993	5	16
Ponasterone A	464	90.9	34.8	465.1 → 447.0	$y = 3561x$	.9987	3	10
Poststerone	362	98.3	22.8	363.1 → 345.0	$y = 1652x$	.9990	2	8
Taxisterone	464	97.9	28.4	461.1 → 429.0	$y = 3045x$	.9998	3	10
Turkesterone	496	99.0	8.9	496.9 → 461.1	$y = 230.3x$	.9996	18	59

Note: the transitions used are different for each ecdysteroid, but some may occur in the fragmentations of other ecdysteroids too (i.e. they are not fully specific).

[a]Two isomers (25*R*/25*S*) possible; the elution sequence has not been established for this HPLC system.

MW, molecular weight; LOD, limit of detection; LOQ, limit of quantification.

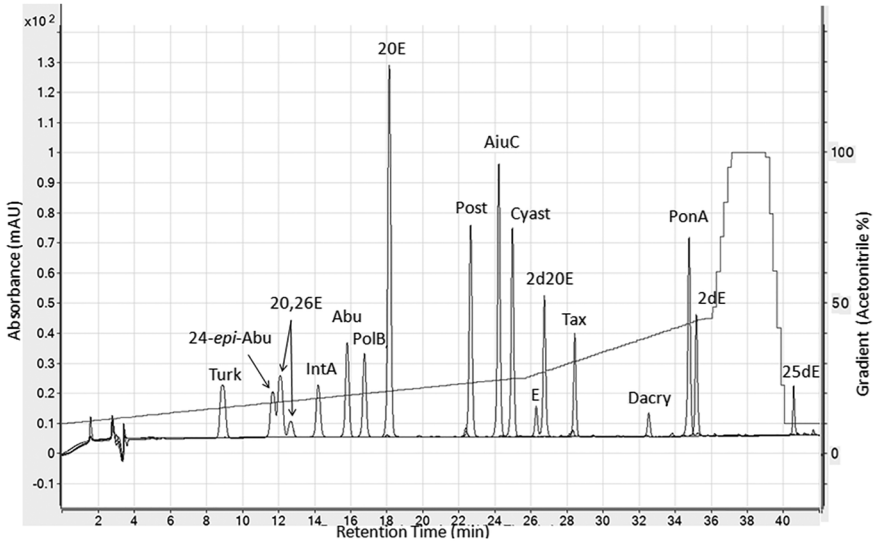


FIGURE 2 Chromatogram of the separation of 17 ecdysteroid standards on an Acquity column (CSH; 150 mm × 2.1 mm; 1.7 μm particle size) with a gradient of 0.1% (*v*/*v*) formic acid/acetonitrile in 0.1% (*v*/*v*) formic acid/water at 0.3 mL/min and 30°C, monitored at 254 nm. Details of the gradient are included in the text. The abbreviations for the ecdysteroids are given in Figure 1

acetonitrile in water (with 0.1% formic acid in both) over 6 min with isocratic elution at 100% acetonitrile for a further 4 min. Furthermore, 20E was monitored and quantified by selective reaction monitoring (SRM) (transition 481.1 → 371.2) in comparison to a calibration curve prepared with pure reference 20E (5–5000 ng/mL). In the case that the amount of 20E detected exceeded the upper limit of the calibration curve (5 μg/mL), the analysis was repeated at an appropriate dilution.

2.8 | Quantification of 17 ecdysteroids by HPLC-MS/MS

Samples of 20E-positive extracts (5 μL, dissolved in 50% aqueous methanol and containing 20 ng 20E) were separated using the system described earlier. The relevant transitions for monitoring each of the ecdysteroids are given in Table 1.

2.9 | Identification of ecdysteroids by HPLC-DAD-MS

Samples (5 μL, dissolved in 50% aqueous methanol and containing 0.1–0.25 μg 20E) were separated on an analytical-scale (2.1 mm × 150 mm) 1.7 μm Acquity CSH fluoro-phenyl RP column, supplied by Waters. The column was kept at 30°C. The following gradient system was used with water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B): 0 min, 90%A/10%B; 25–25.5 min, 75%A/25%B; 35–35.5 min, 55%A/45%B; 36–37 min, 0%A/100%B, with linear gradients in the intervening periods. The re-equilibration time at starting conditions was 10 min. The flow-rate was 0.15 mL/min. Acquisition of the mass spectral data between *m/z* 100 and 700 was performed in the positive- and negative-ion electrospray modes.

2.10 | Sensitivity of detection of ecdysteroids in biological samples

The 100% methanol RP-SPE (solid-phase extraction) fractions of seed extracts of *Silene laciniata angustifolia* (0.40 mg 20E/g), *Silene multiflora* (2.85 mg 20E/g), *Ourisia macrophylla* (9.1 mg 20E/g), *Serratula coronata* (18.4 mg 20E/g) and *Chenopodium giganteum* (0.56 mg 20E/g) were prepared as serial two-fold dilutions in 50% aqueous methanol and 5 μL aliquots separated for analysis by HPLC-DAD/-MS. The quality and information content of the UV- and MS-spectra for each ecdysteroid peak were compared for each dilution to identify at which dilution it became impossible to record reliable spectra. These seed extracts were selected to represent those containing moderate, medium, high and very high levels of total ecdysteroids relative to other components of the biological matrix, and to contain a range of other ecdysteroids in addition to 20E.

2.11 | Strategy for identification and quantification of ecdysteroids in seed extracts

Seeds were micro-extracted and RP-SPE purified as described earlier. An aliquot (5 μL) of the 100% methanol SPE fraction was assessed in triplicate for the amount of 20E present by HPLC-MS/MS. If the quantification was above the range of the calibration curve (5 μg/mL), the quantification was repeated after appropriate dilution of the sample. For the 20E-containing extracts, a sample of each corresponding to 20 ng 20E/5 μL was prepared and separated by HPLC-MS/MS for the separation and quantification of the 17 ecdysteroids, monitoring all the relevant transitions (Table 1), which permitted quantification of those 17 ecdysteroids (when present) and recognition of peaks possessing those transitions, but at other retention times than those of the reference ecdysteroids, which could indicate the presence of additional ecdysteroids. Samples of the ecdysteroid-positive extracts were then prepared at a known concentration of between 20 and 50 μg 20E/mL and 5 μL was separated by HPLC-DAD-MS to obtain UV- and MS-spectra for each peak at the retention times of the known ecdysteroids and to those of the other peaks demonstrating an ecdysteroid-related transition. Data were tabulated to assess conformity of the retention time, UV-spectrum and mass spectral information ($[M + H]^+$, $[M + Na]^+$ and number of sequential H_2O molecules lost from the pseudo-molecular ion in positive-ion electrospray mode) with the putative identity of the ecdysteroid, or to predict the possible identity in the case of unknown peaks. In the latter cases, Ecdybase[3] was searched for all known ecdysteroids with the same molecular weight (MW) and these were assessed for suitability on the basis of λ_{max}, number of hydroxyl groups and predicted polarity. Candidate reference ecdysteroids were then separated in the same system and the UV-/MS-spectra and retention times were compared with those of the peaks in the biological extracts.

2.12 | Further reference ecdysteroids

To assist in the identification of unknown ecdysteroids present in seed extracts, the retention times, UV- and MS-spectra of a further 17 ecdysteroids were determined by HPLC-DAD-MS (Supporting Information Table S2).

2.13 | Statistics

Ecdysteroid quantifications were performed in triplicate and are expressed as means ± standard deviation (SD).

3 | RESULTS AND DISCUSSION

3.1 | Method validation

It was found, by spiking samples with known amounts of 20E, that quantification of the 50% ethanol extracts by HPLC-MS/MS was not fully additive, indicating that the sample matrix depressed quantification. Addition of a straightforward and fast RP-SPE partial purification step markedly increased the level of 20E detected in ecdysteroid-positive samples and provided additivity.

The method was finally validated with a seed extract of *Chenopodium quinoa*, which has been previously extensively studied for its content and profile of ecdysteroids.[14,15] Previous studies had shown that the seeds contain ca. 0.4 mg 20E/g as the clearly predominant ecdysteroid, with significant amounts of MakA, 24(28)-dehydroMakA, 24-*epi*-MakA and PolB, smaller amounts of MakC, 2d20E, 2d2026E and traces of several other identified ecdysteroids.[14] The qualitative and quantitative analyses of seeds of *Chenopodium quinoa* by the method developed here are described later.

3.2 | Sensitivity of ecdysteroid detection in seed extracts

The lowest levels of 20E, PolB, AjuC, 2026E and IntA which gave useful UV-spectra were similar, being 0.5 to 1.5 ng injected, reflecting the identical chromophoric groups and similar MWs in these ecdysteroids. The amounts required to give fully usable mass spectra ($[M + H]^+$ and/or $[M + Na]^+$ and sequential dehydration from $[M + H]^+$) were somewhat higher in the range of 1.2 to 3.5 ng, with PolB being most readily detected (1.2 ng) and 2026E (3 ng) and IntA (3.5 ng) being the least readily detected of these ecdysteroids. The sensitivity of detection by both methods did not vary noticeably in relation to the concentration of total ecdysteroids in the seeds, but this was assessed after partial purification after RP-SPE as prior studies had shown that quantification by HPLC-MS is suppressed by the biological matrix unless SPE is performed (see earlier).

3.3 | Analysis of seed extracts

3.3.1 | The method

Micro-analysis methods were developed about 20 years ago for assessment of the presence/absence of ecdysteroids in small samples of seeds, based on micro-extractions coupled with off-line qualitative/quantitative assessment with an ecdysteroid-specific insect cell-based assay (detecting the presence of compound with biological activity similar to 20E) and ecdysteroid-specific radioimmunoassays (RIAs) (for structural similarity to 20E),[16,17] which permitted the screening of ca. 5000 species of plants.[12,13,17] While this approach was a considerable methodological advance on what had been previously possible in terms of useful information obtained per man-hour, it required three dedicated and skilled researchers to catalogue the plant material, coordinate the extractions and analyses, and process and analyse the data. Time and technology have moved on and HPLC-DAD-MS offers the possibility for the on-line analysis of ecdysteroid profiles and quantification with sequential and continuous automated delivery of extracts of many samples, providing physico-chemical data (retention time UV- and MS-spectra) for each separated major component and the potential to quantify selected identified components (by UV, MS or MS/MS).

3.3.2 | Identification of ecdysteroid-positive species

More than half (148/215; Tables 2 and 3) of the species tested here are not recorded in the 'Compilation of the literature reports for the screening of vascular plants, algae, fungi and non-arthropod invertebrates for the presence of ecdysteroids: Version 5' (Ecdybase[3]), so whether they contain ecdysteroids or not has not been previously published. Among the 55 selected species (Table 2), seeds were found to be ecdysteroid-positive for 23 species. Most of the positive species belong to the Caryophyllaceae (in the genera *Lychnis* and *Silene*; 19 positive from 38 tested), which accords with the previous finding that about 50% of the species in this large family are ecdysteroid-positive.[18] The 20E levels in the positive species vary from very low (*Silene alpestris*, *Lychnis yunnanensis*) to rather high (*Silene otites*). Seeds of two of the species in the Asteraceae (*Leuzea carthamoides* and *Serratula coronata*) contain high levels of ecdysteroid, but the *Cyanus* species are all negative, as are the seven species in the Ranunculaceae and both tested species of *Amaranthus*. Of the 160 randomly selected species six (4%) were found to be positive for 20E, which is slightly lower to the 5–6% found to be positive when screening with ecdysteroid-specific RIA and bioassay,[17] but this is to be expected because the frequently positive genera *Silene* and *Lychnis* were excluded from the otherwise random selection. Overall there is excellent agreement with previous findings (Ecdybase) as to whether these species are ecdysteroid-positive or not.

3.3.3 | Comparison between the HPLC-MS/MS data and previous RIA data

Tables 2 and 3 incorporate data obtained for seeds of many of the same species (112/215 species) previously obtained after assessment of 70% methanolic micro-extracts with the DBL-1 antiserum, which is ecdysteroid-specific, but recognises various ecdysteroid analogues to different extents (cross-reactivities are given in Dinan[17]) and it, importantly, has a greater affinity for E than 20E, whereas the HPLC-MS/MS data in the two tables quantifies 20E specifically. Thus, comparison of the results of the two methods should be largely qualitative and can, at best, only be viewed semi-quantitatively. Further, the two data sets derive from seed samples from different suppliers. With these caveats in mind, the two data sets concord very well, with only a few species showing marked differences. *Silene multiflora* is clearly positive by HPLC-MS/MS, but was negative by RIA. Previous studies by others present contradictory results for this species, as two report it as ecdysteroid-positive,[19,20] while two found it to be ecdysteroid-negative,[18,21] implying variation according to the source of the plant material. Similarly, *Atriplex hortensis* was found to be negative for 20E by HPLC-MS/MS, while analysis of 13 separate samples by RIA revealed five to be significantly positive and eight to be negative.[22] A sample of seeds of *Cucubalus baccifer* had previously been found to be negative by RIA, but a separate sample was positive by HPLC-MS/MS, which is in accord with results obtained by others for plants of this species.[23, 24] This is also the case for *Sida cordifolia*, since an earlier study had detected ecdysteroids in plants of this species.[25]

3.3.4 | Ecdysteroid profiles in seeds of ecdysteroid-positive species

The qualitative and quantitative findings are summarised in Supporting Information Table S1, combining the data from HPLC-MS/MS and HPLC-DAD-MS analyses. HPLC chromatograms for six

TABLE 2 Assessment of seeds of species in selected plant genera for the presence of ecdysteroids by HPLC-MS/MS

Species	Family	Common name	Ecdybase[a]	Exeter survey[b]	20E µg/g by HPLC-MS/MS	Other ecdysteroids identified
Amaranthus caudatus	Amaranthaceae	Love-lies-bleeding	✓	—	—	
A. gangeticus	Amaranthaceae	Elephant-head amaranth	✓	2.8	—	
Centaurea cyanus	Asteraceae	Cornflower	✓	—	—	
C. macrocephala	Asteraceae	Giant knapweed	✓	—	—	
C. ruthenica	Asteraceae	Russian knapweed	✓	—	—	
C. uralensis	Asteraceae		Npp	Nt	—	
Leuzea carthamoides	Asteraceae	Maral root	✓	731	7190	PolB, AjuC, IntA
Lychnis alpina (Silene suecica)	Caryophyllaceae	Arctic campion	✓	—	—	
L. x arkwrightii	Caryophyllaceae	Arkwright's campion	✓	1170	4166	PolB, E
L. chalcedonica	Caryophyllaceae	Maltese-cross	✓	1054	1652	PolB, IntA, tax
L. coronaria	Caryophyllaceae	Rose campion	✓	521	3750	PolB, IntA
L. flos-cuculi	Caryophyllaceae	Ragged robin	✓	1375/287	3547	PolB, IntA
L. flos-jovis	Caryophyllaceae	Flower of Jove	✓	647	1253	PolB, 2026E
L. viscaria (Silene viscaria)	Caryophyllaceae	Sticky catchfly	✓	14	—	
L. yunnanensis	Caryophyllaceae		✓	—	51	
Ourisia macrophylla	Plantaginaceae	Mountain foxglove	✓	2800	9143	PolB, 2026E, Tax
Paris quadrifolia	Melanthiaceae	Herb Paris	✓	2796	3952	PolB
Serratula coronata	Asteraceae		✓	602	18430	PolB, AjuC, IntA
Silene alpestris	Caryophyllaceae	Alpine catchfly	✓	2.3	9	Not analysed
S. armeria	Caryophyllaceae	Sweet William catchfly	✓	—	—	
S. asterias	Caryophyllaceae	Cherry drumsticks	✓	—	—	
S. atigraca	Caryophyllaceae		Npp	Nt	—	
S. atropurpurea	Caryophyllaceae		Npp	Nt	—	
S. bellidiodes	Caryophyllaceae		✓	—	—	
S. caroliniana	Caryophyllaceae	Carolina pink	✓	—	—	
S. compacta	Caryophyllaceae	Rock campion	✓	Nt	—	
S. delavayi	Caryophyllaceae		✓	Nt	1437	Tax
S. dioica	Caryophyllaceae	Red campion	✓	—	—	
S. fimbriata	Caryophyllaceae	Fringed campion	Npp	Nt	391	PolB
S. hookeri	Caryophyllaceae	Hooker's campion	Npp	Nt	630	PolB, 2026E
S. italica	Caryophyllaceae	Italian catchfly	✓	1086	2844	PolB, IntA,2d20E, 2026E
S. keiskei	Caryophyllaceae	Japanese campion	✓	200	2107	PolB
S. laciniata angustifolia	Caryophyllaceae	Cardinal catchfly	✓	2000	396	PolB, 2026E
S. latifolia	Caryophyllaceae	White campion	✓	0.4	—	
S. lerchenfeldiana	Caryophyllaceae		Npp	Nt	—	
S. maritima (S. uniflora)	Caryophyllaceae	Sea campion	✓	—	—	
S. mexicana	Caryophyllaceae	Mexican catchfly	Npp	Nt	—	
S. multiflora	Caryophyllaceae		✓	—	2847	IntA, PolB, Abu, 2026E
S. nutans	Caryophyllaceae	Nottingham catchfly	✓	1147	4963	PolB, IntA, 2026E
S. otites	Caryophyllaceae	Spanish catchfly	✓	260/2688	9038	2d20E, IntA
S. pusilla	Caryophyllaceae	Alpine catchfly	✓	4.4	—	
S. saxifraga	Caryophyllaceae	Saxifrage catchfly	✓	250	1054	PolB, IntA

(Continues)

TABLE 2 (Continued)

Species	Family	Common name	Ecdybase[a]	Exeter survey[b]	20E μg/g by HPLC-MS/MS	Other ecdysteroids identified
S. schafta	Caryophyllaceae	Autumn catchfly	✓	586	1299	PolB, Abu, IntA
S. suecica (Lychnis alpina)	Caryophyllaceae	Red alpine catchfly	✓	—	—	
S. viridiflora	Caryophyllaceae		✓	Nt	1925	PolB, 2026E, IntA
S. viscaria (Lychnis viscaria)	Caryophyllaceae	Sticky catchfly	✓	14	—	
S. waldsteinii (S. clavata, S. marocarpa)	Caryophyllaceae	Waldstein's campion	✓	Nt	—	
S. zawadskii	Caryophyllaceae	Zawadski's catchfly	✓	—	—	
Trollius chinensis	Ranunculaceae	Jin lian hua	✓	—	—	
T. europaeus	Ranunculaceae	European globeflower	✓	0.5	—	
T. ircuticus	Ranunculaceae	Siberian globeflower	Npp	Nt	—	
T. laxus	Ranunculaceae	American globeflower	✓	—	—	
T. pumilus	Ranunculaceae	Dwarf globeflower	✓	1.0	—	
T. vaginatus	Ranunculaceae		Npp	Nt	—	
T. yunnanensis	Ranunculaceae	Chinese globeflower	Npp	Nt	—	

[a]Ecdybase[3] contains a compilation of the literature reports for the occurrence of ecdysteroids in plant species. Entries can refer to any part (or all) of the plant; ✓ = an entry for the species exists in the database: Npp = nothing previously published.

[*]The Exeter Survey (1995–2001) screened the seeds of ca. 5000 species of plants for the presence of ecdysteroids by means of an ecdysteroid-responsive cell-based bioassay (to detect ecdysteroid biological activity) and up to three ecdysteroid-specific RIAs (to detect for chemical similarity to ecdysteroids). The data for the DBL-1 antiserum are presented here and where values are given they correspond to the μg E equivalent/g seed (where multiple values are given, they correspond to the results for replicate seed samples); — = none detected; Nt = not tested. Note: the data for the Exeter Survey and the current HPLC-DAD-MS data derive from separate seed samples from different suppliers.

of the species are presented in Figure 3. The results are briefly discussed below in relation to plant family, species and previous literature.

Agapanthus praecox (Amaryllidaceae)

Seeds of this species contain moderate levels of ecdysteroids (ca. 1.2 mg/g), but unusually E is the major ecdysteroid in a complex profile in which 20E could be conclusively identified and Abu and Turk are tentatively identified. Several unidentified ecdysteroids are also present. The complex ecdysteroid profile, containing a limited amount of 20E had already been indicated by RP-HPLC-RIA for seeds of this species.[26]

Chenopodium giganteum (Amaranthaceae)

Seeds of *Chenopodium giganteum* contain moderate to low levels of ecdysteroids (ca. 900 μg/g), with 20E, PolB and 2026E being definitely identified by HPLC-DAD-MS and MakA and 24(28)-dehydroMakA being identified by co-chromatoraphy with further reference ecdysteroids. A previous report had detected between 0.48–1.03 mg E equivalent/g by RIA in three samples of seeds of *Chenopodium giganteum*.[22]

Chenopodium quinoa (Amaranthaceae)

The seed of the sample of *Chenopodium quinoa* examined contained low amounts of ecdysteroids (ca. 75 μg/g). HPLC-MS/MS revealed the presence of 20E as the major ecdysteroid (67.5 μg/g) with much smaller amounts of PolB (5 μg/g) and 20,26E (1.6 μg/g; first eluting isomer only). HPLC-DAD-MS identified a further ecdysteroid peak (ca. 10 μg/g), corresponding to MakA and/or 24-*epi*-MakA which co-elute in the RP-HPLC system used and have identical UV- and MS-spectra. Ecdysteroid levels vary significantly in seeds of *Chenopodium quinoa* depending on origin and cultivar.[14, 22,27] Previous examination of *Chenopodium quinoa* seeds[15] identified 20E as the major ecdysteroid, with smaller amounts of MakA, 24-*epi*-MakA, 24(28)-dehydroMakA and MakC. PolB and 20,26E, along with other analogues, have also been identified as minor ecdysteroids in seeds of this species.[14, 27]

Cucubalus baccifer (Caryophyllaceae)

Moderate levels of ecdysteroid (ca. 1.4 mg/g) are present in seeds of this species, with 20E being the major component along with much smaller amounts of PolB, IntA and Tax. From the whole plant of *Cucubalus baccifer*, 20E (0.0014% of the dry weight), 24(28)-dehydroMakA, Tax, 25-hydroxypanuosterone, rubrosterone and 2,22-dideoxy-3β-glucoside had previously been detected.[23]

Ipheion uniflorum (Amaryllidaceae)

Although seeds of this species contain only low levels of ecdysteroids (ca. 50 μg/g), it was possible to identify and quantify not only 20E as the major ecdysteroid present (47 μg/g), but also PolB (2.4 μg/g) and 2d20E (3.0 μg/g) as the minor ones. A previous study[28] had identified 20E (0.012% of the fresh weight) from bulbs of this species.

TABLE 3 Assessment of seeds of randomly-selected plant species of other genera for the presence of ecdysteroids by HPLC-MS/MS

Species	Family	Common name	Ecdybase[aa]	Exeter survey[bb]	20E μg/g by HPLC-MS/MS	Other ecdysteroids identified
Abelmoschus esculenta	Malvaceae	Okra, ladies' fingers	Npp	Nt	–	
Acaena saccaticupula	Rosaceae	Blue goose leaf	Npp	–	–	
Acer palmatum	Sapindaceae	Japanese maple	Npp	–	–	
Aconitum vulparia	Ranunculaceae	Wolf's bane	✓	–	–	
Acrocarpus fraxinifolius	Fabaceae	Pink cedar	Npp	Nt	–	
Agapanthus praecox	Amaryllidaceae	African lily	✓	1.5/1.7/0.6/–/3.8	517	E, Abu?, Turk?
Agastache foeniculum	Labiatae	Blue giant hyssop	✓	–	–	
Alliaria petiolata	Brassicaceae	Jack-by-the-hedge	Npp	–	–	
Allium ampeloprasum	Amaryllidaceae	Wild leek	Npp	Nt	–	
A. canadense	Amaryllidaceae	Canadian garlic	Npp	Nt	–	
Amelanchier alnifolia	Rosaceae	Pacific serviceberry	Npp	Nt	–	
Anemonopsis macrophylla	Ranunculaceae	False anemone	Npp	Nt	–	
Annona muricata	Annonaceae	Soursop	Npp	–	–	
Anthriscus cerefolium	Apiaceae	Chervil	Npp	–	–	
Anthyllis montana rubra (Vulneraria montana)	Fabaceae	Mountain kidney vetch	Npp	–	–	
A. vulneraria	Fabaceae	Woundwort	Npp	–	–	
Aquilegia skinneri	Ranunculaceae	Mexican columbine	Npp	Nt	–	
Arabis collina rosea (A. muralis)	Brassicaceae	Arabette des collines	Npp	Nt	–	
A. cypria	Brassicaceae		Npp	–	–	
A. pumila	Brassicaceae	Dwarf rockcress	Npp	Nt	–	
Aralia cachemirica	Araliaceae	Kashmir aralia	Npp	Nt	–	
Artemesia dracunculus	Asteraceae	Tarragon	✓	–	–	
Asparagus acutifolius	Asparagaceae	Wild asparagus	Npp	Nt	–	
A. myriocladus	Asparagaceae	Ming fern	Npp	Nt	–	
A. verticillatus	Asparagaceae	Climbing asparagus	✓	–	–	
Atriplex hortensis rubra	Amaranthaceae	Garden orache	✓	186/460/799/991/1113/–/–/–/–/–/–/–/–	–	
Averrhoa carambola	Oxalidaceae	Carambola, star fruit	✓	Nt	–	
Barbarea verna	Brassicaceae	Land cress	Npp	Nt	–	
Blechnum chilense	Blechnaceae	Chilean hard fern	Npp	Nt	–	
Bloomeria crocea	Asparagaceae	Goldenstar	Npp	Nt	–	
Boweia volubilis	Asparagaceae	Sea onion	Npp	–	–	
Boykinia jamesii (Telesonix jamesii)	Saxifragaceae	Alumroot brookfoam	Npp	–	–	
Brugmansia suaveolens	Solanaceae	White angel trumpet	Npp	Nt	–	
Caltha palustris	Ranunculaceae	Kingcup	✓	–	–	
Camassia quamash	Asparagaceae	Camas	Npp	Nt	–	
Campanula carpatica	Campanulaceae	Carpathain harebell	Npp	–	–	
C. makaschvilii	Campanulaceae	Makaschvili's bellflower	Npp	Nt	–	
Carex trifida	Cypeaceae	New Zealand sedge	✓	–	–	
Carum carvi	Apiaceae	Caraway	Npp	–	–	
Carya illinoinensis	Juglandaceae	Pecan	Npp	Nt	–	
Castilleja miniata	Orobanchaceae	Giant red Indian paintbrush	Npp	–	–	

(Continues)

TABLE 3 (Continued)

Species	Family	Common name	Ecdybase[aa]	Exeter survey[bb]	20E μg/g by HPLC-MS/MS	Other ecdysteroids identified
Centaurium scilloides	Gentianaceae	Perennial centaury	Npp	Nt	—	
Cephalotaxus fortunei	Cephalotaxaceae	Chinese plum yew	Npp	Nt	—	
Chaerophyllum bulbosum	Apiaceae	Parsnip chervil	Npp	—	—	
Chenopodium giganteum	Amaranthaceae	Tree spinach	✓	1697	559	PolB, 2026E, MakA, 24(28)-dehydroMakA
C. quinoa	Amaranthaceae	Quinoa	✓	456/612/769/955/1292	67.5	PolB, 2026E, MakA/24-*epi*-MakA
Chionochloa rubra	Poaceae	Red tussock grass	Npp	Nt	—	
Colchicum neapolitanum	Colchicaceae	Autumn crocus	Npp	Nt	—	
Commelina coelestis	Asparagaceae	Blue spider wort	✓	—	—	
C. dianthifolia	Asparagaceae	Birdbill dayflower	Npp	—	—	
C. virginica	Asparagaceae	Virginia dayflower	Npp	Nt	—	
Cucubalus baccifer	Caryophyllaceae	Berry-bearing catchfly	✓	—	1340	PolB, IntA, tax
Cypripedium parviflorum pubescens	Orchidaceae	Yellow lady's slipper	Npp	Nt	—	
Dactylorhiza fuchsii	Orchidaceae	Common spotted orchid	Npp	—	—	
Danae racemosa	Asparagaceae	Alexandrian laurel	Npp	Nt	—	
Dianthus monspessulanus	Caryophyllaceae	Fringed pink	Npp	Nt	—	
Digitalis davisiana	Plantaginaceae	Davis' foxglove	Npp	Nt	—	
Diospyros lotus	Ebenaceae	Date plum	Npp	—	—	
Disporum smithii	Colchicaceae	Smith's fairy bell	Npp	Nt	—	
Echinops tienschanicus	Asteraceae		Npp	—	—	
Echium pininana	Boraginaceae	Giant viper's bugloss	Npp	—	—	
Edraianthus pumilio	Campanulaceae	Silvery dwarf harebell	Npp	—	—	
Epilobium angustifolium	Onagraceae	Rosebay willowherb	Npp	Nt	—	
Erysimum perofskianum	Brassicaceae	Afghan bittercress	Npp	—	—	
Euphorbia characias wulfenii	Euphorbiaceae	Mediterranean spurge	Npp	—	—	
E. cognata	Euphorbiaceae		Npp	Nt	—	
E. myrsinites	Euphorbiaceae	Myrtle spurge	Npp	—	—	
Gentiana asclepiadea	Gentianaceae	Willow gentian	Npp	—	—	
G. tibetica	Gentianaceae		Npp	—	—	
Geranium renardii	Geraniaceae	Renard geranium	Npp	Nt	—	
G. versicolor	Geraniaceae	Pencilled crane's-bill	Npp	Nt	—	
Globularia bisnagarica	Plantaginaceae	Common ball flower	Npp	Nt	—	
G. repens	Plantaginaceae	Globulaire rampants	Npp	Nt	—	
G. trichosantha	Plantaginaceae	Blue globe daisy	Npp	Nt	—	
G. valentina	Plantaginaceae	Globulaire de valence	Npp	Nt	—	
Haplopappus rehderi	Asteraceae	Rehders Scheinsonnenhut	Npp	Nt	—	
Helleborus foetidus	Ranunculaceae	Stinking hellebore	✓	0.6	—	
Hemerocallis lilioasphodelus (H. flava)	Asphodelaceae	Yellow daylily	Npp	Nt	—	
H. middendorfii	Asphodelaceae	Amur daylily	Npp	Nt	—	
Heuchera americana	Saxifragaceae	American alumroot	Npp	—	—	
Holboellia coriacea	Lardizabalaceae	Sausage vine	Npp	Nt	—	
Hylocereus undatus	Cactaceae	White-fleshed pitahaya	Npp	Nt	—	

(Continues)

TABLE 3 (Continued)

Species	Family	Common name	Ecdybase[aa]	Exeter survey[bb]	20E μg/g by HPLC-MS/MS	Other ecdysteroids identified
Hypericum perforatum	Hypericaceae	St. John's wort	✓	0.9	–	
Ipheion uniflorum	Amaryllidaceae	Spring starflower	✓	Nt	47.1	PolB, 2d20E
Ipomoea lindheimeri	Convulvulaceae	Lindheimer's morning-glory	Npp	Nt	–	
Iris vicaria	Iridaceae		Npp	Nt	–	
I. warleyensis	Iridaceae		Npp	Nt	–	
Jasione laevis (J. perennis)	Campanulaceae	Sheep's bit scabious	Npp	–	–	
Kitaibelia vitifolia	Malvaceae	Chalice flower	Npp	–	–	
Kniphofia typhoides	Asphodelaceae	Brown poker	Npp	Nt	–	
Lachenalia reflexa	Asparagaceae	Yellow soldier	Npp	Nt	–	
Lansium parasiticum	Meliaceae	Langsat	Npp	Nt	–	
Lathyrus sativus	Fabaceae	Grass pea	Npp	–	–	
L. tuberosus	Fabaceae	Earthnut pea	Npp	Nt	–	
Leibnitzia anandria (Gerbera anandria)	Asteraceae	Leibnitz lily	Npp	Nt	–	
Leopoldia caucasica	Asparagaceae	Grape hyacinth	Npp	Nt	–	
Libertia chilensis	Iridaceae	Satin flower	Npp	Nt	–	
Linaria purpurea	Plantaginaceae	Purple toadflax	Npp	–	–	
Litchi chinensis	Sapindaceae	Lychee	Npp	Nt	–	
Lupinus chamissonis	Fabaceae	Dune bush lupine	Npp	Nt	–	
Lycium barbarum	Solanaceae	Himalayan goji	✓	–	–	
Lysmachia clethroides	Primulaceae	Gooseneck loosestrife	Npp	Nt	–	
Magnolia champaca	Magnoliaceae	Champak	Npp	Nt	–	
Matricaria chamomile	Asteraceae	Chamomile	Npp	Nt	–	
Meconopsis superba	Papaveraceae		Npp	Nt	–	
Melothria scabra	Cucurbitaceae	Mouse melon	Npp	Nt	–	
Morinda citrifolia	Rubiaceae	Noni	Npp	Nt	–	
Muscari leucostomum	Asparagaceae	Grape hyacinth	Npp	Nt	–	
Myrciaria cauliflora	Myrtaceae	Brazilian grapetree	Npp	Nt	–	
Nectaroscordum siculum (Allium siculum)	Amaryllidaceae	Scicilian honey garlic	Npp	–	–	
Nicotiana langsdorffii	Solanaceae	Langdorff's tobacco	✓	–	–	
Oxytropis vicida (O. viscidula)	Fabaceae	Viscid locoweed	Npp	Nt	–	
Paeonia delavayi	Paeoniaceae	Delavay poppy	Npp	Nt	–	
Papaver nudicaule (P. croceum, P. amurense, P. macounii)	Papaveraceae	Iceland poppy	Npp	–	–	
P. sendtneri	Papaveraceae	Sendtner's alpine poppy	Npp	Nt	–	
Paradisea lusitanica	Asparagaceae		Npp	–	–	
Passiflora quadrangularis	Passifloraceae	Giant granadilla	Npp	–	–	
Penstemon alpinus (P. glaber var. alpinus)	Plantaginaceae	Alpine sawsepal	Npp	Nt	–	
P. lyallii	Plantaginaceae	Lyall's beardtongue	Npp	–	–	
Phoenix dactylifera	Arecaceae	Date palm	Npp	–	–	
Phyllanthus acidus	Phyllanthaceae	Malay gooseberry	Npp	Nt	–	

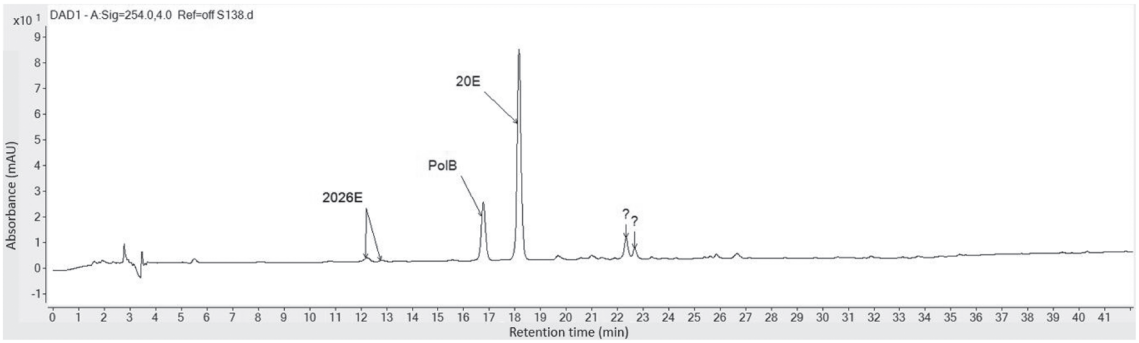
(Continues)

TABLE 3 (Continued)

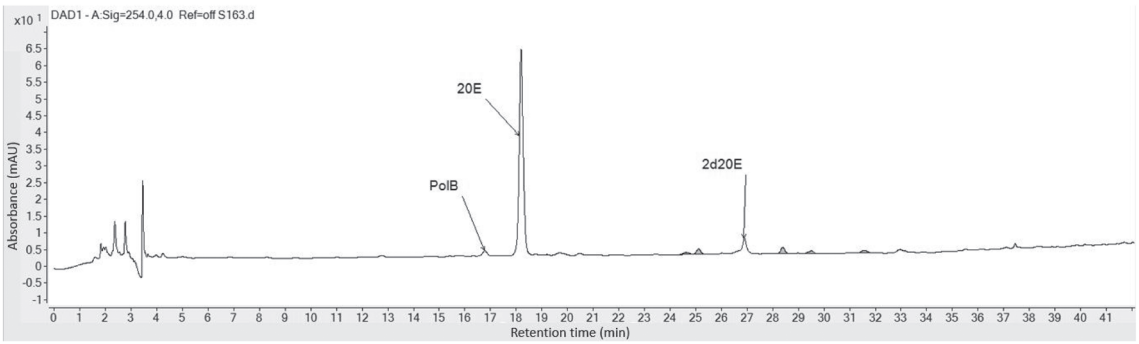
Species	Family	Common name	Ecdybase[aa]	Exeter survey[bb]	20E µg/g by HPLC-MS/MS	Other ecdysteroids identified
Physalis ixocarpa	Solanaceae	Tomatillo	✓	—	—	
Phytolacca americana	Phytolaccaceae	Pokeweed	✓	0.3	—	
Plantago coronopus	Plantaginaceae	Buck's-horn platain	Npp	—	—	
Primula heucherifolia	Primulaceae	Heuchera primrose	Npp	Nt	—	
P. pulverulenta	Primulaceae	Candelabra primrose	Npp	—	—	
P. vialii	Primulaceae	Orchid primrose	Npp	—	—	
Prostanthera cuneata	Lamiaceae	Alpine mint bush	Npp	—	—	
Protea coronata	Proteaceae	Green sugarbush	Npp	Nt	—	
Prunus armeniaca	Rosaceae	Apricot	Npp	—	—	
P. avium	Rosaceae	Wild cherry	Npp	—	—	
Pulsatilla halleri (Anemone halleri)	Ranunculaceae	Haller's anemone	✓	—	—	
Punica granatum	Lythraceae	Pomegranate	Npp	—	—	
Puya chilensis	Bromeliaceae		Npp	Nt	—	
P. coerulea	Bromeliaceae	Pink torch	Npp	Nt	—	
Rheum moorcroftiana	Polygonaceae	Moorcroft's rhubarb	Npp	Nt	—	
Ribes nigrum	Grossulariaceae	Blackcurrant	Npp	Nt	—	
Robinia pseudoacacia	Fabaceae	Black locust	Npp	—	—	
Roscoea scillifolia	Zingiberaceae		Npp	Nt	—	
Rubus ludwigii	Rosaceae	Silver bramble	Npp	Nt	—	
Ruscus aculeatus	Asparagaceae	Butcher's-broom	Npp	—	—	
Sedum verticillatum (Hylotelephium verticillatum)	Crassulaceae	Stonecrop	Npp	Nt	—	
Senecio polyodon	Asteraceae		Npp	7.1	—	
Sida cordifolia	Malvaceae	Bala	✓	—	3926	PolB, Abu
Sidalcea candida	Malvaceae	Prairie mallow	Npp	—	0.87	
Solanum melanocerasum	Solanaceae	Garden huckleberry	✓	1.1	—	
S. villosum	Solanaceae	Hairy nightshade	Npp	Nt	—	
Stachys macrantha	Lamiaceae	Big betony	Npp	Nt	—	
Stipa calamagrostis (Achnatherum calamagrostis)	Poaceae	Spear grass	Npp	Nt	—	
S. lessingiana	Poaceae	Feather grass	Npp	Nt	—	
Swertia kingii	Gentianaceae		Npp	Nt	—	
Talinum calycinum	Talinaceae	Flameflower	Npp	Nt	—	
Trichosanthes cucumerina	Cucurbitaceae	Snake gourd	Npp	Nt	—	
T. tricuspidata	Cucurbitaceae	Bitter snake gourd	Npp	Nt	—	
Tropaeolum majus	Tropaeolaceae	Garden nasturtium	Npp	—	—	
T. minus	Tropaeolaceae	Dwarf nasturtium	Npp	Nt	—	
T. polyphyllum	Tropaeolaceae	Wreath nasturtium	Npp	Nt	—	
Veronica spicata	Plantaginaceae	Spike speedwell	Npp	—	—	
Vigna radiata	Fabaceae	Mung bean	Npp	Nt	—	
Weigela florida	Caprifoliaceae	Old-fashioned weigela	Npp	Nt	—	
Wyethia angustifolia	Asteraceae	Narrowleaf mule-ears	Npp	Nt	—	

[a]See the corresponding footnote to Table 2.
[b]See the corresponding footnote to Table 2.

(A) *Chenopodium giganteum*



(B) *Ipheion uniflorum*



(C) *Lychnis coronaria*

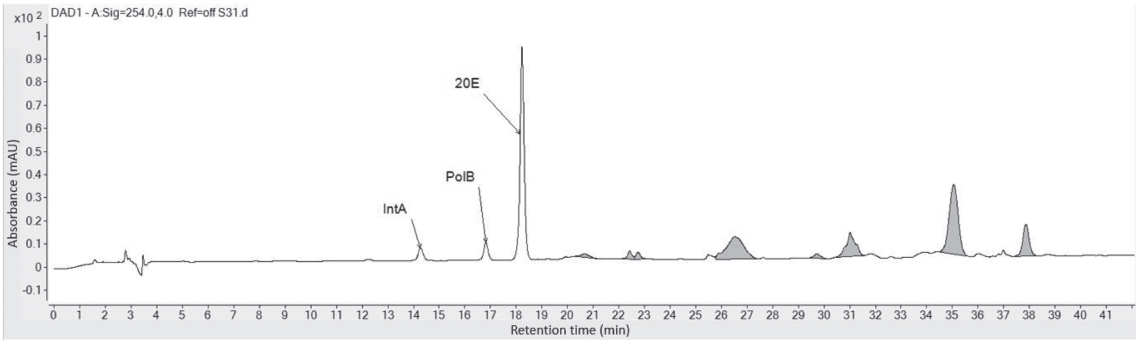


FIGURE 3 RP-HPLC chromatograms of extracts of the seeds of selected ecdysteroid-positive species with UV-monitoring at 254 nm: (A) *Chenopodium giganteum*; (B) *Ipheion uniflorum*; (C) *Lychnis coronaria*; (D) *Sida cordifolia*; (E) *Silene hookeri*; (F) *Silene multiflora*. The chromatographic conditions are the same as those described in the legend of Figure 2. Peaks resulting from the presence of ecdysteroids are labelled with the abbreviated name or '?', Indicating unknown ecdysteroid. Shaded peaks correspond to those for which no evidence was obtained that they are ecdysteroidal

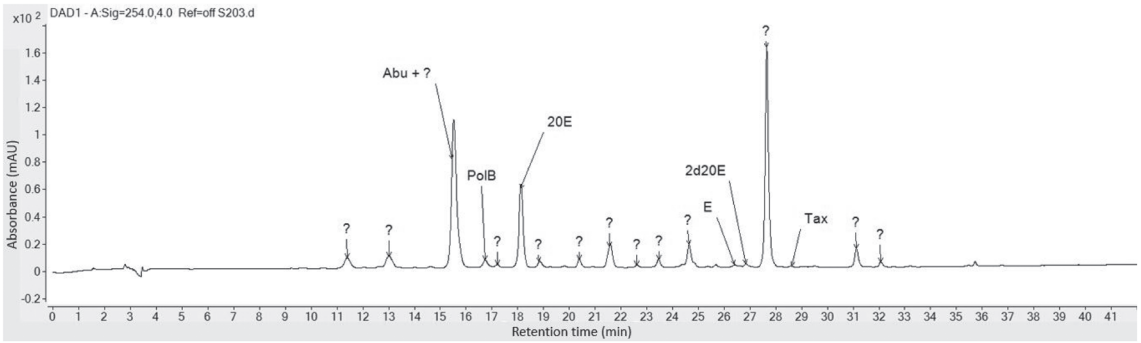
Leuzea carthamoides (Asteraceae)

Leuzea carthamoides is a recognised high producer of ecdysteroids, and this study identified 20E (7.2 mg/g), PolB (1.5 mg/g), AjuC (0.42 mg/g) and IntA conclusively in seeds. A further probable ecdysteroid (MW = 480) eluted at 24.5 min, and a peak for the transition 481.1 → 445.0 is observed at 20.6 min. Previous studies have focussed on the ecdysteroids present in the rhizome/root, as this is the portion of the plant which is used medicinally, and over 50 ecdysteroid analogues have been isolated and identified. However, 20E, E, lesterone, rapisterones B, C and D, rapisterone D 20-acetate, 24(28)-dehydroamarasterone B, 24(28)-dehydroMakA, PolB 22-benzoate and carmathosterones A and B have been previously isolated from seeds (reviewed in Kokoska and Janovska[29]).

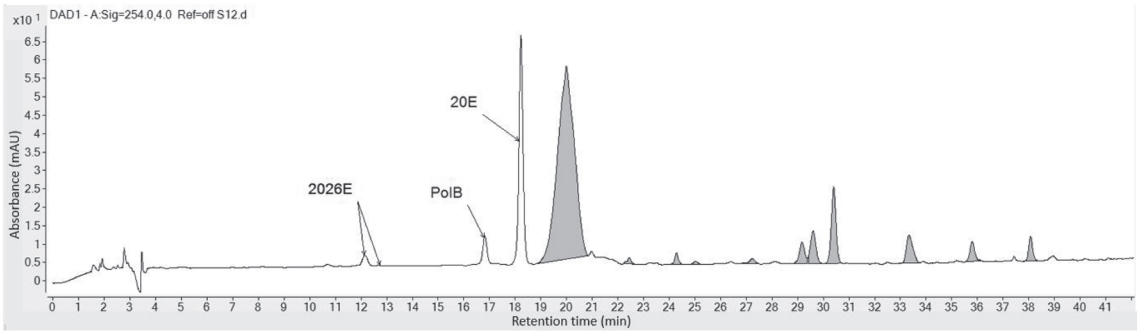
Lychnis arkwrightii (Caryophyllaceae)

Seeds contain moderate to high levels of ecdysteroids (ca. 8.4 mg/g), with 20E (4.2 mg/g) and PolB (3.8 mg/g) being present in almost equal amounts and with about one-tenth of the amount as E (0.4 mg/g).

(D) *Sida cordifololia*



(E) *Silene hookeri*



(F) *Silene multiflora*

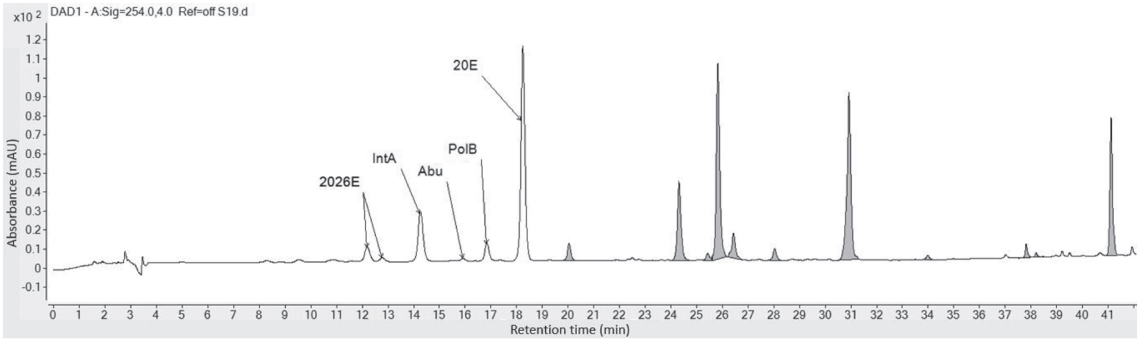


FIGURE 3 (Continued)

Assessment of ecdysteroid levels in seeds of this species by RIA with the DBL1 antiserum gave 1.17 mg E equivalent/g [21] and 20E has been identified from the plant.[30]

Lychnis chalcedonica (Caryophyllaceae)

Seeds contain moderate levels of ecdysteroids (4.1 mg/g) with PolB (2.0 mg/g) and 20E (1.7 mg/g) predominating. IntA (0.35 mg/g) and Tax (0.13 mg/g) were also identified. RIA (with the the DBL1 antiserum) assessed ecdysteroid levels at 1.1 mg E equivalent/g [21] and 20E, PolB, IntA, Ptero, 24(28)-dehydroMakA, E, viticosterone E (20E 25Ac) and stachysterone D have been isolated from plants of this species.[30]

Lychnis coronaria (Caryophyllaceae)

Here, 20E, PolB and IntA could be conclusively identified in the moderately high accumulating seeds of this species (total = ca. 4.4 mg/g), with 20E clearly being the predominant analogue present (3.75 mg/g), with smaller amounts of PolB (664 μg/g) and IntA. Ecdysteroid levels were assessed as 582 μg/g by RIA using the DBL1 antiserum.[21] The ecdysteroid profiles in seeds or plants of this species do not appear to have been investigated previously.

Lychnis flos-cuculi (Caryophyllaceae)

Seeds of this species accumulate moderate to high levels of ecdysteroids (ca. 8.2 mg/g), mainly as PolB (4.6 mg/g) and 20E

(3.6 mg/g). IntA could also be conclusively identified, but not quantified owing to the high LOQ for this analogue in the HPLC-MS/MS method (Table 1). Previous analysis of plant material of *Lychnis flos-cuculi* identified 20E (0.17% of the dry weight) and PolB as the major ecdysteroids present.[31] More extensive analysis of plants of this species[32] identified nine further (minor) ecdysteroids, but IntA was not amongst them, indicating that seeds possess a simpler, and different, profile.

Lychnis flos-jovi (Caryophyllaceae)

Moderate levels of 20E (1.3 mg/g) together with lower levels of PolB (0.38 mg/g) and 2026E (0.05 mg/g) could be conclusively identified, giving a total of 1.68 mg ecdysteroids/g. The ecdysteroid levels in two batches of seeds were found previously to be 647 and 712 μg E equivalent/g by RIA with the DBL1 antiserum[21] and 20E has been isolated from the plant.[30]

Ourisia macrophylla (Scrophulariaceae)

Seeds of this species contain moderate levels of ecdysteroids (ca. 0.98 mg/g), of which 93% is 20E. The rest is composed of PolB (301 μg/g), 2026E (324 μg/g; first- and second-eluting isomers in the ratio 6:4) and Tax (50 μg/g), the presence of all of which could be confirmed. The identification of the three more major ecdysteroids corresponds to previous results for seeds of this species.[33]

Paris quadrifolia (Melanthaceae)

Seeds of this species contain moderate levels of ecdysteroids (ca. 7.7 mg/g) which are almost equally divided between 20E and PolB. 20E and PolB have previously been identified from whole plants of this species.[34, 35]

Serratula coronata (Asteraceae)

Ecdysteroids are accumulated to a very high extent in seeds of *Serratula coronata* (ca. 30 mg/g; 3%), of which 63% is 20E, 25.5% is PolB and 11.2% is AjuC. The presence of IntA could also be confirmed, and some evidence is presented for the presence of Dacry and Tax, although the identities of these last two need confirmation. Growing plants of *Serratula coronata* and juice pressed from growing plants have been extensively studied for their ecdysteroid content, but the profile of ecdysteroids in seeds of this plant does not appear to have been published previously. The most abundant ecdysteroids isolated from pressed juice from aerial portions of the plant[36] were 20E (1.5% of the dry weight of aerial portions), E (0.13%), PolB (0.07%), AjuC (0.06%), 20E 22-acetate (0.09%), Tax (0.01%) and 3-*epi*-20E (0.01%), but many other minor ecdysteroids have been isolated as well. The major differences to the profile in seeds seem to be the absence of E from seeds and the enhanced contributions of PolB and AjuC.

Silene delavayi (Caryophyllaceae)

Seeds of this species contain moderate levels of ecdysteroids (1.5 mg/g) with a simple profile, comprising 20E as the major component (95%) and Tax as the minor (5%). The ecdysteroid profile of this species has not been investigated previously, although it was known to be ecdysteroid-positive.[37]

Sida cordifolia (Malvaceae)

Seeds of *Sida cordifolia* possess a moderate level of ecdysteroids (ca. 6.1 mg/g) and a complex ecdysteroid profile, amongst which 20E (3.9 mg/g) and PolB (0.2 mg/g) could be definitively identified and Abu (1.9 mg/g) was quantified by MS/MS. Co-chromatography with authentic reference also identified the presence of 24-*epi*-abutasterone at a retention time of 11.2 min, which was supported by mass spectral data for the peak ($[M + H]^+$ = 497.3, with three sequential losses of H_2O). The peak at 15.7 min was hypothesised to be (5α-H)20-hydroxyecdysone, but this was not supported by co-chromatography with a verified reference standard. A previous study had found the seeds of *Sida cordifolia* from a different suppler to be ecdysteroid-negative,[38] but a study of the ecdysteroids in an extract of whole plants of the species had found relatively low levels of 20E (0.001% of the dry weight) and 25-acetoxy-20E 3-glucoside (0.003% of the dry weight).[39]

Silene fimbriata (Caryophyllaceae)

The total level of ecdysteroids in seeds of this species are relatively low (0.55 mg/g) with a simple two-component profile of 20E (391 μg/g) and PolB (145 μg/g). This species has not been investigated previously.

Silene hookeri (Caryophyllaceae)

This species has not been examined before. The seeds contain low to moderate levels of ecdysteroid (ca. 0.85 mg/g), being composed of 20E as the major component (630 μg/g) and smaller amounts of PolB (168 μg/g) and 2026E (48 μg/g). The first- and second-eluting isomers of 2026E are in the proportion of 19:1.

Silene italica (Caryophyllaceae)

Seeds of this species possess a more complex ecdysteroid profile, consisting of significant amounts of 20E (2844 μg/g), polB (811 μg/g) and IntA (469 μg/g), together with small amounts of 2d20E (138 μg/g) and 2026E (23 μg/g; first- and second-eluting isomers in the ratio of 68:32) (total ecdysteroids = ca. 4.3 mg/g). Analysis of aerial portions of the plants had previously given 20E (0.53 mg/g dry weight) with significantly smaller amounts of (5α-H)2dIntA, IntA, 22dIntA, (5α-H)20E and 9β,20-dihydroxyecdysone,[40] (5β-H)2dIntA[41] and 2d20E 22-glucoside.[42]

Silene keiskei (Caryophyllaceae)

Seeds contain moderate levels of ecdysteroid (ca. 4.8 mg/g), with a simple profile, but unusually the amount of PolB (2.7 mg/g) exceeds that of 20E (2.1 mg/g). Seeds of *Silene keiskei* had previously been shown to accumulate ecdysteroids (200 μg E equivalent/g by RIA[21]), but no ecdysteroid profile had been determined.

Silene laciniata angustifolia (Caryophyllaceae)

The seeds contain lowish levels of ecdysteroids (ca. 0.5 mg/g) with a three-component profile consisting of 20E (396 μg/g) and smaller amounts of PolB (85 μg/g) and 20206E (11 μg/g), with the two isomers being in the ratio of 1:1. Seeds of *Silene laciniata* had previously been shown to accumulate ecdysteroids (2000 μg E equivalent/g by RIA[21]), but no ecdysteroid profile had been determined.

Silene multiflora (Caryophyllaceae)

Ecdysteroid levels are moderate in seeds of this species (ca. 4.8 mg/g) with significant proportions of 20E (2.85 mg/g) and IntA (1.0 mg/g) and lower levels of PolB (518 μg/g), Abu (44 μg/g) and 2026E (392 μg/g, with the first- and second eluting isomers in the ratio of 4:1). There are conflicting previous reports as to whether *Silene multiflora* is ecdysteroid-positive or not (see Lafont *et al*.[3]).

Silene nutans (Caryophyllaceae)

The seeds of this species are relatively high accumulators of ecdysteroids. The total ecdysteroid level is 8.6 mg/g. The data here show the presence of significant amounts of 20E (4.96 mg/g) and PolB (2.61 mg/g), with lower amounts of IntA (844 μg/g) and 2026E (188 μg/g, with the first- and second eluting isomers in the ratio of ca. 8:2). Dry plants of this species had yielded 20E (0.27%), PolB, 26-hydroxyPolB, IntA and 2026E.[31]

Silene otites (Caryophyllaceae)

Seeds of this species contain high levels of 20E (ca. 9 mg/g) with much smaller amounts of 2d20E (239 μg/g) and IntA. From plants, 20E (0.98% of the dry weight), IntA, 2dE, 2d20E and 2dIntA have been identified as the most abundant ecdysteroids[31] along with an array of minor ecdysteroids,[43–46] which required considerable enrichment for detection and identification.

Silene saxifraga (Caryophyllaceae)

Seeds of this species contain moderate levels of ecdysteroids (ca. 1.4 mg/g). 20E (1054 μg/g), PolB (342 μg/g) and IntA could be identified conclusively, with indications that two other unidentified ecdysteroids could be present (eluting at 19.9 and 20.6 min). Seeds of this species were previously assessed for the presence of ecdysteroids and found to contain 250 μg E equivalent/g as determined by RIA with the DBL1 antiserum.[21] Furthermore, 20E has also been identified from this species.[47]

Silene schafta (Caryophyllaceae)

Seeds of this species contain moderate levels of ecdysteroids (2.65 mg/g) with 20E (1.3 mg/g) and PolB (1.1 mg/g) being almost equally prevalent. Lower amounts of Abu (41ug/g) and IntA (215ug/g) could also be conclusively identified, along with an ecdysteroid of MW = 512 eluting at 9.6 min, which co-chromatographed with 26-hydroxyPolB (MW = 512). Peaks corresponding to the transition 481.1 → 445.0 occurring at 19.9 and 20.6 min may indicate the presence of further ecdysteroids, but these co-elute with a broad impurity peak which prevented obtaining useful UV- and MS-spectra. RIA assessment with the DBL1 antiserum of ecdysteroid levels in seeds of this species revealed the presence of 586 μg E equivalent/g [21] and 20E and PolB have been identified from plants of this species.[48]

Silene viridiflora (Caryophyllaceae)

The total ecdysteroids in these seeds was ca. 3.3 mg/g, which was mainly accounted for by 20E (1.9 mg/g) and PolB (1.2 mg/g), with much smaller amounts of 2026E (128 μg/g; with the first- and second-eluting isomers in the ratio of 87:13) and IntA being also conclusively identified. The dried herb of *Silene viridiflora* had previously yielded 20E, PolB, 2d20E, IntA, silenosides A and D and 26-hydroxyPolB[49] along with other minor 26-hydroxylated ecdysteroids (including 2026E[50]) and ecdysteroid acetates[51] and acetonides.[52]

3.4 | Applications

The method described here is appropriate for the rapid screening of small samples of seeds and other plant parts for the detection, quantification and identification of several ecdysteroid analogues simultaneously. It can be used to identify high producers of 20E or other commercially-expensive or -unavailable ecdysteroid analogues, or for dereplication of plant extracts to identify those which have a high probability of containing new analogues in adequate amounts for isolation and full identification. The method allows comparison of ecdysteroid profiles in related species to assess the significance and usefulness of phytoecdysteroids as chemotaxonomical markers.

4 | SUMMARY

The validated method described here permits the sensitive, quantitative detection of multiple ecdysteroids in simply prepared samples. The micro-extraction and C_{18}-Sep Pak partial purifications are straightforward and rapid. One person can perform 50 micro-extractions on one day and comfortably carry out the corresponding Sep Pak purifications on a second day. Initial screening for samples by HPLC-MS/MS for the presence of 20E is rapid (4 samples/h). The gradient separation of the ecdysteroids by HPLC-DAD-MS takes longer because of the need for good resolution of the analogues, permitting, with re-equilibration time, the separation of 1 sample/h, but this is only applied to the relatively few ecdysteroid-positive extracts. As described here, the HPLC-MS/MS method was used to detect and quantify 17 common phytoecdysteroids, but other analogues (e.g. C_{17}-, C_{21}-, C_{24}-analogues or conjugates) could readily be added, depending on the specific purpose of the analyses. The 5 mL 100% methanol fraction deriving from a 25 mg plant sample containing as little as 50 μg/g 20E is (e.g. the *Ipheion uniflorum* seeds studied here) readily

provided enough material for the quantification and identification of all the significant ecdysteroids present. The method is suitable for screening plant samples for the identification of high accumulators of specific ecdysteroid analogues, dereplication to identify extracts containing novel analogues, characterisation of ecdysteroid profiles and chemotaxonomic studies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Supplementary Information; Table 1: Identification of ecdysteroids in seeds of ecdysteroid-positive plant species by HPLC/DAD/MS

Species	Rt (min)	Transition	λmax (nm)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	No. H_2O Lost	Identity	Amount (µg/g) from HPLC-MS/MS
Agapanthus praecox	8.9	496.9→461.1	250	-	-	-	Turk?	3.15
	15.8	497.0→371.0	-	-	-	-	Abu?	14.9 (LOQ low)
	18.2	481.1→371.2	248	481.2	503.3	4	20E	517
	20.6	481.1→445.0 (AjuC)	250(Sh)	481.1	-	2	TBI	
	24.8	481.1→445.0 (AjuC)	258	481.2	503.2	3	TBI	
	26.2	447.0→429.0	250 (Sh)	447.2	-	2	E	631
	38.8	449.0→413.0 (2dOE); 447.0→429.0 (E)	-	465.2	487.2	4	TBI	
	39.6	449.0→413.0 (2dE); 447.0→429.0 (E)	-	465.2?	-	?	TBI	
	41.5	449.0→413.0 (2dE)	250(Sh)	447.2 $[M+H-H_2O]^+$?	-	3	TBI	
Chenopodium giganteum	12.1/12.7 (8:2)	495.2→175.0	250 (sh)	497.1	519.1	3	2026E	20.8
	16.8	497.4→351.3	246	497.2	519.2	4	PolB	361
	18.2	481.1→371.2	248	481.2	503.1	4	20E	559
	22.3		248	495.1	517.2	3	TBI	
	22.8		248	493.2	515.2	3	TBI	
C. quinoa	12.1	495.2→175.0	-	-	-	-	2026E	1.57
	16.8	497.4→351.3	-	-	-	-	PolB	5.03
	18.3	481.1→371.2	248	481.2	503.2	4	20E	67.5
	22.5		-	495.2	517.2	3	MakA/24-*epi*-MakA	
Cucubalus bacciferi	14.3		248	461.2 $[M+H-2H_2O]^+$	519.2	4	IntA	nd (LOQ high)
	16.8	497.4→351.3	246	497.2	519.1	3	PolB	59.0
	18.2	481.1→371.2	248	481.2	503.1	4	20E	1340

	28.4	461.1→429.0	246	465.3	487.2	3	Tax	18.6
Ipheion uniflorum	16.8	497.4→351.3	-	497.1	519.0	4	PolB	2.36
	18.2	481.1→371.2	248	481.2	503.2	4	20E	47
	26.7	465.3→429.0	248	465.2	487.1	3	2d20E	2.98
Leuzea carthamoides	14.2		?	479.2 $[M+H-H_2O]^+$	519.1	3	IntA	nd (LOQ high)
	16.8	497.4→351.3	246	497.1	519.1	3	PolB	1495
	18.2	481.1→371.2	248	481.2	503.2	4	20E	7190
	20.6	481.1→445.0 (AjuC)	250(Sh)	-	-	-	No ID	
	24.2	481.1→445.0	244	481.2	-	2	AjuC	420
	24.5	481.1→445.0 (AjuC)	242	481.2	-	2	TBI	
Lychnis arkwrightii	16.8	497.4→351.3	246	497.2	519.0	5	PolB	3748
	18.2	481.1→371.2	248	481.1	503.2	4	20E	4166
	26.2	447.0→429.0	250	447.2 $[M+H-H_2O]^+$	487.2	3	E	470
L. chalcedonica	14.3	497.1→386.9	246	479.1 $[M+H-H_2O]^+$	519.2	4	IntA	345
	16.8	497.4→351.3	246	497.2	519.2	3	PolB	2008
	18.2	481.1→371.2	248	481.2	503.1	4	20E	1652
	28.4	461.1→429.0	248	465.2	487.2	3	Tax	134
L. coronaria	14.3	497.1→386.9	246	479.3 $[M+H-H_2O]^+$	519.2	4	IntA	nd (LOQ high)
	16.8	497.4→351.3	246	497.2	519.1	4	PolB	664
	18.2	481.1→371.2	248	481.2	503.2	4	20E	3750
L. flos-cuculi	14.2		246	479.2 $[M+H-H_2O]^+$	519.2	3	IntA	nd (LOQ high)
	16.8	497.4→351.3	246	497.2	519.2	4	PolB	4600
	18.2	481.1→371.2	248	481.2	503.2	4	20E	3547
L. flos-jovi	12.1/12.7 (94:6)	495.2→175.0	250	497.3	-	2	2026E	46.2
	16.8	497.4→351.3	246	497.2	519.1	4	PolB	381
	18.2	481.1→371.2	246	481.2	503.2	4	20E	1253
	19.9	481.1→445.0 (AjuC)	-	-	-	-	No ID	

Ourisia macrophylla	12.1/12.7 (6:4)	495.2→175.0	250 (sh)	497.2	519.2	4	2026E	324
	16.8	497.4→351.3	246	497.2	519.2	3	PolB	301
	18.2	481.1→371.2	248	481.2	503.2	4	20E	9143
	28.4	461.1→429.0	246	465.1	-	3	Tax	50.2
Paris quadrifolia	16.8	497.4→351.3	246	497.2	503.2	5	PolB	3755
	18.2	481.1→371.2	248	481.2	519.1	4	20E	3952
Serratula coronata	14.2		246	479.2 [M+H-H_2O]$^+$	519.2	4	IntA	nd (LOQ high)
	16.8	497.4→351.3	246	497.2	519.2	5	PolB	7484
	18.2	481.1→371.2	248	481.2	503.1	4	20E	18430
	24.2	481.1→445.0	246	481.2	503.2	4	AjuC	3303
	28.4	461.1→429.0	-	447.0 [M+H-H_2O]	-	2	Tax?	71.3
	32.4	462.9→299.0	246	-	-	-	Dacry?	87.1 (LOQ low)
Sida cordifolia	11.4		248	497.2	-	4	24-*epi*-Abu	trace
	13.3	497.1→371.0 (Abu)	248	497.2	-	4	TBI	
	15.7	481.1→445.0 (AjuC); 447.0→429.0 (E)	248	481.2	-	4	TBI	
	15.8	497.1→371.0	248				Abu?	1918
	16.8	497.4→351.3	246	497.2	519.2	3	PolB	197
	17.3		250	481.2	-	3	TBI	
	18.2	481.1→371.2	248	481.2	503.2	4	20E	3926
	18.3	481.1→445.0 (AjuC); 465.0→429.0 (2d20E); 447.0→429.0 (E)					TBI	
	18.9	497.1→371.0 (Abu)	248	497.2	519.2	4	TBI	
	20.6	465.0→429.0 (2d20E)	248	465.2	479.1	3	TBI	
	21.7	481.1→445.0 (AjuC)	248	481.2	-	3	TBI	
	23.6	447.0→429.0 (E)	250	447.2	-	2	TBI	
	24.8	481.1→445.0 (AjuC)	248	481.2	-	2	TBI	
	25.8	465.0→429.0 (2d20E)						

	26.2	447.0→429.0					E?	259
	26.5	447.0→429.0 (E)						
	26.7	465.3→429.0	248				2d20E?	110
	27.0	465.0→429.0 (2d20E)						
	27.7	465.0→429.0 (2d20E); 465.0→447.0 (PonA); 447.0→429.0 (E)	248	505.2	-	3	TBI	
	28.4	461.1→429.0					Tax?	39.4
	28.6	465.0→429.0 (2d20E)						
	31.1	465.0→429.0 (2d20E); 465.0→447.0 (PonA)	248	463.2?	-	2	TBI	
	32.2		248	463.2	-	2	TBI	
Silene delavayi	18.2	481.1→371.2	248	481.2	503.2	4	20E	1437
	28.4	461.1→429.0	249	465.2	487.1	3	Tax	76.2
S. fimbriata	16.8	497.4→351.3	248	497.2	519.2	4	PolB	145
	18.2	481.1→371.2	248	481.2	503.2	4	20E	391
S. hookeri	12.1/12.7 (19:1)	495.2→175.0	246	497.2	519.2	3	2026E	48.1
	16.8	497.4→351.3	246	497.1	519.2	4	PolB	168
	18.2	481.1→371.2	248	481.2	503.2	4	20E	630
S. italica	12.1/12.7 (68:32)	495.2→175.0	250	497.2	519.2	3	2026E	22.5
	14.3	497.1→386.9	246	479.2 $[M+H-H_2O]^+$	519.1	3	IntA	469
	16.8	497.4→351.3	246	497.2	519.2	4	PolB	811
	18.2	481.1→371.2	248	481.2	503.2	4	20E	2844
	26.7	465.3→429.0	248	465.2	487.2	3	2d20E	138
S. keiskei	16.8	497.4→351.3	246	497.2	519.2	4	PolB	2715
	18.2	481.1→371.2	248	481.2	503.3	4	20E	2107
S. laciniata angustifolia	12.1/12.7 (1:1)	495.2→175.0	-	497.0	519.4	3	2026E	10.6
	16.8	497.4→351.3	244	497.1	519.2	4	PolB	84.7
	18.2	481.1→371.2	248	481.2	503.2	4	20E	396

S. multiflora	12.1/12.7 (4:1)	495.2→175.0	248	497.2	519.2	4	2026E	392
	14.3	497.1→386.9	248	479.2[M+H-H_2O]$^+$	519.2	4	IntA	1012
	15.8	497.0→371.0	249	497.3	519.1	3	Abu	44.1
	16.8	497.4→351.3	248	497.2	519.2	4	PolB	518
	18.2	481.1→371.2	248	481.2	503.1	4	20E	2847
S. nutans	12.1/12.7 (78:22)	495.2→175.0	248	497.2	519.2	3	2026E	188
	14.3	497.1→386.9	248	479.1 [M+H-H_2O]$^+$	519.2	3	IntA	844
	16.8	497.4→351.3	246	497.2	519.2	3	PolB	2605
	18.2	481.1→371.2	248	481.2	481.2	4	20E	4963
S. otites	14.2		246	479.2 [M+H-H_2O]$^+$	519.1	3	IntA	nd (LOQ high)
	18.2	481.1→371.2	248	481.2	503.2	4	20E	9038
	26.7	465.3→429.0	-	465.1	487.3	3	2d20E	239
S. saxifraga	14.2		246	479.1 [M+H-H_2O]$^+$	519.1	3	IntA	nd (LOQ high)
	15.9		248	-	-	-		
	16.8	497.4→351.3	248	497.2	519.2	4	PolB	342
	18.2	481.1→371.2	248	481.2	503.2	4	20E	1054
	19.9	481.1→445.0 (AjuC)	270&335 (under peak)	-	-	-	No ID	
	20.6	481.1→445.0 (AjuC)	270&335 (under peak)	481.0	-	2	TBI	
S. schafta	9.6		248	513.2	535.2	4	26OHPolB	trace
	14.3	497.1→386.9	248	479.1 [M+H-H_2O]$^+$	519.2	4	IntA	215
	15.8	497.0→371.0	249	497.2	-	3	Abu	41.3
	16.8	497.4→351.3	246	497.2	519.2	4	PolB	1099
	18.2	481.1→371.2	247	481.2	503.2	4	20E	1299
	19.9	481.1→445.0 (AjuC)	270&335 (under peak)	-	-	-	No ID	
	20.6	481.1→445.0 (AjuC)	270&335 (under	-	-	-	No ID	

			peak)					
S. viridiflora	5.5		248	-	-	-	No ID	
	12.1/12.7 (87:13)	495.2→175.0	248	497.3	519.1	4	2026E	128
	14.3		248	479.1 [M+H-H_2O]$^+$	519.2	4	IntA	nd (LOQ high)
	16.8	497.4→351.3	246	497.2	519.1	3	PolB	1223
	18.2	481.1→371.2	247	481.2	503.2	4	20E	1925

TBI: ecdysteroid to be identified

Supplementary Information; Table 2: Data for additional reference ecdysteroids

Ecdysteroid	MW	Publ. λmax (nm)	Rt§	Found λmax (nm)	$[M+H]^+$ m/z	$[M+Na]^+$ m/z	No. H_2O lost
24-*epi*-abutasterone	496	242	12.22	248	497.2	519.2	3
dacrysterone	510	240	21.49	246	511.2	533.1	3
Makisterone A	494	243	22.54	248	495.3	517.2	4
24-*epi*-makisterone A	494	242	22.5	248	495.2	517.3	3
24,28-dehydromakisterone A	492	245	22.51	248	493.2	515.3	4
Muristerone A	496	236?	22.58	240	497.4	-	4
Polysterone	362	242	22.57	246	363.3	385.1	2
Rubrosterone	334	240?	14.05	244	335.2	-	2
(5α)Rubrosterone	334	240?	11.65	242	335.2	357.2	2
Stachysterone C	462	242	31.17	248	463.2	485.2	2
Viticosterone E (20E 25-acetate)	522	243	30.77	248	523.2	545.3	3
(5α)20-Hydroxyecdysone	480	242	16.07	246	-	503.3	4
(5α)2-deoxyecdysone	448	242	33.91	245	-	471.3	3
25-deoxyecdysone	448	241	40.26	248	449.3	471.2	2
5,20,26E (26-hydroxyPolB)	512	242	10.19	246	513.3	535.2	3
3-dehydroE	462	242	28.2/29.0&	248	-	485.3	3
3-dehydro20E	478	242	20.0/20.6&	248	-	501.1	4
Sidisterone	416	240	28.45	246	417.2	439.1	2

§Rt for 20E = 17.61min

&distorted peak shape with leading front, owing to 3-oxo/2-oxo tautomerisation and/or H_2O adduct formation (see [53]).

Reference 53. Girault J-P, Blais C, Beydon P, Rolando C, Lafont R. Synthesis and nulear magnetic resonance study of 3-dehydroecdysteroids. *Arch Insect Biochem Physiol* 1989; 10: 199-213.