Phytoecdysteroid Levels and Distribution during Development in *Limnanthes alba* Hartw. ex Benth. (Limnanthaceae)

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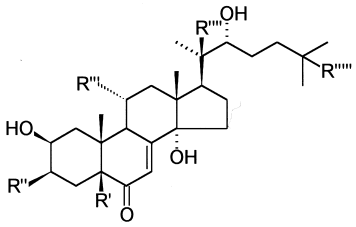
Phytoecdysteroid (PE) production and accumulation in *Limnanthes alba* Hartw. ex Benth. is associated with flowering. PE content per plant remains fairly constant during the primary growth phase of the plant and only begins to increase significantly above amounts found in the seed once the development of the flower stalk has begun. Both content and concentration increase concomitantly from this point. Distributions in individual plants also associated the highest levels of PE accumulation with the reproductive tissues. This substantiates the association of PE with tissues of greatest fitness value and therefore the hypothesis that they contribute to defence. Analysis of extracts of *L. alba* tissues by reversed-phase HPLC coupled with ecdysteroid-specific RIA was used to monitor ecdysteroid profiles. RIA-positive peaks co-chromatographing with 20-hydroxyecdysone, ecdysone and ponasterone A were detected and several tissues also contain PE conjugates. Seedmeal of *L. alba* appears to be a convenient and promising source for the commercial isolation of the potent PE ponasterone A.

Introduction

Shortly following the structural elucidation of the insect moulting hormone ecdysone (E; Fig. 1: structure **1**) (Huber and Hoppe, 1965), analogues (phytoecdysteroids; PEs) were isolated from plants in much higher amounts than those present in arthropods (reviewed in Dinan, 2001). Approximately 250 PE structural analogues have been identified to date (Lafont and Wilson, 1996). The favoured hypothesis for the function of these PEs is defence against unadapted insect herbivores (Lafont *et al.*, 1991). A range of evidence now exists in support of this hypothesis (summarised in Dinan, 1998; 2001). For example, the ingestion of 20-hydroxyecdysone (20E; **2**) with the diet results in the disruption of moulting cycles in several insect species leading to reduced body weights or larval fatalities (Blackford and Dinan, 1997a). However, some polyphagous insect species are remarkably tolerant to dietary PEs. In these tolerant species, pathways for the deactivation and rapid excretion of exogenous dietary ecdysteroids are present (Blackford *et al.*, 1996; Blackford and Di-



	R'	R''	R'''	R''''	R'''''
1: ecdysone	H	OH	H	H	OH
2: 20-hydroxyecdysone	H	OH	H	OH	OH
3: ponasterone A	H	OH	H	OH	H
4: muristerone A	OH	OH	OH	OH	H
5: limnantheoside A	H	β-D-xylose	H	OH	OH
6: limnantheoside B	H	β-D-xylose	H	OH	H

Fig. 1. Ecdysteroid structures.

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nan, 1997b). The distribution of PEs observed in a range of annual and perennial plants suggests that the highest concentrations are associated with those tissues which contribute the most to plant fitness such as young leaves and shoots, and the reproductive tissues, particularly of annuals (Ripa *et al.*, 1990; Grebenok and Adler, 1991; Dinan, 1992a,b). This can also be viewed as supporting a defensive role for PEs.

Plant preparations containing high levels of PE have been used in Central/Eastern Europe and Central Asia for many years as therapeutic treatments for neurosis, depression and hypotension among other things (Sláma and Lafont, 1995) and appear to be non-toxic to vertebrates (Sláma *et al.*, 1996). Currently, the PEs ponasterone A (PoA; **3**) and muristerone A (**4**) are of particular interest for future pharmaceutical/agronomic applications, since they can be successfully used as ligands to induce the expression of target genes in transgenic plant and animal systems regulated by ecdysteroid-activated receptors (Fussenegger, 2001); thus, ecdysteroid systems are of interest as a gene switching mechanism in gene therapy. However, the very limited world supply of the rare phytoecdysteroid muristerone A is already causing difficulties and the identification of ready sources of this and other potent ecdysteroids is highly desirable.

Our survey of ca. 5000 terrestrial plant species for ecdysteroid agonist and antagonist activities (Dinan, 1995a; Dinan *et al.*, 1999) revealed the presence of important amounts of phytoecdysteroids in seeds of all the studied species of the Limnanthaceae, a small family with undefined relationships to other plant families. A study of the ecdysteroids in *L. douglasii* (poached egg plant; Sarker *et al.*, 1997) identified the ecdysteroids present in seeds as 20E (**2**), PoA (**3**), limnantheoside A (20E 3β-D-xyloside; **5**) and limnantheoside B (PoA 3β-D-xyloside; **6**). *L. alba* (meadowfoam) is grown in the Pacific states of the U. S. A. as an oil seed crop. The oil, which is valued for its stability and high content of long-chain fatty acids (Jolliff *et al.*, 1981), is extracted from seedmeal with a non-polar solvent. The extracted seedmeal has largely been used as a low-value mulch. In view of the increasing interest in ecdysteroids for medical and pharmaceutical applications, the meal after extraction of the oil might be a readily available source of the polar ecdysteroids, including the biologically potent ponasterone A.

Wide variations in PE levels were observed among seeds of *L. douglasii* and in seeds of *L. alba* seeds from different sources (38.4–572.2 μg E eq./g seed with the DBL-1 antiserum; Sarker *et al.*, 1997). If this variation is genetically determined, it would be possible to select lines of high and low PE-containing plants. These would allow direct comparison of levels of herbivory and feeding behaviour in insects and, potentially, to provide agronomically important lines of *L. alba* with greater resistance to insect predators.

Thus, the aims of this investigation were as follows: 1) to track the changing levels over the life-cycle of the crop species *L. alba*, 2) to determine the distribution of PEs within plants during development, 3) to investigate the levels of variation observed among individual seeds and plants from a single population of *L. alba*, so as to indicate whether *L. alba* was a potential species for the selection of high and low level PE lines and 4) to examine the PE profile of *L. alba* in order to establish whether it contains PoA.

Materials and Methods

Plant material

Seeds of *L. alba* (cv. Floral) were supplied by OMG/Natural Plant Products LLC, Oregon, USA. Plants were germinated under controlled conditions of <14 °C and in darkness to prevent secondary dormancy. Following emergence, they were grown on in trays of John Innes No. 2 compost in an unheated greenhouse.

Plant extractions

Whole plants were collected at various stages of development (measured in days post-emergence) and their roots carefully washed clean of soil. Five whole plants were collected at each time point to observe content, concentration and variability in whole plants during development. For the observations of distributions within individuals, 3 plants were collected at 17, 24 and 35 days post-emergence, but only single individuals at 55 and 67 days, owing to the increased number of plant parts. Plants were dissected rapidly into root ball, adventitious roots, 1 cm stem sections, entire pinnate

leaves and, where appropriate, 1 cm flower stem sections, growing tips, flower buds, open flowers, flowers containing green unripened seed, flower stem leaves, flower stalks and flowering side-shoots. Stem sections, leaves and side-shoots were numbered in sequence from the base upwards. Three individual leaves were also dissected into individual leaflets, numbered bottom left to top right, and 1 cm petiole sections, numbered from base to tip. Having recorded the fresh weights, plant samples were freeze-dried for 3–4 days (until constant weight was achieved). The dry weights were then recorded.

Plant parts, together with 7-, 14-, 21- and 28-day whole plants, were extracted using 3 × 1 ml methanol at 55 °C for 3 h (Dinan, 1992a). Whole plants aged 35, 42, 48, 57 and 67 days were extracted in 3 × 10 ml methanol as above, while the dissected leaf parts were extracted in 3 × 0.5 ml methanol as above. After pooling the three extracts, water was added to produce a 70% aqueous methanol solution, which was then partitioned against hexane to remove apolar lipids and plant pigments. The aqueous methanol phase was used for analysis of ecdysteroids.

Seed extractions

A sample of 100 seeds was separated into endosperm and testa and finely ground in a pestle and mortar (the testa sample was contaminated with no more than 2% endosperm). Samples (3 × 25 mg) of each tissue were then extracted as for the plant parts above.

Individual seeds were weighed and cut into fragments inside their extraction tubes to avoid loss of oil or other material. Again the samples were extracted using 3 × 1 ml methanol at 55 °C. However, each of the three extractions was subject to 90 min sonication, followed by 60 min heating in a water bath and a further 90 min sonication to ensure efficient extraction from the relatively solid endosperm. The pooled extracts were then processed as above.

Phytoecdysteroid quantification

Duplicate aliquots of the extracts underwent radioimmunoassay (RIA) using DBL-1 antiserum, as described in Dinan (1992a), producing PE levels in ecdysone equivalents (E eq.; the cross-reactivities of E:20E:PoA with DBL-1 being 1:1.3:6.9, respectively [Dinan, 1995a]).

Ecdysteroid profiles

RP-HPLC was performed on root, stem, leaf, flower bud, open flower, individual seed, endosperm and testa extracts using a Waters Spherisorb ODS2 column (25 cm × 4.6 mm internal diameter) eluted at 1 ml/min with a linear gradient from 30% methanol water to 100% methanol over 30 min followed by 10 min at 100% methanol. Fractions of 1-min duration were collected. Suitable aliquots of the fractions were then subjected to RIA (Dinan, 1992a).

Conjugate hydrolyses were performed on HPLC fractions using a *Helix pomatia* gut hydrolase mixture (Dinan *et al*., 2001). Aliquots (200 µl) of the HPLC fractions were prepared in duplicate and dried. One set was used as a control, to which 200 µl sodium acetate buffer (0.1 M, pH 5.4) was added, while 200 µl *Helix* enzymes (10 mg/ml sodium acetate buffer) were added to the experimental set. Following incubation at 37 °C for 48 h, the reaction was terminated with 800 µl ethanol. Subsequent to chilling and centrifugation, suitable aliquots of the supernatant were subjected to RIA.

Results and Discussion

Whole plant levels and distribution within individual plants throughout development

Prior to germination, seeds contain on average 63.0 µg E eq./g fr.wt. (n = 60; 0.57 ± 0.10 µg/seed, average weight 8.9 mg/seed). The seeds, by weight, are divided into 77% endosperm and 23% testa. PE within the seed is allocated in the proportions 91% to the endosperm and 9% to the testa. Following germination, the concentration of whole plants initially increases to ca. 9 µg E eq./g and then decreases to ca. 1.5 µg E eq./g at day 42 (Fig. 2). In contrast to the continued decrease in concentration up to 28–35 days, the amount in the plant changes little from around 0.2 µg to 1 µg E eq. during this same period. It appears that the seed supplies much of the PE allocation to the plant during this early stage of growth resulting in declining concentrations, owing to rapid tissue expansion and low PE production, although there is evidence of PE synthesis between days 7–14.

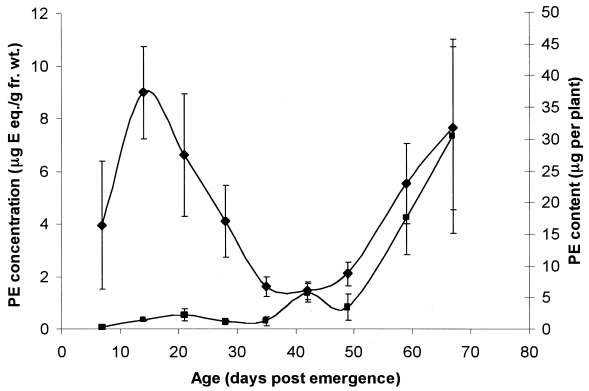

Fig. 2. Changes in phytoecdysteroid (PE) content (♦) and concentration (■) in whole plants over time. Error bars represent the SE of 5 individual plants at each time point. Quantification by RIA with DBL-1 antiserum.

After 35 days however, when the flower stem and buds have begun to develop, PE content starts to increase concurrently with a rapid increase in plant biomass. Concentration, on the other hand, does not begin to increase until the biomass stabilises, owing to the flower stem reaching full extension by 42 days, enabling PE production to overtake growth. PE content and concentration then continue to increase right up to seed set.

In accord with the whole plant observations, the distribution of PEs in single plants during development also shows the majority of the PE being associated with flowering. Early on in development (17 days post-emergence) higher levels of PE are associated with the growing tip (30–60 µg E eq./g fr. wt.) and younger leaves, similar to the distribution seen in Fig. 3. The roots, stem and lower leaves have comparatively low concentrations (10 µg E eq./g). A gradient is observed in the stem and the leaves increasing with proximity to the apex which to some degree is sustained throughout development.

By 24 days, the overall concentration in the plant had fallen, owing to the rapid expansion of rosette leaves. Roots, stems and lower leaves in general possess lower concentrations than the younger leaves and growing tip, which consistently accumulates higher concentrations than the rest of the stem, but not always than the newest leaf. The cotyledons retain a comparatively high concentration compared with the true leaves right up to the point of senescence.

When plants reach the 35-day stage (Fig. 3), the point of lowest PE concentration, they have developed a 2–4 cm main flower stem with small flowering side-shoots at the base. While the concentration of the roots remains relatively low, the remaining cotyledon retained a comparatively high concentration. Again a consistent gradient increasing from base to tip is observed in the flowering stem (2–9 µg E eq./g). Flower buds at the end of this stem contain the highest ecdysteroid levels in both content and concentration. There is a general increase in leaf PE concentration ascending the plant. However, this situation is not so well defined in all individuals. Leaves at the

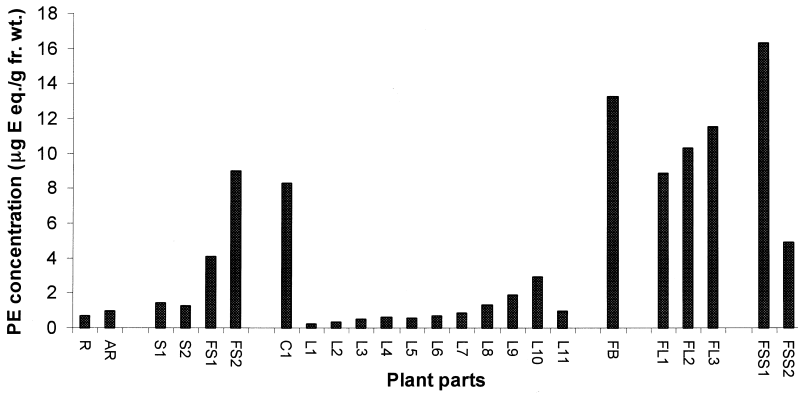

Fig. 3. Typical distribution of phytoecdysteroid (PE) in an individual plant of *L. alba* at 35 days post-emergence; R: roots, AR: adventitious roots, S: 1 cm stem sections, FS: 1 cm flower stem sections, C: cotyledon, L: true leaves numbered from base of plant upwards, FB: flower buds, FL: leaves at base of flower stalks, FSS: flowering side-shoot. Quantification by RIA with DBL-1 antiserum.

base of the flower stalks (designated FL) maintain a higher concentration than the rosette leaves. In general, the highest concentrations at this growth stage accumulate in tissues which are associated with flowering.

Well into the main flowering period by 55 days, the stem continues to maintain an upwardly increasing gradient at levels twice those of before (5–19 μg E eq./g). The concentration in the flower buds remains high at 15 μg E eq./g fr.wt., but falls to about half of this when the flowers open. Concentrations observed in the side-shoots relates to development, the highest concentration and content being recorded in the oldest most developed shoots.

Seed set occurs between 55 and 67 days and by this stage all the leaves (except FL) have senesced and abscised. Senescent leaves have low PE levels (ca. 1 μg E eq./g), indicating transport out of, or breakdown in, the leaves (also observed in *Leuzea carthamoides*; Zeleny *et. al.*, 1997). There is no longer any clear gradient in the flower stem and the adventitious roots contain the highest concentration, although (excluding side-shoots) the green seed contained the highest absolute amount. These un-ripened green seeds contained around the same concentration of PE as the open flowers, having a total content of 2.4 μg E eq. This is surprisingly low, since 4 of these flowers were included in the extract with a possible 5 seeds per flower. This allows only 0.6 μg per flower, the equivalent of the content of a single seed, but because the concentration is determined by the number of seeds, it is likely that few seeds had set in these flowers giving a lower value than might be expected.

RIA analysis of the PE distribution in pinnate leaves showed that, although a gradient occurred in the petiole increasing from base to tip, there was little difference between the PE concentrations in the individual leaflets (Fig. 4).

PE distributions relate to the predictions of optimal defence theory. Optimal defence theory suggests that plants invest defence most heavily in those parts of the plant which contribute the most to plant fitness (Van Dam *et al.*, 1996). Numerous examples exist of high accumulation of defensive secondary metabolites accumulating in young tissues and declining as the tissues age (Bennett and Wallsgrove, 1994). Annual plants, such as *L. alba*,

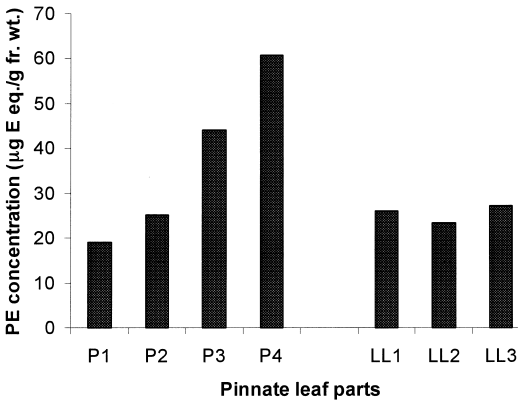

Fig. 4. Typical distribution of phytoecdysteroids in a single pinnate leaf; P: 1 cm petiole section, LL: leaflet. Quantification by RIA using DBL-1 antiserum.

must complete their life-cycle in less than a year and so depend upon setting seed for survival into the next generation. Therefore, the flowers and their associated structure are critically important to the fitness of the plant. It is more cost-effective for short-lived species to use defensive compounds which are more mobile with rapid turnover rates (Coley, 1988), i.e. qualitative compounds such as the triterpenoids (which include the phytoecdysteroids), rather than quantitative compounds (such as lignans and tannins) which perennials tend to accumulate in older tissues using qualitative defences to protect their new shoots in the short-term. The distribution of PEs in the woody perennial *Taxus cuspidata,* where they are associated with the new shoot tips and levels decline with age as tannins accumulate (Ripa *et al.*, 1990), is also in accord with defence theory. Similar within-plant distributions have also been observed for spinach (Dinan, 1995b; Grebenok & Adler, 1993), other annuals in the Chenopodiaceae (Dinan, 1992b & 1995b) and *Silene* species (Zibareva, 2000), where the higher concentrations were associated with the newest leaves, growing tips and reproductive tissues.

PE concentrations in *L. alba* (1–20 μg E eq./g fr.wt.) are relatively low when compared with species such as spinach (5–100 μg E eq./g fr.wt.), yet are higher than some other members of the Chenopodiaceae (Dinan , 1992a,b; Dinan, 1995b). The levels of dietary PE which induce deleterious effects in insects depend on the insect species, stage of development and the type of PE (Dinan, 1998).

However, 2–25 ppm 20E appear to be effective against non-adapted phytophagous insects, so the more elevated concentrations in young plants and during flowering can be expected to be effective. Further, the presence of PoA, which is 50–100-fold more effective than 20E (Dinan, 1989), will considerably enhance the defence against non-adapted predators. Few invertebrates pests have been associated with fields of *L. alba*; those that have been found include dipterans (*Scaptomyza* spp.) of commercial concern, the spotted cucumber beetle, nitidulid beetle, seed bug, and seed-feeding Carabidae beetle (Oelke *et al.,* 1990). This general lack of pest species suggests that reasonably effective defence mechanisms operate, whether they are PE-related, alternative defence compounds or habitat situation.

Seed variation

To examine the correlation between individual seed PE content and seed weight, individual seeds ($n = 60$) from the same population were extracted and analysed by RIA. The results revealed no correlation, the R^2 value being 0.051. The majority of the sample, 85%, contains between >0 and 1 μg E eq./seed, only a few outliers containing >1 μg, up to 3.5 μg E eq. (concentrations >50 μg E eq/g seed). Similar results were also obtained from seed produced by greenhouse-grown plants raised from the first batch of seeds, which ranged from >0–7 μg E eq, again the majority lying below 1 μg E eq. ($R^2 = 0.000$). This low level of variation in *L. alba* seeds may be a consequence of selection for a good seed oil variety. Wild populations, on the other hand, may show considerably greater variation, as a previous study indicated (Sarker *et al.*, 1997). There seems to be a positive correlation between oil yield and size of the seeds (Pierce and Jain, 1977; Johnson *et al*., 1980).

Ecdysteroid profiles

RP-HPLC coupled with RIA was used to identify the ecdysteroid profiles of *L. alba* root, stem, leaf, flower-bud, open flower, individual seed, seed endosperm and seed testa extracts. All extracts contained RIA-positive peaks corresponding to the retention times for the major ecdysteroids 20E, E and PoA in varying proportions (Table I), together with various smaller ecdysteroid-positive peaks. RIA with the DBL-1 antiserum under-represents the amount of PoA relative to the other two compounds. Once the cross-reactivities are taken into account, the leaf sample contains primarily PoA, with approximately equal amounts of 20E and E. The root and stem sample also contain primarily PoA, but less E than 20E, while the other tissues (flowers and seeds) contain five times as much 20E as PoA and even smaller amounts of E (Table I). Identification of ecdysteroids is based on co-chromatography of RIA-positive material with reference ecdysteroids and analogy to the compounds unequivocally identified from seeds of *L. douglasii* (Sarker *et al*., 1997), but should be confirmed by spectroscopic means for *L. alba*.

The hydrolysis of a range of plant extracts taken from the different tissues of various plants at dif-

Table I. Relative concentrations of the major phytoecdysteroid components of *L. alba* tissues determined by RP-HPLC/RIA (DBL-1 antiserum). Concentrations adjusted for cross-reactivities (20-hydroxyecdysone [20E], ecdysone [E] and ponasterone A [PoA], 1.3:1:6.9, respectively) with DBL-1 antiserum are shown in brackets.

Tissue sample	μg E eq./g fr. wt.						
	Fraction 17 (20E)	Adjusted for CR	Fraction 20 (E)	Fraction 24 (PoA)	Adjusted for CR	Total PE μg/g fr. wt.	% as PoA
Root[a]	2.2	(2.8)	0.5	0.5	(4.0)	7.3	55
Stem[a]	4.2	(5.4)	1.9	1.9	(9.5)	16.8	57
Leaf[a]	0.6	(0.8)	0.7	0.7	(1.7)	3.2	53
Flower buds[a]	9.6	(12.4)	1.6	1.6	(2.6)	16.6	17
Open flowers[a]	3.4	(4.5)	0.9	0.9	(3.2)	8.6	37
Whole seed[a]	404.5	(525.9)	10.2	10.2	(61.5)	597.6	10
Endosperm[b]	51.7	(67.2)	2.6	2.6	(10.7)	80.5	13
Testa[b]	7.5	(9.8)	0.3	0.3	(2.6)	12.7	20

a: extract of individual plant parts.
b: extract of pooled sample (from 100 seeds).

ferent growth stages was carried out using a *Helix pomatia* gut hydrolase mixture. Following comparison of the RIA results from the controls and hydrolysed samples, the RIA response of the seed extracts (whole and separate parts) was found to double on hydrolysis. In general, no consistency between samples from the same tissue type or growth stage was observed and conjugates appeared to be present in at least one sample from all tissue types including cotyledons close to senescence. Hydrolysis of the root, stem, leaf and open flower HPLC fractions (identified as containing ecdysteroid conjugates) was again carried out using the *Helix* hydrolases. Increased RIA response upon RIA analysis revealed the presence of conjugates in all four tissues. However, different profiles were observed in each tissue. For example, the stem sample (Fig. 5) contained conjugates in fraction 17 and 24, (20E- and PoA-containing fractions), but no conjugates were detected in fraction 20, the E-containing fraction. The leaf sample, on the other hand, contained no conjugates in fraction 17, but did in fractions 20 and 24, while the open flower sample revealed conjugates in all three of these fractions. The conjugate-positive fractions detected in the *L. alba* samples lie in the same area of RIA-positive material as the conjugates identified from *L. douglasii*, from which ecdysteroid 3β-xylosides were isolated (Sarker *et al.*, 1997).

Conclusions

The levels throughout development and the distributions show that higher PE concentrations and the initiation of the major burst of PE production are associated with flowering. This study established that the variation in PE levels among seeds and among plants from this population of *L. alba* is relatively low, hence this population is unlikely to be suitable for the selection of phytoecdysteroid-containing lines, but natural populations may be suitable for this purpose. Finally, all *L. alba* tissues contain material co-chromatographing with 20E, E and PoA. Seeds have a lower proportion of PoA, but because the overall PE levels in seeds are so much higher, seeds are the best source of this compound. Therefore, the seedmeal could potentially provide a commercial source of PoA for medical/pharmaceutical applications. The seeds of other oil seed crop species (cotton seed, groundnut, linseed, mustard, rape, soybean, sunflower) do not contain phytoecdysteroids (Dinan, unpubl. results).

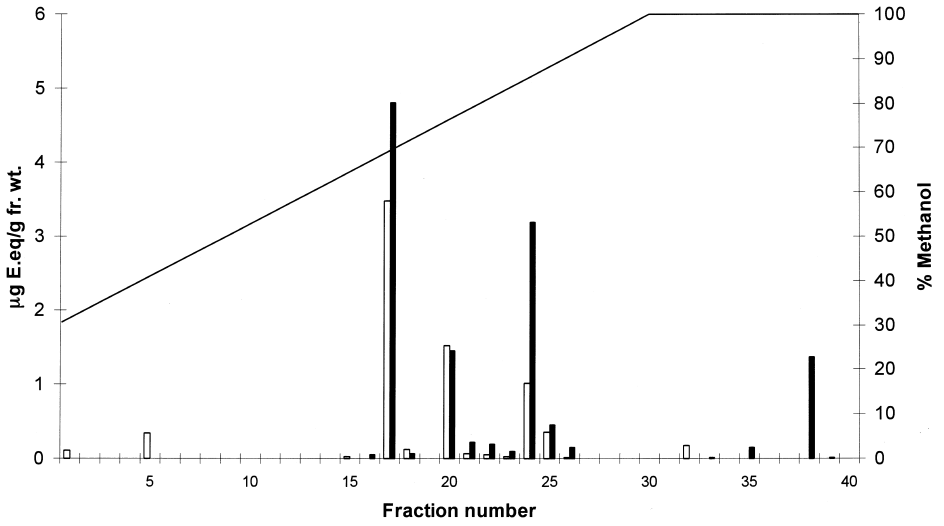

Fig. 5. RP(C_{18})-HPLC plus ecdysteroid conjugate hydrolysis of an *L. alba* stem sample. Ecdysteroids were analysed in 1-minute fractions collected from the column. Ecdysteroid levels in unhydrolysed (□) and hydrolysed (■)fractions are shown, as quantified by RIA with DBL-1 antiserum. The reference ecdysteroids 20-hydroxyecdysone, ecdysone and ponasterone A elute in fractions 17, 20 and 24, respectively. In this HPLC system, ecdysteroid 3β-D-xylosides co-elute with the corresponding free ecdysteroid (e.g. limnantheoside A co-chromatographs with 20E: Sarker *et al.*, 1997).

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