Separation Methods for Phytoecdysteroids

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Ecdysteroids are insect moulting hormones that regulate metamorphosis. Coincidentally, these compounds also show therapeutic benefits in both humans and animals. Some, particularly 20-hydroxyecdysone, are found at surprisingly high levels in certain plants, which have subsequently been subjected to intensive research as the possibility of their use in health preparations is investigated. Research on plant ecdysteroids (phytoecdysteroids) includes the analysis of known and new substances (hitherto unknown ecdysteroids), and isolation of known and new compounds. In this article the use of high performance liquid chromatography, thin-layer chromatography and classic column chromatography of ecdysteroids will be discussed.

Introduction

The effectiveness of natural health preparations is based on the amount/ratio of their active ingredients. This can differ in plants depending on the species variant, the part of the plant used, the season or the micro-climate. Moreover, the method of harvest, the drying and extraction techniques used, and any further processing also influence the final composition of medicinal health preparations. Several plants yield a series of active compounds with similar chemical structures, and some compounds may be subjected to chemical degradation or structural transformation during their processing. Analytical methods support the reliable control of medicinal health preparations, and the control of choice has always been chromatography.

Analysis of 20-Hydroxyecdysone

Health-improvement preparations with 20-hydroxyecdysone (Figure 1) content have been widely described (1–5). Table 1 lists some commercially available products, together with their characteristics. The overground part (herba) of *Silene otites* (L.) Wib. (Figure 2) can be an adequate source of ecdysteroids, as its 20-hydroxyecdysone content is high and several other ecdysteroids are also present.

Research on phytoecdysteroids comprises four basic types of analytical procedure: analysis of known compounds, analysis of new compounds, isolation of known compounds and isolation of new compounds. Chromatography plays an essential role in all of these procedures (Table 2).

Table 3 lists several methods used for the determination of 20-hydroxyecdysone from either crude samples or purified preparations (6–12).

Our method of choice for 20-hydroxyecdysone determination was thin-layer chromatography (TLC) combined with ultraviolet (UV) densitometry (TLC–UV). The method used automated spot application, development by an adequate mobile phase, and UV absorbance scanning densitometry of the fluorescent-quenched spots of samples and standards. Quantitative evaluation was performed by reflectance measurement at 254 nm. The optimum mobile phase was found to contain benzene, chloroform and

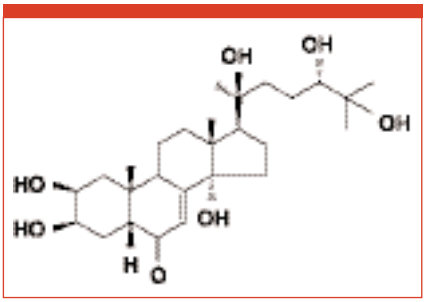

Figure 1: *The chemical structure of 20-hydroxyecdysone. It contains the whole steroid skeleton with a sterine side chain. Characteristic substituents are the six hydroxyl groups at the positions of C2, C3, C14, C20, C22 and C25 and a 7-en-6-one conjugation.*

Figure 2: *The blossoming Silene otites* (L.) Wib.

methanol. The separation was performed on 20 × 20 cm silica F_{254} plates (Merck, Darmstadt, Germany), which had been previously washed with methanol via a single development. A Linomat IV Sample Applicator (CAMAG, Muttenz, Switzerland) was used to load both the standards and the samples. The vapour phase of the developing chamber was pre-equilibrated for 30 min. An IBM PC-controlled CS-9301 PC Scanner (Shimadzu, Osaka, Japan) was used in the absorbance-reflectance mode at 254 nm. The slit was 1.2 × 1.2 mm, and zig-zag scanning mode was used. The peak area was recorded and used for

Table 1: *Health Improvement Preparations with 20-Hydroxyecdysone.*

Name	Indication	Source	Country	Company	20E Content	Dosage Form
Victory Beta Ecdysone	Fitness, anabolic body building	*Pfaffia paniculata* (Sumax™)	USA	General Nutrition Centers	10 mg/capsule	Capsule
Immunectare®	Adaptogenic for HIV patients	*Pfaffia paniculata*	USA	Nature's Plus Product	150 mg Suma®,	Bewerage
Changing Times™	Adaptogenic	*Pfaffia paniculata* + Vitamins + Antioxidants	USA	Nature's Plus Product		
Retibol	Anabolic	*Pfaffia paniculata*, *Leuzea carthamoides*	USA	Atletica Sport International		Tablet
Prime One® Prime Plus® Prime Perfect, Prime Quest® Gold®	Shields the body from stress to naturally heighten mental, physical and sexual performance, builds lean tissue	*Leuzea carthamoides* + protein	USA	Prime Firm		Liquid, tablet
Ecdysten®	Anabolic, general tonic, prevention from infectious diseases, neurosis, asthenics, astheno depressive states, intoxications	*Leuzea carthamoides*	Russia	Thermo Life	5 mg/tablet	Tablet (pills)
Robofit Drops	Adaptogenic, against stress improvement of psychical and physical conditions, increase appetite	*Leuzea carthamoides*, *Mentha piperita*, *Urtica dioica*, *Rubus caesius*	Hungary	Research Institute of Medicinal Plants	n.g.	Alcoholic extract (40%)
Maralan	Stimulant, removal of fatigue, improvement of physical condition, improvement of digestion, resistance against stress, stimulation of functions of CNS	*Leuzea carthamoides*	Slovak Republic	J. Kren Firm	0.08–0.22%	Green tea
Leveton	Approaches to sexual adaptation, increases physical work capacity of athletes, broad-spectrum adaptogenic	*Leuzea carthamoides* + tocoferol + vitamin C	Russia	Bipharm	n.g.	Flower powder
Sumax,	Anabolic	*Pfaffia paniculata*			5%	Capsules
Triboxin	Anabolic, builds muscle		USA	Atletica Sport International	n.g.	Capsules
VitiCom N VitiCom P	Adaptogenic	*Leuzea carthamoides*	Czech Republic		n.g.	n.g.

Table 2: *Aims, Basic Steps, Requirements, Preferred and Peculiar methods, and Novelties for Chromatography of Phytoecdysteroids.*

Aims	Steps	Requirements	Preferred Methods	Peculiar Methods, Novelty
Analysis of known ecdysteroids	Essential clean-up in one (or several) steps + analysis	Validation	Silica octadecyl-silica, cyano-silica	Clean-up on polyamide; solid-phase extraction on C18
Analysis of unknown ecdysteroids	Essential clean-up in one (or several) steps + analysis	Reliability	Silica, octadecyl-silica, cyano-silica	2-dimensional TLC, displacement TLC, TLC with in-situ UV spectrum HPLC–MS, TLC–MS
Isolation of known ecdysteroids	See Figure 1	Isolation of the active (native) compound with high recovery	Aluminium oxide, silica, DCCC (classic column chromatography) silica (HPLC)	Unified separation scheme
Isolation of new ecdysteroids	See Figure 1	Isolation without structural change	Combination of several methods	Unified separation scheme, DCCC

quantification. Calibration and determinations were performed in six parallel measurements. To ensure selectivity, the following five mobile phases were checked:

A: Dichloromethane–ethanol 8:2, v/v
B: Ethyl acetate–methanol–ammonia (25% solution), 85:10:5, v/v/v
C: Toluene–acetone–ethanol(96%)–ammonia solution, 100:140:32:9, v/v/v/v
D: Chloroform–methanol–benzene 25:5:3, v/v/v
E: Ethyl acetate–ethanol(96%)–water, 16:2:1, v/v/v

Mobile phases A and B did not provide adequate resolution, as certain components overlapped with the 20-hydroxyecdysone spot. However, mobile phases C, D and E gave good resolution. Mobile phase D was selected for the routine determinations of 20-hydroxyecdysone as it gave not only good resolution, but also compact spots for quantitative evaluation. Quantification was performed by the use of an external standard. In quantitative TLC determinations, the results were highly influenced by the quality of calibration (13). In order to find a suitable range, calibration was performed over a wide range, and the linear range was determined according to this plot. The calibration curve for 20-hydroxyecdysone was constructed by plotting the peak area (*y* axis) against the respective amount of standard (*x* axis) in the range 0.09–36.00 µg. A linear range was found from 0.45–9.00 µg, while the working range was from 0.90–6.75 µg. Correlation coefficients were over 0.999, indicating good linearity in the working range (Figure 3).

Linearity was also demonstrated by spotting the residuals against the quantity of 20-hydroxyecdysone applied (Figure 4). As the fitted statistical mode is correct, residuals are distributed at random around the regression 0 line (13). In the working range from 0.90–6.75 µg, the calibration can be assumed to be linear.

The level of plant 20-hydroxyecdysone was studied in five different phenophases: rosette stage, stem and shoot formation, bud development and blossoming, after blossoming and fruit ripening. The results of the replicate analyses are given in Table 4, together with the mean square error, relative standard deviation and variation coefficient.

The stability of ecdysteroid spots was checked during development. Two-dimensional TLC was performed using mobile phase D, in both dimensions in consecutive developments. As every spot was located at the major diagonal of the chromatogram, no degradation occurred through the development.

We also investigated the stability of spots on the developed plate. Six loads of the standard and 10 µL of the extract were used. Repeat scanning of the chromatogram after 24 h of development indicated no, or only minor, changes in the 20-hydroxyecdysone content of the *Silene otites* samples (Figure 5). The results show the stability of spot intensity when stored for one day after development.

The suitability of this system was confirmed by comparing a complete UV spectra of both sample and standard peak, directly on the TLC plate (not shown here). The accuracy of the proposed method was validated by determining recoveries from the sample solution, spiked with a known amount of 20-hydroxyecdysone. A comparison of the results of the samples and spiked samples yielded an accuracy of 94–99%. The spiking experiment was repeated six times.

The seasonal dependence of 20-hydroxyecdysone content in *Silene otites* varied greatly: 0.7% in May compared with 0.13% and 0.22% in June and July, respectively.

The TLC–UV method permits the quantitative determination of 20-hydroxyecdysone content, and could also be used for purity testing, and for stability control. In addition to the analysis of 20-hydroxyecdysone, determination of the UV spectrum of potential impurities was also possible. TLC permits the

Table 3: *Methods for the Determination of 20-Hydroxyecdysone.*

Determination method	MDA (minimum detectable amount)	Reference
Bioassay: in vivo test	5ng	6
in vitro test	10^{-8} M	6
RIA	10 pg	6
TLC	500 ng	7
TLC–MS–MS		8
HPLC	10 ng	9
HPLC–MS	1 ng	10
GC–ECD	50 pg	7
GC–FID	5 ng	7
GC–MS–MS	10 pg	7
Spectrophotometry	1 µg	11
MEKC	100 pg	12
Fluorescence detection	10 pg	6

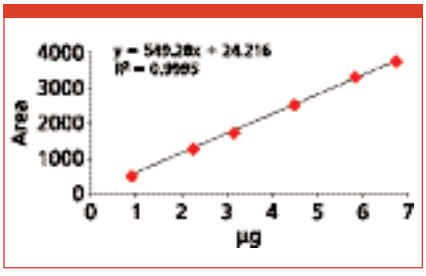

Figure 3: *Calibration plots for 20-hydroxyecdysone in the working range. (0.9–6.75 µg/spots).*

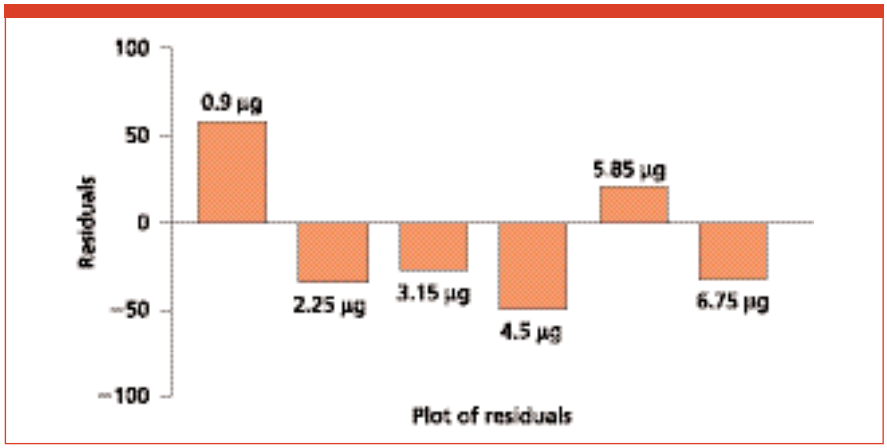

Figure 4: *Test for linearity of calibration graph by plotting the residuals in the working range.*

"Various possibilities exist for the analysis of plant-derived ecdysteroids."

Table 4: *20-Hydroxyecdysone Content of the Dried Herba of Silene otites (L.) Wib. in its Different Phenophases (n = 6).*

Date	20-Hydroxyecdysone content (%)	Mean square error	RSD	Variation coefficient (%)
May 31	0.7	0.2599	0.0002	0.98
June 27	0.13	0.0019	3.92×10^{-5}	0.30
July 26	0.22	0.0067	0.0004	0.54
August 12	0.59	0.0104	0.0009	2.20
September 26	0.27	0.0009	7.84×10^{-6}	0.46

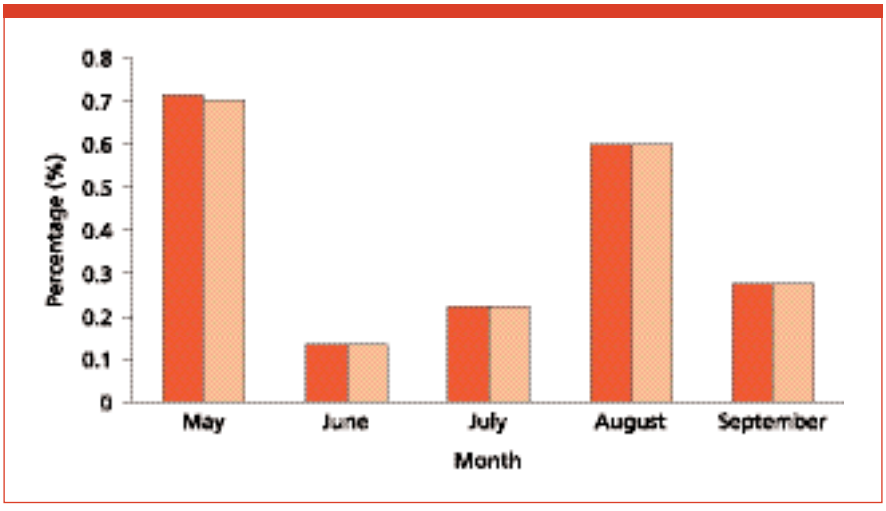

Figure 5: *Stability of 20-hydroxyecdysone in situ on the plate. ■ = 20E content after 24 h; ■ = 20E content after the separation.*

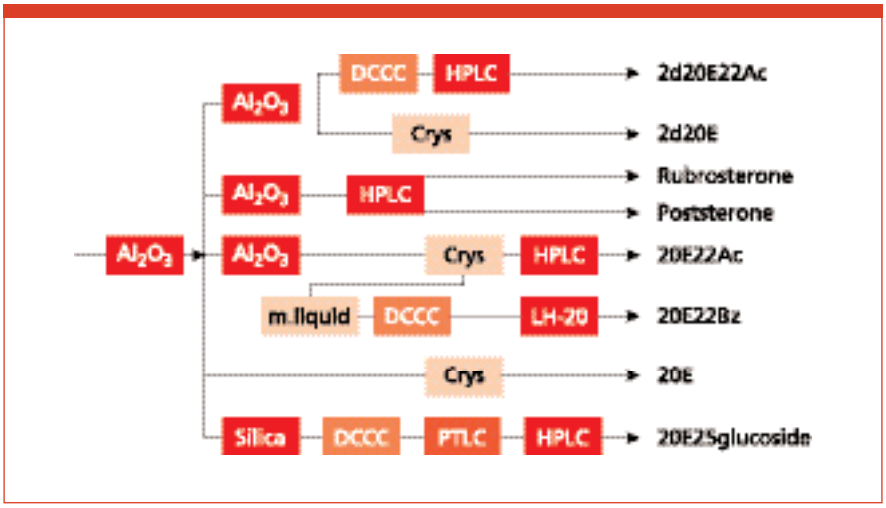

Figure 6: *Unified isolation scheme for phytoecdysteroids. Al_2O_3 = column chromatography on alumina; silica (column chromatography on silica); DCCC = droplet countercurrent chromatography; HPLC = high performance liquid chromatography; PTLC = preparative thin-layer chromatography on silica; LH-20 = column chromatography on Sephadex LH-20; Crys = crystallization; m.liquid = using the mother liquid for further isolation; 20E = 20-hydroxyecdysone; 2d = 2-deoxy; Ac = acetate; Bz = benzoate.*

simultaneous determination of several samples, and the method is economical when compared with the cost of high performance liquid chromatography (HPLC) and gas chromatography (GC) analyses.

Chromatographic Detection of Ecdysteroids

Various possibilities exist for the analysis of plant-derived ecdysteroids. Lafont (14) introduced HPLC for such investigations, using both normal-phase and reversed-phase HPLC. A basic conclusion from the normal-phase HPLC of ecdysteroids was that the presence of a trace amount of water in the mobile phase highly improved the separation power. In this way, peaks became symmetrical and resolution improved. At the same time, however, the majority of silica stationary phases that were used accumulated water from the mobile phase. As a consequence, the retention time values of ecdysteroids changed (showed a constant shift) from injection to injection. The exception was Zorbax, Sil, which was fairly resistant to water accumulation and gave stable retention of ecdysteroids. The reversed-phase HPLC analyses completed the results obtained on normal-phase HPLC columns. The retention time values of certain ecdysteroids are given in Table 5.

Another method of choice was TLC followed by triple detection (15), such as (a) dark spots at 254 nm on silica F_{254} plates; spraying the TLC plate with a vanillin–sulfuric acid reagent (without any heating) and observing the plates both (b) in daylight and (c) under fluorescence at 366 nm. The spots had specific colours (e.g., 20-hydroxyecdysone gave turquoise spots), and ecdysteroids also gave characteristic fluorescent spots at 366 nm (e.g., 20-hydroxyecdysone showed violet fluorescence, while the 22-deoxy-ecdysteroids showed orange fluorescence). The triple detection gives a high probability that the spot represents ecdysteroids. Identification is greatly facilitated with two-dimensional TLC (16), displacement TLC (D-TLC) (17) and forced flow TLC (FF-TLC) (17).

Two-dimensional TLC adequately separates the ecdysteroids from other compounds (e.g., from flavonoids), and from each other (16). Both normal-phase and reversed-phase TLC plates can be used; however, silica (normal-phase) plates provide better separation of 20-hydroxyecdysone and polypodine B than C18 (reversed-phase) plates. A specific sequence of developing systems was favourable for two-dimensional TLC of ecdysteroids on silica plates. The use of water-containing mobile phase in the first

dimension followed by a water-free mobile phase in the second dimension was suggested. This procedure resulted in the saturation of the silica stationary phase with water. The analysis of ecdysteroids can be simplified by D-TLC (17) and also by forced-flow of the mobile phase (17). A detailed description of D-TLC and FF-TLC can be found in the literature (17–19).

Isolation of Ecdysteroids

Dried plant parts were extracted at room temperature with a high excess of methanol (e.g., 10-fold). Evaporation, fractional precipitation and solvent–solvent distribution permitted the removal of the abundant amounts of both apolar and polar contamination. A unified scheme has been established for processing the plant extracts to isolate both the known and new ecdysteroids. With this procedure, numerous ecdysteroids have been isolated, and the same scheme is used today to find further new ecdysteroids (Figure 6). The first chromatographic step of isolation is low-pressure column chromatography on alumina, which yields 20-hydroxyecdysone in a single-step purification. After crystallization, the purity of 20-hydroxyecdysone is over 99%. To isolate a minor component (e.g., 2-deoxy-20-hydroxyecdysone, about 30-fold less then 20-hydroxyecdysone) requires the double employment of column chromatography, on alumina and/or silica. As certain components are present at a trace level (e.g., 20-hydroxyecdysone-22-benzoate, 20-hydroxyecdysone-25-glucoside, etc.) their isolation requires the correct combination of various chromatographic procedures (15), such as classic column chromatography on alumina and silica, droplet countercurrent chromatography, HPLC and, sometimes, either preparative TLC or size-exclusion chromatography on an LH-20 Sephadex column. Using this method, over 50 ecdysteroids have been isolated from various plant sources, 32 of which were previously unknown.

The key point to note is that consecutive stages in the purification process should be based on different ecdysteroid properties, such as lipophilicity, hydrophilicity, polar/apolar substituents, molecular size, H-bonding, etc.

Table 5: *HPLC of Ecdysteroids Isolated by Báthori et al. from Plants. The structures are related to 20-hydroxyecdysone. The stationary phases were Spherisorb®, 5 ODS-2 (System RP-1) and Zorbax® Sil (in Systems NP-2–4). The mobile phases were water-acetonitrile (77:23, v/v) also containing 0.1% trifluoroacetic acid in RP-1, dicloromethane-isopropanol-water (125:40:3, v/v/v) in NP-2, cyclohexane-isopropanol-water (100:40:3, v/v/v) in NP-2 and isooctane-isopropanol-water (50:15:1, v/v/v) in NP-3. Experimental conditions are given in reference 15.*

Change in the structure of 20-hydroxyecdysone	No. of OH-groups	Name of ecdysteroids	RP-1	NP-2	NP-3	NP-4
			Retention time (min)			
	6	20-hydroxyecdysone (20E)	5.2	16.9	21.5	51.7
Dehydroxylation and hydroxylation						
−OH at C2 and C20 +OH at C21	5	2d21E	n.d.	n.d.	14.8	n.d.
−OH at C2 +OH at C1	6	2-Deoxy-integristerone A	n.d.	13.5	16.0	n.d.
−OH at C22 +OH at C1	6	22-Deoxy-integristerone A	n.d.	13.8	n.d.	n.d.
Hydroxylation						
+OH at C23	7	Gerardiasterone	n.d.	22.8	n.d.	n.d.
−OH at C5 +OH at C26	8	26-OH-Polypodine B	4.7	30.9	n.d.	n.d.
Derivatization	Substituent					
Oxydation at C22	Oxo-group	22-Oxo-20E	12.4	n.d.	15.2	38.8
Esterification Acetylation						
at C3-OH	Acetyl-	20E 3-Acetate	10.4	8.7	14.91	29.4
at C2 and C22-OH	Acetyl-	20E 2, 22-Diacetate	n.d.	n.d.	n.d.	22.9
at C3 and C22-OH	Acetyl-	20E 3, 22-Diacetate	n.d.	n.d.	n.d.	24.1
Esterification and glycosilation						
at C22 and at C25	Benzoyl- and Glucose-	20E 22-Benzoate-25-Glucoside	4.2	24.6	n.d.	n.d.
Isomerization						
Trans A/B ring junction		5α-20E	4.9	n.d.	n.d.	n.d.
Epimerization at C22		22-Epi-20E	4.7	25.4	25.8	n.d.

*n.d. = no data observed, d = deoxy.

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