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Chromatography in Plant Hormone Analysis

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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SPECIAL VOLUMES

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JOURNAL OF CHROMATOGRAPHY A

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For Contents see p. VII.

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SPECIAL ISSUE

CHROMATOGRAPHY IN PLANT HORMONE ANALYSIS

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CONTENTS

CHROMATOGRAPHY IN PLANT HORMONE ANALYSIS

Journal of Chromatography A, 658 (1994) 1
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Preface

This special issue on "Chromatography in Plant Hormone Analysis" is devoted to a chemically heterogeneous group of natural products, having in common their hormonal activity in plants. Although the insect molting hormones have not been demonstrated to have hormonal effects in plants, I have chosen to include them in this issue for two reasons. Firstly, they are chemically and biosynthetically related to the brassinosteroids, which are known plant hormones, and secondly, I believe that compounds which have profound effects on the molecular biology of animal cells will probably sooner or later be found to produce analogous cellular responses in plants.

Be this as it may, it has been personally rewarding for me to participate vicariously, long after having retired from active research, in the impressive progress in plant hormone analysis. The chemical analysis of the minute quantities of hormones in plants is, of course, a prerequisite for understanding their mechanism of action and for a rational approach to the chemical regulation of such vital processes as plant reproduction. In fact, the painstaking and ingenious research of plant biochemists is already beginning to "bear fruit".

This collection of papers on various aspects of plant hormone research will give our readers some idea of the extent to which chromatography has contributed to plant hormone analysis. If it inspires further progress along these lines, the authors and editor will be amply rewarded for the labor that has gone into producing this special issue.

Orinda, CA (USA) Erich Heftmann

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Review

Brassinosteroids: distribution in plants, bioassays and microanalysis by gas chromatography–mass spectrometry

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ABSTRACT

During the past decade, studies on brassinosteroids (BRs) have greatly widened the knowledge of new steroidal plant hormones. This review summarizes studies on BRs from the viewpoints of distribution in plants, bioassays and a microanalytical method using gas chromatography–mass spectrometry with selected ion monitoring (GC–MS-SIM). A highly sensitive and specific bioassay employed to isolate brassinolide from rape pollen is bean-second internode assay. The rice-lamina inclination test and wheat leaf unrolling test are now widely and routinely used mainly in Japan as highly sensitive and specific bioassays during the purification steps of BRs from the plant sources. When a highly purified fraction containing a very small amount of BRs is obtained, the fraction is derivatized with methaneboronic acid to form a bismethaneboronate of BRs and then analysed by GC–MS-SIM. The rice-lamina inclination test and the GC–MS-SIM microanalytical method have contributed greatly to studies on the identification of many natural BRs and also to their screening and distribution in the plant kingdom. So far about 30 natural BRs have been characterized in a number of higher plants (phanerogams) and also in some lower plants (cryptogams). These data strongly suggest that BRs occur widely in the plant kingdom, as in the case of other known plant hormones, and that BRs play some physiological functions in plant growth and development.

CONTENTS

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1. INTRODUCTION

Brassinolide (BL), present at 0.1 mg/kg in rape (*Brassica napus* L.) pollen, was the first steroidal plant growth promoter to be isolated, in 1979 [1]. In 1982, another new BL-related steroid termed castasterone (CS) was isolated from the insect galls of chestnut (*Castanea crenata*) [2]. CS differs from BL only in B-ring functionality. BL, CS and their structurally related steroidal compounds are known collectively as brassinosteroids (BRs). After the discovery of BL, the chemical synthesis of BL and its related compounds, the physiological study of them, the study of structure–activity relationships and screening of natural BRs in the plant kingdom were intensively and extensively carried out by many scientists. Following the basic investigations, some BRs (mainly BL, 24-epiBL and 28-homoBL) have been tested for many years for their practical applications in agriculture. These BRs have been shown to possess the following characteristics: promotion of germination and plant growth, raising of ripening, thickening promotion, recovery from stresses under various conditions unfavourable for plants and effects on flowering or its differentiation. Several excellent reviews on these subjects are available [3–14].

Microanalytical methods for BRs have also been developed: (i) gas chromatographic–mass spectrometric (GC–MS) analysis of BRs as bismethaneboronate derivatives [15] or methaneboronate–trimethylsilyl derivatives [16], (ii) high performance liquid chromatographic analysis of BRs as bisboronate derivatives having a fluorophore or an electrophore [17] and (iii) radioimmunoassay for BRs [18]. Among these microanalytical methods, GC–MS analysis has contributed greatly to the study of the identification and characterization of a number of natural BRs. At present about 30 BRs (Fig. 1) have been chemically identified from plants sources. Some reviews on microanalytical methods of BRs are also available [17,19–21].

This review summarizes research on BRs with respect to the distribution of BRs in the plant kingdom, bioassays for BRs and microanalytical methods for BRs using GC–MS with selected ion monitoring (SIM).

2. DISTRIBUTION OF BRASSINOSTEROIDS IN PLANT KINGDOM

Since the discovery of BL and CS, intensive and extensive studies on the isolation of new BRs from plant sources and on screening of BRs in the plant kingdom have been made mainly by Japanese scientists. In these studies, a sensitive and specific bioassay, the rice-lamina inclination test [22], and a GC–MS analysis [15,16] have been very effective and useful. So far about 30 BRs have been isolated and their structures have been chemically characterized [5,14]. It is now believed that BRs are ubiquitously distributed in the plant kingdom.

2.1. Brassinosteroids in higher plants (dicots)

The occurrence of BRs in a number of dicots plants has been reported: BL, CS, 28-norBL and 28-homoCS from the sheaths and immature seeds of Chinese cabbage (*Brassica campestris* L. var. *pekinensis*) [23,24], CS from the seeds of persimmon (*Diospyros kaki* Thunb.) [25], BL and CS from the pollen of European alder, *Alnus glutinosa* (L.) Gaertn [26], BL, CS and 28-norCS from the pollen of sunflower (*Helianthus annuus* L.) [27], BL from the stems of *Solidago altissima* L. [28], BL and CS from the pollen of *Cistus hirusutum* [29], BL and CS from the crown gall cells of *Catharanthus roseus* Don [30], BL and CS from the pollen of buckwheat (*Fagopyrum esculentum* Moench) [31], BL, CS, 28-homoCS, 28-norCS, typhasterol (2-deoxyCS, TyS) and teasterone (TeS, 3-epimer of TyS) from the leaves of green tea (*Thea sinensis* L.) [24,32,33], CS from the pollen and anther of green tea [34], CS from the flower buds of loquat (*Eriobotrya japonica* Lindl.) [35], CS and 28-norCS from the immature seeds of *Pharbitis purpurea* Voigt [36], dolichosterone (DS) from the pollen of *Eucalyptus marinata* [29], BL from the pollen of *Eucalyptus calophylla* [29], BL, CS and 6-deoxo-CS from the insect galls [2,37,38] and CS and 6-deoxoCS from the shoots, leaves and flower buds [38] of chestnut (*Castanea crenata* Sieb. et Zucc.), dolicholide (DL), DS, 28-homoDL, 28-homoDS, 6-deoxoCS and 6-deoxoDS from the immature seeds of *Dolichos*

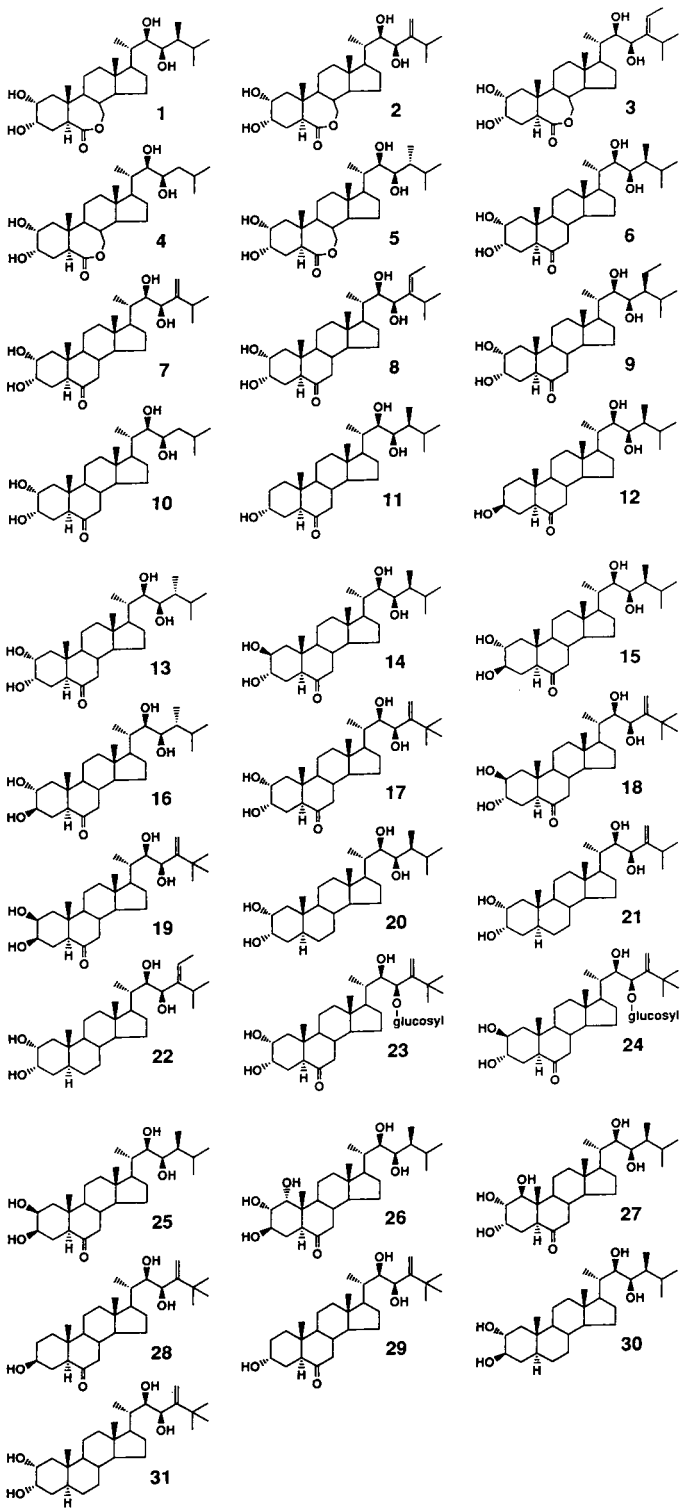

Fig. 1. Structures of natural brassinosteroids. **1** = Brassinolide; **2** = dolicholide; **3** = 28-homodolicholide; **4** = 28-norbrassinolide; **5** = 24-epibrassinolide; **6** = castasterone; **7** = dolichosterone; **8** = 28-homodolichosterone; **9** = 28-homocastasterone; **10** = 28-norcastasterone; **11** = typhasterol; **12** = teasterone; **13** = 24-epicastasterone; **14** = 2-epicastasterone; **15** = 3-epicastasterone; **16** = 3,24-diepicastasterone; **17** = 25-methyldolichosterone; **18** = 2-epi-25-methyldolichosterone; **19** = 2,3-diepi-25-methyldolichosterone; **20** = 6-deoxocastasterone; **21** = 6-deoxodolichosterone; **22** = 6-deoxo-28-homodolichosterone; **23** = 23-O-β-D-glucopyranosyl-25-methyldolichosterone; **24** = 23-O-β-D-glucopyranosyl - 2 - epi - 25 - methyldolichosterone; **25** = 2,3-diepicastasterone; **26** = 3-epi-1α-hydroxycastasterone; **27** = 1β-hydroxycastasterone; **28** = 3 - epi -2 - deoxy - 25 - methyldolichosterone; **29** = 2-deoxy-25-methyldolichosterone; **30** = 3-epi-6-deoxocastasterone; **31** = 6-deoxo-25-methyldolichosterone.

lablab L. [39–42], BL and CS from the immature seeds [43] and BL, 24-epiBL, CS and 28-norCS from the pollen [44] of broad bean (*Vicia faba* L.), BL, CS, 28-homoCS, 6-deoxo-28-homoCS and 6-deoxoCS from the immature seeds of *Piophocarpus tetragonolobus* [45], CS from the shoots of *Pisum sativum* L. cv. Holland [46], CS and 28-norCS from the insect galls and BL, CS, 28-norBL and 28-norCS from the leaves of *Distylium racemosum* Sieb. et Zucc. [47], BL, CS, TyS and TeS from the pollen of *Citrus unshiu* Marcov. [34], BL and CS from the pollen of orange (*Citrus sinensis* Osbeck) [48], BL and CS from the pollen of *Echium piantagineum* [29], BL and CS from the pollen of *Banksia grandis* [29] and BL and CS from the seeds of *Raphanus sativus* var. Remo [49]. The occurrence of more than 30 BRs in the immature seeds of *Phaseolus vulgaris* L. has been reported [50–56] and BRs whose structures have been established are BL, CS, DL, DS, 6-deoxoCS, 6-deoxoDS, 6-deoxo-28-homoDS, 2-epiCS, 3-epiCS, 2,3-diepiCS, 3,24-diepiCS, 1β-hydroxyCS, 3-epi-1α-hydroxy-CS, 3-epi-6-deoxoCS, 25-methylDS, 2-epi-25-methylDS, 2,3-diepi-25-methylDS, 2-deoxy-25-methylDS, 3-epi-2-deoxy-25-methylDS and 6-deoxo-25-methylDS. Among them, 25-methylDS is an interesting compound because it is the first 25-methylated BR and its biological activity is about ten times higher than that of its non-25-methylated counterpart, DS. Two conjugated BRs, 23-O-β-D-glucopyranosyl-25-methylDS and 23-O-β-D-glucopyranosyl-2-epi-25-methylDS have also been isolated from the *Phaseolus vulgaris* L. seeds.

Biological activity characteristic to BRs has also been detected by the bean second-internode assay and the rice-lamina inclination test, which are sensitive and specific bioassays for BRs, in the following dicot plant extracts: the pollens of *Sinapis arvensis* L. (=*Brassica kaber* L.) [3], *Sisymbrium irio L.* [3], *Rhus* sp. [3], *Leucopogon conostephioides* [29], thistle (*Carduus nutans* L.) [3], horse chestnut (*Aesculus hippocastanum* L.) [3], elm (*Ulmus* spp.) [3], pear (*Pyrus communis* L.) [3], hawthorn (*Crataegus sp.*) [3], *Robinia pseudo-acacia* L. [57], *Echium vulgare* L. [3] and rye (*Secale cereal* L.) [3].

2.2. Brassinosteroids in higher plants (monocots)

The presence of BRs in monocots has been demonstrated; TyS and TeS from the pollen of cat-tail (*Typha latifolia* L.) [34,58], CS and DS from rice shoots (*Oryza sativa*) [59], CS, TyS and TeS from the pollen (dent corn) [60] and CS from the immature seeds (sweet corn) [61] of corn (*Zea mays* L.), BL, CS and 28-homoCS from the immature seeds of wheat (*Triticam aestivum* L.) [62], BL, CS and TyS from the pollen of lily (*Lillium longiflorum* cv. Georgia) [34], BL, CS, TyS and TeS from the pollen of lily (*Lilium elegans* Thumb.) [63] and TyS from the pollen of tulip (*Tulipa gesneriana* L.) [34].

2.3. Brassinosteroids in higher plants (gymnosperms)

The occurrence of BRs in gymnosperms has been reported: TyS and CS from the pollen of Japanese black pine (*Pinus thunbergii* Parl.) [64], CS and TyS from the shoots of sitka spruce (*Picea sitchensis* Bong Carr) [65], and BL and CS from cambial scrapings of Scots pine (*Pinus silverstris*) [66] and DL and several unknown BRs from the pollen and anthers of a Japanese cedar (*Cryptomeria japonica* D. Don) [67].

2.4. Brassinosteroids in lower plants

Some lower plants (cryptogams) have been investigated for the presence of BRs. Green alga, *Hydrodictyon reticulatum* (L.) Lagerheim, has been reported to contain 24-epiCS and 28-homoCS [68], and in fern, *Equisetum arvense* L., specific biological activity for BRs has been obtained by the rice-lamina inclination test and some BRs have been tentatively identified by high-performance liquid chromatographic analysis [69]. The presence of BR-like bioactive substances in a highly purified fraction from *Chrollera pyrenoidosa*, which was obtained by the rice assay, has recently been reported [70]. Although these three studies strongly suggest that BRs also occur in lower plants, the wide occurrence of BRs in lower plants remains to be clarified.

2.5. Distribution in plants

From the above-described work on the identification of BRs in higher plants (phanerogams), it has been proved that BRs are contained in as many as 22 families and 39 genuses (27 of dicots, 8 of monocots and 4 of gymnosperms). In addition, the occurrence of BR-like bioactive substances has been suggested in 13 families and 17 genuses from the specific bioassays and chromatographic behaviour. Some studies on the occurrence of BRs in lower plants have also reported. Therefore, these screening data on BRs strongly suggest that BRs occur widely in the plant kingdom, as in the case of other known phytohormones, and that BRs play some physiological functions in plant growth and development.

Among the plants so far investigated, CS occurs most frequently, followed by BL. Therefore, these two BRs are believed to be important. In most plants, several kinds of BRs are found. In this respect, it is interesting that more than 30 BRs including unknown compounds (partial structures being determined by GC–MS analysis) occur in immature seeds of *Phaseolus vulgaris* [51]. Based on the data, it is likely that the number of natural BRs will increase in the future.

2.6. Content of brassinosteroids in plants

As far as the amount of BRs contained in plant tissues is concerned, pollens are the richest sources of BRs (*ca.* 10–100 μg/kg), immature seeds have also high contents of BRs (*ca.* 1–100 μg/kg) while shoots and leaves have lower levels (*ca.* 10–100 ng/kg) [3,4,7]. Although roots have not yet been examined, it has recently been suggested by the rice-lamina inclination test that a tuber of potato (*Solanum tuberosum*), a tap root of carrot (*Daucus carota* var. *sativus*) and a tuberous root of sweet potato (*Ipomoea batatas*) contain 6-keto-type (CS-type) BR-like bioactive substances [71]. Another interesting tissue is insect gall. The galls of *Castanea crenata* and *Distylium racemosum* have higher levels of BRs (several μg/kg) than the normal tissues [38,47]. Another tissue with a BR content is the crown gall (nopaline type) cells of *Catharanthus roseus* [30]. The crown gall cells have higher contents of BL and CS (*ca.* 30–40 μg/kg) than the normal cells.

It is known that in the same plant tissues, the young growing tissues are likely to have higher contents of BRs than old tissues. In *Dolichos lablab* immature seeds, the BR content is higher at a younger stage of the seed [4]. In the pollens of green tea (*Thea sinensis*) and lily (*Lillium longiflorum*), the bioactivity by rice-lamina inclination test increased as pollens grew mature and reached a maximum value just before anthesis; after the anthesis, the activity decreased [34]. These results suggest the possibility that BRs play an important role in regenerative growth regulation. Another interesting study was the comparative quantification of BRs with seeds of *Raphanus sativus* var. Remo [49]. It has been found by a GC–MS-SIM analysis that the ratio of BL to CS is significantly different in germinated seeds and in resting seeds, indicating an increase in BL formation during germination. The result suggests that BL could play an important role in germination.

2.7. Biosynthesis of brassinosteroids

A structural relationship between phytosterols and BRs could be suggested in which all naturally occurring BRs possess carbon skeletons identical with those of common phytosterols (*e.g.*, campesterol, 24-methylenecholesterol, isofucosterol, sitosterol and cholesterol). Therefore, BRs may be speculatively regarded as the enzymatic oxidation products of phytosterols with the corresponding carbon skeletons. Studies of the biosynthesis of BRs has just started. Yokota *et al.* [72] have proved that BL is biosynthesized from CS in crown gall cells of *Catharanthus roseus*. By employing the feeding experiment using deuterium-labelled BRs, it has recently been proved that the biosynthetic pathway TeS → TyS → CS → BL operates in both the crown gall cells and the normal cells of *Catharanthus roseus* [73,74]. However, a major part of the biosynthesis from phytosterols remains to be investigated.

3. BIOASSAYS USED TO GUIDE FRACTIONATION OF BRASSINOSTEROIDS

Since the isolation of BL, BL and its related compounds have been tested by a number of bioassays originally designed for known plant hormones. BRs have been shown to have a broad spectrum of biological activities [3,4,8]. Structure–activity relationships of BRs have also been clarified by bean second-internode bioassay, bean first-internode bioassay, raphanus test, tomato test and rice-lamina inclination test [75–78].

The development of bioassays for the isolation of bioactive compounds from natural sources has played an important role in recent natural product chemistry. For the isolation and purification of BRs from plant sources, highly sensitive and specific bioassays are essential, because of the very low concentration of BRs in plants. The bean-second internode assay was used to isolate BL from rape pollen [1], and the rice-lamina inclination test was used to isolate CS from chestnut insect galls [2]. After these studies, the latter bioassay has been widely employed in Japan to isolate successfully many BRs from a number of plant sources, because of its simplicity, high sensitivity and specificity for BRs.

The following three bioassays have been employed for the BR purification procedure to guide the fractionation: (i) bean second-internode bioassay, (ii) rice-lamina inclination test and (iii) wheat leaf unrolling test. Although the bean second-internode bioassay has historical significance (by employing the assay, BL was isolated from the rape pollen in 1979 [1]), the most frequently employed rice-lamina inclination test and a convenient wheat leaf unrolling test are described in this section.

3.1. Rice-lamina inclination test

It has been found by Wada *et al.* [22] that the rice-lamina inclination test is a highly sensitive and specific bioassay for BRs. The bioassay has been used to guide the fractionation during the purification procedure of the plant extracts, successfully resulting in the isolation and identification of a number of BRs with both lactone and ketone groups in the B-ring. 2-DeoxyBRs (TyS and TeS) have also been isolated by this bioassay [33,58,64]. The rice test is routinely employed in the purification steps mainly by Japanese scientists. In the rice test with cultivars Arborio J-1 and Nihonbare, a linear correlation was obtained between $5 \cdot 10^{-3}$ and $5 \cdot 10^{-5}$ μg/ml for BL and CS [79]. The induced angles leveled off at higher concentrations. BRs including 2-deoxy compounds showed very strong and specific activity at very low concentrations. Indole-3-acetic acid (IAA) was tested and was found to produce only a weak effect, five orders of magnitude less than BL. Cytokinins were inactive and actually counteracted the effect of BL. Abscisic acid (ABA) also counteracted the effect of BL. This assay is therefore highly specific for BRs and is also the most sensitive, concentrations as low as 0.05 ng/ml of BL being readily detected.

3.2. Wheat leaf unrolling test

The wheat leaf unrolling test has been found to be a convenient bioassay [80], in which BRs show strong activity. BL and CS dramatically stimulated wheat leaf unrolling, their activity being dose dependent. At 0.5 ng/ml both compounds markedly stimulated unrolling and, at 0.01 μg/ml or higher, BL produced complete unrolling of the leaf segments to about 3.6 mm. This assay is about one tenth as sensitive as the rice-lamina inclination test, but it is much simpler to carry out. GA_3 produced only slight unrolling at 0.1–10 μg/ml, as did the cytokinin 6-(3-methyl-2-butenyl)aminopurine. However, zeatin, 6-(4-hydroxy-3-methyl-2-butenyl)aminopurine, caused complete unrolling at 1 μg/ml and had a measurable effect at 0.001 μg/ml. ABA, IAA and indoleacetonitrile inhibited unrolling of leaf segments.

There have been some reports of the isolation of BRs from plant sources, employing the wheat leaf unrolling test as a bioassay: BL was isolated in pure form from the stems of *Solidago altissima* L. [28] and CS was identified by GC–MS analysis of the highly purified fraction of the immature seeds of corn [61]. Similarly, BL, CS and 28-homoCS were identified in the immature seeds of wheat (*Triticam aestivum* L.) [62]. These

reports suggest that isolation and purification of BRs from plants are successfully guided by the wheat leaf unrolling test. Because of its simpler manipulation than the rice-lamina inclination test, the wheat leaf unrolling test will replace the rice assay.

Highly sensitive and specific bioassays are essential for studies of the isolation of BRs from plant sources, because the contents of BRs in plants are extremely low. The above-described bioassays, in particular the rice-lamina inclination test, have greatly contributed to the study of BRs and will continue to do so in the future.

4. GC–MS-SIM OF BRASSINOSTEROIDS

It is well known that the content of BRs in plants is very low. In most instances the isolation of BRs in pure form is time consuming and tedious work. BRs are highly polar and involatile compounds. Therefore, in gas-phase analysis, conversion of BRs into volatile derivatives makes it easy to characterize BRs in a partially purified bioactive fraction by GC–MS or GC–MS-SIM. The GC–MS-SIM microanalytical method for BRs was developed by Takatsuto and co-workers [15,16,47].

4.1. GC–MS-SIM

BRs are converted into volatile derivatives based on the presence of two sets of vicinal diol groups in BRs. Considering its application to the analysis of natural BRs, the desired derivatives of BRs are bismethaneboronates (BMBs), because methaneboronic acid is a specific reagent for a vicinal diol function, allowing easy separation from other contaminants originating from plant sources. The derivative is suitable for gas-phase analysis and also for the analysis of fragment ions in electron impact (EI) mass spectra. The BMBs of BL, 28-homoBL, 28-norBL and their corresponding 6-keto analogues were well separated by GC using packed and capillary columns (capillary columns showed better resolution) and gave sharp peaks. A pair of 24-epimers of BL and that of CS were completely separated by GC using a capillary column.

MS fragmentation patterns of BMBs of typical BRs are summarized in Fig. 2. For BMBs of saturated BRs such as BL and CS, the fragment ions resulting from C_{23}–C_{24} fission, C_{20}–C_{22} fission and C_{17}–C_{20} fission are characteristic ions. The 6-ketone derivatives generally showed stronger molecular ions than the 7-oxalacetone derivatives. The ions at m/z 374 (lactones) and m/z 358 (ketones) were accompanied by hydrogen transfer. Thus, the fragment ions at m/z 457, 374, 345 and 177 (the assignment is shown in Fig. 2) are common for lactone-type BRs, whereas ions at m/z 441, 358 and 329 are common for ketone-type BRs. The ketone derivatives also gave another common fragment ion at m/z 287 resulting from C_{14}–C_{15} and C_{13}–C_{17} fissions. The ions corresponding to the cyclic boronate moiety of the side-chain part (C_{20}–C_{22} fission) are base peaks in both lactone- and ketone-type BRs. However, in the case of BMBs of unsaturated BRs such as DL and DS, which possess a C-24(28) double bond, different fragments ions were observed and most of them resulted from the cleavages of the cyclic boronate moiety of the side-chain part. These ions are m/z 427, 403, 385, 124 and 82 for the DL derivative and m/z 411, 387, 369, 124 and 82 for the DS derivative, as shown in Fig. 2. Another remarkable difference is that hydrogen transfer observed from C_{20}–C_{22} fission in the saturated series is not recorded in the case of the unsaturated series, but two-hydrogen transfer from the C_{17}–C_{20} fission is observed for the unsaturated series. The BMBs of 28-homoDL and 28-homoDS showed similar fragmentation patterns to those of DL and DS, respectively. However, salient differences in the relative intensities of the ions resulting from C_{24}–C_{25} fission are observed; m/z 497 (relative intensity 100%) for 28-homoDL BMB *vs.* m/z 483 (2.6%) for DL BMB and m/z 481 (100%) for 28-homoDS BMB *vs.* m/z 467 (5.3%) for DS BMB. Similar fragment patterns in EI-MS were reported for the BMBs of 6-deoxoCS and 6-deoxoDS [50]. In addition to the characteristic fragment ions derived from the side-chain cleavages as described above, the ions at m/z 288, 273 and 205 resulting from ring C and D fissions were observed. More detailed data on the fragmentation of BMBs of

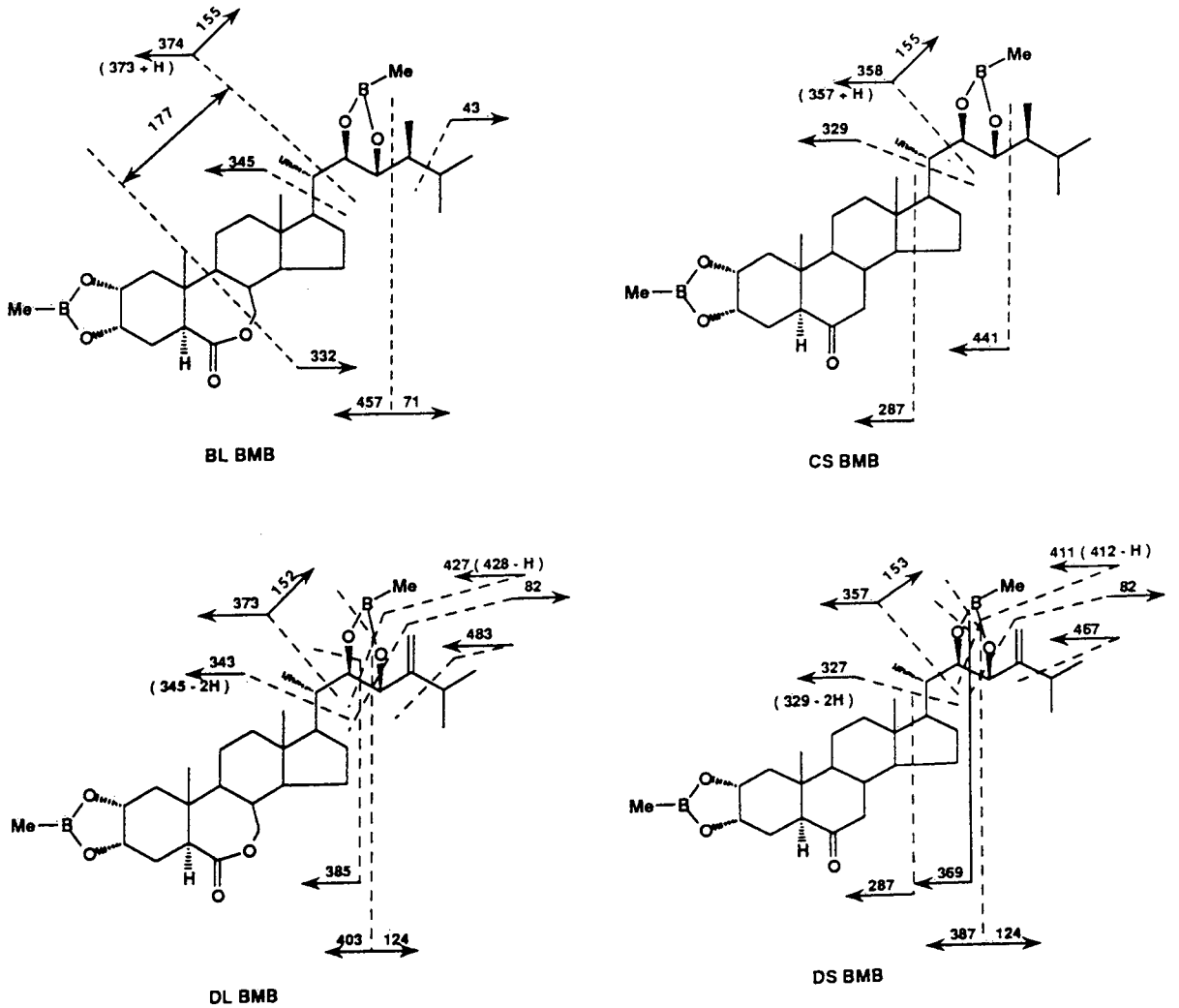

Fig. 2. Mass fragmentation of typical BR BMBs.

BRs in EI/MS were described in a previous review [19].

GC–MS analysis of 2-deoxyBRs (*e.g.*, TeS, TyS) has also been developed [16]. Since the 2-deoxyBRs have a vicinal diol function in the side-chain and an isolated hydroxyl group in the A-ring, the diol was first methaneboronated and then the remaining 3-hydroxyl group was trimethylsilylated. The resulting methaneboronate–trimethylsilyl (MB–TMS) derivative is also suitable for gas-phase analysis and also for analysis of fragment ions in EI-MS. The MB–TMS derivatives of TeS, TyS and their 28-homo and 28-nor analogues were well separated by GC using packed and capillary columns and gave sharp peaks. The fragmentation patterns in the EI mass spectra of MB–TMS derivatives of several representative 2-deoxyBRs have been summarized in a previous paper [16].

In the chemical ionization (CI) mass spectra of BMBs of BRs, the derivatives afforded ions of M + 1 as a base peak, M + 1 − 60, and also weak

ions resulting from C_{17}–C_{20} and C_{20}–C_{22} fissions. There is not much difference between the saturated and unsaturated BR derivatives.

4.2. Applications

In order to determine very small amounts of BRs in a bioactive fraction from plant sources, computerized SIM using a GC–CI-MS system is suitable and effective in detecting BRs, because the molecular ions of the BMBs of BRs are base peaks, making it easy to monitor these ions in GC–MS-SIM for screening of natural BRs. The presence of these molecular ions could be used to detect BRs. The GC–MS-SIM method was capable of detecting BRs at the picogram level. GC–CI-MS-SIM has been successfully applied to the identification of BRs with two sets of vicinal diols. Using this technique, BL (detected as its BMB, *m/z* 529) and CS (BMB, *m/z* 513) were found in the crude bioactive fractions obtained from the immature seeds and sheaths of Chinese cabbage, *Brassica campestris* var. *pekinensis* [23], the leaves of green tea, *Thea sinensis* [32], the insect galls of chestnut tree, *Castanea* spp. [37] and the leaves and insect galls of *Distylium racemosum* Sieb. et Zucc [47]. In the bioactive fraction from the aerial part of rice plant, *Oryza sativa*, CS and DS were detected as their BMBs [59]. From the historical points of view, it is of interest to note that BRs are contained in *Distylium racemosum* Sieb. et Zucc. GC–MS analysis coupled with the rice-lamina inclination test has made it clear that the *Distylium* factors obtained from the leaves of *Distylium racemosum* Sieb. et Zucc. in 1968 by Marumo and co-workers [81] are unequivocally BRs (BL, 28-norBL, CS and 28-norCS) [47]. A detailed story of this is described in a review [82]. Our microanalytical method was very effective in the structure determination of BRs in the immature seeds of *Phaseolus vulgaris* cv. Kentucky Wonder [50]. As the amounts of isolated BRs were very small and they were mixed with structurally closely related compounds, their structures were determined, only by GC–MS as their BMB derivatives, to be 6-deoxoCS, 6-deoxoDS, CS and DS. In screening for new BRs, TeS and TyS have been identified for the first time as MB–TMS derivatives in the leaves of green tea (*Thea sinensis*) [33].

GC–MS has been applied successfully to identify traces of natural BRs from plant sources, as described above. When a sufficient amount of purified BRs was obtained from plants, the full mass spectrum is taken by EI-MS. A recent example is the identification of three BRs in the pollen of sunflower (*Helianthus annuus* L.) [27]. The methanol extract of the pollen was subjected to solvent partitioning to obtain a chloroform-soluble neutral fraction. This fraction was successively purified by silica gel column chromatography, normal-phase preparative thin-layer chromatography (p-TLC) and Sephadex LH-20 column chromatography to give a highly purified bioactive fraction. The fraction was derivatized with methaneboronic acid and the resulting BMBs were analysed by GC–EI-MS, using a capillary column. A total ion chromatogram is presented in Fig. 3. The retention times of 12.13, 13.20 and 15.06 min and the full mass spectra of the peaks were identical with those of the BMBs of authentic 28-norCS, CS and BL, respectively. The amounts of these BRs are 65, 21 and 106 ng/g, respectively. Hence the bioactive BRs contained in the sunflower pollen were rigorously identified.

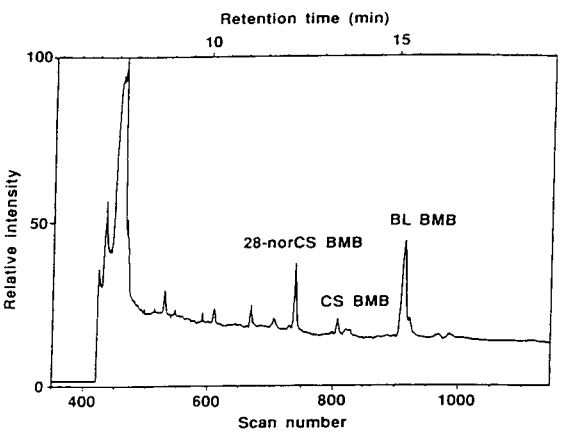

Fig. 3. Total ion chromatogram obtained in GC–MS of the methaneboronated highly purified fraction obtained from sunflower pollen.

Rigorous identification was also achieved by GC–high-resolution (HR)-MS-SIM. This technique, capable of detecting BRs at the picogram level, was applied to the identification of BRs in the pollens of broad bean (*Vicia faba* L.) [44] and buckwheat (*Fagopyrum esculentum* Moench) [31]. A recent example is the identification of BRs in the buckwheat pollen [31]. As the content of BRs in the buckwheat pollen was found to be very small by the bioassay and the BRs were extremely contaminated with unknown natural products, several chromatographic methods, including silica gel adsorption chromatography, normal-phase p-TLC, activated charcoal chromatography and reversed-phase p-TLC, were employed. The bioactive fraction thus obtained was derivatized and analysed by the GC–HR-MS-SIM method using a capillary column in the EI-MS mode. Under our GC–MS conditions, authentic BMBs of CS and BL were eluted at 13.56 and 15.16 min, respectively. The SIM results for the derivatized samples obtained from the active fraction are presented in Fig. 4. Monitoring of the molecular ion of CS BMB at *m/z* 512.3842 and that of BL BMB at *m/z* 528.3791 exhibited sharp peaks with the same retention times as those of authentic BMBs, thereby establishing rigorously the presence of BL (5.0 ng/g) and CS (7.1 ng/g) in the buckwheat pollen.

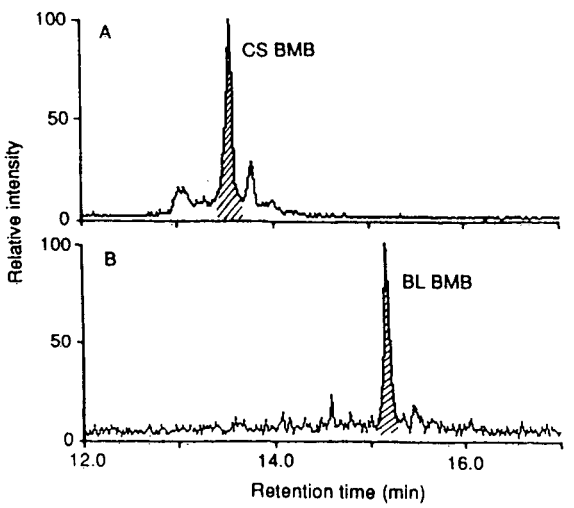

Fig. 4. GC–HR-MS-SIM of the BMBs of CS and BL obtained from buckwheat pollen. (A) Detection of CS BMB by monitoring the molecular ion at *m/z* 512.3842; (B) detection of BL BMB by monitoring the molecular ion at *m/z* 528.3791.

Deuterium-labelled BRs, [26,28-$^2H_6$]BL, [26,28-$^2H_6$]CS, [26,28-$^2H_6$]TyS and [26,28-$^2H_6$]TeS, have been synthesized for use as internal standards [83]. Quantitative analysis of natural BRs by GC–MS employing the deuterated BRs has been carried out [46]. In order to understand the growth retardation mechanism of (*S*)-uniconazole, the shoots *of Pisum sativum* L. treated with (*S*)- and (*R*)-uniconazoles were analysed in terms of the levels of the endogenous GAs, BRs and phytosterols. Only referring to BRs, it is of interest to examine whether uniconazoles modify the biosynthesis of BRs. BRs contained in the shoots *of P. sativum* L. were extracted, purified and analysed by GC–MS. GC–MS analysis of the active fraction led to the identification of CS: *m/z* (relative intensity) 512 (M^+, 54%), 155 (100%). GC–MS-SIM determination using an internal standard (d_6-CS) revealed that the content of CS in the control plants was 0.9 ng/g fresh mass and, after treatment with (*S*)- and (*R*)-uniconazoles, reduced to 54% and 34% of the controls, respectively. The result suggests that the altered metabolism of BRs is probably involved in the action mechanism of (*S*)-uniconazole.

Deuterium-labelled BRs have also been used in the biosynthetic study of natural BRs [73,74,84]. When [26,28-$^2H_6$]TyS, which is a hypothetical precursor of CS, was fed to cultured crown gall cells of *Catharanthus roseus*, conversion of [26,28-$^2H_6$]TyS into [26,28-$^2H_6$]CS and [26,28-$^2H_6$]BL was shown by GC–MS analysis of the metabolite [73,84]. In a similar feeding experiment employing [26,28-$^2H_6$]TeS and [26,28-$^2H_6$]TyS, it has recently been proved that the biosynthetic pathway TeS → TyS → CS → BL operates in both the crown gall cells and the normal cells of *Catharanthus roseus* [74]. Thus, our GC–MS method is useful and effective for the biosynthetic study of natural BRs.

An alternative highly sensitive GC–MS technique involves tandem MS (MS–MS). In this technique, the first mass filter is used to select the ion of interest from all the other ions

produced from the matrix. This ion is usually the molecular ion in the EI mode or the protonated molecule (MH^+) in the CI mode. These selected ions then undergo collisionally activated dissociation to produce daughter ions, which are then separated by a second mass filter and analysed. GC–MS–MS has been applied to the identification of BRs in the pollen of European alder, *Alnus glutinosa* (L.) Gaertn [26]. The crude bioactive fraction obtained from the pollen was derivatized with methaneboronic acid and the resulting derivatives were analysed by GC–MS–MS in the CI mode, because the protonated molecular ions of BR BMBs are produced as base peaks in the CI mode. BL and CS have been identified in the pollen as their BMBs.

The GC–MS-SIM microanalytical method for BRs has most frequently been employed in analytical studies of trace levels of BRs in plants and it has greatly contributed to studies of the identification of many natural BRs and also their distribution in the plant kingdom.

5. CONCLUSIONS

Studies on BRs have greatly widened our knowledge of the chemistry and plant physiology of BRs in the past 10 years and more. With respect to the microanalysis of BRs, highly sensitive and specific bioassays and microanalytical methods including GC–MS-SIM, HPLC and radioimmunoassay have been developed. Combination of the bioassays and the GC–MS-SIM microanalysis has led successfully to the identification of more than 30 naturally occurring BRs. So far, in as many as 22 families and 39 genuses BRs have been identified and, in addition, in 13 families and 17 genuses, the occurrence of BR-like bioactive substances has been suggested. These screening data strongly indicate a ubiquitous distribution of BRs in the plant kingdom. In these studies the rice-lamina inclination test and our GC–MS method have been effectively employed. The microanalytical methods in combination with the bioassays will contribute to more detailed studies of the physiological mechanism of BRs, because they are essential for the identification and determination of endogenous BRs which are involved in plant growth and development.

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Review

Liquid chromatographic assay of brassinosteroids in plants

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ABSTRACT

The liquid chromatographic assay of brassinosteroids (BRs) in plants as boronate derivatives with UV, fluorimetric and electrochemical detection is described. Several boronic acid derivatives proved to be satisfactory for use in the derivatization of BRs with respect to reactivity and sensitivity. BRs were readily condensed with the boronic acid derivatives under mild conditions to provide the corresponding boronates, which exhibit strong absorption, fluorescence or maximum amperometric sensitivity with detection limits of *ca.* 20–100 pg, depending on the prelabelling reagents. The method was successfully applied to the determination of natural BRs. BRs were identified in the pollen of broad bean (*Vicia faba* L.), sunflower (*Helianthus annuus* L.) and buckwheat (*Fagopyrum esculentum* Moench). The results demonstrate that the liquid chromatographic microanalytical method is useful for the screening of BRs in the plant kingdom.

CONTENTS

1. INTRODUCTION

Since the discovery of brassinolide (BL) in 1979 [1], studies on BL and its related steroids (brassinosteroids, BRs) have greatly widened our knowledge of this class of steroidal plant hormone. It has been found that BRs show a wide range of biological activities when compared with other known phytohormones, and

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that BRs promote plant growth and increase crop yields [2]. Intensive and extensive studies on the isolation of new BRs from plant sources and on their screening in plants have been carried out, mainly in Japan. It has been found that a number of BRs occur in a wide variety of higher plants and also in some lower plants [2].

Because of their high biological activity and their low concentrations in plants, bioassays to guide fractionation during purification steps and microanalytical methods are necessary for the screening and identification of BRs in plants. A rice-lamina inclination test [3] and microanalysis by gas chromatography–mass spectrometry (GC–MS) [4–7] have been very effective in these studies. In fractionating and isolating very small amounts of BRs from plants, sensitive and specific bioassays for BRs are indispensable. A bean-second internode assay was used to isolate BL from rape (*Brassica napus* L.) pollen [1], and the rice-lamina inclination test was used to isolate castasterone (CS) from chestnut (*Castanea* spp.) insect galls [8]. Subsequently, the latter bioassay has been widely employed to isolate many BRs from a number of plant sources, because of its simplicity, high sensitivity and specificity for BRs.

Isolation of BRs in pure form is a time-consuming and tedious task because of their very low concentrations in plants. Therefore, in order to determine BRs in a partially purified fraction, they should be converted into suitable derivatives. In the GC–MS microanalysis method, natural BRs have been measured as their bismethaneboronate or methaneboronate–trimethylsilyl derivatives [4–7]. GC–MS analysis has contributed to the identification and characterization of more than 30 natural BRs in many higher plants and some lower plants [7]. Among the natural BRs, CS occurs most frequently, followed by BL, suggesting that these two BRs are important in plant growth and development. With respect to the content of BRs, pollen is the richest source. Immature seeds also have a high content of BRs, whereas shoots and leaves have lower levels [2].

High-performance liquid chromatography (HPLC) is a useful tool for the separation and determination of trace amounts of naturally occurring compounds. The study of micro-scale analyses for BRs includes some basic problems: (1) highly sensitive methods of detection are required because the levels of BRs in plants are the lowest among the known phytohormones; and (2) highly selective separation methods are needed because several analogues of BRs coexist in plants. These two problems must be solved and the highest level of analytical sensitivity must be achieved.

In HPLC, UV–Vis, fluorimetric and electrochemical detection methods are commonly used to achieve the highly sensitive and selective determination of many kinds of analytes. Prelabelling derivatization methods in HPLC are frequently used to enhance the sensitivity and selectivity of detection of compounds that do not have a suitable chromophore, fluorophore or electrophoric group in the molecules. Because of the lack of a UV-active chromophore or a fluorophore in BRs, derivatization is essential for their LC microanalysis. BRs could be derivatized to highly detectable derivatives because they usually have four hydroxyl groups as two sets of vicinal diols, in the A-ring ($2\alpha,3\alpha$-position) and in the side-chain ($22R,23R$-position).

Since 1988, we have been developing the liquid chromatographic assay of natural BRs according to the prelabelling method as their boronate derivatives [9]. In this review we describe several kinds of boronic acid derivatives as prelabelling reagents for BRs (Fig. 1), and we also demonstrate the potential of the HPLC method by the separation of standard BRs and its application of the determination of natural BRs in some pollens.

2. DERIVATIZATION

Poor selectivity was observed in the prelabelling of four hydroxyl groups of BRs by naphthoyl chloride [10] or anthroyl nitrile [11], which are excellent prelabelling reagents for hydroxyl groups, because the derivatives afforded complex peak patterns in the liquid chromatograms under reversed-phase conditions.

Boronic acid derivatives give high selectivity and reactivity for the *cis*-1,2-diols of BRs. The

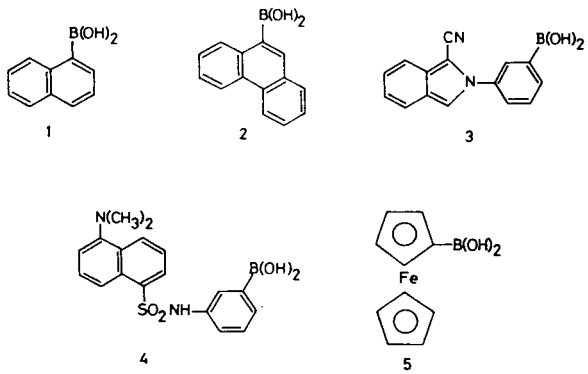

Fig. 1. Boronic acid derivatives for derivatization of brassinosteroids. 1 = Naphthaleneboronic acid; 2 = phenanthreneboronic acid; 3 = 1-cyanoisoindole-2-*m*-phenylboronic acid; 4 = dansylaminophenylboronic acid; 5 = ferroceneboronic acid.

design of a derivatization reagent for the LC separation of BRs requires two structural features: a functional group highly reactive towards the 1,2-diol group and a very responsive fluorophore or electrophore for detection. For this purpose, we synthesized 1-cyanoisoindole-2-*m*-phenylboronic acid and dansylaminophenylboronic acid. Information on early developments in the formation of cyclic boronates was reported in general reviews on cyclic derivatives of bifunctional compounds by Poole and Zlatkis [12].

Previous derivatizations using methaneboronic acid in GC analysis have usually been carried out under dry pyridine. After investigation of the reaction conditions, it was found that a catalytic amount of pyridine was essential to achieve boronate formation. Accordingly, the optimum reaction conditions were as follows: a standard mixture or a biologically active BR fraction was dissolved in a small amount of acetonitrile (100 μl), 100 μl of boronic acid derivatives (1 mg/ml) in 1% (v/v) pyridine–acetonitrile were added (an excess of the reagents over BRs) and the mixture was heated at 70°C for 10–20 min. The general scheme of boronate formation using BL as a substrate is illustrated in Fig. 2. Different reactivities between the boronic acid derivatives has not been observed. The boronate derivatives formed have been found to be stable for at least 2–3 months in a freezer.

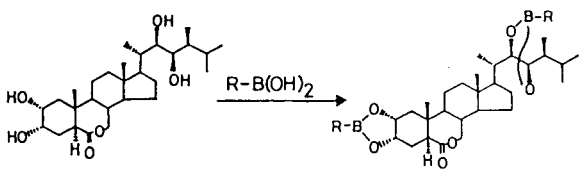

Fig. 2. General scheme for the formation of brassinolide boronates using boronic acid derivatives [R–B(OH)$_2$].

3. SEPARATION MODE

In the LC analysis of steroid compounds, adsorptive separation using silica or reversed-phase separation using an ODS- or CN-bonded column has generally been selected. We selected the reversed-phase mode for the analysis of BRs. The main reason was that reversed-phase columns, especially ODS columns, have good stability and durability, and a wide range of mobile phases are available for the separation of analytes.

A comparison of a number of ODS columns revealed that some of them showed peak tailing in the chromatograms of the series of 6-ketone compounds (*e.g.*, CS). This reflected the content of silanol groups remaining on the surface of ODS-derivatized silica. Although many studies have been carried out to produce highly efficient ODS columns, the problems of end-capping treatment of the silanol on ODS-silica still remains. Throughout this work we selected ODS columns whose silanol groups are sufficiently excluded from the ODS-silica. Attempted separations of BRs using C_8- or CN-bonded columns failed to give good separation patterns on the chromatograms.

4. UV DETECTION

There has been no report of the application of naphthaleneboronic acid as a derivatization reagent for HPLC. We examined the derivatization of BRs with this reagent [13]. The electron impact (EI) mass spectrum of the boronate of BL afforded a molecular ion at *m/z* 752, which confirmed the formation of the bis-naphthaleneboronate. The other BRs were also derivatized to the corresponding bisboronates.

TABLE 1

RETENTION TIMES OF NAPHTHALENEBORONATES OF BRASSINOSTEROIDS

HPLC conditions: Shim-pack CLC-ODS column (15 cm × 6.0 mm I.D.); mobile phase, acetonitrile–water (75:25, v/v); flow-rate, 1.2 ml/min; temperature, 45°C; detection, 280 nm.

Compound	t_R (min)
28-Norbrassinolide	6.24
Dolichosterone	7.87
28-Norcastasterone	8.14
Brassinolide	8.54
Castasterone	11.66
28-Homobrassinolide	11.81
28-Homocastasterone	16.68

Separation of these boronates was examined using a conventional reversed-phase ODS column. When a Shim-pack CLC-ODS column was used at 45°C with acetonitrile–water (75:25, v/v) as the mobile phase, the boronates of BL and its side-chain analogues were detected at 280 nm and exhibited sharp peaks. The retention times of the boronates of seven authentic BRs are given in Table 1.

A narrow-bore column such as a Shim-pack SBC-ODS afforded better resolution, sharper peaks and shorter separation times. We examined the detection limit of the BL naphathaleneboronate using the column. The result showed that BL can be detected down to at least 100 pg per injection (signal-to-noise ratio = 2) as its boronate derivative.

5. FLUORIMETRIC DETECTION

As a continuation of our studies on highly sensitive detection methods for BRs, we screened fluorescent boronic acid derivatives as derivatization reagents in the hope of developing a more sensitive and selective method.

First, we examined the prelabelling derivatization of BRs with phenanthreneboronic acid (commercially available) [14]. Although Poole *et al.* [15] reported its application as a derivation reagent to detect ecdysteroids by TLC, no data were available for its use in HPLC. The EI mass spectrum of the boronate derivative of BL confirmed the formation of the bisphenanthreneboronate.

For the separation of the phenanthreneboronates of BRs, an STR ODS-H column was found to afford better resolution than several conventional ODS columns tested. We examined the detection limits of these PBs using this column and acetonitrile–water as the mobile phase. The boronates were monitored at an emission wavelength of 375 nm when excited at 305 nm. As shown in Fig. 3, the phenanthreneboronates of seven BRs afforded sharp peaks. The method gave a detection limit for BL 50 pg per injection (signal-to-noise ratio = 3). A fivefold increase in detectability was obtained in comparison with UV absorption detection of the naphthaleneboronates.

Second, we examined 1-cyanoisoindole-2-*m*-phenylboronic acid, which is readily prepared from *o*-phthalaldehyde (OPA) and *m*-aminophenylboronic acid in one step, as a prelabelling reagent [16]. The reagent has an isoindole fluorophore and a large signal was observed with

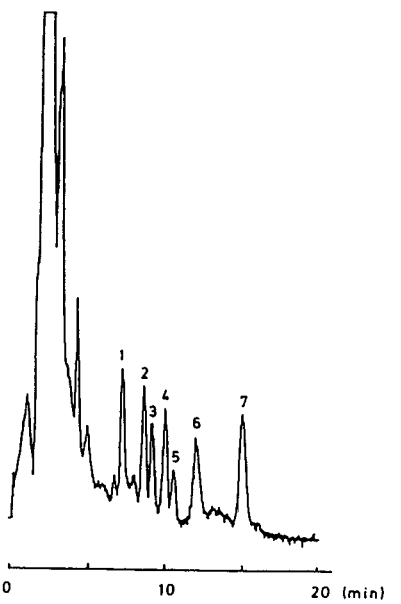

Fig. 3. Chromatogram of phenanthreneboronates of authentic brassinosteroids. Peaks: 1 = 28-norbrassinolide; 2 = brassinolide; 3 = dolichosterone; 4 = 28-norcastasterone; 5 = 28-homobrassinolide; 6 = castasterone; 7 = 28-homocastasterone. Conditions: STR ODS-H column (15 cm × 4.0 mm I.D.); mobile phase, acetonitrile–water (9:1); flow-rate, 0.8 ml/min; temperature, 45°C.

a detector monitoring the fluorescence intensity at 400 nm, when excited at 330 nm. The detection limit for this boronate derivative of brassinolide was 20 pg (signal-to-noise ratio = 3). One of the advantages of this reagent is its high sensitivity in comparison with other boronic acid derivatives used for prelabelling. The separation of five BR boronates, which were formed with the boronic acid in quantitative yield, was successfully carried out with a variety of solvent mixtures under isocratic conditions. The boronates of BRs exhibited sharp peaks, as shown in Fig. 4. The cause of the slightly enhanced broadening of the peak of a CS derivative is not clear.

Third, the prelabelling derivatization of BRs as their dansylaminophenylboronates using fluorimetric detection was studied [17]. The boronates were easily prepared by the reaction of BRs with dansylaminophenylboronic acid, which was newly synthesized as a fluorogenic reagent for BRs.

The excitation maximum, which is representative of the other BR derivatives, is at 345 nm and the emission maximum is at 515 nm. As an example, the detection limit for the boronate derived from BL was 25 pg (signal-to-noise ratio = 2) in the reversed-phase mode. A twofold increase in detectability was observed when the fluorescence of the dansylaminophenylboronates was compared with that of phenanthreneboronates.

The separation of six BRs was successfully performed with a variety of solvent mixtures under isocratic conditions. When a reversed-phase column was used at 45°C with acetonitrile–water (8:2, v/v) as the mobile phase at a flow-rate of 1.0 ml/min, the boronates of BRs exhibited sharp peaks and the derivatives could be separated as shown in Fig. 5.

The relationships between the peak areas and the amounts of the individual BRs were linear from 25 pg to 40 ng. The precision was established by repeated determinations ($n = 8$) using a mixture of BL and CS. The relative standard

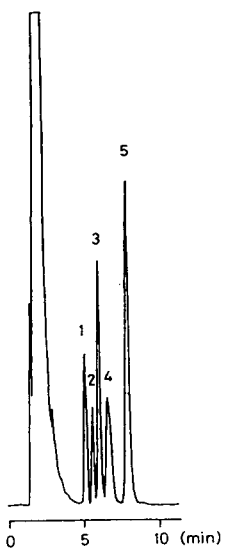

Fig. 4. Chromatogram of 1-cyanoisoindole-2-*m*-phenylboronates of authentic brassinosteroids. Peaks: 1 = brassinolide; 2 = 28-norcastasterone; 3 = 28-homobrassinolide; 4 = castasterone; 5 = 28-homocastasterone. Conditions: STR ODS-H column (15 cm × 4.0 mm I.D.); mobile phase, acetonitrile–1% acetic acid (9:1); flow-rate, 0.8 ml/min; temperature, 45°C.

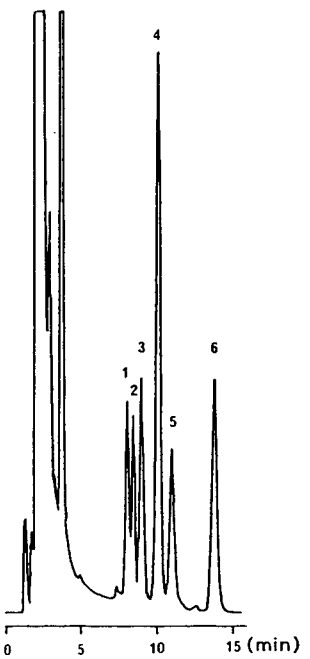

Fig. 5. Chromatogram of dansylaminophenylboronates of authentic brassinosteroids. Peaks: 1 = brassinolide; 2 = dolichosterone; 3 = 28-norcastasterone; 4 = 28-homobrassinolide; 5 = castasterone; 6 = 28-homocastasterone. Conditions: Shim-pack CLC-ODS(M) column (15 cm × 4.6 mm I.D.); mobile phase, acetonitrile–water (8:2); flow-rate, 1.0 ml/min; temperature, 45°C.

deviations (R.S.D.s) were 2.5% and 3.1%, respectively.

As described above, it is evident that the boronic acid derivatives incorporating a fluorophore are suitable and highly sensitive derivatization reagents for BRs that have 1,2-diol groups in the molecules.

6. ELECTROCHEMICAL DETECTION

Electrochemical detection in LC, which was presented by Kissinger *et al.* [18], was applied to the determination in biological samples of catecholamines, catecholestrogens, etc., having a phenolic hydroxyl group or a catechol moiety in the molecules. Further interest in the analysis of BRs prompted us to investigate electrochemical detection in the hope of developing a more sensitive and selective method.

Ferrocene derivatives are readily oxidizable and selectively detected in the presence of other electroactive compounds. Brooks and Cole [19,20] proposed ferroceneboronic acid (FBA) for the prelabelling of glycol compounds for GC with electron-capture detection. We have developed a micro-scale method for the determination of BRs as ferroceneboronate derivatives by HPLC with electrochemical detection [21].

On treatment with FBA, authentic BRs were quantitatively derivatized. The separation of the ferroceneboronates could be performed successfully on a reversed-phase column using acetonitrile–water (85:15, v/v) containing 1 *M* sodium perchlorate as the mobile phase. The hydrodynamic voltammogram of the BL ferroceneboronate derivative showed a constant value above +0.6 V *vs.* a silver–silver chloride reference electrode owing to oxidation of the ferrocenyl moiety. The authentic BR ferroceneboronates were clearly separated on a Shim-pack CLC-ODS(M) column, as illustrated in Fig. 6. The relationships between the peak areas and the amounts of the individual BRs were linear from 50 pg to 5 ng. The method with FBA gave a detection limit for BL of 25 pg per injection (signal-to-noise ratio = 3). A twofold increase in detectability was observed when compared with the fluorimetric detection of phenanthereneboronates. The detection limits of the derivatives with electrochemical detection were found to be comparable to those for other derivatives with fluorimetric detection.

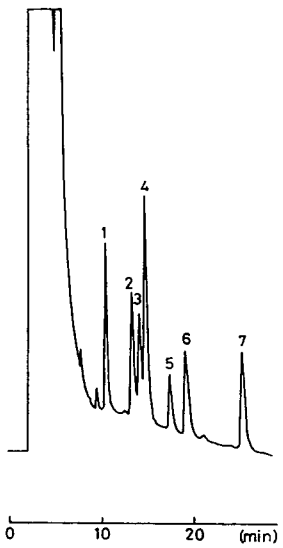

Fig. 6. Chromatogram of ferroceneboronates of authentic brassinosteroids. Peaks: 1 = 28-norbrassinolide; 2-brassinolide; 3 = dolichosterone; 4 = 28-norcastasterone; 5 = 28-homobrassinolide; 6 = castasterone; 7 = homocastasterone. Conditions: Shim-pack CLC-ODS(M) column (15 cm × 4.6 mm I.D.); mobile phase, acetonitrile–water (85:15) containing 1 *M* $NaClO_4$; flow-rate, 1.0 ml/min; temperature, 40°C.

7. SEPARATION OF C-24 EPIMERIC PAIRS OF BRASSINOSTEROIDS

For the microanalysis of BRs, there is an important consideration with respect to the stereochemistry of BR biosynthesis. Much interest has been directed to the stereochemistry at C-24 of BRs in plants, in connection with the stereochemistry of phytosterols. Identification of 24-epiBL in the pollen of broad bean (*Vicia faba* L.) was achieved by means of the selected-ion monitoring technique in GC–MS [22]. We have reported a simple and effective method for the separation of the C-24 epimeric pairs of BL and CS by HPLC with precolumn labelling via the two diol groups and subsequent postcolumn

fluorescence detection [23]. The standard C-24 epimeric mixture of the BRs was derivatized with *m*-aminophenylboronic acid. The boronate formed was injected into a Shim-pack RPC-ODS column. The optimum mobile phase for the separation of the boronates was acetonitrile–1% (v/v) acetic acid (75:25, v/v) containing 20 m*M* 18-crown-6 at a flow-rate of 1.0 ml/min. The BR *m*-aminophenylboronates were monitored by fluorescence detection using a postcolumn reaction system [24], with OPA in the presence of cyanide ion. The first postcolumn reagent is OPA in ethanol/carbonate–borate buffer and the second reagent is potassium cyanide in the same buffer. The boronate OPA derivatives were determined with high response by monitoring the fluorescence intensity at 400 nm, with excitation at 330 nm.

The capacity factors (k'), separation factors (α) and resolution values (R_s) of C-24 epimeric BRs on a Shim-pack RPC-ODS column are listed in Table 2. The chromatogram is shown in Fig. 7. It is evident from the data that a good separation of C-24 epimers of BL and CS was attained. Although no plausible explanation can be given for the elution order of each pair of the epimers (24*S*-isomers were eluted before the 24*R*-isomers), it is likely that an aminophenyl group in the boronate introduced at the side-chain (C-22,23) plays an important role in the conformational discrimination of the C-24 epimeric pairs of BRs in the mobile phase containing a crown ether.

TABLE 2

LC SEPARATION OF THE C-24 EPIMERIC PAIRS OF BRASSINOSTEROIDS AS *m*-AMINOPHENYLBORONATES

For LC conditions, see Fig. 7.

Compound	k'	α	R_s
Brassinolide (24*S*)	2.68	1.07	1.18
24-Epibrassinolide (24*R*)	2.84		
Castasterone (24*S*)	4.31	1.08	1.48
24-Epicastasterone (24*R*)	4.53		

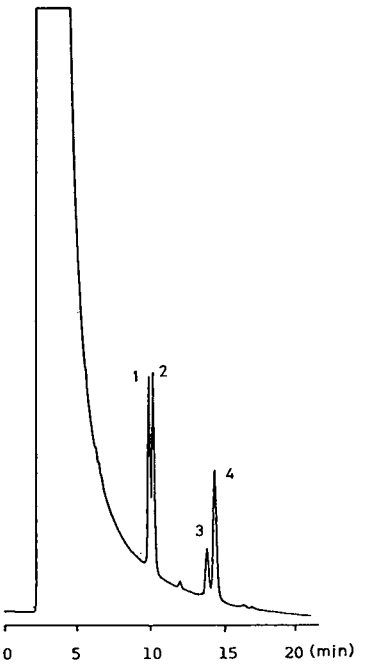

Fig. 7. Chromatogram of the *m*-aminophenylboronate of C-24 epimeric pairs of brassinosteroids. Peaks: 1 = brassinolide; 2 = 24-epibrassinolide; 3 = castasterone; 4 = 24-epicastasterone. Conditions: Shim-pack RPC-ODS column (25 cm × 4.6 mm I.D.); mobile phase, acetonitrile–1% acetic acid (75:25) containing 20 m*M* 18-crown-6; flow-rate, 1.0 ml/min; temperature, 45°C.

8. APPLICATION TO NATURAL BRASSINOSTEROIDS

As it is well known that the content of BRs in plants is very low, the isolation of BRs in a pure form is time consuming and tedious. Therefore, HPLC of trace amounts of BRs contained in partially purified fractions has been investigated. BRs should be converted into suitable derivatives. Our HPLC method was based on esterification of BRs with boronic acid derivatives such as naphthaleneboronic acid [13], 9-phenanthreneboronic acid [15], 1-cyanoisoindole-2-*m*-phenylboronic acid [16], dansylaminophenylboronic acid [17] and ferroceneboronic acid [21]. The derivatized BRs were effectively separated by a reversed-phase column and they were monitored by UV, fluorimetric or electrochemical detection with detection limits of *ca.* 20–100

pg, depending on the prelabelling reagent. The HPLC method is very convenient, highly sensitive and specific. Some examples of its application to the determination of natural BRs are presented below.

When a sufficient amount of BRs was found to be present in a plant material by the rice-lamina inclination test, a crude BR fraction could be used to identify known BRs as boronic acid derivatives. As an example, the identification of BRs in the pollens of broad bean (*Vicia faba* L.) [15] and sunflower (*Helianthus annuus* L.) [25] has been reported. The methanol extract of the respective pollen was subjected to solvent partitioning to obtain a chloroform-soluble neutral fraction. Purification of the fraction by silica gel column chromatography was followed by normal-phase preparative thin-layer chromatography (p-TLC) to give a highly bioactive fraction. The fraction was derivatized with 9-phenanthreneboronic acid and the resulting boronate derivative was subjected to reversed-phase HPLC with fluorimetric detection of (excitation at 305 nm, emission at 375 nm). Four BRs, BL (181 ng/g), dolichosterone (537 ng/g), 28-norCS (628 ng/g) and CS (134 ng/g), were identified in broad bean pollen (Fig. 8) and three

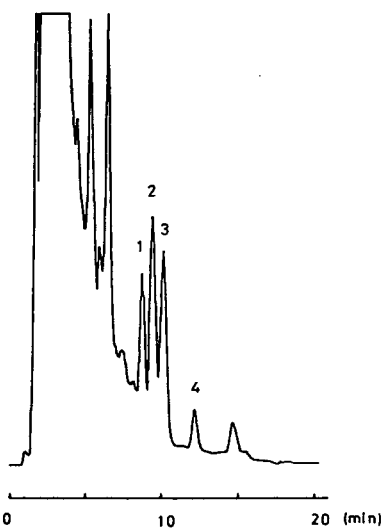

Fig. 8. Chromatogram of the 9-phenanthreneboronates of natural brassinosteroids extracted from the pollen of broad bean. Peaks: 1 = brassinolide; 2 = dolichosterone; 3 = norcastasterone; 4 = castasterone. Conditions as in Fig. 3.

BRs, 28-norCS (65 ng/g), CS (21 ng/g) and BL (106 ng/g), in sunflower pollen.

For comparison of the three fluorescence prelabelling reagents, 9-phenanthreneboronic acid, 1-cyanoisoindole-2-*m*-phenylboronic acid and dansylaminophenylboronic acid, and an electrochemical prelabelling reagent, ferroceneboronic acid, these four reagents were used in analysis for BRs contained in the bioactive fraction obtained from sunflower pollen [9]. Judging from the chromatograms obtained from the derivatized fractions, dansylaminophenylboronic acid is the most effective, because BR derivatives derived from this reagent can be detected at longer wavelengths than those from the other fluorescence reagents. A chromatogram obtained from the derivatives is less susceptible to interference from the matrix than that from the other derivatives, including ferroceneboronate derivatives [9].

As an example of the analysis of a plant material with a low content of BRs, the identification of BRs in buckwheat (*Fagopyrum esculentum* Moench) pollen [26] is described. As the content of BRs in the pollen was found to be very low by bioassay and the BRs were extremely contaminated with unknown natural products, several chromatographic methods, including silica gel adsorption chromatography, normal-phase p-TLC, activated charcoal chromatography and reversed-phase p-TLC, were employed. Aliquots of the bioactive fraction obtained were derivatized with 9-phenanthreneboronic acid and dansylaminophenylboronic acid, respectively. The resulting respective boronate derivatives were subjected to reversed-phase HPLC with fluorimetric detection (excitation at 305 nm, emission at 375 nm and excitation at 345 nm and emission at 515 nm, respectively). As shown in Fig. 9, BL (5.0 ng/g) and CS (7.1 ng/g) were determined in buckwheat pollen.

The results obtained for the three pollens showed a good correlation with those given by GC–MS and BRs identified in the three pollens were also rigorously characterized as bismethaneboronate derivatives by GC–MS [15,25,26]. Our HPLC method using prelabelling reagents is a convenient microanalytical method

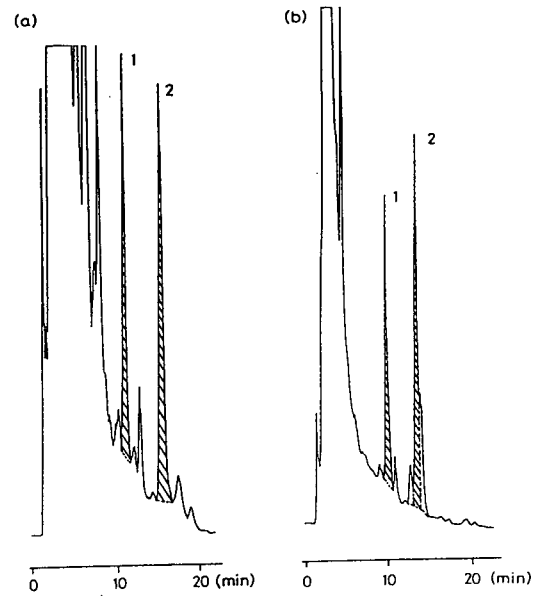

Fig. 9. Chromatograms of (a) 9-phenanthreneboronates and (b) dansylaminophenylboronates of natural brassinosteroids extracted from the pollen of buckwheat. Peaks: 1 = brassinolide; 2 = castasterone. Conditions as in Figs. 3 and 5.

and could be applicable to the identification and determination of trace levels of known BRs in small amounts of plant material because of its high sensitivity and specificity.

9. CONCLUSION

This review has summarized work on HPLC methods for the determination of BRs with UV, fluorimetric and electrochemical detection using boronic acid derivatives as derivatization reagents. We have demonstrated their usefulness in the identification and determination of several BRs in plants. As boronate derivatives were found to be highly sensitive, specific and suitable derivatives, this derivatization method for BRs in HPLC is very useful and ideally suited to this type of sample-limited natural product analysis and may be a suitable tool for use by agricultural and biological chemists interested in natural small amounts of BRs and their trace analysis.

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CHROMSYMP. 2820

Identification of brassinolide and castasterone in the pollen of orange (*Citrus sinensis* Osbeck) by high-performance liquid chromatography

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ABSTRACT

Brassinosteroids contained in the pollen of *Citrus sinensis* Osbeck were investigated. By using a rice lamina inclination test, a highly sensitive and specific bioassay for brassinosteroids, as a guide for purification, the pollen was extracted and the extract was subjected to solvent partitioning and subsequent purification by three chromatographic procedures. Two highly purified bioactive fractions were obtained and they were reacted with dansylaminophenylboronic acid. Each derivatized fraction was then analysed by reversed-phase high-performance liquid chromatography with fluorimetric detection. The bioactive compounds contained in the fraction were identified as brassinolide and castasterone by co-chromatography using authentic samples.

INTRODUCTION

Brassinosteroids (BRs) are a new class of plant growth hormone [1,2]. To date more than 30 natural BRs have been chemically characterized. It has been found that BRs occur in a wide variety of higher plants and also in some lower plants, and that pollens are the richest sources of BRs. In screening investigations, our microanalytical methods of analysing BRs by gas chromatography–mass spectrometry were very effective and they have contributed to a widening of our knowledge of the structural variation and distribution of BRs in the plant kingdom [3,4]. Recently, we have developed microanalytical methods of analysing BRs by high-performance liquid chromatography (HPLC) with fluorescence prelabelling reagents [5–8]. The HPLC method has been successfully applied to the identification of BRs in the pollens of broad bean [5], corn [8], sunflower [9] and buckwheat [10]. Of the prelabelling reagents developed for BRs, dansylaminophenylboronic acid is the most effective, because BR derivatives derived from this reagent can be detected at longer wavelength (excitation 345 nm/emission 515 nm) than those derived from the other fluorescence reagents. Therefore, the chromatogram obtained from the derivatives is subject to less interference from the matrix than those from the other derivatives. The HPLC method is very convenient, highly sensitive and specific for compounds with a vicinal diol function, such as BRs. As part of our research programme into the microanalysis of natural BRs and as an application of our HPLC microanalytical method to the identification of natural BRs, we have now

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investigated the pollen of *Citrus sinensis* Osbeck for the presence of BRs. The results are described in this paper.

EXPERIMENTAL

Plant material and chemicals

The bee-collected pollen of *Citrus sinensis* Osbeck was obtained from Spain and was kindly supplied by Tama Biochemical (Tokyo, Japan). Microscopic identification of the pollen was carried out by Dr. S. Ogawa of Joetsu University of Education. Authentic brassinolide and castasterone were synthesized as described previously [11].

Dansylaminophenylboronic acid was prepared as described in a previous paper [8]. All the other reagents were obtained from Wako (Osaka, Japan) and were of analytical grade.

Bioassay

Chromatographic purification of BRs was guided by the rice lamina inclination test. The bioassay was carried out according to the reported method [12], using etiolated seedlings of rice (*Oryza sativa* L. cv. Koshihikari).

Extraction of brassinosteroids

The pollen (100 g) of *Citrus sinensis* Osbeck was extracted with methanol (500 ml) for a week. The pollen residue was again extracted with methanol (300 ml) for another week. The combined extracts were concentrated *in vacuo* below 30°C to give an aqueous residue, to which was added ethyl acetate (250 ml) and water (250 ml). The mixture was partitioned and the organic phase was collected. The resulting water phase was then extracted twice with ethyl acetate (2 × 100 ml). The combined organic phases were concentrated to give an oil. The oil was dissolved in *n*-hexane (100 ml) and then partitioned with 90% methanol (100 ml). The separated aqueous methanol phase was collected. The remaining hexane phase was partitioned twice more with 90% methanol (2 × 50 ml). The combined aqueous methanol phases were concentrated and subsequently partitioned between chloroform (100 ml) and saturated sodium bicarbonate solution (100 ml). The aqueous phase was twice extracted with chloroform (2 × 50 ml). The combined chloroform phases were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to give an oily material (680 mg), which showed significant biological response in the bioassay. No other fractions were found to be biologically active.

Silica gel adsorption chromatography

One-half of the active oily material was charged onto a column (30 cm × 3.0 cm I.D.) of silica gel (E. Merck, Kieselgel 60, 70–230 mesh, Art. 7734). Elution was carried out stepwise with chloroform (200 ml) and then with chloroform–methanol (98:2, v/v, 200 ml; 95:5, 200 ml; 90:10, 200 ml; 85:15, 200 ml; 80:20, 200 ml; 70:30, 200 ml). The fractions were collected in 100-ml quantities, and an aliquot (1.0 ml) of each fraction was subjected to the bioassay. Biological activity appeared in the 10–15% methanol in chloroform eluates, and these fractions were combined and concentrated *in vacuo* to give an active fraction (50 mg).

Preparative thin-layer chromatography

The active fraction (50 mg) was applied to two precoated plates of reversed-phase silica gel (E. Merck, Kieselgel $60F_{254}$, 20 cm × 20 cm, 0.25-mm film thickness, Art. 5746). The plate was developed with methanol–water (3:1, v/v) as the developing solvent. The plate was dried and silica gel was scraped off into seven bands, and each was eluted with chloroform–methanol (15:1, v/v). Each eluate was adjusted to 50-ml quantities and one-hundredth of each fraction was subjected to the bioassay. Activity appeared in the regions of $R_F = 0.49–0.62$, this R_F value being in good agreement with those of authentic brassinolide and castasterone by co-chromatography. These fractions were combined and concentrated to give an active fraction (1.8 mg). This was then applied to a precoated plate of normal-phase silica gel (E. Merck, Kieselgel $60F_{254}$, 20 cm × 10 cm, 0.25-mm film thickness, Art. 5744). The plate was developed with chloroform–methanol (15:1, v/v) as the developing solvent. The plate was dried and silica gel was scraped off into ten bands, and each was eluted with chloroform–methanol (15:1, v/v). Each

eluate was adjusted to 50-ml quantities and one-hundredth of each fraction was subjected to the bioassay. Strong and weak activities appeared in the regions of $R_F = 0.20–0.28$ and $R_F = 0.28–0.35$, respectively, these R_F values being in good agreement with those of authentic brassinolide and castasterone, respectively, by co-chromatography. Each fraction was concentrated to give two highly purified bioactive fractions, fractions I (0.7 mg) and II (0.8 mg).

Derivatization procedure

As described previously [8], one-fifth of the active fractions I and II was derivatized with 100 μl of a solution of dansylaminophenylboronic acid (1.0 mg/ml) in pyridine–acetonitrile (1:99, v/v) at 70°C for 20 min. After cooling, an aliquot of the resulting solution was injected into the analytical column.

HPLC analysis

Our previous method [8] was slightly modified in terms of analytical column and the flow-rate of a mobile phase. A Shimadzu Model LC-6A chromatograph equipped with a fluorimetric detector (Shimadzu Model RF-530) was employed (excitation 345 nm, emission 515 nm). A reversed-phase column of STR ODS-H (150 mm × 4.6 mm I.D.) (Shimadzu Techno Research, Kyoto, Japan) was used at 45°C. Samples were injected into the column using a Rheodyne 7125 rotary valve syringe-loading injector. The mobile phase was acetonitrile–water (80:20, v/v) at a flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

The pollen of *Citrus sinensis* Osbeck was extracted with methanol and the extract was concentrated *in vacuo* below 30°C to give an aqueous residue. The residue was extracted with chloroform and concentrated to give an oil. The oil was partitioned between *n*-hexane and 90% methanol. The aqueous methanol phase was concentrated and further partitioned between chloroform and a saturated sodium bicarbonate solution. In a rice lamina inclination test, which is highly sensitive and specific to BRs, biological activity appeared only in the chloroform phase. The bioassay was used to guide the subsequent fractionation at each purification step. The bioactive phase was concentrated and then chromatographed on silica gel, eluting stepwise with increasing concentrations of methanol in chloroform. The eluates with 10–15% methanol in chloroform were found to be biologically active. These fractions were combined and concentrated, and further purified by reversed-phase preparative thin-layer chromatography (p-TLC), using methanol–water (3:1, v/v) as the developing solvent. Biological activity appeared in the region of $R_F = 0.49–0.62$, which corresponded to the R_F values of typical brassinosteroids (brassinolide and castasterone). These fractions were combined and concentrated to give a bioactive fraction. This fraction was then purified by normal-phase p-TLC, using chloroform–methanol (15:1, v/v) as the developing solvent. Strong and weak biological activities were found to be in the regions of $R_F = 0.20–0.28$ and $R_F = 0.28–0.35$, respectively, and these R_F values were in good agreement with those of authentic brassinolide and castasterone, respectively. Each fraction was concentrated to give two bioactive fractions, fractions I and II.

One-fifth of each bioactive fraction I and II was derivatized with dansylaminophenyl boronic acid and the resulting boronate derivatives were analysed by reversed-phase HPLC with a fluorimetric detection of excitation 345 nm/emission 515 nm, as previously described [8]. Under our HPLC conditions, the relationships between the peak areas and the amounts of the boronate derivatives of authentic brassinolide and castasterone were linear from 50 pg to 50 ng. The precision was established by repeated determinations ($n = 8$) and the relative standard deviations (R.S.D.) were 2.2 and 2.7%, respectively. As shown in Fig. 1a and b, peaks 1 and 2 were identified as those of the brassinolide and castasterone derivatives, respectively, by co-chromatography using the corresponding authentic dansylaminophenylboronate derivatives. The amounts of brassinolide and castasterone in the bioactive fractions were calculated by use of authentic samples for calibration and they were 36.2 ± 1.0 and 29.4 ± 0.7 μg per kg of pollen

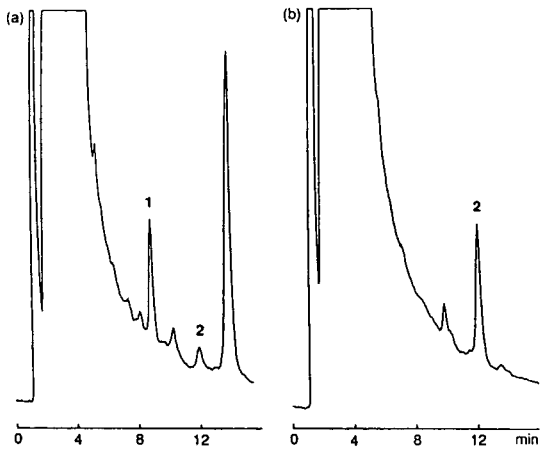
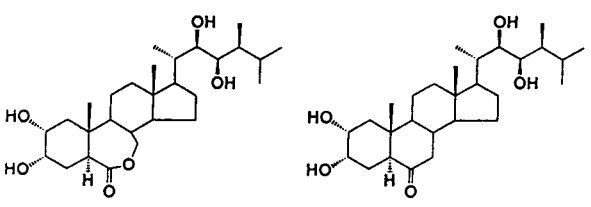


Fig. 1. Chromatograms of dansylaminophenylboronates of brassinosteroids, (a) bioactive fraction I; (b) bioactive fraction II, which were obtained from the pollen of *Citrus sinensis* Osbeck. Peaks: 1 = brassinolide, 2 = castasterone.

($n = 4$, mean ± S.D.), respectively. A recovery test was carried out by adding a mixture of 4 ng of brassinolide and 5 ng of castasterone to the divided bioactive fraction. The samples were derivatized as described above and analysed by HPLC. The recovery of the added steroids was more than 94% ($n = 4$; R.S.D. = 2.9%). It is evident from these data that the HPLC method is satisfactory in both accuracy and precision and that brassinolide and castasterone (Fig. 2) are unequivocally identified in the pollen of *Citrus sinensis* Osbeck.

With respect to the clean-up of the bioactive fraction, it was found that purification by reversed-phase p-TLC of bioactive fraction obtained from column chromatography on silica gel was very effective. The fraction obtained from the reversed-phase p-TLC seems to be sufficiently highly purified to be subjected to HPLC analysis, because it was found that an efficient purification was not attained by the subsequent purification by normal-phase p-TLC. Thus, for the small scale purification of BRs from natural sources, we recommend the use of the reversed-phase p-TLC, subsequent to column chromatography on silica gel.



Brassinolide Castasterone

Fig. 2. Structures of brassinosteroids identified in the pollen of *Citrus sinensis* Osbeck.

In conclusion, we were able to identify brassinolide and castasterone in the pollen of *Citrus sinensis* Osbeck by an HPLC analytical method using dansylaminophenylboronic acid as a prelabelling reagent. From the chemotaxonomical points of view, the present work is the first report of identification of brassinosteroids in *Rutaceae*. In addition to our previous works, this report describes a further demonstration that our HPLC method is convenient, highly sensitive, specific and effective for determining trace amounts of naturally occurring BRs.

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Review

Chromatographic procedures for phytoecdysteroids

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ABSTRACT

The complexity of the mixtures of ecdysteroids in plants and the close similarities in their chemical structures have challenged chemists to find suitable ways to separate and identify them. Great ingenuity has been applied to these problems and consequently a wide range of separation and methods are available today. These methods have been reviewed with assessment of their strengths and limitations, with the intention to guide investigators towards the methods most useful to their purpose.

CONTENTS

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1. INTRODUCTION

The short period between 1965 and 1967 was one of remarkable advances in the study of the insect moulting hormones or ecdysteroids. In 1965, eleven years after their initial isolation of the compound, Karlson *et al.* [1], using an improved isolation procedure, obtained sufficient crystalline ecdysone from silkworm pupae for Huber and Hoppe to determine that it was a polyhydroxysterol [2]. The next year Hampshire and Horn [3] showed their crustecdysone, isolated from a crayfish, *Jasus lalandii* was 20-hydroxyecdysone and Hocks and Wiechert [4] isolated 20-hydroxyecdysone from the silkworm *Bombyx mori*, but more important for our present subject, the data becoming available made others realize that compounds they were examining from plants had the same or similar structures. Very quickly Nakanishi *et al.* [5] reported ponasterone A from the leaves of the coniferous tree *Podocarpus nakai*, and Galbraith and Horn [6] isolated 20-hydroxyecdysone from the Australian pine *Podocarpus elatus*. In the following year Takemoto *et al.* [7] isolated 20-hydroxyecdysone and inokosterone from the common Japanese weed *Achyranthes fauriei* and Jizba *et al.* [8] reported the isolation of 20-hydroxyecdysone from the roots of the fern *Polypodium vulgare*.

Thus began the remarkable series of investigations of plants for ecdysteroids, which still continues today. Galbraith and Horn [6] suggested in their paper that the ecdysteroids in plants, or *phytoecdysteroids* may protect plants from insect attack. Thus, too began the controversy, still unsettled, over the function, if any, of ecdysteroids in plants.

The isolations referred to above were all carried out using solvent extractions, partitions and low-pressure column chromatography of various kinds, with only a little help from thin layer chromatography. The best of these methods are listed here. The most efficient methods of separation and the most sensitive methods of detection were developed subsequently, largely stimulated by the search through insect and plant materials for ecdysteroids.

2. SAMPLE PREPARATION

2.1. General considerations

The ecdysteroids form a group of rather polar compounds and as a consequence the initial extraction step is best performed using a polar solvent such as methanol (MeOH). Alternative solvents include ethanol (EtOH), acetone, acetonitrile and methanol–water mixtures. Supercritical fluid extraction would also be adequate, but it was not used up to now in this case. Having obtained and concentrated an extract, the next step usually involves one or more solvent partitions with the aim of removing the bulk of polar and non-polar contaminants prior to chromatography (Fig. 1).

2.2. Partition techniques

2.2.1. Solvent partitioning

In the early studies [9], the initial partition was made between an aqueous concentrate and *n*-butanol (BuOH). The BuOH residue, into which the ecdysteroids were extracted, was then partitioned between aqueous MeOH and hexane to remove non-polar material such as lipids. However, reversing this order of operation was found to be beneficial and led to reduced emulsion formation during the partition step and fewer problems with frothing during evaporation.

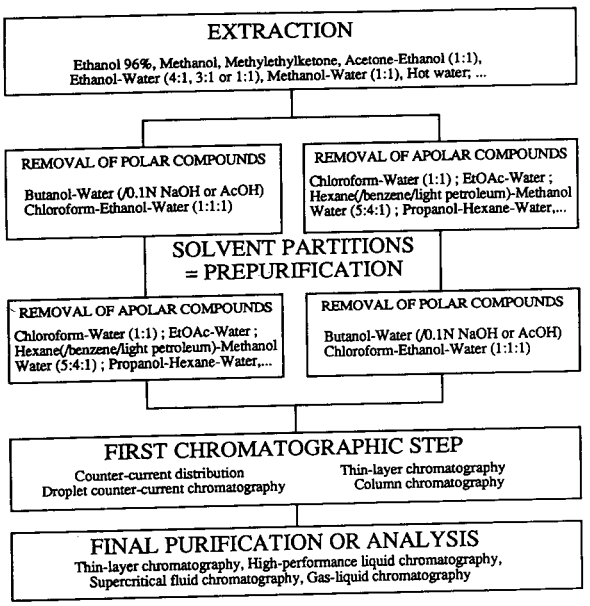

Fig. 1. General extraction and purification chart for ecdysteroids.

Other suitable partitioning systems for removing lipids include hexane–aqueous methanol (7:3, v/v) or light petroleum (b.p. 40–60°C)–aqueous methanol. Mixtures of water–propanol (PrOH) (3:1, v/v) and hexane can also be used to remove non-polar contaminants, the ecdysteroids remaining in the aqueous phase. The addition of $(NH_4)_2SO_4$ to promote the formation of the two phases may be required with the PrOH mixture. The separation of polar impurities from the ecdysteroids can be achieved by partition between water and BuOH (ecdysteroids partition into the organic phase) and water and ethyl acetate (EtOAc) (ecdysteroids remain in the aqueous phase).

The major factors governing the choice of solvent partition system are the type of contaminants to be removed (*i.e.* mainly lipids or mainly polar, etc.) and the nature of the ecdysteroids to be isolated. Thus the addition or removal of one OH group, or conjugation to polar (*e.g.* sulphate) or non-polar (*e.g.* acetate or fatty acyl) groups can significantly affect partition ratios. Partition coefficients of representative ecdysteroids and precursors in a number of systems are given in Table 1.

TABLE 1

PARTITION COEFFICIENTS OF ECDYSTEROIDS AND VARIOUS PRECURSORS

K = Concentration in the non-polar phase/concentration in the polar phase.

Ecdysteroid	K	Ref.
Cyclohexane–n-butanol–water (5:5:10)		
Ecdysone	3.54	71
Makisterone A	1.27	72
20-Hydroxyecdysone	0.52	71
3-Epi-20-hydroxyecdysone	0.52	72
26-Hydroxyecdysone	0.39	73
20,26-Dihydroxyecdysone	0.06	72
n-Butanol–water (1:1)		
Ecdysone	*ca.* 10	74
20-Hydroxyecdysone	5.3	9
Ethyl acetate–water (1:1)		
2,22-Dideoxyecdysone	20	116
2-Deoxyecdysone	4	
Ecdysone	0.4	
20-Hydroxyecdysone	0.1	
Chloroform–methanol–water (2:1:1)		
2,22-Dideoxyecdysone	13	116
2-Deoxyecdysone	2.7	
Ecdysone	0.4	
20-Hydroxyecdysone	0.1	
Chloroform–ethanol–water (1:1:1)		
Ecdysone	4.6	116
20-Hydroxyecdysone	1.5	
Chloroform–water (1:1)		
2,22,25-Trideoxyecdysone (Ketodiol)	90	116
2,22-Dideoxyecdysone	20	
2-Deoxyecdysone	2.9	
Ecdysone	0.06	
20-Hydroxyecdysone	0.015	
Hexane–acetonitrile (1:1)		
Cholesterol	1.5	116
2,22,25-Trideoxyecdysone	0.06	
2,22-Dideoxyecdysone	<0.01	
2-Deoxyecdysone	<0.01	
Ecdysone	<0.01	

2.2.2. Liquid–liquid partition techniques

2.2.2.1. Counter-current distribution (CCD). Counter-current distribution between BuOH and water is effective for removing polar contami-

nants (the addition of a small amount of salt reduces emulsion formation) and $CHCl_3$–MeOH–water or hexane–PrOH–water.

This technique, as is the case for all other liquid–liquid partitions systems, has the advantage of a 100% sample recovery. The technique is not of wide use at the present time. It has been advantageously replaced by the more recent DCCC and RLCC techniques (see below).

2.2.2.2. Droplet counter-current chromatography (DCCC). This technique provides an efficient means for purifying samples up to the gram range. It belongs to the family of liquid–liquid partition chromatographic methods.

DCCC equipment is compact, it can be used with rather crude samples, and in favourable cases it can allow the preparation of "pure" compounds [10–13]. In our hands, it seemed however to require a subsequent HPLC step in order to get fully pure ecdysteroids. Several systems in the ascending or descending mode have been described (Table 2).

Rotation locular counter-current chromatography (RLCC) was designed as an alternative to DCCC [14]. It was applied to the purification of *Vitex strickeri* methanolic extracts [15], in combination with recycling HPLC.

Although efficient, DCCC and RLCCC are rather time-consuming, and processing a single sample usually requires 1–5 days [14]. This is because of the need to allow good exchange to proceed between the mobile droplets and the stationary phase. To overcome this drawback, HSCCC (high-speed counter-current chromatography) was proposed [14]. In that case, the column is a multi-layer coil and efficient mixing is achieved through planetary rotation. Centrifugal force replaces normal gravity and allows separation to be achieved in hours rather than days.

2.3. Low-pressure column chromatography

2.3.1. Analytical scale: disposable cartridges for sample clean-up

2.3.1.1. Normal-phase cartridges. The use of low pressure chromatography on a small column has been used very early in phytoecdysteroid research for the fractionation of crude extracts. It was at that time either silica or alumina which was used in such columns (normal phase systems), generally eluted with binary mixtures —a step-gradient of alcohol in chloroform or benzene. Use of these disposable cartridges is now generally called solid-phase extraction (SPE).

2.3.1.2. Reversed-phase cartridges. The availability of hydrophobic phases (resins like Amberlite or hydrocarbon-bonded silica) had led to a complete renewal of the procedures [16]. Moreover, the design of small cartridges or syringes containing 0.2–1 g of HPLC phase has led to an ideally suited material for a rapid clean-up of small samples.

Among them, Sep-Pak cartridges from Waters are the most widely used (Fig. 2 [17–21]. In fact their use has been extended not only for biological extracts but also for desalting purposes, *e.g.* direct adsorption of ecdysteroids from cul-

TABLE 2

SOLVENT SYSTEMS FOR DROPLET COUNTER-CURRENT CHROMATOGRAPHY

A = Ascending mode; D = descending mode.

Solvent system	Mode	Ref.
$CHCl_3$–MeOH–water (13:7:4)	A	10, 11, 13, 50, 75
$CHCl_3$–C_6H_6–EtOAc–MeOH–water (45:2:3:60:40)	D	23
C_6H_6–$CHCl_3$–MeOH–water (5:5:7:2)	D	24
$CHCl_3$–MeOH–water (65:20:20)	D	24, 50

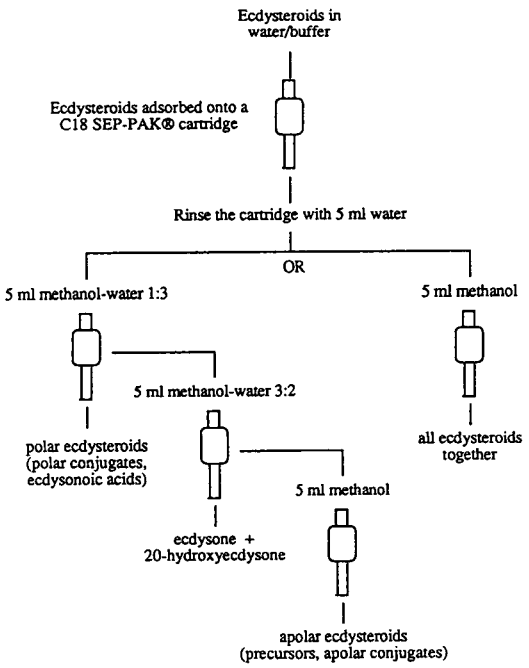

Fig. 2. Utilization of reversed-phase cartridges for ecdysteroid purification.

ture media or from reverse phase HPLC fractions containing an involatile buffer.

The use of C_{18} cartridges may be extended to more refined separations, provided that rigorous protocols are used regarding solvent composition and volume. By that way, it appears possible to separate ecdysteroids that differ by the presence or absence of a single –OH group (provided that its position on the molecule evokes a significant polarity change).

2.3.1.3. Immobilized phenylboronic acid (PBA) cartridges. Recently, cartridges filled with immobilized phenylboronic acid on silica, agarose or cellulose have become available. Such systems are able to retain selectively some compounds bearing vicinal diols. Ecdysteroids may contain such diol functions, *i.e.* at C2–C3, C20–C22 and/or C25–C26. Up to now, experiments have been made with compounds bearing no 26-OH, and therefore were limited to the two first cases [22].

It appears that the 2,3-diol does not readily form cyclic boronates (at least with a 2-OH_{ax} and a 3-OH_{eq}), and the adsorption process is essentially determined by the presence or absence of the 20,22-diol, *e.g.* it is particularly efficient to distinguish between ecdysone and 20-hydroxyecdysone series. It is thus possible to adsorb ecdysteroids and to elute them sequentially, first ecdysone-type and then 20-hydroxyecdysone-type compounds (Fig. 3).

This procedure can be combined with other solid phase systems to separate various classes of ecdysteroids [22] and it could have several specific developments.

2.4. Preparative scale

Low-pressure column chromatography uses either normal phases or (less often) reversed phases (Table 3). The size of the column has to be related to the size of samples (at least ten times the dry weight of the sample), and elution is usually performed with a step-gradient. Most often, silica or alumina are used with a step-gradient of methanol or ethanol in a chlorinated solvent (chloroform or methylene chloride). In the reversed-phase mode, Sephadex LH-20 may be used to remove non-polar contaminants, and polyamide is used specifically to remove yellow pigments.

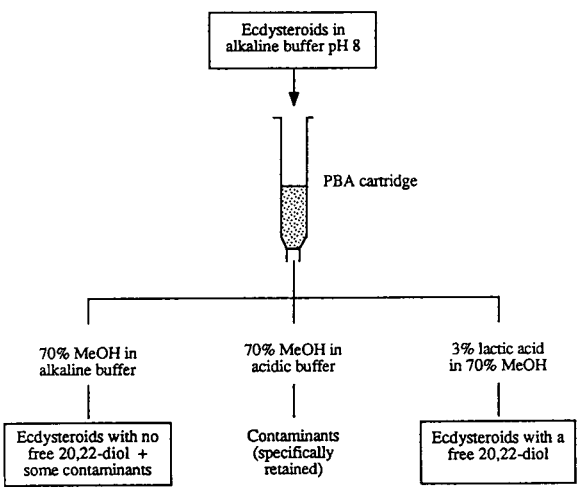

Fig. 3. Extraction procedure for ecdysteroids using immobilized phenylboronic acid cartridges.

TABLE 3

USE OF LOW-PRESSURE CHROMATOGRAPHY FOR MEDIUM- OR LARGE-SCALE PURIFICATION

Type of stationary phase	Solvent system	Ref.
Normal phases		
Silica (silica gel, silicic acid or celite)	$CHCl_3$–MeOH (step gradient)	76
	$CHCl_3$–MeOH (step gradient)	77
	$CHCl_3$–MeOH (step gradient)	78
	$CHCl_3$–MeOH (step gradient) (+1% CH_3COOH)	79
	$CHCl_3$–MeOH (8:2)	80
	$CHCl_3$–MeOH (95:5)	81
	$CHCl_3$–MeOH (100:3)	82
	$CHCl_3$–EtOH (19:1)	83
	Me_2CO–CH_2Cl_2–water (2:8:1), then EtOH	84
	$CHCl_3$–MeOH–water (8:2:1 or 7.5:2:1, lower phase)	85
	CH_2Cl_2–EtOH (step gradient)	49
	EtOAc	86
	EtOAc–MeOH (step gradient)	87
Alumina (containing usually 10% water)	$CHCl_3$–MeOH (step gradient)	77
	$CHCl_3$–MeOH (step gradient)	78
	$CHCl_3$–MeOH (step gradient)	88
	$CHCl_3$–MeOH (step gradient)	89
	$CHCl_3$–MeOH (2:1)	90
	$CHCl_3$–EtOH (step gradient)	91
	CH_2Cl_2–EtOH (step gradient)	92, 93
	EtOAc–EtOH (step gradient)	94
	Me_2CO–CH_2Cl_2–water (62.5:15:10)	84
	CH_2Cl_2–EtOH (9:1)	95
	EtOAc–MeOH (1:1)	76
	EtOAc–EtOH (1:1)	83
	EtOAc–EtOH (2:1)	96
Sephadex LH-20	CH_2Cl_2–Me_2CO	83, 97
	CH_2Cl_2–MeOH (step gradient)	83, 97
	$CHCl_3$–EtOH (88:12)	96
Reversed-phases[a]		
Amberlite XAD-2 (medium-pressure LC)	Water–MeOH (step gradient)	98, 99
Sephadex LH-20	EtOH–water (7:3)	81
	MeOH	85, 92, 93
Celite impregnated with BuOH + cyclohexane	Water (saturated with BuOH + cyclohexane)	76
Polyamide (for removal of flavonoids)	Water	80, 93, 100

[a] RP is used in a broad sense, to include less conventional systems.

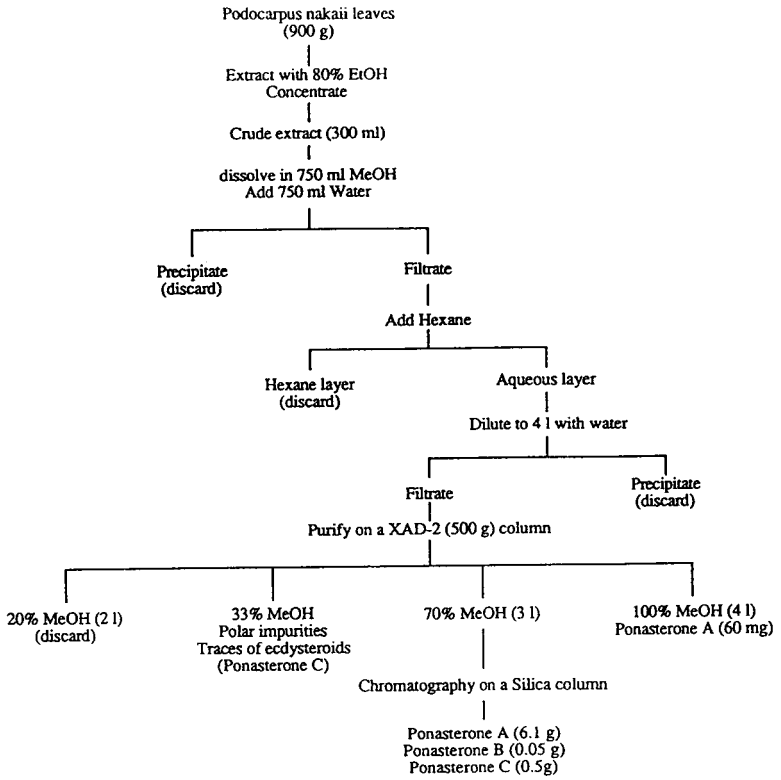

Fig. 4. Isolation of ecdysteroids from *Podocarpus nakaii* leaves [16].

2.5. Selected examples of protocols

Some representative protocols are given in Figs. 4–7, that each use a part of the above techniques. Other complex processing schemes can be found elsewhere [23–25].

3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC is the most popular technique for ecdysteroid separations, both for analytical and preparative purposes. It offers wide choice of techniques, that are adapted for polar or non-polar metabolites. The identification of any ecdysteroid by co-migration with a reference compound must rely on the simultaneous use of several (at least two) different HPLC systems, generally one normal-phase and one reversed-phase system. Given the very large ecdysteroid family, there is no guarantee that these criteria provide unambiguous evidence for the identity of a given compound.

When facing a separation problem it is possible to make a logical choice, according to the results of a preliminary TLC step. Current HPLC techniques have been designed about ten years ago and the HPLC of ecdysteroids may now be considered to be a mature technique (for reviews see refs. 26–31).

3.1. Correspondence between TLC and HPLC

It is known that relationships between TLC and HPLC may help to design adequate HPLC solvent systems. The migration of a given compound by TLC is given by its R_F (=retardation factor). Another parameter also used for TLC is the R_M, defined as

$$R_M = \log(1/R_F - 1)$$

Any change on a molecule evokes a change of

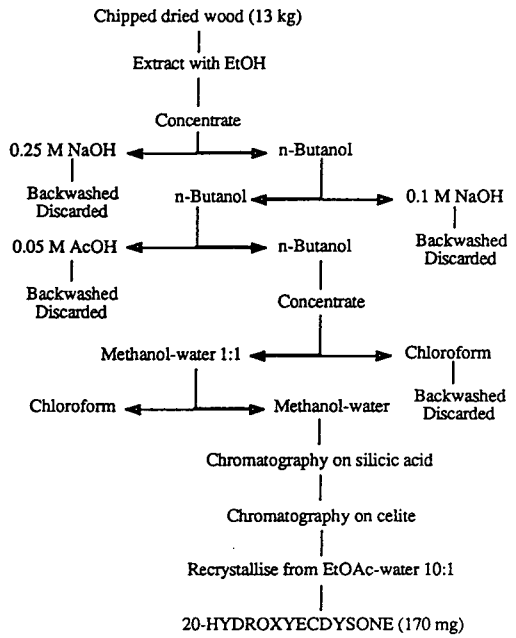

Fig. 5. Isolation of 20-hydroxyecdysone from *Podocarpus* bark [115].

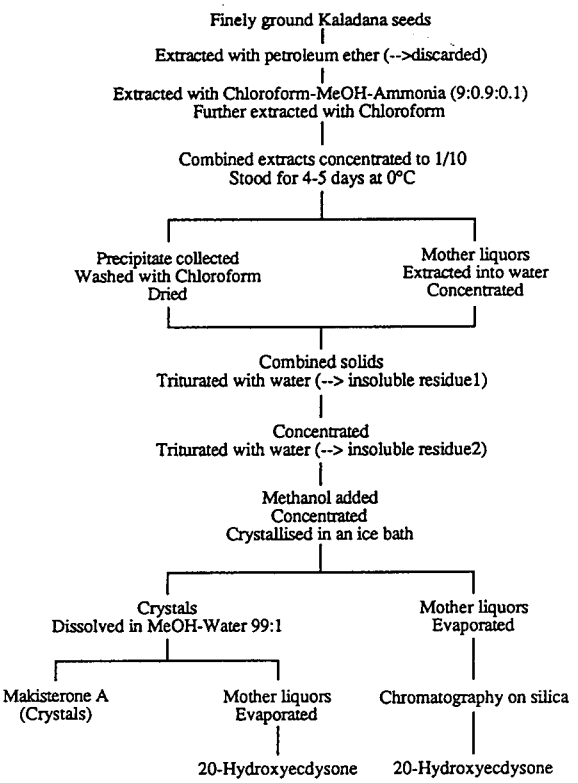

Fig. 6. Isolation of ecdysteroids from *Kaladana* seeds [108].

the R_F or R_M and the same change on two molecules of the same family may evoke the same ΔR_M. Accordingly, the ΔR_M values are cumulative, and two independent changes 1 and 2 evoke a final $\Delta R_M = \Delta R_{M1} + \Delta R_{M2}$.

When using HPLC, the behaviour of a given compound is expressed by its retention time (or volume) t_R or better by its capacity factor k', defined as $k' = (t_R - t_0/t_0)$, where t_0 is the retention time of a compound eluted in the void volume. The correspondence with TLC is easy to make, as

$t_R = t_0/R_F$, $k' = (1/R_F - 1)$ and thus $R_M = \log k'$

Therefore, provided that the chromatographic conditions and the phases are the same, it is easy to deduce the HPLC behaviour from the TLC data, and the relationship between R_F and k' is exemplified in Fig. 8. It is clearly apparent from it that HPLC solvents might be chosen from among those giving R_F between 0.1 and 0.3, in order to avoid a too rapid elution or extended analysis times.

Moreover, the selectivity factor α for a pair of compounds 1 and 2 analyzed by HPLC can be defined as $\alpha = k'_2/k'_1$, and from this

$\Delta R_M = \log k'_2 - \log k'_1 = \log(k'_2/k'_1)$, or

$\Delta R_M = \log \alpha$

The consequence of this is that a given modification (*e.g.* presence or absence of a given –OH group) evokes a similar effect on various compound pairs; for instance the α value for the ecdysone:20-hydroxyecdysone pair is the same as for the 2-deoxyecdysone:2-deoxy-20-hydroxyecdysone one, provided of course that *isocratic* conditions are used. Moreover, it seems possible to determine the expected retention time of a compound where no reference is available [32]. Similar calculations apply to acetates, conjugates, etc. and may prove useful in many cases.

3.2. Chromatographic procedures (Table 4)

3.2.1. Normal-phase systems

Normal-phase (NP) systems generally use silica columns (sometimes aminopropyl- or diol-bonded columns) and a standard system of

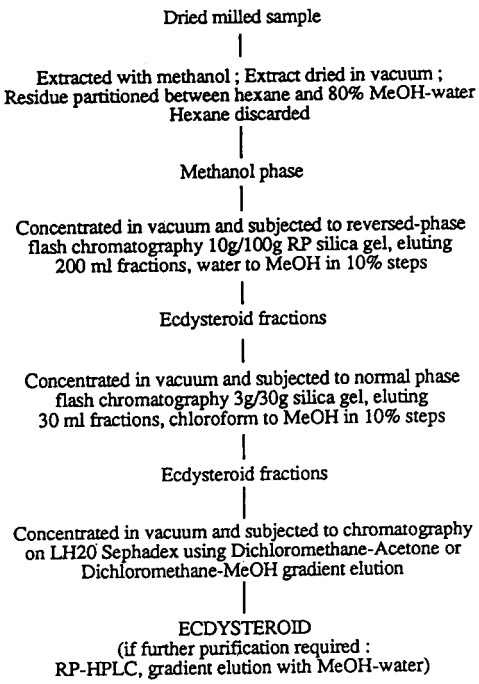

Fig. 7. Isolation of ecdysteroids from plants (after Russel and Greenwood [97]).

dichloromethane–isopropanol–water mixtures as initially designed by Lafont *et al.* [33]. The respective proportions of the three components can vary, according to sample polarity. Thus, specific ternary mixtures can be prepared for non-polar compounds, *e.g.* acetates or ecdysone precursors (*e.g.* 125:15:1, v/v/v) for medium-polarity compounds (125:25:2 or 125:30:2 for ecdysone and 20-hydroxyecdysone) or for more polar ecdysteroids (125:40:3 for 26-hydroxy-ecdysteroids; 100:40:3 for glucosides).

With normal-phase systems, k' is particularly sensitive to temperature. Low temperatures may result in greatly *decreased* retention times, possibly because they reduce ecdysteroid solubility in the stationary water phase adsorbed onto the silica particles. This effect is particularly striking between 10°C and 20°C. As a consequence, it is highly recommended to place the HPLC system in a room where temperature is controlled.

Polar-bonded columns (diol or aminopropyl

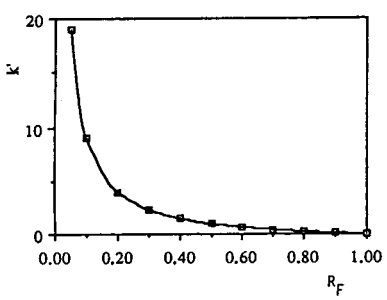

Fig. 8. Inverse relationship between k' (HPLC) and R_F (TLC).

TABLE 4

CHROMATOGRAPHIC SYSTEMS COMMONLY USED FOR THE HPLC ANALYSIS OF ECDYSTEROIDS

See also ref. 26 for a more extensive review of data before 1980.

Mode of chromatography	Ref.
Normal-phase chromatography (silica or diol-, aminopropyl or TMS-bonded silica)	
Chloroform–95% aqueous ethanol	101
Chloroform–ethanol (gradient)	96
Chloroform–isopropanol (gradient)	81
Chloroform–methanol	79
Dichloromethane–tetrahydrofuran–methanol	42
Dichloromethane–methanol	42
Dichloromethane–ethanol–water	33
Dichloromethane–isopropanol–water	33
Dichloromethane–methanol–water–acetic acid	103
Dichloroethane–methanol–isopropanol	
Hexane–ethanol–methanol–acetonitrile	104
Isooctane–isopropanol–water	105
Isooctane–isopropanol–water (with grad. methanol)	38
Cyclohexane–isopropanol–water	32
Reversed-phase chromatography [Amberlite XAD-2 or (mainly) C_{18} bonded silica]	
Ethanol–water	98
Methanol–water	16
Methanol–water	106
Methanol–water	39
Methanol–water	85
Acetonitrile–water	107
Acetonitrile–water	81
Acetonitrile–water	39
Acetonitrile–methanol–water	75
Acetonitrile–0.1% TFA	75
Dioxan–water	39
Tetrahydrofuran–water	39
Methanol–triethylammonium phosphate + 1-butanesulfonic acid	95
Isopropanol–water, 50°C	108
Recycling HPLC (Asahipack GS-320)	
Methanol	15, 40

silica) can also be used instead of silica ones [27,34]. Diol-bonded columns have been used with *Chenopodium album* and *Kochia scoparia* (Chenopodiaceae) extracts [35,36] whereas aminopropyl ones proved particularly efficient for the separation of mixtures of 3α-OH, 3β-OH and 3-oxoecdysteroids [37] —indeed these separations were better than those achieved on silica columns [3]. In addition, bonded phases allow the use of gradients without the problems linked with long re-equilibration times encountered with silica columns.

Non-polar bonded-phase columns can also be used with the above solvents, and they provide somewhat efficient separations with reduced retention times [38]. In this respect, trimethylsilane (TMS) bonded phases seem particularly interesting: they give very symmetrical peaks and a selectivity that differs from silica columns. The retention of ecdysteroids on non-polar bonded columns results from the presence of remaining free silanol groups on bonded phases (this becomes especially evident when columns have previously been used over a long period) which could in theory permit both NP- and RP-HPLC with a single column! There exist some HPLC columns (cyanopropyl or nitrile-bonded) that have been designed both for NP and RP purposes and these might probably be used for normal-phase chromatography of polar (but of course non-ionic) ecdysteroids.

Dichloromethane-based solvents, although very efficient for chromatographic separations, suffer from a high UV-cutoff and quenching properties of this compound, which preclude the use of diode-array detectors or in-line radioactivity monitors. This problem may be overcome with isooctane-based mixtures [29] which in counterpart suffer from a lower efficiency (plate number) and poor ecdysteroid solubility, which may become inconvenient for (1) polar ecdysteroids and (2) preparative purposes. The selectivity of such solvent systems is also very different from that of dichloromethane-based ones. More recently, [32] cyclohexane-based mixtures were tested: they provide much better solubilities and allow the use of diode-array detectors. On the other hand, these mixtures display a significantly higher viscosity and hence working pressure, a problem that can be overcome by increasing solvent temperature.

3.2.2. *Reversed-phase systems*

Reversed-phase HPLC with C_{18}-bonded columns is the most widely used system, and it provides efficient separations. Methanol-water mixtures are usual, and they represent the easiest way to obtain satisfactory separations, even if they are not the most efficient ones. Other water-miscible organic solvents may equally be used, and acetonitrile provides the advantage of forming mixtures with water that have a much lower viscosity. On the other hand, 50% methanol is a much better solvent than 20–25% acetonitrile for preparative purposes.

Selectivity linked with the nature of the organic phase is an important parameter for determining the most adequate solvent mixture for a given separation: depending upon the specific problem, better results may be obtained either with methanol, acetonitrile or THF [32,39].

Recycling HPLC using methanol as eluent may provide an interesting method for the resolution of closely migrating compounds and it was successfully used for the separation of ecdysteroids from the bark of *Vitex strickeri* [15]. This method appears to have several advantages, regarding its ability to separate closely related compounds at the preparative scale and its low solvent consumption [40].

4. PLANAR CHROMATOGRAPHY

Planar chromatography, particularly thin-layer chromatography (TLC) has been used extensively for the qualitative analysis and isolation of phytoecdysteroids. Paper chromatography, whilst now generally considered to be obsolete, has been used for the separation of ecdysteroids [41] and for completeness these results are presented in Table 5.

NP-TLC systems using silica as stationary phase have most frequently been used to separate ecdysteroids. A widely used general solvent system has been chloroform–ethanol (4:1, v/v) with a single ascending development, however, many normal phase solvent systems have been described for these and details of a number of

TABLE 5

R_F OF ECDYSTEROIDS ON PAPER CHROMATOGRAPHY [41]

Solvent systems are prepared by saturating the non-polar component (A) with the mixture (B + C) of polar solvents.

Compound	Solvent system			Ratio B:C	R_F
	A	B	C		
Ecdysone	Benzene	propan-2-ol	water	55:45	0.69
	Toluene	propan-2-ol	water	50:50	0.34
	Toluene	butan-2-ol	water	50:50	0.79
	Water	butan-1-ol		Saturated[a]	0.68
20-Hydroxyecdysone	Benzene	propan-2-ol	water	55:45	0.34
	Benzene	butan-2-ol	water	55:45	0.29
	Toluene	butan-2-ol	water	50:50	0.45
	Toluene	butan-2-ol	water	60:40	0.29
	Toluene	propan-2-ol	water	50:50	0.12
	Water	butan-1-ol		Saturated[a]	0.80
	Butan-1-ol	water		Saturated[a]	0.86
20-Hydroxyecdysone 2-acetate	Toluene	propan-2-ol	water	50:50	0.46
Inokosterone	Benzene	propan-2-ol	water	55:45	0.45
	Benzene	butan-2-ol	water	55:45	0.38
	Toluene	butan-2-ol	water	50:50	0.53
	Toluene	butan-2-ol	water	60:40	0.37
	Toluene	propan-2-ol	water	50:50	0.15
	Water	butan-1-ol		Saturated[a]	0.74
	Butan-1-ol	water		Saturated[a]	0.88
Makisterone A	Benzene	propan-2-ol	water	55:45	0.52
	Toluene	propan-2-ol	water	50:50	0.22
	Toluene	butan-2-ol	water	50:50	0.64
Ponasterone A	Benzene	propan-2-ol	water	55:45	0.93
	Toluene	propan-2-ol	water	50:50	0.74
	Toluene	butan-2-ol	water	50:50	0.92

[a] First liquid saturated with the second.

solvent systems used to separate the ecdysteroids are given in Table 6 [42]. The R_F values obtained for some 20 compounds in the chloroform-ethanol (4:1) solvent system are provided in Table 7 [43]. Following separation by NPTLC the detection of ecdysteroids on TLC plates can be accomplished in a number of ways. Where the plates incorporate a suitable fluorescence indicator that can provide a general, if rather non-specific method of detection. Spray reagents have been used to provide a higher degree of specificity, and the vanillin-sulphuric acid reagent has been particularly widely employed. This reagent gives a range of colour with different ecdysteroids as indicated in Table 8. The colours produced range from pink (*e.g.* cyasterone) and red (*e.g.* 20-hydroxyecdysone) to dark green (*e.g.* ecdysone), but like many such colour reactions are subject to quite wide inter-laboratory variations. Table 8 also lists a number of additional chromogenic reactions which have been utilised for these compounds. Scanning densitometry now provides a convenient method for obtaining *in situ* UV spectra, and this can provide increased confidence in the identification of sample components as ecdysteroids (*e.g.* see

TABLE 6

SOLVENT SYSTEMS FOR TLC OF ECDYSTEROIDS ON SILICA GEL

Data from ref. 42.

Solvent system	Composition	R_F	
		Ecdysone	20-Hydroxyecdysone
$CHCl_3$–95% aqueous EtOH	7:3	0.39	0.34
$CHCl_3$–95% aqueous EtOH	13:7	–	0.31
$CHCl_3$–EtOH	3:2	–	0.5
$CHCl_3$–MeOH	9:1	0.10	0.07
$CHCl_3$–MeOH	5:1	–	0.23
$CHCl_3$–MeOH	3:2	–	0.40
$CHCl_3$–MeOH	1:1	–	0.55
$CHCl_3$–MeOH–25% aqueous NH_3–water	12:7:1:1	–	–
$CHCl_3$–MeOH–AcOH	4:1:0.05	–	0.36
$CHCl_3$–propan-1-ol	9:5	0.21	0.12
$CHCl_3$–MeOH–Me_2CO	6:2:1	–	0.48
$CHCl_3$–MeOH–Me_2CO	6:2:1	–	0.33
CH_2Cl_2–Me_2CO–MeOH	2:1:1	0.69	0.62
CH_2Cl_2–Me_2CO–EtOH	16:4:5	0.32	0.10
CH_2Cl_2–MeOH–C_6H_6	25:5:3	–	0.19
CH_2Cl_2–Me_2CO–water	15:62.5:10	0.65	–
CH_2Cl_2–MeOH–water	7.9:15.1	0.32	0.19
CH_2Cl_2–MeOH–25% aqueous NH_3–water	77:20:2:1	0.47	0.40
CH_2Cl_2–MeOH	7:3	–	–
C_6H_6–MeOH	7:3	0.40	–
EtOAc–EtOH	4:1	–	0.60
EtOAc–EtOH	4:1	0.49	0.46
EtOAc–EtOH	4:1	–	0.32
EtOAc–EtOH–water	2:8:1	–	–
EtOAc–MeOH–NH_3–water	10:2:1:1	–	–

Fig. 9). As discussed in more detail later, *in situ* mass spectrometry has also been used to good effect for the identification of phytoecdysteroids following NP-TLC.

RP-TLC chromatography on bonded phases (C_2, C_8, C_{12}, C_{18}, aminopropyl and cyanopropyl) as well as on paraffin-impregnated silica gel (*e.g.* refs. 39, 44 and 45) has also been employed for ecdysteroids and typical results, obtained using various C_{18} bonded phases, for a range of structures are provided in Table 9 [45]. In general, methanol–water (1:1, v/v) solvent systems provide good separations but other organic modifiers can be used to achieve different selectivities [44]. Reversed-phase systems, like their HPLC equivalents are poor at separating 5β-OH compounds from their 5β-H analogues (*e.g.* polypodine B from 20-hydroxyecdysone). The effects of various substitution patterns on the RP-TLC of ecdysteroids are discussed in detail elsewhere [45]. Detection methods following RP-TLC are essentially the same as those employed for NP-TLC separations, although RP-TLC–MS has not proved to be so readily achieved as NP-TLC–MS.

Separations can also be modified, in both NP- and RP-TLC systems by esterifying 20,22-diol-containing ecdysteroids with boronic acids (*e.g.* phenylboronic acid) [46,47]. This has the effect of reducing the polarity of such esters with a consequent effect on the chromatographic separation of an ecdysteroid mixture. This can be

TABLE 7

R_F AND $R_{ecdysone}$ VALUES OBTAINED FOR ECDYSTEROIDS BY NP-TLC ON SILICA GEL TLC PLATES USING CHLOROFORM–ETHANOL (4:1)

Data from ref. 43.

Compound	R_F	$R_{ecdysone}$	Compound	R_F	$R_{ecdysone}$
Poststerone	0.32	152	22-Isoecdysone	0.13	62
2-Deoxyecdysone	0.38	180	Calonysterone	0.42	200
Ecdysone	0.21	100	Pterosterone	0.32	152
20-Hydroxyecdysone	0.15	71	Kaladasterone	0.49	233
Muristerone A	0.27	129	Pinnasterol	0.56	267
Dacrysterone	0.27	129	20-Hydroxyecdysone 2-cinnamate	0.53	252
2-Deoxy-20-hydroxyecdysone	0.31	141	Polypodine B 2-cinnamate	0.56	267
Makisterone A	0.20	95	Acetylpinnasterol	0.68	324
Polypodine B	0.22	104	Ponasterone C	0.38	181
Inokosterone	0.17	77	Ponasterone C 2-cinnamate	0.65	310
2-Deoxy-3-epiecdysone	0.44	209	Ponasterone A	0.42	200
Ajugasterone C	0.22	104	Carpesterol	0.86	410
Cyasterone	0.33	157			

used to good effect to resolve ponasterone A and 2-deoxyecdysone, which are otherwise difficult to separate [4,6].

Of the more recently developed techniques for planar chromatography, automated multiple development (AMD) and over-pressure layer chromatography (OPLC) have been applied to the resolution of ecdysteroids [48,49]. AMD provided good separations but was somewhat time-consuming [48]. The use of OPLC for the separation of phytoecdysteroids of *Silene nutans* [49] enabled very rapid analysis to be performed, and it could easily be used for preparative work. However, the time required for plate preparation, etc., means that this is unlikely to supplant conventional separation methods.

The current practice of TLC for the analysis of phytoecdysteroids is to use it for qualitative applications such as monitoring extractions and purification (see refs. 50 and 51 for some recent examples). In these applications the ease of TLC and the generally rugged nature of the separation system are well suited to a rapid and high throughput of often difficult samples. However, there is no practical reason why scanning densitometry could not be used for quantitative analysis of plant extracts expecially with the high concentrations of material frequently encountered in such samples. It therefore seems likely that, with the wider availability of scanning densitometers that the use of TLC and HPTLC for quantitative analysis of these compounds will increase.

5. GAS CHROMATOGRAPHY

Ecdysteroids are too polar and have too little thermal stability to be suitable subjects for gas chromatography (GC) but by protecting some or all of the hydroxyl groups as silyl ethers they can be thermally stabilized and their polarity reduced so that they can be chromatographed in the gaseous phase. The gas chromatography of ecdysone as a trimethylsilyl ether derivative was first described by Katz and Lensky [52], although the exact nature of the product was not stated. Morgan and Woodbridge [53,54] developed conditions for the preparation of trimethylstyl ether O-methyloximes for the quantitative study of ecdysteroids, but later it was found that protection of the ketone group as an O-methyloxime was not necessary. Poole *et al.* [55] and Borst

TABLE 8

REACTIONS OF ECDYSTEROIDS AND THEIR DERIVATIVES WITH SPRAY REAGENTS

Compound	Reagent	Colour	Ref.
Cyasterone	Vanillin–sulphuric acid	Pink	9
Dacrysterone	Vanillin–sulphuric acid	Mauve-brown	109
2-Deoxy-20-hydroxyecdysone	Vanillin–sulphuric acid	Olive green then yellow	9
Ecdysone	Vanillin–sulphuric acid	Blue then red brown	9
	Vanillin–sulphuric acid	Turquoise	9
	Vanillin–sulphuric acid	Red-brown	110
	2,4-Dinitropheny hydrazine, then $K_3Fe(CN)_6$	Slightly yellow	110
	Phosphotungstic acid	Blue	110
20,26-Dihydroxyecdysone	Vanillin–sulphuric acid	Turquoise	111
20-Hydroxyecdysone	Vanillin–sulphuric acid	Olive green then brown	9
	Vanillin–sulphuric acid	Yellow-green	9
	Vanillin–sulphuric acid	Turquoise-grey blue	110
	2,4-Dinitrophenyl hydrazine, then $K_3Fe(CN)_6$	Slightly yellow	
	Phosphotungstic acid	Blue	
20-Hydroxyecdysone 2-cinnamate	Vanillin–sulphuric acid	Olive green	112
Inokosterone	Vanillin–sulphuric acid	Orange	9
Makisterone A	Vanillin–sulphuric acid	Mauve-brown	9
	Vanillin–sulphuric acid	Mauve-brown	109
Makisterone C	Vanillin–sulphuric acid	Dark green	9
	Vanillin–sulphuric acid	Green	109
Ponasterone A	Vanillin–sulphuric acid	Mauve then grey-green	9
Ponasterone B	Vanillin–sulphuric acid	Blue-mauve then brown	9
Ponasterone C	Vanillin–sulphuric acid	Green then brown	9
	Vanillin–sulphuric acid	Dark green	112
Ponasterone C 2-cinnamate	Vanillin–sulphuric acid	Dark green	112
Polypodine B 2-cinnamate	Vanillin–sulphuric acid	Olive green	112
Pterosterone	Vanillin–sulphuric acid	Dark green	112
		Green	9
Rubrosterone	Vanillin–sulphuric acid	Brown	9
20-Hydroxyecdysone 2-acetate	Vanillin–sulphuric acid	Olive green then yellow	113
20-Hydroxyecdysone 3-acetate	Vanillin–sulphuric acid	Olive green then yellow	113
20-Hydroxyecdysone 22-acetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 2,3-diacetate	Vanillin–sulphuric acid	Olive green then yellow	113
20-Hydroxyecdysone 2,22-diacetate	Vanillin–sulphuric acid	Dark green	113

TABLE 8 (*continued*)

Compound	Reagent	Colour	Ref.
20-Hydroxyecdysone 3,22-diacetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 2,3,22-triacetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 2,3,22,25-tetracetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 20,22-acetonide	Vanillin–sulphuric acid	Olive green then brown	113
20-Hydroxyecdysone 2,3,20,22-diacetonide	Vanillin–sulphuric acid	Olive green then brown	113

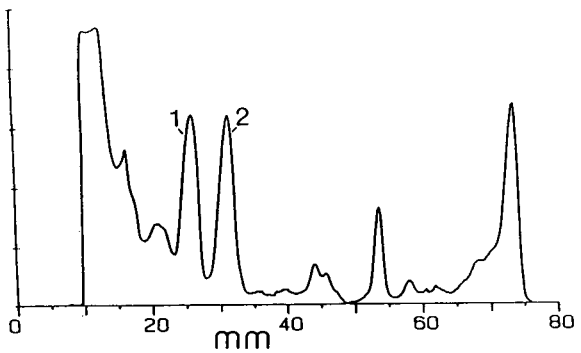

Fig. 9. HP-TLC of an extract from the plant *Silene schafta*, silica gel, chloroform–ethanol (4:1, v/v), 254 nm. Peaks: 1 = 20-hydroxyecdysone; 2 = polypodine B.

and O'Connor [56] almost simultaneously discovered that ecydsone trimethylsilyl ethers were suitable subjects for the electron capture detector which made the sensitivity and selectivity of detection of ecdysteroids much greater. This work was done with packed columns, and was reviewed in 1976 [42], but the difficulties of preparing trimethylsilyl ethers in a reproducible and quantitative manner [57] have discouraged the use of gas chromatography in ecdysteroid work.

The introduction of fused-silica capillary columns increased the resolution of GC enormously so that the resolving power of GC and the sensitivity of electron-capture detection have yet to be bettered by any physical methods for ecdysteroids. Because other chromatographic methods described here which do not require derivative formation have replaced GC we are not aware of the use of GC in any phytoecdysteroid work in the past decade.

An example of capillary column operating conditions for trimethylsilylated ecdysteroids is given by Evershed and co-workers [58,59]. They used a 25 m × 0.22 mm fused-silica capillary column, coated with 0.1 μm film of BP-1 (a non-polar dimethylsiloxane polymer) with oven temperature 50°C at injection, then raised immediately to 200°C and programmed at 8°C min^{-1} to 320°C and held at that temperature. Using helium as the moving phase at a linear velocity of 100 cm s^{-1}, they found ecdysone trimethylsilyl ether had a retention time of 15.2 min, 20-hydroxyecdysone 16.0 min, makisterone A 17.2 min and 20,26-dihydroxyecdysone 18.2 min [58].

6. SUPERCRITICAL FLUID CHROMATOGRAPHY

Separation of complex mixtures can be achieved by chromatography using a moving liquid or gaseous phase. It was pointed out by Lovelock in 1958 (see ref. 60) that a supercritical fluid should also act as a mobile phase for chromatography. The supercritical fluid used in most cases is carbon dioxide or carbon dioxide "modified by" (*i.e.* mixed with) methanol, or some other small-molecule polar substance to increase the polarity of the supercritical fluid.

The instrumentation is either an adaptation of GC, using fused-silica capillary columns and a flame ionization detector or an adaptation of HPLC, using packed columns and a UV detec-

TABLE 9

RETENTION OF PHYTOECDYSTEROIDS ON RP-TLC PLATES OF DIFFERENT ORIGINS

Solvent system: methanol–water (50:50, v/v). Data from ref. 45.

Compound	Merck C_{18} bonded TLC plates		Whatman C_{18} bonded TLC plates		Macherey–Nagel C_{18} bonded TLC plates	
	hR_F	$\%R_F$ ecdysone	hR_F	$\%R_F$ ecdysone	hR_F	$\%R_F$ ecdysone
Ecdysone	29	100.0	28	100.0	32	100.0
20-Hydroxyecdysone 2-cinnamate	4	13.8	3	10.7	5	15.6
Inokosterone	44	151.8	37	132.0	45	140.6
20-Hydroxyecdysone	44	151.8	38	135.7	49	153.1
Muristerone A	32	110.3	31	110.7	45	140.6
Carpesterol	0	0.0	0	0.0	0	0.0
2-Deoxy-20-hydroxyecdysone	21	72.4	29	103.6	29	90.6
Ponasterone C 2-cinnamate	0	0.0	0	0.0	0	0.0
Pterosterone	29	100.0	38	135.7	37	115.6
Abutasterone	42	144.8	56	200.0	52	162.5
Integristerone A	45	155.2	63	225.0	52	162.5
Calyonysterone	20	68.9	37	132.1	24	75.0
Kaladasterone	17	58.6	30	107.1	30	93.8
Ponasterone A	16	55.2	18	64.3	24	75.0
Makisterone A	31	106.9	40	142.9	40	125.0
Dacrysterone	31	106.9	37	132.1	40	125.0
Polypodine B	42	144.8	44	157.1	49	153.1
Cyasterone	40	137.9	51	182.1	49	153.1
Acetylpinnasterol	0	0.0	0	0.0	0	0.0
2-Deoxyecdysone	15	51.7	17	60.7	17	53.1
Ajugasterone C	38	131.0	38	135.7	44	137.5
Ponasterone C	29	100.0	37	132.1	40	125.0
Poststerone	37	127.6	38	135.7	46	143.8

tor. The flame detector precludes the use of organic modifiers but has great sensitivity and resolution. Raynor *et al.* [61] have used capillary supercritical fluid chromatography (SFC) to chromatograph some relatively non-polar ecdysteroid precursors but the retention times of ecdysone and hydroxyecdysone were too great for practical purposes. Perhaps these experiments would be worth repeating using higher pressures and possibly slightly higher temperature. Now that the electron capture detector is becoming adaptable to SFC conditions, it becomes more important to attempt capillary SFC of ecdysteroids. SFC with electron-capture detection could become a superior method for ecdysteroids in terms of resolution, sensitivity and selectivity. Morgan *et al.* [62] have shown that packed-column SFC using carbon dioxide–methanol mixtures is a practical procedure, both for pure ecdysteroids and for plant extracts (see Fig. 10) [63]. Very short columns can be used with advantages of short retention times and high resolution. The sensitivity of detection is greater than with HPLC probably as a result of the sharper peaks and greater UV transparency of the fluid [64]. Packed-column SFC has the retention characteristics of a NP-HPLC system. A variety of columns from silica to ODS can be used. In practice, aminopropyl silica and cyanopropyl silica seem best for ecdysteroids. Some conditions are given in Table 10. A further advantage of SFC over HPLC is the avoidance of costly high purity solvents and the problems of their recovery and disposal. Since many ecdysteroids contain vicinal diols, particularly 2,3- and 20,22-diols, reagents specific for vicinal diols

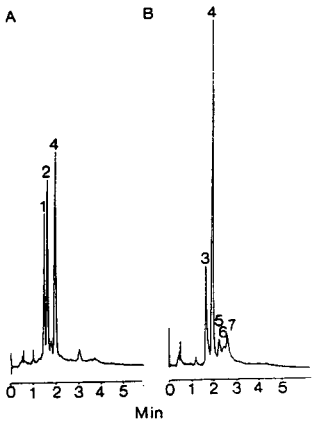

Fig. 10. Supercritical fluid chromatograms of plant extracts. (A) *Silene otites* and (B) *Silene nutans* [63]. Peaks: 1 = 2-deoxyecdysone; 2 = 2-deoxy-20-hydroxyecdysone; 3 = polypodine B; 4 = 20-hydroxyecdysone; 5 = 26-hydroxypolypodine B; 6 = integristerone A; 7 = 20,26-dihydroxyecdysone. Conditions: column 5 μm cyanopropyl silica (20 × 4.6 mm) CO_2–MeOH (9:1) at 3 ml min^{-1}, 60°C and 290 bar, detection at 235 nm.

can be useful in selecting out such compounds from mixtures. We have described the use of solid-phase extraction with immobilized boronic acids for the selective retention of ecdysteroids containing 20,22-diols [22]. We have recently extended this to the preparation and separation by SFC of methyl, butyl and phenylboronic esters of ecdysteroids with 20,22-diol groups [65].

7. CAPILLARY ELECTROPHORESIS

Capillary zone electrophoresis (CZE) has been shown to provide very efficient separations for a wide range of substances. As the bulk of the ecdysteroids are uncharged, simple CE, which relies on the presence of an ionised group to attain separations, cannot be used. However, micellar capillary electrophoresis (MCE), whereby a molecule such as sodium dodecyl sulphate is added to the buffer at a concentration above its critical micelle concentration, has been shown to be suitable for such compounds. In a series of preliminary studies we have evaluated MCE [66,67] for a range of sample types, including plant extracts of various degrees of purity. These studies clearly demonstrated that MCE could be used for the separation of phytoecysteroids. MCE was performed using fused-silica polyimide-coated capillaries of 50 or 75 μm I.D. and 72 or 50 cm in length, respectively. The run buffer employed was 40 m*M* sodium dihydrogen phosphate–20 m*M* disodium borate–20 m*M* sodium dodecyl sulphate–methanol (5%, v/v) at pH 9.4. Depending upon the system, separations were performed at 20 or 8.5 kV and a run temperature of 50°C. Increasing either the applied voltage or temperature reduced migration times. The organic modifier content of the mobile phase also affected the migration of the ecdysteroids with elution time increasing with organic modifier content.

The migration times of a number of common phytoecdysteroids, in the two CZE systems examined, with the run buffer described above are given in Table 11. Using a 50 μm I.D. capillary, migration times ranged from 7.2 min for cyasterone up to 25.9 min for 2-deoxyecdysone. The observed elution order is similar to that seen in RP-HPLC. This is not surprising as in MCE the separation mechanism for ecdysteroids is based on hydrophobic partitioning. It is interesting to note that there is an excellent separation between ponasterone A (16.5 min) and 2-deoxyecdysone (25.9 min) which have been difficult to separate by RP-HPLC, TLC, etc. The results obtained with a 75 μm capillary were similar. MCE gave a linear calibration curve over the range 0 to 560 μg/ml, corresponding to 0 to 2.8 ng injected on-column. The sensitivity of the instrument was such that the detection of *ca.* 175 pg (35 μg/ml) on-column of a sample of pure ecdysone was possible without difficulty. Ecdysteroid-rich extracts from both plant and insect sources were subjected to analysis by MCE. Samples with a range of purities were examined with different degrees of success. Attempted MCE of simple methanol extracts of *Silene nutans* were unsuccessful because large and distorted peaks were obtained, apparently due to column overloading and the presence of interfering compounds. The analysis of plant extracts after more extensive purification gave good results as illustrated in Fig. 11 for an extract of *Silene otites*. This shows the sample to contain 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdy-

TABLE 10

EXAMPLES OF CONDITIONS USED FOR SUPERCRITICAL FLUID CHROMATOGRAPHY OF ECDYSTEROIDS

1 p.s.i. = 6894.76 Pa. For many other conditions, see ref. 114.

Column type	Column dimensions	Mobile phase	Flow-rate	Pressure or density	Temperature (°C)	Compound	t_R (min)	Ref.
Capillary								
Cyanopropyl–dimethylsiloxane (1:1)	10 m × 5 μm	CO_2	–	0.4–0.71 g cm^{-3}	120	14,22,25-Trideoxyecdysone	20	61
						2-Deoxyecdysone	31	61
Packed								
Cyanopropyl Spherisorb 5 μm	250 × 4.6 mm	CO_2–MeOH (90:10)	3.25 l min^{-1} gas	300 bar	50	14,22,25-Trideoxyecdysone	1	61
						2-Deoxyecdysone	2.3	61
						Ponasterone A	2.5	61
						Ecdysone	3.2	61
						Polypodine B	3.2	61
						Makisterone A	3.2	61
						20-Hydroxyecdysone	4.0	61
						Cyasterone	7.0	61
Hypersil 5 μm	100 × 4.6 mm	CO_2–MeOH (80:20)	4 ml min^{-1}	300 bar	80	Ecdysone	1.68	62
						20-Hydroxyecdysone	1.88	62
						Inokosterone	2.10	62
						Cyasterone	1.71	62
Cyanopropyl Spherisorb	250 × 46 mm	CO_2–MeOH (90:10)	3 ml min^{-1}	290 bar	60	2-Deoxyecdysone	1.5	63
						2-Deoxy-20-hydroxy-ecdysone	1.6	63
						Polypodine B	1.8	63
						20-Hydroxyecdysone	2.0	63
						26-Hydroxypolypodine B	2.2	63
						Integristerone A	2.4	63
						20,26-Dihydroxyecdysone	2.6	63
Silica 5 μm	250 × 4.6	CO_2–MeOH (75:25)	3.0	2000 p.s.i.	60	Ecdysone	3.0	64
						20-Hydroxyecdysone	3.2	64
		(80:20)	2.0	2000 p.s.i.	60	Ecdysone	7.1	64
						20-Hydroxyecdysone	8.1	64
ODS 5 μm	250 × 4.6	CO_2–MeOH (75:25)	3.0	2000 p.s.i.	60	Ecdysone	2.3	64
						20-Hydroxyecdysone	2.3	64
		(86:14)	3.0	2000 p.s.i.	60	Ecdysone	4.3	64
						20-Hydroxyecdysone	4.7	64
Aminopropyl	250 × 4.6	CO_2–MeOH (80:20)	2.0	2000 p.s.i.	60	Ecdysone	4.5	64
						20-Hydroxyecdysone	4.8	64
Cyanopropyl	250 × 4.6	CO_2–MeOH (80:20)	2.0	2000 p.s.i.	80	Ecdysone	5.2	64
						20-Hydroxyecdysone	6.0	64
Aminopropyl	250 × 4.6	CO_2–MeOH (80:20)	2.0	2000 p.s.i.	40	Ecdysone	11.0	64
						20-Hydroxyecdysone	13.9	64

sone and 2-deoxyecdysone as major components. The electropherogram obtained for the analysis of an extract of the roots of *Silene nutans* is shown in Fig. 12. This extract contained 20-hydroxyecdysone, polypodine B and 2-deoxy-20-hydroxyecdysone. Under these conditions 20-hydroxyecdysone and polypodine B co-migrate (Fig. 12a). This lack of separation between

TABLE 11

RETENTION TIME DATA FOR ECDYSTEROIDS FOR MICELLAR CAPILLARY ELECTROPHORESIS

MCE conditions as in text, data from ref. 67. A = ABI 270A CZE system; B = Beckman P/ACE 2000 CZE system.

Compound	Retention time (min)	
	A	B
Calonysterone	14.43	–
Cyasterone	7.17	–
2-Deoxyecdysone	25.91	21.74
2-Deoxy-20-hydroxyecdysone	12.84	11.90
Ecdysone	11.30	10.65
20-Hydroxyecdysone	7.35	7.34
20-Hydroxyecdysone 2-cinnamate	13.57	–
Kaladasterone	13.19	–
Polypodine B	–	7.34
Ponasterone A	16.45	16.02
Pterosterone	10.07	–
Makisterone A	8.89	9.00
Muristerone	8.37	–

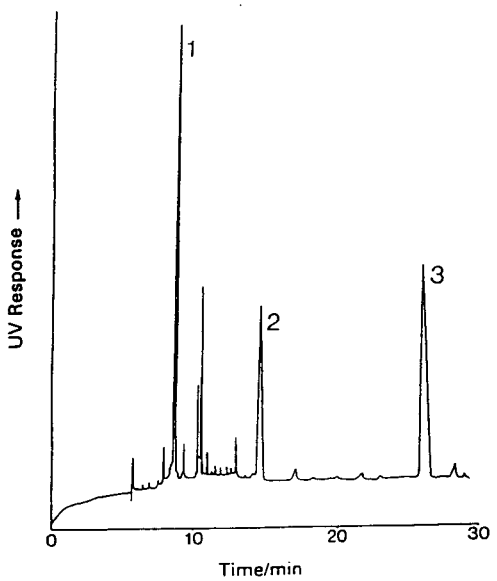

Fig. 11. MCE of an ecdysteroid-rich extract of the plant *Silene otites* using the ABI 270A. The extract contained, in order of elution, (1) 20-hydroxyecdysone, (2) 2-deoxy-20-hydroxyecdysone and (3) 2-deoxyecdysone. Conditions: UV absorption at 240 nm, mobile phase 5% methanol and 95% buffer containing 40 m*M* NaH_2PO_4, 20 m*M* Na_2HBO_3 and 20 m*M* sodium dodecyl sulphate, pH 9.4, 20 kV.

polypodine B and 20-hydroxyecdysone is similar to that found in RP-HPLC. However, the resolution of these compounds is possible by MCE providing that run buffers containing over 50% (v/v) of methanol are used, albeit with long analysis times (over 30 min). Increasing the applied voltage did enable analysis times to be reduced without loss of resolution as shown in Fig. 12b.

MCE therefore is capable of providing efficient separations of phytoecdysteroids in plant extracts. On-column sensitivity is excellent, and clearly adequate for these concentrated samples. MCE can provide an alternative to HPLC or TLC in this type of application and the small on-column sample requirements may also prove useful. However, relatively pure extracts seem to be required for good peak shape. Sensitivity is limited by the amount of material (a few nanolitres) that can be introduced into the capillary. The relatively low concentration, as opposed to on-column sensitivity of MCE, may therefore be a source of difficulty for some

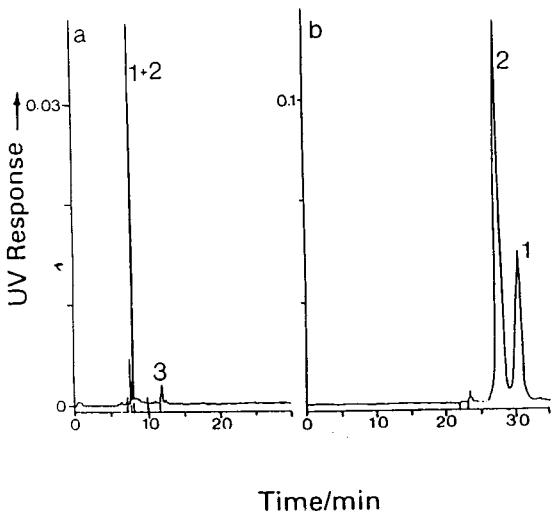

Fig. 12. MCE, using the Beckman P/ACE, of an ecdysteroid-rich extract of the plant *Silene nutans* using (a) standard conditions in which polypodine B (1) and 20-hydroxyecdysone (2) co-migrate, but are resolved from 2-deoxy-20-hydroxyecdysone (3); and (b) 52.5% of methanol in the run buffer when 20-hydroxyecdysone elutes before polypodine B. Conditions as in Fig. 11, but varying the mobile phase in (b) and using 8.5 kV throughout.

sample types. Whilst these initial studies are promising it is too early to say whether or not MCE will be generally useful for the analysis of phytoecdysteroids. Further work is required to enable a rigorous comparison with other analytical techniques to be made.

8. HYPHENATED TECHNIQUES OF CHROMATOGRAPHY AND MASS SPECTROMETRY

The large number of phytoecdysteroids that have been encountered in plant extracts clearly poses problems in their identification based merely on chromatographic properties alone. Whilst selectivity can be attained by the use of a number of different chromatographic systems (see also Lafont *et al.* [32]), linked chromatography–mass spectroscopy provides an alternative system for unequivocally establishing identity. The use of GC–MS for ecdysteroids is well established for insect and other arthropod samples but has not been widely applied to plant samples. The reason for this is that in the case of arthropod samples it is the sensitivity as well of the specificity of the mass spectrometer as a detector which is the driving force for its use. In the case of plant samples the quantities of material are usually such that it is considered to be easier to isolate material for mass spectrometry rather than go through the process of derivatisation which is necessary before GC can be used. The introduction of SFC has also enabled the ecdysteroids to be introduced into the mass spectrometer without the need for derivatisation, and SFC–MS has been used for the identification of a number of ecdysteroids in extracts of *Silene nutans* and *Silene otites* [63]. The results obtained were either chemical ionization- or electron-impact-like, depending upon the temperature of the jet block, with higher temperatures associated with greater fragmentation. Conditions could thus be varied to give molecular mass or diagnostic fragmentation data.

In the case of HPLC–MS for phytoecdysteroids progress has been limited by the available interfaces. In Fig. 13 the chromatograms obtained for HPLC–MS of an extract of *Silene otites* with UV and MS data, monitoring the ions

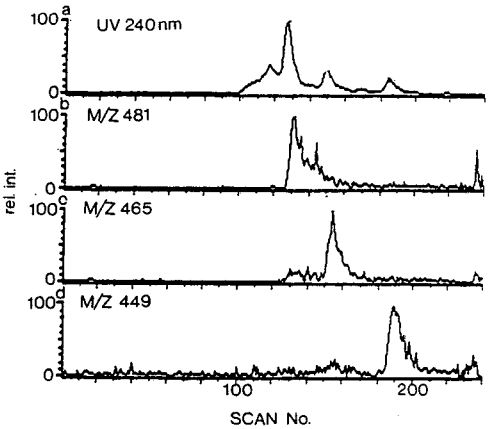

Fig. 13. HPLC–MS of ecdysteroids present in an extract of *Silene otites* separated by RP-HPLC on a C_{18} bonded column (ODS Spherisorb 20 × 0.46 cm) using acetonitrile–0.1 *M* ammonium acetate (40:60) at 1 ml min^{-1} with a thermospray interface (VG Quatro, capillary temperature 270°C). (a) UV trace at 240 nm; (b) ion current for *m/z* 481 for 20-hydroxyecdysone; (c) ion current *m/z* 465 for 2-deoxy-2-hydroxyecdysone and (d) ion current *m/z* 449 for 2-deoxyecdysone.

at *m/z* 481, 465 and 449 are shown using the thermospray interface. The resulting spectrum for 20-hydroxyecdysone (*m/z* 481) is given in Fig. 14A. It is typical of the results obtained with this interface following reversed-phase HPLC (methanol–ammonium acetate buffer, see caption for details). A number of ions are associated with the 20-hydroxyecdysone peak corresponding to the protonated molecular (MH) ion (481), MH − 18 (463) and MH − 2 × 18 (445) (loss of one or two molecules of water) and an ion corresponding to $M + MeCN + NH_4^+$ (539), highlighting the difficulties of interpretation encountered with this mode of ionisation. In order to characterise more fully such compounds with this interface we have recently employed MS–MS techniques. An example of the results obtained is shown in Fig. 14B where the daughter ion spectrum obtained for the ion at *m/z* 481 is shown. The weak ion at *m/z* 463 (not marked) and ions at *m/z* 445 and 427 correspond to loss of 1, 2 and $3H_2O$ respectively, but no simple explanation can be given for the other ions. Similar spectra were obtained for 2-deoxy-20-hydroxyecdysone and 2-deoxyecdysone.

In addition to the hyphenation of SFC and

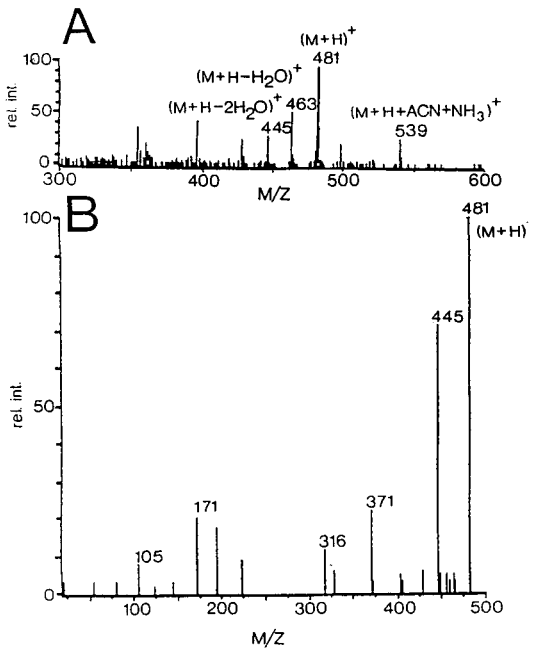

Fig. 14. (A) The mass spectrum obtained for 20-hydroxyecdysone using the conditions outlined in Fig. 13. (B) The daughter ion spectrum obtained for the M + 1 ion (*m/z* 481) for 20-hydroxyecdysone.

HPLC with MS and MS–MS techniques we have also experienced considerable success in combining TLC separations with fast atom bombardment MS [68] and MS–MS [69,70], using both "in-line" and "off-line" techniques. The inherent simplicity of this approach (especially the off-line technique) makes it an attractive and readily implemented alternative to the method of hyphenation outlined above. Furthermore, as the TLC plate effectively stores the separation, confirmation of identity by MS need not be performed at the time of separation (or indeed at the same site).

9. FUTURE PROSPECTS

It would appear that all the stratagems and inventions of the chromatographer have been applied to the separation of phytoecdysteroids from their matrix of plant substances. Techniques are still developing quickly and we can expect to see these advances being applied to phytoecdysteroids too. The prospects of supercritical fluid extraction combined with supercritical fluid chromatography and electron capture detection is a particularly attractive long term goal. In the shorter term we should see advances in LC–MS to make it a more sensitive and diagnostically more useful system and we should also see greater use of TLC with UV scanning and mass spectral analysis. A further system, not yet discussed in detail will be the combination of HPLC and NMR spectroscopy. We can begin to foresee the day when ecdysteroids will be separated, quantified and structurally identified all in one operation without ever having been isolated as pure substances.

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Selectivity in the high-performance liquid chromatography of ecdysteroids

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ABSTRACT

Phytoecdysteroids are a large family of plant compounds structurally related to ecdysone, and the complete HPLC separation of all these compounds requires the development of various complementary chromatographic systems. This paper describes a set of reversed-phase (RP) and normal-phase (NP) HPLC systems that can be used to separate ecdysteroids efficiently. A suitable combination of these NP- and RP-HPLC systems may allow the complete resolution of complex mixtures, but clearly the use of only two systems is not sufficient. Several original solvent systems are described and some examples are given to illustrate their selectivity towards the most common modifications of the ecdysteroid molecule.

INTRODUCTION

HPLC is by far the most widely used separation technique for many natural compounds, including phytoecdysteroids (*e.g.*, [1–6]). Many HPLC systems have been applied to the separation of ecdysteroids [7–11]. Both reversed-phase (RP) and normal-phase (NP) systems are used, and it is generally considered that a combination of one NP and one RP system will allow the complete resolution of individual components of complex ecdysteroid mixtures, as is most often the case with plant extracts. Usually, plants contain one or a few major components (mainly 20-hydroxyecdysone and, *e.g.*, polypodine B) and, in addition, a wide range of minor components, which seem to result from a random combination of a limited number of individual modifications [12,13]. Each structural modification may result in a significant change in the chromatographic behaviour, which may be more or less marked when using RP or NP systems. As a consequence, when individual peaks collected during RP analysis are run on an NP system, previously co-eluting compounds are usually resolved [14]. For this reason, it is generally accepted that co-migration of a given ecdysteroid with the same reference compound in two solvent systems (generally one RP and one NP) can be considered as sufficient evidence for establishing their identity.

However, the size of the ecdysteroid family has increased continuously in recent years, and over 150 different compounds have now been described [12,13]. As a consequence, it has become necessary to develop several RP and NP systems to ensure all the required separations, because in a given HPLC system many ecdysteroids may elute very close together. Of course, very efficient chromatographic systems are required, but efficiency alone is not sufficient

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to ensure good resolution, and selectivity is a major parameter to be considered. The selectivity of a given chromatographic system results from both the stationary phase (column type) and the mobile phase. Changing one or both parameters may result in dramatic changes in separations (*i.e.*, the elution order of a given set of ecdysteroids). This important topic has already been addressed [11]. Published data concern mainly RP systems, where columns from various suppliers and mobile phases that contained either methanol, acetonitrile, tetrahydrofuran or dioxane were compared [15]. This problem has nevertheless not led to systematic developments that would provide general recipes to be used when faced with a given problem of separation. Moreover, this approach has not concerned NP systems. This paper reviews published data and includes original data, paying special attention to NP systems.

EXPERIMENTAL

Ecdysteroids

Ecdysone, 20-hydroxyecdysone (Fig. 1) and makisterone A were obtained from Simes (Milan, Italy). 2-Deoxyecdysone, 2-deoxy-20-hydroxyecdysone and ponasterone A were generous gifts from Dr. D. Horn (Acheron, Australia). Abutasterone was a gift from Dr. M. Pinheiro (Manaus, Brazil). Turkesterone was a gift from Dr. A. Suksamrarn (Bangkok, Thailand). 11α-Hydroxyecdysone (and its dehydration by-product) was a gift from Professor I. Kubo (Berkeley, CA, USA). Makisterone C, rubrosterone, poststerone, polypodine B, taxisterone, 20,26-dihydroxyecdysone, 22-oxo-20-hydroxyecdysone, 20-hydroxyecdysone 25-acetate, dacryhainansterone and 24,28-dehydromakisterone A were isolated from various plant sources [16–19]. 24-Epimakisterone was prepared by reduction of 24,28-dehydromakisterone A [20]. 3-Dehydro-20-hydroxyecdysone was prepared from 20-hydroxyecdysone by chemical oxidation [21]. 22-Epi-20-hydroxyecdysone and 3-epi-20-hydroxyecdysone were prepared from the 22-oxo and 3-oxo compounds, respectively, by chemical reduction [22]. Monoacetates of 20-hydroxyecdysone were prepared according to Horn [23]. 5α-Isomers were obtained from the corresponding 5β-compounds on equilibration under alkaline conditions [23]. 25-Deoxyecdysone and various dehydration products of ecdysone and 20-hydroxyecdysone were prepared according to Heinrich [24–26].

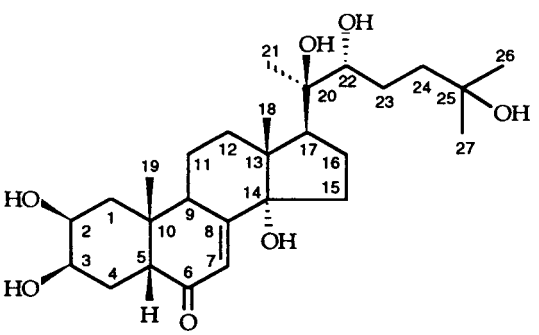

Fig. 1. Structure and carbon numbering of 20-hydroxyecdysone.

HPLC systems

HPLC equipment from Waters or DuPont was used. Isocratic conditions were always used, so that they can more readily be reproduced in other laboratories. Analytical columns were either (1) a Spherisorb 5 ODS-2 (Biochrom), 25 cm × 4.6 mm I.D., eluted with water–0.1% (final concentration) trifluoroacetic acid and either 23% acetonitrile (solvent system 1), 50% methanol (solvent system 2), 30% ethanol (solvent system 3), 18% 2-propanol (solvent system 4), 23% acetonitrile–2-propanol (5:2) (solvent system 5), 30% acetonitrile–methanol (1:1) (solvent system 6), or (2) a Zorbax-Sil column (DuPont), 25 cm × 4.6 mm I.D., eluted with either dichloromethane–2-propanol–water (125:30:2) (solvent system 7) or (125:40:3) (solvent system 8), cyclohexane–2-propanol–water (100:40:3) (solvent system 9) or (80:40:3) (solvent system 10), or isooctane–2-propanol–water (100:40:3) (solvent system 11) or (100:30:2) (solvent system 12). The flow-rate was 1 ml min^{-1} for all systems. Solvents (HPLC grade) were obtained from Carlo Erba (2-propanol, methanol, trifluoroacetic acid), Scharlau (iso-

octane, acetonitrile), Janssen (cyclohexane) and Prolabo (dichloromethane). Ultrapure water was obtained from a standard Millipore Milli-RO/Milli-Q system.

RESULTS AND DISCUSSION

Fundamental structural changes

Usually, 20-hydroxyecdysone (Fig. 1) represents the major phytoecdysteroid found in most ecdysteroid-containing plants. It is therefore logical to consider how variations in the basic structure of 20-hydroxyecdysone result in changed chromatographic properties. Any combination of several of these individual changes (Table I) might be expected to be found in plants, even if not yet described. Mainly phytoecdysteroids will be considered here (for a more extensive list of known ecdysteroids, see ref. 27).

Towards general rules for the HPLC behaviour of ecdysteroids

Both isocratic RP and NP systems can separate complex ecdysteroid mixtures, and the use of solvent gradients would increase the possibilities of these systems. In order to evaluate the effects of single modifications on the HPLC behaviour of ecdysteroids, we selected a set of available reference ecdysteroids and used four different HPLC systems, *i.e.*, two RP and two NP. The results of this study are given in Table II.

Effect of changing the number of OH groups. We examined most of the already described possibilities, *i.e.*, structural variations at positions 1, 2, 5, 11, 20, 22, 24, 25 and 26, in NP- and RP-HPLC, and several conclusions can be drawn. First, it is clear that the addition of one OH group generally results in increased polarity, which is not surprising. 5β-OH is an exception to this rule, and indeed polypodine B shows unusu-

TABLE I

FUNDAMENTAL CHANGES TO THE 20-HYDROXYECDYSONE MOLECULE

Type	Positions on the molecule
Hydroxyl groups:	
Additional	1, 5, 11, 19, 23, 24, 26
Less	2, 20, 22, 25
Oxidation ($\rangle$CHOH→ $\rangle$C=O)	3, 22
Epimerization	3α/β,5α/β
Alkyl substitution	24 (methyl, ethyl, methylene,...)
Esterification:	
Acetates	2, 3, 22, 25
Benzoates	20, 22, 25
Cinnamates	2
Coumarates	3
Sulphates	22
Etherification:	
Intramolecular	Between C-22 and C-25
Methoxy ether	25
Galactosides	3, 22
Glucosides	3, 25
Acetonides	2-3, 20-22
Dehydration	$\Delta^{9(11)}$, $\Delta^{14(15)}$, $\Delta^{24(25)}$, $\Delta^{25(26)}$
Side-chain cleavage	C-20/C-22, C-17/C-20
Presence of a lactone ring	Concerns essentially C_{28} or C_{29} ecdysteroids

TABLE II

INFLUENCE OF SOME REPRESENTATIVE ELEMENTARY STRUCTURAL CHANGES ON THE HPLC BEHAVIOUR OF VARIOUS ECDYSTEROIDS USING TWO RP AND TWO NP SYSTEMS

Relative retention times (20-hydroxyecdysone = 100). Retention times of 20-hydroxyecdysone = 5.15 min (system 1), 5.40 min (system 2), 28.9 min (system 7) and 21.3 min (system 9). For solvent systems, see Experimental.

Ecdysteroid	System 1	System 2	System 7	System 9
20-hydroxyecdysone	100	100	100	100
Hydroxylation/dehydroxylation at various positions				
Integristerone A (+1β-OH)	81	81	134	135
2-Deoxy-20-hydroxyecdysone (−2β-OH)	243	219	48	55
Polypodine B (+5β-OH)	105	100	65	101
Turkesterone (+11α-OH)	86	68	311	171
Ecdysone (−20-OH)	188	155	65	71
Taxisterone (−22-OH)	262	217	75	72
Abutasterone (+24-OH)	86	89	123	127
Ponasterone A (−25-OH)	636	324	23	37
20,26-Dihydroxyecdysone (+26-OH)	72	75	311	221
Oxidation/isomerization at C-3				
3-Epi-20-hydroxyecdysone	108	100	89	92
3-Oxo-20-hydroxyecdysone	127	107	32	76
Oxidation/isomerization at C-22				
22-Epi-20-hydroxyecdysone	90	87	173	121
22-Oxo-20-hydroxyecdysone	241	181	58	71
Isomerization of A/B ring junction				
5α,20-Hydroxyecdysone	95	89	86	113
Alkyl substitutions at C-24				
Makisterone C (+24α-C_2H_5)	326	238	39	53
Makisterone A (+24α-CH_3)	136	128	71	83
24-Epimakisterone A (+24β-CH_3)	152	127	58	73
$\Delta^{24(28)}$-Makisterone A (+24-=CH_2)	155	138	47	64
Side-chain cleavage				
Rubrosterone	109	142	29	24
Poststerone	181	112	29	23
Acetylation				
20-Hydroxyecdysone 2-acetate	283	210	37	65
20-Hydroxyecdysone 3-acetate	202	143	39	70
20-Hydroxyecdysone 22-acetate	233	141	60	81
20-Hydroxyecdysone 25-acetate	349	175	30	54

al behaviour. In the RP mode, it may elute slightly before or after 20-hydroxyecdysone, whereas in the NP mode it may migrate close to either 20-hydroxyecdysone or ecdysone. In the latter instance (solvent system 7, Table II), this means that adding one OH group may result in a decrease in polarity, possibly owing to hydrogen bonding with the 6-ketone.

The changes in polarity induced by additional hydroxyl groups are strongly dependent on their position in the molecule (Fig. 2). Clearly positions 11, 25 and 26 have the greatest effects, owing to their location in hydrophobic parts of the molecule, whereas other positions, *e.g.*, 1 or 24, which are in close vicinity to α-diols at C-2/C-3 or C-20/C-22, have more limited effects. The combined effects of two elementary changes depend whether they are located in adjacent or remote areas, so that they might be considered as linked or independent. In the latter instance the effect (in isocratic systems) can be approximated by a calculation that takes into account

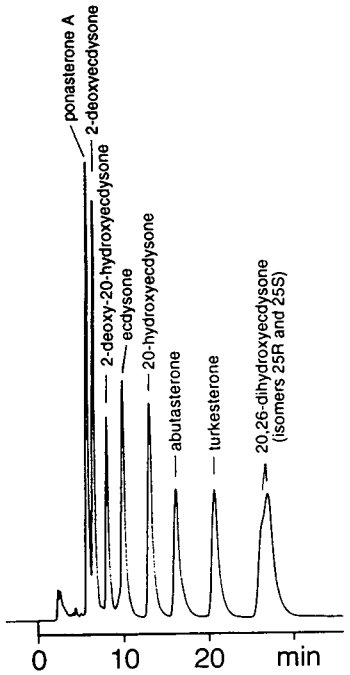

Fig. 2. Separation of a mixture of ecdysteroids (differing in the number of hydroxyl groups) by NP-HPLC (solvent system 10).

the equivalent of the property of additivity of elementary ΔR_m values used in TLC [28,29]. Some examples are given in Table III.

Effect of changing functions at C-3 (3β-OH, 3α-OH and 3-oxo). Oxidation of the secondary hydroxyl group to a ketone results in a significant decrease in polarity when studied by NP-HPLC, especially with solvent system 7 (Fig. 3). Using RP-HPLC, the decrease in polarity is less pronounced, and indeed when using methanol–water mixtures some workers were unable to obtain any separation of 3-oxo from 3β-OH compounds [30]. More generally, oxidation or epimerization at C-3 has only a limited effect on RP-HPLC when using methanol in the mobile phase. On the other hand, 3β-OH, 3α-OH and 3-oxo compounds are baseline resolved using acetonitrile in the mobile phase [21], with elution in that order. On NP columns, the separation is usually more efficient: 3-oxo compounds show a much decreased polarity, and the elution order is 3-oxo, 3α-OH, 3β-OH using silica [21].

Effect of changing functions at C-22 (22R-OH, 22S-OH and 22-oxo). Changes in stereochemistry at C-22 (to 22-isoecdysteroids) have so far not been described for naturally occurring ecdysteroids, although their occurrence cannot be excluded, as 22-oxoecdysteroids have recently been discovered [18,19]. Clearly changes at C-22 result in significant changes in polarity when studied using NP-HPLC, and the 22-epimer (22-iso-20-hydroxyecdysone) elutes much later than the parent compound.

Effect of changing the stereochemistry of the A/B ring junction (5β = cis; 5α = trans). With 20-hydroxyecdysone, this change results in limited effects on polarity, which vary according to the solvent system (Table II). In fact, the magnitude of these effects is more or less pronounced, depending on the number and stereochemistry of OH groups present on ring A at C-2 (or C-1; data not shown), and solvent selectivity is also important (see below).

Effect of alkyl substituents at C-24. The presence of substituents at C-24 results in significant decreases in polarity with both NP and RP systems (effect of ethyl > effect of methyl or methylene), as expected from the introduction of an additional hydrophobic group (Fig. 4).

Effect of side-chain cleavage (between C-20/C-22 or C-17/C-20). Side-chain cleavage at C-17/C-20 has little effect on the RP-HPLC behaviour (Table II). This can be interpreted as some compensation between the effects of the simultaneous removal of both the polar hydroxyls and the hydrophobic groups present on the side-chain. On the other hand, it results in a large decrease of polarity when studied by NP-HPLC, which is of the same order as that produced by the absence of a 25-OH group when the side-chain is present.

Effect of esterification: example of acetates. Acetylation results in a significant decrease in polarity, which varies according to the position involved (Fig. 5) On NP-HPLC, esterification, absence of the corresponding OH group or its conversion into a ketone seem to have similar effects (Table II). Acetates at C-2 and C-3 are poorly separated by NP-HPLC, whereas this separation is easily achieved by RP-HPLC (Table II). The effect of esterifying a given OH group may depend on the presence or absence of

TABLE III

EFFECT OF CHANGING THE NUMBER OF –OH GROUPS ON k' AND α (RELATIVE TO 20-HYDROXYECDYSONE) OF ECDYSTEROIDS ON NP-HPLC (SOLVENT SYSTEM 8)

k' = Capacity factor $[=(t_r - t_0)/t_0]$; α = selectivity factor $(=k'_2/k'_1)$. Ln α is equivalent to ΔR_m used in TLC [26]. According to the same additivity rules as those used in the case of TLC with the elementary ΔR_m, we can calculate the expected ln α in HPLC when double changes are made to the 20-hydroxyecdysone molecule.

Position/compound	k'	Ln α	
20-Hydroxyecdysone	5.00		
		observed	
Single changes			
−C-2 (2-deoxy-20-hydroxyecdysone)	2.35	−0.752	
−C-20 (ecdysone)	3.29	−0.420	
−C-22 (taxisterone)	3.78	−0.278	
−C-25 (ponasterone A)	0.88	−1.740	
+C-1β (integristerone A)	7.53	+0.410	
+C-5 (polypodine B)	3.29	−0.420	
+C-11α (turkesterone)	14.7	+1.077	
+C-24 (abutasterone)	6.14	+0.205	
+C-26 (20,26-dihydroxyecdysone)	14.7	+0.077	
		Observed	Calculated
Double changes: independent?			
−C-20 − C-2 (2-deoxyecdysone)	1.57	−1.157	−1.172
−C-20 − C-25 (25-deoxyecdysone)	0.63	−2.071	−2.160
+C-26 + C-5 (26-hydroxy-polypodine B)	9.9	+0.682	+0.657
+C-26 − C-20 (26-hydroxyecdysone)	7.8	+0.445	+0.657
+C-26 − C-22 (22-deoxy-26-hydroxyecdysone)	10.6	+0.750	+0.799
+C-11α − C-20 (11α-hydroxyecdysone)	7.53	+0.410	+0.657
+C-11α − C-25 (ajugasterone C)	2.27	−0.789	−0.663
Double changes: linked?			
+C-1 − C-2 (2-deoxy-integristerone A)	3.82	−0.269	−0.342
−C-20 − C-22 (22-deoxyecdysone)	1.43	−1.25	−0.698
−C-25 + C-24 (pterosterone)	2.62	−0.644	−1.535
−C25 + C-26 (inokosterone)	4.48	−0.110	−0.663

another OH group in its close vicinity. For instance, when studied by RP-HPLC (solvent system 1 in Table II), 20E22Ac elutes *between* 20E3Ac and 20E2Ac, whereas in the ecdysone series (data not shown) E22Ac elutes well *after* E3Ac and E2Ac. Methanol and acetonitrile result in differences in the order of elution of acetates, and in the former system 20E3Ac and 20E22Ac are not resolved.

Effect of an additional double bond. The presence of an additional double bond either on the nucleus [$\Delta^{9(11)}$] or on the side-chain [$\Delta^{24(25)}$ and $\Delta^{25(26)}$] results in limited changes in the retention times, with the exception of solvent system 2 (Table IV). The presence of a $\Delta^{9(11)}$ double bond results in a slightly increased polarity in all the tested systems, whereas double bonds on the side-chain have more pronounced effects. Clearly, NP-HPLC solvents based on dichloromethane are very poor in that case. Surprisingly, the 24-methylene group appears less polar than the methyl group in NP-HPLC [compare makisterone A and its $\Delta^{24(28)}$ derivative].

Selectivity in NP-HPLC

Selectivity due to the column. Usual NP-HPLC columns are packed with either silica (*e.g.*, Partisil, Zorbax-Sil) or polar-bonded silica (*e.g.*,

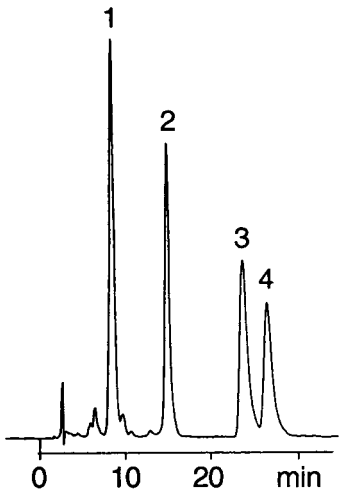

Fig. 3. Separation of a mixture of ecdysteroids modified at position 3 or 22 by NP-HPLC on a Zorbax-Sil column (solvent system 7). Peaks: 1 = 3-dehydro-20-hydroxyecdysone; 2 = 22-oxo-20-hydroxyecdysone; 3 = 3-epi-20-hydroxyecdysone; 4 = 20-hydroxyecdysone.

diol, Polyol, NH_2). Using diol instead of silica columns does not seem to introduce any large changes; the retention times may vary, but the elution order usually remains the same; diol columns provide the advantage of allowing gradients to be used. On the other hand, NH_2 (or APS) columns may interact in a different way with some ecdysteroids and therefore introduce a different selectivity [30]. We have also used TMS-bonded phases [31]; they behave very like silica columns but with less peak tailing. However, these data were obtained with an "old" column and they could not be reproduced with a new column. Clearly, the properties of such columns depend strongly on the percentage of free silanol groups, and a lower percentage is connected with shorter retention times. Nevertheless, changing the usual solvents to less polar types still allows the efficient use of "new" TMS columns.

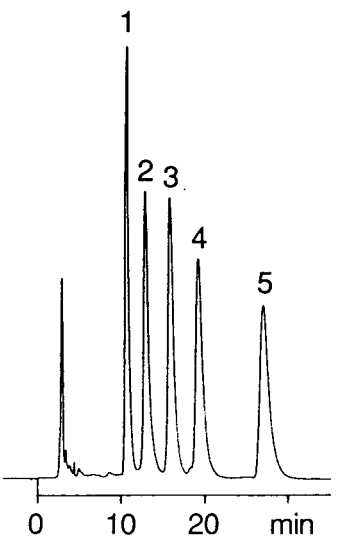

Fig. 4. Separation of a mixture of ecdysteroids bearing substitutions at C-24 by NP-HPLC on a Zorbax-Sil column (solvent system 7). Peaks: 1 = makisterone C; 2 = 24(28)-dehydromakisterone A; 3 = 24-epi-makisterone A; 4 = makisterone A; 5 = 20-hydroxyecdysone.

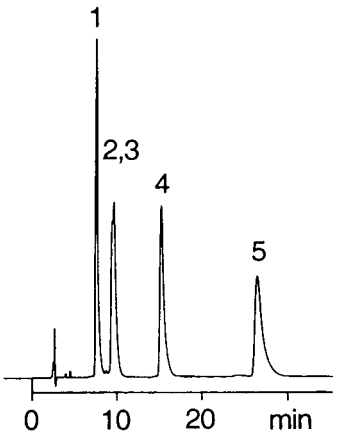

Fig. 5. Separation of a mixture of 20-hydroxyecdysone monoacetates by NP-HPLC on a Zorbax-Sil column (solvent system 7). Peaks: 1 = 20-hydroxyecdysone 25-acetate; 2 = 20-hydroxyecdysone 2-acetate; 3 = 20-hydroxyecdysone 3-acetate; 4 = 20-hydroxyecdysone 22-acetate; 5 = 20-hydroxyecdysone.

The retention times obtained with silica columns are not completely stable. They may decrease on prolonged use, especially with water-containing solvents which will slowly deactivate the column. In this event, reactivation with anhydrous solvents allows the complete recovery of previous retention times. Among the columns tested, it seems that Zorbax-Sil is the least affected by the prolonged use of water-containing solvents.

Other types of NP stationary phases are also available (alumina, graphitic carbon), but there

TABLE IV

SEPARATION OF COMPOUNDS DIFFERING BY THE PRESENCE/ABSENCE OF DOUBLE BONDS

Retention times in minutes. For solvent systems, see Experimental.

Compound	System 1	System 2	System 7	System 9	System 12
Ponasterone A (25d20E)	6.45[a]	17.6	6.85	7.4	15.1
Dacryhainansterone [$\Delta^{9(11)}$]	5.6[a]	14.3	7.3	7.7	15.9
Stachysterone C [$\Delta^{24(25)}$]	5.25[a]	13.4	7.3	8.1	16.8
"Iso"-stachysterone C [$\Delta^{25(26)}$]	5.25[a]	12.2	7.3	8.2	16.8
25-Deoxyecdysone (25dE)	12.2[a]	32.8	6.1	6.4	11.6
$\Delta^{24(25)}$-25dE	9.5[a]	24.5	6.2	6.8	12.9
$\Delta^{25(26)}$-25dE	8.8[a]	21.8	6.3	6.9	13.5
Ecdysone (E)	9.45	8.4	18.8	15.1	–
$\Delta^{9(11)}$-E	8.1	7.2	20.9	16.9	–
Makisterone A	7.7	7.0	20.5	17.7	–
$\Delta^{24(28)}$-makisterone A	6.9	7.5	13.6	13.6	–

[a] 35% instead of 23% acetonitrile.

are no descriptions of their use with ecdysteroids.

Selectivity due to solvents. Many solvent systems have already been described for the NP-HPLC of ecdysteroids [7–11,29]. They are less numerous than those described for TLC, however, simply because UV detection (242 or 254 nm) precludes the use of solvents with a high UV cut-off (*e.g.*, ethyl acetate, benzene or acetone). Therefore, the usual basis for the solvent is a chlorinated hydrocarbon (chloroform, methylene chloride, ethylene dichloride [29]), and the modifier is an alcohol (methanol, ethanol, propanol or 2-propanol). Adding water just below saturation is useful, because it results in reduced peak tailing [32]. We proposed some years ago [32] the use of such tertiary mixtures based on dichloromethane, 2-propanol and water (*e.g.*, 125:25:2, v/v/v) to separate complex ecdysteroid mixtures. Later, we proposed the replacement of dichloromethane with isooctane, as the latter allowed the in-line use of diode-array detectors or radioactivity monitors [10]. However, isooctane is a poor solvent for ecdysteroids, and we encountered recovery problems, which were overcome by using cyclohexane.

Cyclohexane-based ternary mixtures have a significantly different selectivity, and their combination with dichloromethane-based solvents allows many separations to be achieved (Table II). They are highly viscous, and working pressures are above 100 bar with analytical columns at a flow-rate of 1 ml min^{-1}. Raising the temperature to 50°C can overcome this problem, however, as it results in a *ca.* 40% decrease in working pressure without affecting the separation.

Some examples. Clearly dichloromethane- or cyclohexane-based solvents (Table II) provide very different selectivities, as exemplified by the separation of ecdysone, 20-hydroxyecdysone and polypodine B, or by turkesterone and 20,26-dihydroxyecdysone. When considering the separation of 5α–5β pairs (Table V), the differences are striking.

The separation of 3-oxo, 3β-OH and 3α-OH compound mixtures is achieved on silica columns, but with aminopropyl-bonded phases 3α-OH compounds elute *after* 3β-OH compounds [30] and the overall separation is more efficient (Table VI).

The separation of compounds with or without double bonds has clearly shown (see above) that NP-HPLC is inefficient in this respect, whereas RP-HPLC allows their easy resolution.

Another way of modifying chromatographic

TABLE V

CHROMATOGRAPHIC DATA FOR 5α–5β PAIRS OF ECDYSTEROIDS

Relative retention times (20-hydroxyecdysone = 100). Retention times of 20-hydroxyecdysone: 5.15 min (system 1), 5.40 min (system 2), 5.80 min (system 3), 6.0 min (system 4), 7.5 min (system 6), 28.9 min (system 7), 21.3 min (system 9) and 27.8 min (system 11). For solvent systems, see Experimental. 2dE = 2-deoxyecdysone; 2d20E = 2-deoxy-20-hydroxyecdysone; E = ecdysone; 20E = 20-hydroxyecdysone.

Solvent	2dE		2d20E		E		20E	
	5α	5β	5α	5β	5α	5β	5α	5β
RP-HPLC								
System 1	586	620	225	237	181	189	95	100
System 2	401	419	198	219	135	155	89	100
System 3	650	721	240	260	167	207	84	100
System 4	639	735	216	248	165	215	84	100
System 6	869	901	311	320	195	232	89	100
NP-HPLC								
System 7	32	33	47	48	52	65	86	100
System 9	47	42	64	55	76	71	113	100
System 11	47	40	62	53	77	74	110	100

mobility in NP-HPLC, specific for 20,22- but not 2,3-diols, is the formation of cyclic boronates as described by Pis and Harmatha [33].

Selectivity in RP-HPLC

Selectivity due to the column. The user is faced with a profusion of available columns that differ according to the bond chain length or type (C_6, C_8, C_{18}, C_{22}, phenyl, CN, . . .), the extent of bonding (percentage of carbon load, usually 5–15%) which may be indicated by the commercial names (*e.g.*, ODS-1, ODS-2, ODS-3) or the porosity of silica used. All these parameters may change the selectivity of the column, and for that reason it is not always possible to reproduce data from the literature except by using exactly the same conditions, including the column type. Comparison of columns from various suppliers has been addressed previously [15] and slightly

TABLE VI

SELECTIVITY CHANGES OF APS COLUMNS WITH VARIOUS TERNARY SOLVENT MIXTURES

After ref. 30. Column, APS-Hypersil (5 μm), 25 cm × 4.6 mm I.D., eluted at 1 ml min^{-1} with various dichloromethane–methanol–2-propanol mixtures. Retention times relative to 20-hydroxyecdysone = 100.

Mobile phase composition	2dE		E			20E		
	3α	3β	3α	3β	3-Oxo	3α	3β	3-Oxo
95:1:4	28	21	110	63	19	190	100	28
95:2:3			105	68	18	169	100	
95:3:2				67			100	
95:4:1				67		124	100	

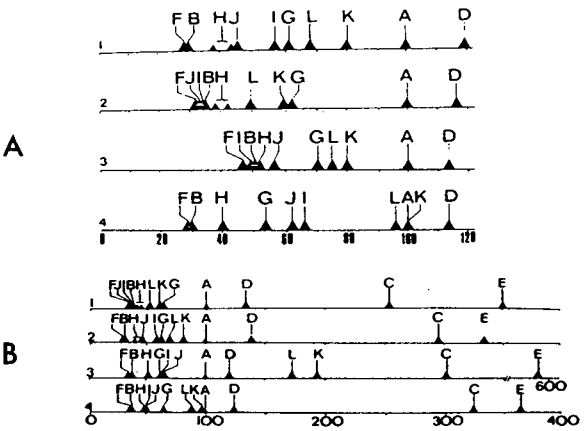

Fig. 6. Selectivity of RP-HPLC is linked to both the columns and the solvent systems (from ref. 15). (A) Selectivity differences between Spherisorb ODS eluted with (1) acetonitrile–water (15:85) or (2) methanol–water (35:65) and Nucleosil-ODS eluted with (3) methanol–water (50:50) or (4) acetonitrile–water (20:80). (B) Comparison of different solvents with a Spherisorb ODS column: (1) methanol–water (35:65); (2) acetonitrile–water (15:85); (3) tetrahydrofuran–water (10:90); (4) dioxane–water (20:80). Retention is given relative to ecdysone (A) which is given a value of 100. A = ecdysone; B = 20-hydroxyecdysone; C = 2-deoxyecdysone; D = 2-deoxy-20-hydroxyecdysone; E = ponasterone A; F = polypodine B; G = makisterone A; H = inokosterone; I = cyasterone; J = poststerone; K = ajugasterone C; L = muristerone A.

different separations were obtained with standard ecdysteroid mixtures (Fig. 6A).

The most usual columns for the separation of ecdysteroids are the C_{18} (or ODS) bonded type, but C_8 columns are also used. Recently, the use of β-cyclodextrin-bonded silica was proposed [34]. Such stationary phases are expected to interact in a specific way with some ecdysteroids and thus display new kinds of selectivities, but so far they have been used with only a few ecdysteroids and this clearly requires further investigations.

Selectivity due to the solvents. RP-HPLC solvents consist of water and a water-miscible organic modifier (*e.g.*, acetonitrile, methanol, ethanol, 2-propanol, tetrahydrofuran, dioxane). When tested with ecdysteroid mixtures, these solvent systems gave significantly different results (Fig. 6B).

Methanol and acetonitrile are the most widely used organic modifiers. Acetonitrile–water mixtures may give excessive peak tailing, which can be suppressed by replacing water with a buffer or simply by adding trifluoroacetic acid (0.1%, v/v). Recently, very interesting separations with 2-propanol have been reported [11,35] and, when applied to 20-hydroxyecdysone–polypodine B mixtures, they clearly resulted in very efficient separations (Table VII).

Similarly, we tested several RP solvent systems on compounds differing by one OH group, and the results are given in Table VIII. Clearly, the retention is greatly affected by changing the organic modifier in the mobile phase. 2-Propanol–water provides the best separation for 5α–5β pairs (Table V). Methanol is particularly

TABLE VII

EFFECT OF ORGANIC MODIFIERS ON THE SEPARATION OF 20-HYDROXYECDYSONE AND POLYPODINE B USING RP-HPLC

After ref. 11. Column, Spherisorb 10 ODS-2, 25 cm × 4.6 mm I.D.; mobile phase flow-rate 1 ml min^{-1}. k' = Capacity factor; α = selectivity factor (k' ratio); N = efficiency (theoretical plates per metre column length); R_s = resolution $\left(=\frac{1}{4}\sqrt{N}\cdot\frac{k'}{1+k'}\cdot\frac{\alpha-1}{\alpha}\right)$.

Organic modifier	k'		α	N	R_s
	PolB	20E			
20% acetonitrile	7.73	7.73	1	13751	0
45% methanol	2.21	2.21	1	1860	0
35% methanol	7.95	8.45	1.06	5261	0.60
15% 2-propanol	2.22	2.63	1.18	16920	2.85
11% 2-propanol	5.42	6.90	1.27	5009	2.93

TABLE VIII

RP-HPLC OF ECDYSTEROIDS USING FIVE DIFFERENT RP SYSTEMS

Relative retention times (20-hydroxyecdysone = 100). Retention times of 20-hydroxyecdysone: 5.15 min (system 1), 5.8 min (system 3), 6.0 min (system 4), 5.95 min (system 5) and 7.5 min (system 6). For solvent systems, see Experimental.

Ecdysteroid	System 1	System 3	System 4	System 5	System 6
20-Hydroxyecdysone	100	100	100	100	100
Integristerone A (+1β-OH)	81	69	84	77	68
2-Deoxy-20-hydroxyecdysone (−2β-OH)	243	260	247	250	320
Polypodine B (+5β-OH)	105	97	92	97	100
Turkesterone (+11α-OH)	86	59	62	63	53
Ecdysone (−20-OH)	188	207	216	201	232
Taxisterone (−22-OH)	262	324	308	297	373
Abutasterone (+24-OH)	86	83	84	85	81
Ponasterone A (−25-OH)	636	679	814	678	687
20,26-Dihydroxyecdysone (+26-OH)	72	69	72	69	61

efficient towards extra double bonds (Table IV), as it may baseline separate $\Delta^{24(25)}$ and $\Delta^{25(26)}$ pairs, but on the other hand it cannot separate 3α–3β isomers, which acetonitrile can do. Extra OH groups generally increase the polarity (but see polypodine B) and their effect depends on both their position *and* the solvent system used (Table VIII). Positions 11α and 26 are located in hydrophobic regions of the molecule, and introducing an extra OH group in these positions results in the most conspicuous effects.

Among the solvents tested, we used acetonitrile–methanol–water and acetonitrile–2-propanol–water mixtures. As can be seen from Table VIII, these mixtures do not exactly possess intermediate properties between those which these organic solvents display when used alone, but they have their own selectivity towards some ecdysteroids.

Effects of temperature. A temperature increase results in increased efficiency, decreased pressure and a decrease in the capacity factor, k'. This has been particularly investigated using 2-propanol–water mixtures [11,35] and it allowed the calculation of the temperature dependence of k' for a set of reference ecdysteroids (Table IX). There were only small differences in the exponents for the various ecdysteroids, which means that selectivity is not much affected.

CONCLUSIONS

It is necessary to be very cautious regarding the conclusions that can be drawn from the co-

TABLE IX

VARIATIONS IN THE CAPACITY FACTOR (k') WITH TEMPERATURE (T) (ADJUSTED CURVES)

From ref. 35. Column, Spherisorb 5 ODS-2, 10 cm × 4 mm I.D.; mobile phase, 2-propanol–water (7:93); flow-rate, 1 ml min^{-1}.

Ecdysteroid	Equation of exponential decay	$T_{1/2}$	r
29-Norsengosterone	$k' = 21.6\ e^{-0.045T} + 3.0$	15.20	0.999
29-Norcyasterone	$k' = 32.0\ e^{-0.049T} + 3.3$	14.07	0.999
Polypodine B	$k' = 19.3\ e^{-0.38T} + 3.7$	17.87	0.998
20-Hydroxyecdysone	$k' = 29.1\ e^{-0.042T} + 4.1$	16.20	0.999
Cyasterone	$k' = 39.0\ e^{-0.046T} + 4.8$	15.06	0.999
Makisterone A	$k' = 75.7\ e^{-0.044T} + 7.1$	15.60	1
Ajugalactone	$k' = 76.8\ e^{-0.044T} + 3.0$	15.61	1

migration of ecdysteroids in only one or even two solvent systems. As shown here (Fig. 7), this may result in erroneous identifications (or provide too optimistic conclusions regarding the purity of a compound). Clearly, using one NP and one RP system gives better selectivity than using two NP or two RP systems.

In order to overcome the problem connected with the existence of a very large family of such closely related compounds, it seems important to recommend the use of more sophisticated criteria. We can see here (Table II) that when using (1) two solvent systems for NP (dichloromethane- and cyclohexane-based) and (2) two solvent systems for RP (acetonitrile- and methanol- or 2-propanol-based), the probability of the identification being incorrect becomes very low.

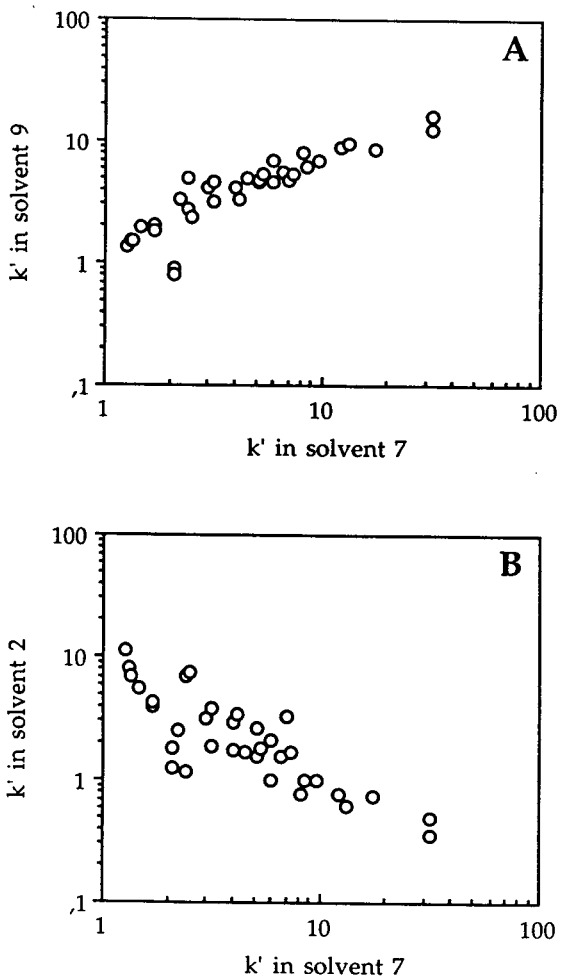

Fig. 7. The use of two different solvent systems increases the possibility of resolving complex ecdysteroid mixtures, but it does not allow a complete resolution of the 35 ecdysteroids tested in this study. (A) Use of two solvents systems of the same type (here two NP systems): some selective effects are apparent, although restricted to a few ecdysteroids, and overlapping of many compounds is observed. (B) Use of one RP and one NP system increases the efficiency of separations, but still a few overlapping compounds remain.

In fact, what we describe here is only a transposition of what has been used for a long time in the TLC analysis of steroid molecules, for which many solvent systems have been proposed to achieve the resolution of specific pairs of compounds. In HPLC, the use of in-line UV detection precludes the use of many of the usual TLC solvents (*e.g.*, ethyl acetate, acetone, pyridine, toluene), but this is counter-balanced by a greater efficiency. In spite of this, a single system is clearly unable to resolve all compounds, and it remains of interest to take advantage of selective effects of the mobile phases to improve the efficiency of HPLC separations.

The approach may provide a basis from which to develop a computer program that would predict the behaviour of any new ecdysteroid in various solvent systems and therefore help to identify ecdysteroids when no reference compounds are available, and when amounts are inadequate for MS or NMR analyses.

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Phytoecdysteroids in *Kochia scoparia* (burning bush)

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ABSTRACT

Seed and plants of *Kochia scoparia* have been analyzed for phytoecdysteroids by high-performance liquid chromatography coupled to ecdysteroid-specific radioimmunoassay (RIA) or bioassay. Relatively low levels of RIA-positive material are found in seed and throughout the plant. Evidence is presented that the major phytoecdysteroids present in seed are 20-hydroxyecdysone and polypodine B (5β,20-dihydroxyecdysone), but significant amounts of other, as yet unidentified, phytoecdysteroids also occur. Extracts of whole plants contain a similar mix of phytoecdysteroids, but also contain a large amount of an apolar conjugate of ecdysone which is not present in seed. Concentrations of RIA-positive material vary throughout the plant, with higher concentrations being present in the root and leaves. Results are discussed in relation to the phytoecdysteroid distribution in other members of the Chenopodiaceae and with regard to the relationships between *K. scoparia* and its insect predators/pollinators.

INTRODUCTION

Insect steroid hormone analogues (phytoecdysteroids) are detectable in many, but not all, species of plant. The concentrations occurring in phytoecdysteroid-positive species are often 100- to 1000-fold higher than those typically found in insects. While it has been suggested that ecdysteroids might have a phytohormonal rôle, it seems more probable that they act to deter insect predation, either by acting as antifeedants or by creating a hormonal imbalance in predatory insects, resulting in developmental disruption or even death [1]. While there is some supporting evidence for this latter rôle, it has not been conclusively proven and the issue is clouded by (1) certain polyphagous insect species being able to tolerate very high levels of ingested phytoecdysteroids without impaired development, (2) relationships between plants and insects being complex, since insects may be beneficial as well as detrimental to plant survival and (3) the diversity of allelochemicals elaborated by plants which probably provides several options for deterring a predator species and provides potential for synergism with regard to defence chemicals.

In order to resolve the question of the function(s) of phytoecdysteroids we have initiated a study focussed on one family of plants, the Chenopodiaceae which include many species of agronomic importance. Consideration is being given to the quantitative and qualitative differences in phytoecdysteroids between species of the family, within individual plants and between individuals within a population. Ultimately, the intention is to relate the findings to the phylogenetics of the Chenopodiaceae, the biology of the plants, the susceptibility of the plants to insect predation and the co-occurrence of other allelochemicals in each species.

To date, most work has concentrated on the genera *Chenopodium* [2–7], where phytoecdysteroids occur in about one-third of the species, and *Spinacia* [8–10]. Rapid and sensitive micro-analytical methods have been developed for the quantification of phytoecdysteroids in small plant samples (*ca.* 50 mg) using radioimmunoassay (RIA) or bioassay and using high-performance liquid chromatography (HPLC)

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coupled to RIA or bioassay for the characterization of the phytoecdysteroid profile in the extract [5,6]. The phytoecdysteroids of *C. album* comprise predominantly 20-hydroxyecdysone (20E: 69%) and polypodine B (PolB; 5β,20-dihydroxyecdysone: 28%) and a complex mixture of minor ecdysteroids (together 3%) [6]. Phytoecdysteroid concentration varies throughout the plant and during development. A concentration gradient occurs within aerial portions rising from the bottom of the stem [*ca.* 0.16 mg ecdysone (E) equivalents/g dry mass] and the lowest leaves (*ca.* 0.1 mg E eq./g dry mass) and such that the highest concentrations are present in the growing tips (1 to 3 mg E eq./g dry mass) and youngest leaves of non-flowering plants (*ca.* 1 mg E eq./g dry mass) [5]. Even higher levels are associated with the flowers, especially the anther tissue (5.4 mg E eq./g dry mass), but not the enclosed pollen (<0.2 mg E eq./g dry mass) [6]. Fluctuating levels are found in root tissue during development [4]. This pattern is common to the other members of the genus *Chenopodium* which contain phytoecdysteroids [6] and to *Spinacia oleracea* [8,10]. This has led us to suggest that phytoecdysteroids help to deflect insect predators from the nutritionally attractive tender young growths and to protect flowers of wind-pollinated species against predation. On this basis, one might predict at the simplest level that higher phytoecdysteroid levels might correlate with anemophilous pollination and low levels with entomophilous pollination. Unfortunately, the mode of pollination in the Chenopodiaceae has not been extensively studied [11], so it is not currently possible to assess the validity of this hypothesis.

Kochia scoparia is a highly insect-resistant member of the Chenopodiaceae. It has rapid growth potential and will grow on arid, saline soils. It has become a serious weed species in North America [12,13], but it is also of interest as a potential forage crop for livestock [14,15]. A number of potential allelochemicals have been identified from *K. scoparia*: saponins, flavonoids, oxalate, alkaloids and phenolic acids [14–19].

Rangeland grasshoppers (*Melanoplus* spp.) avoid eating *K. scoparia* if given a choice of foods [20], but, if given no choice, they eat the older, less nutritious leaves rather than the tender, younger leaves [21]. Grasshoppers fed on *K. scoparia* experience reduced survival, impaired ovarian development and low fecundity [22], reminiscent of effects one might expect from the application of exogenous ecdysteroids. If a concentration gradient of phytoecdysteroids exists in the aerial portions of *K. scoparia* similar to that found in *C. album*, this might be related to the feeding pattern of the insects of this plant. Phytoecdysteroids have not been reported to be present in *K. scoparia*, although there is a brief report of the presence of "moulting hormone activity" in fruits of *Kochia* [23]. The aims of this research were thus (1) to determine whether ecdysteroids are present in *K. scoparia*, (2) to use chromatographic means to begin to identify which ecdysteroids are present and (3) to quantify ecdysteroid concentrations in various portions of the plant. *K. scoparia* presented some interesting methodological problems in the analysis and quantification of phytoecdysteroids because of the relatively low levels present and because of severe interference from other compounds present in apparently all parts of the plant.

MATERIALS AND METHODS

Source of seed and growth of plants

Seeds of *K. scoparia* were purchased from Mr. Fothergill's Seeds, Kentford, Suffolk, UK. Plants were grown in coir (ICI) to a height of 30–40 cm, at which point they had initiated flowering.

Extraction of plant material

Seed. Saponins were selectively extracted from seed by a modified procedure of Kernan *et al.* [17]. Seed were immersed in 0.1% (w/v) NaOH solution for 15 min with gentle shaking. Seed were then washed with distilled water and freeze-dried for 24 h.

Plant portions. Plants were removed from the pots and the roots cleaned of compost. Plants were dismembered with a sharp scalpel blade.

The fresh weight of each portion was determined and then freeze-dried to constant mass (4 days) before the dry mass was determined.

General extraction procedure. Seed or freeze dried plant material was ground with a pestle and mortar. Samples (25 mg) were extracted three times with 1-ml aliquots of methanol for 3 h at 55°C. The three extracts from each sample were pooled, 1.3 ml water added and partitioned twice against 2 ml hexane to remove pigments and non-polar lipids. The aqueous methanol fraction was used for ecdysteroid determination.

High-performance liquid chromatography

HPLC equipment and general procedures have been described previously [24]. Columns and separation systems were as follows:

RP1. Spherisorb ODS-2 (250 × 4.6 mm I.D., 5-μm particle size) eluted at 1 ml/min with a linear gradient from methanol–water (3:7, v/v) to methanol over 30 min and then isocratically with methanol for a further 10 min.

RP2. The above reversed-phase column eluted at 1 ml/min with a linear gradient from methanol–water (45:55, v/v) to methanol–water (7:3, v/v) over 30 min and then isocratically with methanol–water (7:3, v/v) for a further 10 min.

NP1. Apex II DIOL column (150 × 4.6 mm I.D., 5-μm particle size) eluted isocratically at 1 ml/min with methanol–dichloromethane (4:96, v/v).

All separations were monitored at 242 nm and fractions of 30-s (NP1) or 1-min (RP1 and RP2) duration were collected for further analysis by RIA and/or bioassay.

Ecdysteroid radioimmunoassay and bioassay

Ecdysteroids present in extracts or in HPLC fractions were quantified by radioimmunoassay using the DBL-1 antiserum (bleed F; generously provided by Professor Dr. J. Koolman, Universität Marburg, Marburg, Germany) as described previously [5]. Ecdysone (E) was used as the radiolabelled and reference ligands and results are expressed in ecdysone equivalents. Cross-reactivity factors for 20-hydroxyecdysone and polypodine B with this batch of serum were 0.48 and 2.87, respectively (E = 1). Samples were also assessed using the microplate-based B_{II} cell bioassay for ecdysteroid receptor agonists/antagonists [25,26]. Briefly, cells of the *Drosophila melanogaster* B_{II} cell line [27] are grown in the wells of sterile 96-well plates and their densities measured turbidometrically using a microplate reader. In the presence of ecdysteroid agonists, absorbance values (cell densities) are depressed relative to controls. Results are expressed as 100 $(A_{control} - A_{test})$, where $A_{control}$ = the absorbance at 405 nm of wells containing cells grown for 6 days in the absence of ecdysteroid and A_{test} = the absorbance of wells grown in the presence of the test substance. The microplate reader was zeroed on wells containing Schneider's *Drosophila* medium but no cells. There is a logarithmic relationship between the bioassay response and the amount of agonist, such that ≤0.05 ng 20E eq. produces no response, 1 unit = 0.25 ng 20E eq., 2 units = 0.375 ng 20E eq., 3 units = 0.5 ng 20E eq., 4 units = 1.25 ng 20E eq. and 5 units (maximal response) ≥ 5ng 20E eq. Ecdysteroid antagonists may be assessed by determining the ability of test compounds to prevent the reduction in absorbance brought about by $5 \cdot 10^{-8}$ *M* 20-hydroxyecdysone.

Enzymic hydrolyses

Fractions for enzymic hydrolysis were dissolved in 10 μl ethanol and 200 μl *Helix pomatia* gut hydrolases (Sigma, Type H1; 10 mg/ml 0.1 *M* sodium acetate buffer, pH 5.4) and incubated at 37°C for 5 days [28]. Protein was precipitated by the addition of ethanol (1 mL) and aliquouts of the supernatant after centrifugation were assessed by RIA.

Solid-phase extraction

A portion of the aqueous methanol phase (1 ml) deriving from the extract of whole plants was diluted with water (6 ml) and applied to an activated C_{18}-cartridge (Sep-Pak, Millipore). The cartridge was sequentially eluted with 5 ml each of 10, 25, 60 and 100% methanol in water [29].

RESULTS

Initial studies

Previous studies on other members of the Chenopodiaceae have established generally suitable procedures for the extraction of phytoecdysteroids from small plant samples and their analysis and quantification by RIA, bioassay and HPLC [5,6,30]. However, when these methods were applied to extracts of aerial portions or seed of *K. scoparia*, the presence of low levels (relative to *C. album*) of RIA-positive material (*ca.* 40 μg E eq./g dry mass) was indicated, but there was not a linear relationship between RIA response and aliquot size in the RIA (see Fig. 1 for an example), preventing accurate quantification of ecdysteroid levels. The deviation from linearity appeared to derive from the presence of interfering substances co-occurring in the extracts (saponins being the most probable candidate).

A number of chromatographic and partition approaches were attempted to alleviate this interference, but these failed to separate the interfering substances adequately from the RIA-positive material. It has, however, been reported that saponins may be selectively extracted from seed of *K. scoparia* with 1% (w/v) NaOH [17] and thus attention was initially concentrated on seed.

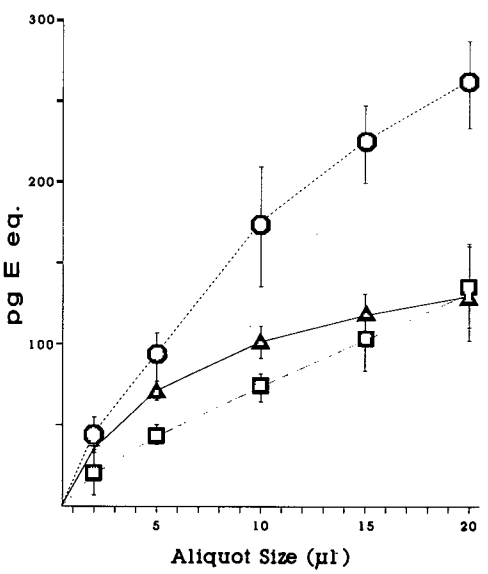

Fig. 1. Radioimmunoassay response in relation to aliquot size for methanolic extracts of *Kochia scoparia* seed: control seed (▲), seed pretreated with 1% (w/v) NaOH (□) and seed pretreated with 0.1% (w/v) NaOH (○). Error bars indicate the standard deviation ($n = 4$).

Analysis of seed

Kernan *et al.* [17] found that *K. scoparia* seed contained 1 to 2% of the dry mass as saponins and demonstrated that washing seed with 1% NaOH selectively extracted 96% of these saponins. Ecdysteroids are, however, alkali-labile [31]. Ten-fold dilution of extracts from seed which had been pre-treated with 1 or 0.1% NaOH were assessed for linearity of RIA response and compared to an extract of untreated seed (Fig. 1). It is clear that pre-extraction with NaOH improves the linearity of the response considerably, and that more than twice as much RIA-positive material is recovered after treatment with 0.1% NaOH than with 1% NaOH. In addition to extracting saponins, 1% NaOH is extracting or degrading a portion of the RIA-positive material. The tangential nature of the initial linear portion of the data for the extract after 0.1% NaOH treatment and the curve for the control extract indicate that ecdysteroids in the seed are not being extracted or degraded by treatment with 0.1% NaOH.

A portion of the extract from 0.1% NaOH-pretreated seed was separated by RP-HPLC (system RP1) and monitored by RIA and bioassay (Fig. 2). RIA-positive material elutes in the region in which many phytoecdysteroids elute and the largest peak elutes at the retention time of 20E and PolB. Significant amounts of less polar RIA-positive material and smaller amounts of more polar RIA-positive material are also present. Bioassay revealed fractions 17 and 23 to be highly active in the ecdysteroid agonist assay; none of the extracts or HPLC fractions showed any antagonistic activity. The breadth of the apolar peak in Fig. 2 indicates that it consists of several components. The seed extract was therefore separated on system RP2, revealing two regions of non-polar RIA-positive material, the first eluting at the retention time of E, and the second with a retention time intermediate between that of E and ponasterone A (PoA). The

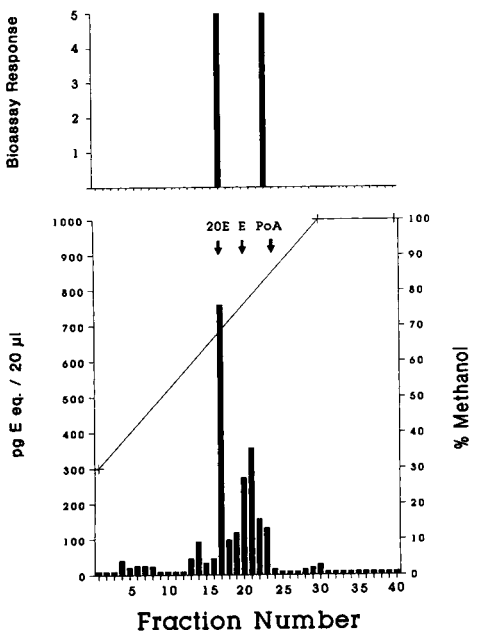

Fig. 2. Reversed-phase HPLC–RIA–bioassay of a methanolic extract of *Kochia scoparia* seed which had been pretreated with 0.1% NaOH to remove saponins. A portion of the extract (equivalent to 4 mg seed) was separated on HPLC system RP1 and fractions of 1 ml were collected. Aliquots (20 µl) of each fraction were subjected to RIA and bioassay: lower panel HPLC–RIA results and upper panel HPLC–bioassay results.

latter peak is still broad and probably consists of at least two components. This is reinforced by biological activity being associated with fractions 20 and 21, but not with fractions 18 and 19. Hydrolysis of portions of the HPLC fractions with *Helix* enzymes prior to RIA revealed no significant increase in RIA response for any of the fractions, indicating that *Helix*-hydrolysable ecdysteroid conjugates are not present in *K. scoparia* seed.

20E and PolB, which have been identified as the major ecdysteroids in other chenopods, generally co-elute on RP-HPLC. The possibility that *K. scoparia* seed contain both 20E and PolB was assessed by separating fraction 9 (RP2: Fig. 3) on system NP1 (Fig. 4). RIA-positive peaks are detected co-chromatographing with both ecdysteroids. Fraction 8–10 and 15 are bioassay-positive. While PolB is less RIA-positive with the DBL-1 antiserum than 20E, it is biologically

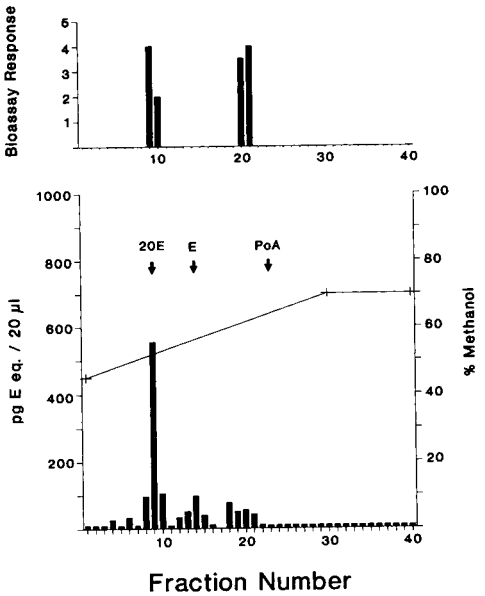

Fig. 3. Reversed-phase HPLC–RIA of an extract of *Kochia scoparia* seed (pretreated with 0.1% NaOH) on system RP2. Details as for Fig. 2.

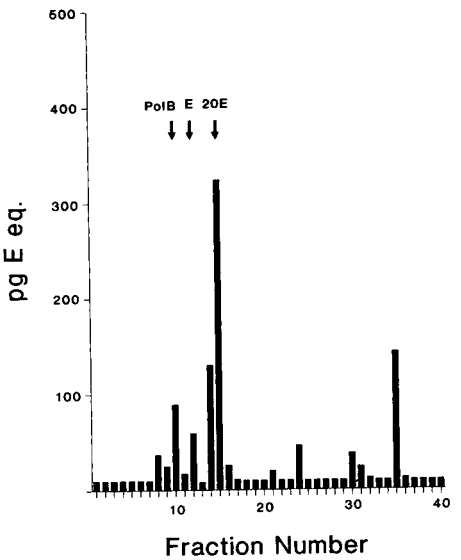

Fig. 4. Normal-phase HPLC–RIA of the RIA-positive material from fraction 9 of the RP-separation (system RP2, Fig. 3) of an extract of *Kochia scoparia* seed. The column was eluted isocratically with 4% methanol in dichloromethane (1 ml/min). Fractions of 0.5 ml were collected and 50 µl aliquots were subjected to RIA.

more potent in the B_{II} bioassay. Several other peaks of RIA-positive material are also detected after normal-phase separation, but these do not have significant biological activity. The identity of these compounds is currently unknown.

Analysis of plants

Partitioning plant extracts between either $CHCl_3$–water or butanol–0.1 *M* NaOH was not effective at removing interfering substances from the ecdysteroids, nor were attempts to hydrolyse saponins with either hesperidinase [32] or *Helix* enzymes. Chromatographic separation on silica was also not effective, but separation on reversed-phase cartridges was partially so. When an extract of whole plants was applied to a C_{18} Sep-Pak and eluted sequentially with 10, 25, 60 and 100% methanol in water, 53 and 33% of the RIA-positive material eluted in the 60 and 100% methanol fractions, respectively. These were analyzed by RP-HPLC (system RP1; Fig. 5); revealing four peaks of RIA-positive material: I–IV. All four peaks were found in the 60% fraction, while the 100% fraction contained only the two non-polar peaks, III and IV. Peak I co-chromatographs with 20E and peak II with E. Peak III has the same retention time as the apolar peak in seed. Peak IV was not observed in extracts of seed. Peaks I and III are biologically active in the B_{II} bioassay. Peak IV is susceptible to hydrolysis by *Helix* enzymes, showing a three-fold increase in RIA-response and being converted to a compound which co-chromatographs with E on systems RP1 and NP1 (data not shown).

Quantification of ecdysteroids

RIA was used to quantify ecdysteroid levels in seed and throughout mature plants (Table I). Seed were pre-extracted with 0.1% NaOH. Ecdysteroid levels in plant extracts were determined by dilution (100-fold) of the extract and assessment of the RIA response with 2- to 20-μl aliquots. At these levels (<100 pg), linearity of RIA response was observed and concentrations were calculated from the gradients of plots of aliquot size *vs.* RIA response.

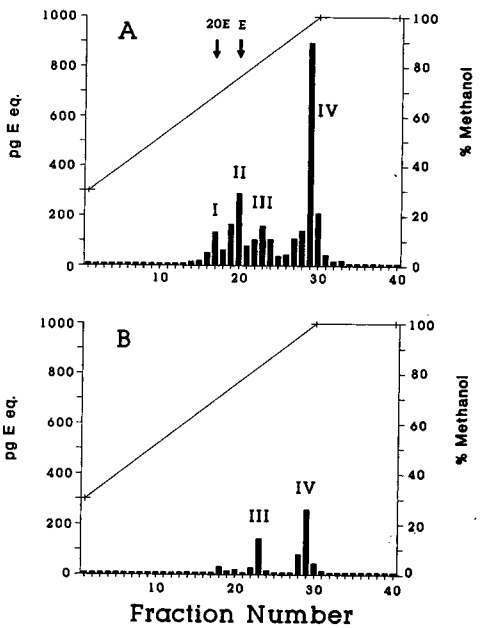

Fig. 5. Reversed-phase HPLC–RIA of fractions deriving from an extract of whole plants of *Kochia scoparia*. Plants were extracted with methanol and partitioned against hexane as described in "Materials and Methods" and then a portion (equivalent to 6 mg dry mass of plant) was separated on a C_{18} Sep-Pak cartridge. The RIA-positive fractions [(A) 60% methanol in water and (B) methanol] were separated on HPLC system RP1, 1-ml fractions collected and aliquots (50 μl) subjected to RIA.

TABLE I

ECDYSTEROID LEVELS IN *KOCHIA SCOPARIA* AS DETERMINED BY RADIOIMMUNOASSAY (DBL-1 ANTISERUM)

	RIA response (μg ecdysone equivalents/ g dry mass)
Seed	30
Roots	143
Lower stem	26
Middle stem	21
Upper stem	37
Lowest leaves	102
Middle leaves	112
Uppermost leaves	44
Flowers	56
Senescing (red) leaves	41

DISCUSSION

The combined use of RIA, bioassay and HPLC provides an effective means of identifying from very small samples which plant species contain phytoecdysteroids and for determining ecdysteroid profiles to identify those species which may contain novel ecdysteroid agonists or antagonists. Since analyses can be performed on such small samples, many samples may be processed rapidly and simultaneously. This approach has often been used in the analysis of ecdysteroids in insects, but has not been extensively used for phytoecdysteroids. In our studies on the Chenopodiaceae, these procedures have allowed the rapid analysis of *ca.* 100 species using seed samples of 50 mg or less. Only with species of the genera *Bassia*, *Corispermum* and *Kochia* were problems encountered [30], owing to interference in the RIA. Since *K. scoparia* provides a rare opportunity to relate phytoecdysteroid levels and distribution to insect feeding preference data, it was worth persevering with the analysis of this species.

The positive responses detected with both RIA and the B_{II} bioassay demonstrate that seed of *K. scoparia* do contain phytoecdysteroids. The major ecdysteroids appear to be 20E and PolB, based on co-chromatography on RP- and NP-HPLC systems and on the presence of appropriate biological activity. The presence of E is indicated by RP-HPLC–RIA, but needs to be confirmed by other means. E has a very low affinity for the ecdysteroid receptor [26], so it is not surprising that this peak does not show activity in the B_{II} bioassay. Several other peaks of RIA-positive material, presumably corresponding to other ecdysteroids, are also present. The identities of these are currently unknown. One of these compounds possesses significant biological activity and deserves to be investigated further.

Analysis of aerial portions of *K. scoparia* plants was hampered by difficulties in separating interfering substances from the ecdysteroids. However, it is clear that ecdysteroids are present in all parts of the plant. Qualitatively, there appears to be some difference between the ecdysteroid profiles of the plants and the seed. Most notable is the presence of relatively large amounts of a *Helix*-hydrolysable conjugate of ecdysone in plant material which is absent from seed. Its absence from seed is not an artefact deriving from the pre-treatment of seed with NaOH, since no evidence for this peak was seen in extracts of seed which had not been pre-treated. Since this conjugate was observed in extracts of whole plants, nothing can be concluded presently about its distribution within the plant. This would be worth investigating.

Quantitatively, levels of RIA-positive material in *K. scoparia* are low and fairly uniformally distributed when compared to *C. album*. Roots showed markedly higher ecdysteroid levels than the other samples and leaves contain higher levels than the stem. Roots may be the site of biosynthesis of phytoecdysteroids. Levels of RIA-positive material should relate directly to the levels of phytoecdysteroid, but is should be borne in mind that the measured RIA-response is a consequence of both the amount of each RIA-positive compound and their relative affinities for the antiserum. Thus, a similar RIA response can be obtained by a small amount of a high-affinity compound or a large amount of a low-affinity compound. Consequently, RIA assessments of complex situations such as might prevail in *K. scoparia*, with the ecdysteroid profile changing throughout the plant should be treated with some caution. However, it is unlikely that such an effect is masking a significant ecdysteroid concentration gradient in leaves of *K. scoparia*, but this possibility cannot be discounted until the phytoecdysteroids in different portions of the plant have been identified and separately quantified.

In conclusion, *K. scoparia* contains several phytoecdysteroids. There are some qualitative differences between ecdysteroids present in seed and in plants. Substantial evidence is presented for 20E and PolB being the major ecdysteroids in seed, as they are in almost all other chenopods which have been analyzed [30]. Although there are quantitative differences in ecdysteroid levels throughout mature plants these are not of the magnitude observed in *C. album*. Also, the distinct concentration gradient found in *C. album* is not present in aerial portions of *K.*

scoparia. Therefore, it seems unlikely that the phytoecdysteroids alone could account for the feeding behaviour of rangeland grasshoppers on leaves of *K. scoparia*. However, phytoecdysteroids may act synergistically with other secondary compounds (*e.g.* saponins) and a gradient of the other class of compound could engender a gradient of phytoecdysteroid potency. *Melanoplus* spp. Have been observed feeding on the stems of large plants of *K. scoparia* [21], and this may be associated with the lower levels of phytoecdysteroid there. Elevated ecdysteroid levels are not associated with flowering in *K. scoparia*. In this context, it is worth mentioning that *K. scoparia* is believed to be insect-pollinated [11].

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Rapid determination of 20-hydroxyecdysteroids in complex mixtures by solid-phase extraction and mass spectrometry

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ABSTRACT

Ecdysteroids possessing a 20,22-diol group react smoothly with arylboronic acids. Cyclic boronates formed in this reaction are stable towards moisture. The changed chromatographic properties of ecdysteroid boronates in comparison with free ecdysteroids allow the efficiency of sample prepurification to be improved using solid-phase extraction. Parent ecdysteroids can easily be released from their boronates by hydrogen peroxide. A rapid and efficient method for the determination of ecdysteroids in samples of biological origin based on solid-phase extraction and fast atom bombardment mass spectrometry is described.

INTRODUCTION

Ecdysteroids are widely distributed natural compounds occurring in both plants and invertebrates. They regulate a series of important physiological functions mostly in insects and other arthropods [1]. However, their function in plants is still not very clear [2,3]. Almost 200 structural analogues have been isolated from plant and ánimal sources so far [4]. They occur in biological material predominantly in complex mixtures. In plants they are often represented as one or two major constituents with admixtures of various minor structurally and biogenetically relative substances. To detect these constituents in either chemical or biological screenings new methods are being developed for their determination or specific extraction [5–7]. For many phytochemical studies there is a need for a rapid method for the determination of ecdysteroids directly in complex mixtures of other extractives. This paper describes such a method, which involves derivatization of ecdysteroids with arylboronic acids followed by solid-phase extraction (SPE) and fast atom bombardment mass spectrometric (FAB-MS) detection of ecdysteroid boronates and/or free ecdysteroids.

Arylboronic acids (aryldihydroxyboranes) have been demonstrated to form stable cyclic boronates with the side-chain diol group of 20-hydroxyecdysteroids (Fig. 1). The versatility of these derivatives has been demonstrated in many instances, *e.g.*, for chromatography [8–10], mass spectrometry [11] and the selective protection of the diol group of ecdysteroids and related compounds [12,13]. Solid-supported phenylboronic acid [6,7] offers the general advantages of solid-supported reagents, *i.e.*, easy removal of non-retained compounds and the possibility of re-using the reagent. On the other hand, it requires an efficient and mild method for releasing retained compounds. A method which is mild with respect to both dihydroxyborane groups and bonded ecdysteroids.

Phenylboronates of ecdysteroids can easily be prepared in various solvents. Several agents, such as diols, hydroxy acids and dicarboxylic acids, have been used for liberating ecdysteroids

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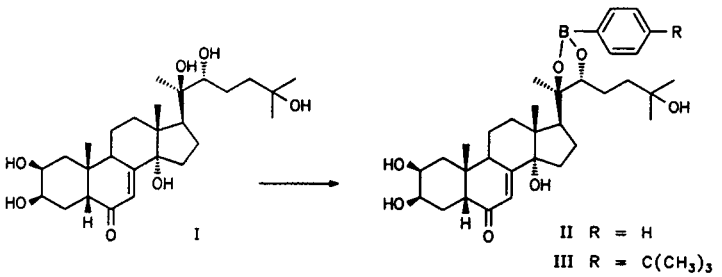

Fig. 1. Structures of 20-hydroxyecdysone (**I**) and its arylboronates **II** and **III**.

from their boronates; however, oxidative splitting of the C–B bond by hydrogen peroxide provides better results [14]. From a practical point of view, it is important that ecdysteroid boronates can also be prepared in water-containing solvents, which are commonly used for the preparation of biological samples.

Changes in the chromatographic properties of boronates permit the use of solid-phase extraction on reversed-phase supports with solvents of higher elutropic strength in comparison with the solid-phase extraction of free ecdysteroids. The efficiency of this purification step is increased when using a solvent with higher elutropic strength to remove polar impurities. In principle, there are two methods to increase the content of ecdysteroids in the sample. One method utilizes the possibility of eluting ecdysteroids as their boronates using a solvent with a higher elutropic strength than is possible when eluting free ecdysteroids. This approach is particularly useful for samples in which the main impurities are eluted with a solvent of the same or slightly higher elutropic strength than for free ecdysteroids. In the other method, the main impurities are eluted with a solvent of lower elutropic strength than is necessary for the elution of ecdysteroid boronates. Subsequently it is possible to release free ecdysteroids from their boronates with a methanolic solution of hydrogen peroxide with an elutropic strength set for the elution of free ecdysteroids. The impurities that would normally be eluted together with free ecdysteroids are thus removed while ecdysteroids are retained as boronates.

Ecdysteroid boronates are formed in complex biological samples in high yields. An excess of arylboronic acid has to be used in order to ensure a quantitative course of the derivatization reaction in water-containing samples and also in samples with a low content of ecdysteroids [14].

Fast atom bombardment mass spectrometry (FAB-MS) has been widely used for the structural identification of ecdysteroids [15–17]. Because it requires only a small amount of sample that, moreover, is applied to a liquid matrix as a solution in protic solvents, FAB-MS appears to be a very efficient method for the determination of ecdysteroids in prepurified samples. Depending on the chosen purification method, ecdysteroids can be determined either as boronates and then released by addition of hydrogen peroxide to the sample dissolved in the matrix on the probe tip, or they can be determined directly in a free form after elution from a solid-phase extraction (SPE) cartridge with a methanolic solution of hydrogen peroxide.

EXPERIMENTAL

Chemicals

Arylboronic acids, *i.e.*, phenylboronic acid and *p*-(*tert.*-butyl)phenylboronic acid, were prepared from the corresponding aryl bromides via Grignard reagents according to a common procedure [18]. 20-Hydroxyecdysone was isolated from roots of *Leuzea carthamoides* (Willd.) DC. The extract from *Leuzea carthamoides* for SPE experiments was prepared from finely ground roots. The roots were extracted with methanol–water (1:1, v/v) four times. The combined extracts were evaporated to half their volume and then extracted with *n*-butanol. The butanolic extract was evaporated to dryness and the residue was dissolved in methanol–water before being subjected to SPE. The content of 20-hy-

droxyecdysone in this extract was *ca.* 10%. Glycerol (Lachema, Brno, Czech Republic) and thioglycerol (Aldrich-Chemie, Steinheim, Germany) were used as the FAB-MS matrix. Hydrogen peroxide (30% aqueous solution) was supplied by Lachema. Methanol (Lachema) was redistilled and water was deionized and distilled.

Derivatization of ecdysteroids using arylboronic acid

20-Hydroxyecdysone 20,22-phenylboronate (**II**) and 20-hydroxyecdysone 20,22-*p*-(*tert.*-butyl)phenylboronate (**III**) were prepared by addition of 1.2 equiv. of arylboronic acid to a solution of 20-hydroxyecdysone (**I**, 10 mg) in methanol (20 μl). The mixture was allowed to react for 5 min. For plant extracts, 5 mg of arylboronic acid were added to the crude extract (20 mg) dissolved in methanol–water mixture (1:1, v/v) (1 ml). The mixture was allowed to react for 10 min.

Solid-phase extraction

SPE of standard compounds **I**, **II** and **III** was performed on Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA). 20-Hydroxyecdysone (**I**) and arylboronates **II** and **III** were applied to Sep-Pak cartridges previously rinsed with water. Methanol–water mixtures of different elutropic strength (2.5 ml) were used for elution. The amounts of eluted compounds **I–III** were determined by weighing of fractions (Fig. 2).

For *Leuzea carthamoides* extract a different method had to be used because of problems with the solubility of crude extracts after the derivatization reaction. A reversed-phase sorbent (40-μm Separon SGX C_{18}; Laboratorní přístroje, Prague, Czech Republic) (40 mg) was added to the solution of derivatized extract and the mixture was evaporated to dryness. The residue was added to a reusable Supelclean SPE tube (Supelco, Bellefonte, PA, USA) filled with the same sorbent (40 mg). The cartridge was enclosed and soaked with water. Methanol–water mixtures (2.5 ml) (containing 60%, 80% and 100% of methanol) were then used for elution.

The ecdysteroid content in the fractions eluted from the SPE cartridge was determined by reversed-phase HPLC. Ecdysteroid boronates

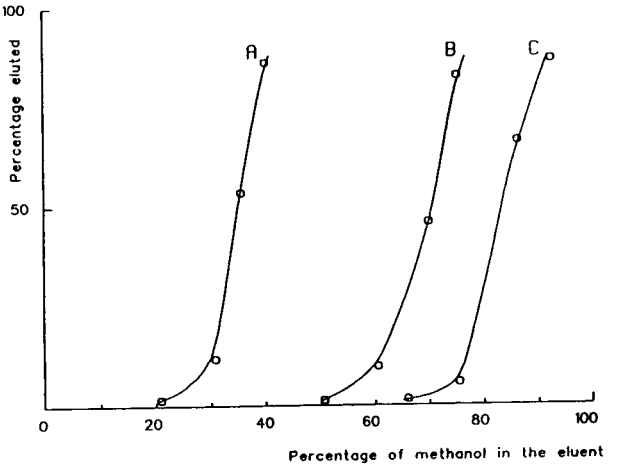

Fig. 2. Elution of (A) 20-hydroxyecdysone (**I**), (B) its phenylboronate (**II**) and (C) *p*-(*tert.*-butyl)phenylboronate (**III**) previously adsorbed on a Sep-Pak C_{18} cartridge. Percentage of 20-hydroxyecdysone (**I**) and its boronates **II** and **III** eluted with 2.5 ml of various methanol–water mixtures.

were first converted into free ecdysteroids by addition of hydrogen peroxide and then the ecdysteroid content in the fractions was determined by reversed-phase HPLC (Table I). A methanolic solution of 20-hydroxyecdysone was used as an external standard.

Mass spectrometry

Mass spectrometric measurements were peformed on a ZAB-EQ reversed-geometry mass spectrometer (VG Analytical, Manchester, UK) with an M-scan FAB gun (xenon, 8 kV, 1 mA) at an accelerating voltage of 8 kV. Glycerol–thioglycerol (3:1, v/v) was used as the matrix. Methanolic fractions from SPE (1 μl) were added to the matrix and mass spectrum was recorded. Release of free ecdysteroids was then accomplished by addition of hydrogen peroxide (3 μl) to a sample dissolved in the matrix on the probe tip. After reaction for 10 min the probe was inserted into the ion source and the spectrum of free ecdysteroid was recorded (Figs. 3 and 4).

RESULTS AND DISCUSSION

The elution curves of 20-hydroxyecdysone (**I**) and its phenylboronate (**II**) and *p*-(*tert.*-butyl)-

TABLE I

RESULTS OF SOLID-PHASE EXTRACTION OF *LEUZEA CARTHAMOIDES* EXTRACT AFTER DERIVATIZATION WITH ARYLBORONIC ACIDS

Content of methanol in eluent (%)	PBA[a]		*t*-BPBA[b]	
	Mass proportion (%)[c]	Content of 20E (%)[d]	Mass proportion (%)[c]	Content of 20E (%)[d]
60	83.4	0.5	67.1	0.3
80	10.2	25.5	23.9	3.0
100	6.4	40.1	9.0	48.7

[a] Extract treated with phenylboronic acid.
[b] Extract treated with *p*-(*tert.*-butyl)phenylboronic acid.
[c] Mass proportion of SPE fractions eluted by the eluent with corresponding elutropic strength.
[d] Content of 20-hydroxyecdysone determined after splitting of boronates.

phenylboronate (**III**) on Sep-Pak C_{18} are shown in Fig. 2. Both boronate curves are shifted to the area of higher methanol content. The presence of bulky *tert.*-butyl group in boronate **III** resulted in even stronger retention in comparison with boronate **II**. As *p*-alkylphenylboronic acids are accessible from related bromides, such aliphatic chains containing arylboronic acids represent an efficient route to modifying the polarity of ecdysteroid boronates. Consequently, the chromatographic properties of boronates can be suitably set for any particular sample. Boronates **II** and **III** showed sufficient stability during the SPE process. When crude extracts of *Leuzea carthamoides* were used, the observed elution curves did not exactly match those of pure boronates **II** and **III**. The major proportion of waste substances (more than 90%) was eluted with the eluent containing up to 80% of methanol. The highest concentration of 20-hydroxyecdysone, *ca.* 40% of the mass of the fraction, was detected (after splitting boronates) in the methanolic fractions. The results are summarized in Table I.

The methanolic fractions were used directly for FAB-MS. The mass spectra of fractions containing boronates **II** and **III** are shown on Figs. 3A and 4A, respectively. The protonated molecular ions of boronates, *m/z* 567 (Fig. 3A)

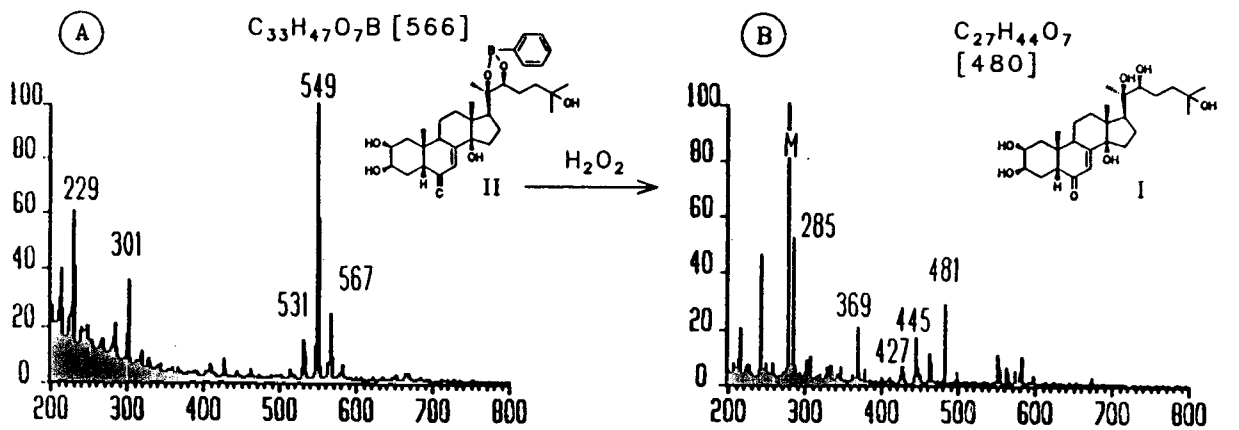

Fig. 3. (A) FAB mass spectra of fraction of *Leuzea carthamoides* extract containing boronate **II**; (B) after the reaction with hydrogen peroxide.

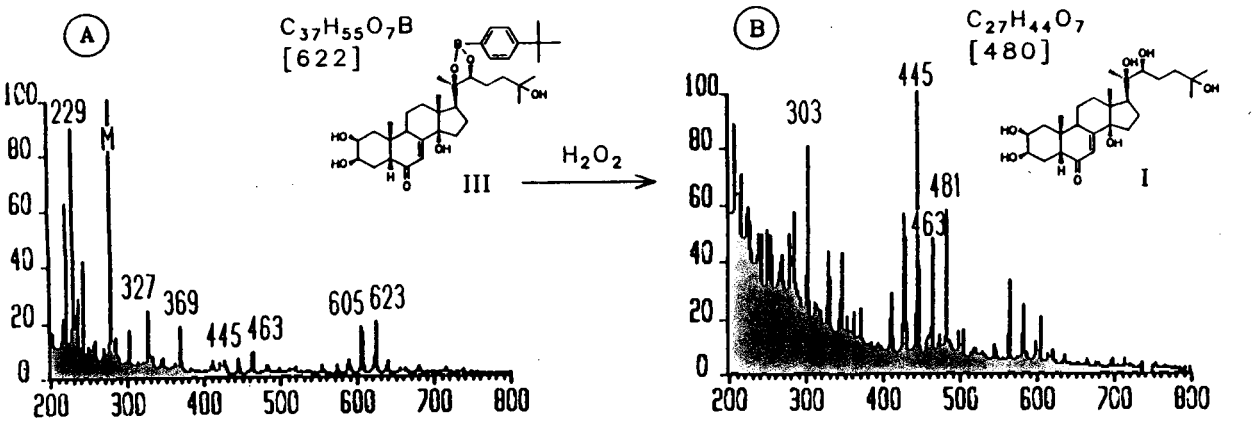

Fig. 4. (A) FAB mass spectra of fraction of *Leuzea carthamoides* extract containing boronate **III**; (B) after the reaction with hydrogen peroxide.

for derivatization with phenylboronic acid and *m/z* 623 (Fig. 4A) for derivatization with *p*-(*tert.*-butyl)phenylboronic acid, were sufficiently abundant to be easily detected. The spectra obtained after releasing 20-hydroxyecdysone (**I**) are shown in Figs. 3B and 4B, respectively. The protonated molecular ion of 20-hydroxyecdysone appeared at *m/z* 481 in both instances (Figs. 3B and 4B). By-products of the cleavage of boronates **II** and **III**, *i.e.*, boronic acid and phenol or *p*-(*tert.*-butyl)phenol, do not interfere in the range of interest for MS measurements. The ecdysteroid content in both samples was sufficiently high to obtain mass spectra of good quality. As cyclic boronate formation is typical of 20,22-diol-containing ecdysteroids, this reaction and also the reverse splitting can be considered as further evidence of the presence of ecdysteroids. The molecular mass of the present ecdysteroid can be determined from mass spectrum. Further information can be obtained from the fragmentation pattern, but this is often limited to losses of water molecules.

Leuzea carthamoides is known to contain, in addition to 20-hydroxyecdysone, several further minor ecdysteroids also possessing a 20,22-diol group [19]. It may be expected that they are also present in the analysed fractions. However, they were not found in the mass spectra of these complex mixtures, probably because of their low abundance. Experiments with MS measurements under various conditions is required for the determination of these trace ecdysteroids. In natural sources of ecdysteroids there could also be present ecdysone-related compounds not containing a 20-hydroxyl group and/or with a vicinal diol group in the side-chain protected as an isopropylidene or ester derivative. As the 2,3-diol moiety is not able to form stable boronates [14], these compounds could not be determined by this method. The method is limited to the major group of 20-hydroxyecdysteroids.

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Abscisic acid content of salt-stressed *Phaseolus vulgaris* L.

Comparison of high-performance liquid chromatography, gas chromatography with electron-capture detection, enzyme-linked immunosorbent assay and radioimmunoassay

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ABSTRACT

Sensitivity, reproducibility and cost effectiveness are important parameters to consider in analyses for abscisic acid (ABA). HPLC, GC with electron-capture detection (ECD), enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) were compared to determine the most appropriate method for the determination of ABA acid in salt-stressed bush bean plants. Determination of ABA by HPLC is better suited for samples with high ABA concentrations owing to the low selectivity of the UV detector. GC–ECD, ELISA and RIA are well suited for the assay of large numbers of samples and show good sensitivity for ABA. Analysis by RIA was the least costly method and required no sample purification process.

INTRODUCTION

Abscisic acid (ABA) (Fig. 1) is a phytohormone present in low concentrations in all higher plant organs, and evidence for its presence in algae and fungi has also been reported. The pathway for ABA synthesis in plants has yet to be fully elucidated, but there is considerable evidence to suggest the existence of two different paths. One results in the direct formation of ABA from farnesyl pyrophosphate and the other is an indirect route, presumably via the cleavage of a xanthophyll. Both pathways however, stem from mevalonic acid [1].

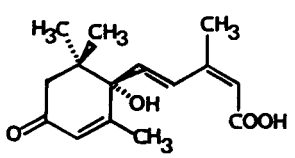

Fig. 1. Structure of (+)-(*S*)-abscisic acid.

ABA has been postulated to be involved in several phases of plant growth and development, although it is difficult to assign a specific role for this hormone owing to the lack of general

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knowledge of the regulation of developmental processes in plants. ABA is considered to be a stress hormone that functions as an endogenous regulator of plant transpiration, ameliorating the effects of water stress [2]. In some species of higher plants there is evidence for an ABA-mediated response to salt stress [3,4].

We are conducting several experiments to determine whether or not ABA is involved in the responses of the glycophyte *Phaseolus vulgaris* L. to conditions of salt stress, with special consideration being given to root–shoot interactions. These studies require extensive analyses of large numbers of samples taken at different time periods, so that we may define more clearly the phytohormone's role in the plant's response and adaptation to saline conditions.

The extraction and determination of ABA are difficult, as the phytohormone is present only in minute concentrations and the extraction and purification procedures are unavoidably extensive and time consuming. In addition, the efficiency of the extraction procedure is very difficult to determine.

ABA can be determined using physico-chemical methods such as HPLC and GC with electron-capture detection (ECD) [5]. Recently, however, the use of enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) methods has gained in popularity for ABA determination, providing a rapid and inexpensive determination of the hormone in plant tissues [6]. These techniques do not require large amounts of plant tissue for the extraction process, and therefore permit the determination of ABA even at the level of an individual plant cell [7]. Nevertheless, these methods share some of the problems that confront the physico-chemical methods: ELISA requires an extensive purification process owing to interferences with other plant compounds, and both ELISA and RIA analyses are occasionally subject to specific interferences that can be difficult to detect. Owing to these problems, ABA determinations via the latter techniques should always be verified by either GC–ECD or GC–MS [8].

The objective of this work was to determine which of the methods tested, HPLC, GC–ECD, ELISA and RIA, was the most suitable for the determination of ABA in tissue samples of bush bean plants.

EXPERIMENTAL

Plant materials

Eight-day-old plants of *Phaseolus vulgaris* L. var. Contender were germinated in vermiculite and transferred to a 50% modified Hoagland nutrient solution (pH 5.5) [9]. To each solution container either 1 or 25 m*M* NaCl was added. The experiments were conducted in a growth chamber (Conviron 15) under conditions that resembled a typical mediterranean spring day.

Twelve hours after salt addition, samples of roots, leaves and xylem sap were taken. Plant tissue was immediately frozen in liquid nitrogen, freeze-dried and stored at −80°C until analysis.

ABA purification for HPLC and GC–ECD

All procedures for ABA extraction and purification were performed under conditions of low light intensity and temperature to minimize the photodegradation and oxidation of the phytohormone. All solvents used were of chromatographic grade.

ABA extraction and purification were carried out by a modification of a procedure for HPLC reported earlier [10]. Freeze-dried plant tissues were homogenized in liquid nitrogen and extracted overnight at 4°C in 80% aqueous methanol, adjusted to pH 8 with $NaHCO_3$. Butylated hydroxytoluene were added as an antioxidant at a concentration of 20 mg l^{-1}.

The same protocol was followed for GC–ECD with a slight modification as the greater sensitivity for ABA provided by this method permitted the use of smaller amounts of sample and lower solvent volumes. For HPLC, 1 g of leaves of stems or 2 g of root tissue were required. For GC-ECD, only 0.25 g of each type of tissue was needed to perform the above extraction procedure.

Approximately 300 Bq of [^{3}H]ABA (2.55 TBq $mmol^{-1}$) (Amersham International, Amersham, UK) were added to determine purification losses. Extraction of ABA from the plant tissue using water [11] was also performed but proved unfeasable for beans, as it was impossible to

separate the organic and aqueous phases in the partitioning step.

The homogenate was centrifuged at 1545 *g* for 20 min. The supernatant was taken to the aqueous phase *in vacuo* at 35°C. The pH of the aqueous phase was then adjusted to 8 with 6 *M* NaOH and partitioned into ethyl acetate three times. The aqueous fraction was then adjusted to pH 2.5 with 6 *M* HCl and again extracted three times with ethyl acetate. The ethyl acetate fraction was dried over Na_2SO_4 (anhydrous) and evaporated to dryness at 35°C. Methylene chloride (2 ml) was added to the dry extract and the solution was loaded on to a Sep-Pak silica cartridge (Waters), prewashed with 5 ml of methylene chloride. The cartridge was washed with aliquots of organic solvents with increasing polarity: (1) 2 ml of 5% acetone in methylene chloride, (2) 5 ml of 4% methanol in methylene chloride and (3) 3 ml 10% methanol in methylene chloride. The ABA was eluted from the Sep-Pak cartridge by solvents with polarities greater than 4% methanol in methylene chloride. Fractions 2 and 3 were bulked and evaporated to dryness *in vacuo* at 35°C.

Quantification by HPLC

The resulting dry residue from the extraction procedure was dissolved in 0.5 ml of methanol and filtered through a 0.45-μm pore size filter.

ABA was determined with the use of a Beckman System Gold HPLC, equipped with a solvent programmable module (Beckman Model 126) and a variable-wavelength detector (Beckman Model 166), set at 254 nm. The column used was a Beckman Ultrasphere (25 cm × 4.6 mm I.D.) of 5.0-μm particle size. ABA was determined using a 0–100% linear gradient from water to methanol for 15 min at a flow-rate of 1 ml min^{-1}. The column was then flushed with methanol for 15 min to remove any remaining compounds. The retention time of ABA was *ca.* 12 min.

To confirm the authenticity of the ABA peak, authentic *cis,trans*-ABA (Sigma) was co-chromatographed with each sample The ABA content was calculated from a calibration graph obtained with known amounts of ABA (r = 0.99).

The efficiency of the extraction process could not be determined as it was not possible to quantify exactly how much of the original *in vivo* hormone pool had been recovered. The efficiency of the purification procedure was determined by scintillation counting of [^{3}H]ABA, removing aliquots of known amount in each step of the purification procedure. Tritium activity was determined with a Beckman LS 1800 liquid scintillation spectrometer. The efficiency for ^{3}H was 81.1 ± 5.56%.

Quantification by GC–ECD

The dry residue obtained at the end of the purification process was methylated with diazomethane (50 μl of methanol + 500 μl of etheral diazomethane). After 15 min the samples were evaporated to dryness in a stream of nitrogen, reconstituted with 0.5 ml of hexane and stored at −30°C for GC–ECD analysis.

The ABA content was determined on a Hewlett-Packard (St. Louis, MO, USA) Model 5790 gas chromatograph equipped with a ^{63}Ni electron-capture detector and a Hewlett-Packard Model 4500 integrator. Splitless injections of 1 μl were made on to a DB-5 capillary column (30 m × 250 mm I.D.) manufactured by J&W Scientific (Folsom, CA, USA). The GC operating conditions were an injector temperature of 250°C with helium as the carrier gas at a flow-rate of 2 ml ml^{-1} and nitrogen as the make-up gas at a flow-rate of 20 ml min^{-1}. The starting oven temperature programme was 100°C and was raised at a rate of 25°C min^{-1} to 240°C, where it was held for 5 min. The retention time of ABA was *ca.* 7 min. The ABA peaks were qualitatively confirmed as *cis,trans*-ABA after chromatography with authentic *cis,trans*-ABA. Peak areas were plotted against known amounts of ABA injected, with a linear working range of 0.5–500 ng for the calibration graph. Based on this calibration curve (r = 0.99), ABA concentrations were calculated, taking into account the specific extraction yield of each particular sample (dpm [^{3}H]ABA recovered).

ABA purification for ELISA

The purification procedure for ELISA was based on methods reported previously [3,4].

Briefly, plant tissue was homogenized in liquid nitrogen for 5 min and extracted overnight at 4°C in 80% aqueous methanol containing 1 mg l^{-1} of butylated hydroxytoluene to avoid oxidation. A trace amount of [^{3}H]ABA was added to each sample to monitor the ABA recovery.

To remove any impurities, the methanolic extract was passed through a Sep-Pak C_{18} cartridge that had been prewashed with 1 ml of 80% methanol. The methanolic phase was removed with a vacuum centrifuge (Savant Speed Vac Plus, Model SC 110A) at 40°C. The resulting aqueous portion was partitioned three times against ethyl acetate, which had previously been adjusted to pH 3.0 with 1 *M* HCl. The ethyl acetate fractions were combined and evaporated to dryness under low pressure. The residue was dissolved in saline Tris buffer (TBS) of pH 7.8 and sonicated for 2–3 min for assay. The efficiency of ABA purification, based on recoveries of added [^{3}H] ABA, was 92 ± 1%.

ABA quantification by ELISA

ABA was determined by ELISA using commercially available ABA assay kits from Idetek (San Bruno, CA, USA), which uses the competitive binding protein method to measure ABA concentrations. In this assay, the sample and the enzyme-coupled antigen must compete for antibody-binding sites.

The ELISA protocol consists of incubation of samples or ABA standards at 4°C for 1 h in a microwell plate precoated with antibodies. A 100-μl volume of tracer (ABA–alkaline phosphatase conjugate) was added to each cell and incubated at 4°C for 3 h. After discarding the solutions, the cells were washed with distilled water and blotted dry with filter-paper. A 200-μl volume of substrate (*p*-nitrophenyl phosphate) was added to the dried cell and incubated at 37°C for 1 h. Absorbances were then read at 405 nm. A series of ABA standards containing from 0.01 to 0.5 ng of ABA ware assayed for each microtitre plate. ABA concentrations were calculated by referring to the calibration graph after linearization using a log–logit function [12]. No evidence of any non-specific interference with the ELISA assay was observed for sample dilutions over a 30-fold range or with the addition of synthetic ABA. Validation of this assay was done by comparing the results with those obtained with physico-chemical methods.

ABA quantification by RIA

Freeze-dried bean tissue was ground to a fine powder and ABA was extracted overnight with chilled distilled water (0°C).

The RIA analysis was carried out as described [13] using the monoclonal antibody AFRC MAC 62 (supplied by Dr. S. Quarrie of the Institute of Plant Research, Cambridge, UK), which is specific for (+)-ABA. Samples were incubated with [^{3}H]ABA and the monoclonal antibody for 45 min at 5°C. A saturated solution of ammonium sulphate was added, incubated for 4 min and then centrifuged for 5 min at 8800 *g* (Eppendorf centrifuge) to separate the free and bound antigen. The pellet was washed by resuspension in 50% ammonium sulphate solution and then centrifuged for 5 min at 8800 *g*. Radioactivity in the resultant solution was determined with a liquid scintillation counter (Beckman LS 1800). Concentrations of ABA in the samples were calculated from a calibration curve constructed from known concentrations of standard *cis,trans*-(+)-ABA after linearization using a logit transformation [13]. ABA concentrations ranged from 0.125 to 2.0 ng per vial that were present in each standard bath. The lack of non-specific interferences in RIA assays for leaves, roots and xylem sap in crude-tissue extracts of beans has been reported recently [14]. Validation of the RIA assay was performed by comparing the results with those obtained by GC–ECD.

RESULTS AND DISCUSSION

Extraction

An extended extraction and purification process was necessary for all of the methods tested, except for the xylem sap samples in ELISA and all plant tissues analysed by RIA, where no sample purification was required.

The experiments involved the determination of ABA in young plants where, as the total ABA content is very low, a large portion of the plant must be sampled to obtain a detectable level of ABA. Consequently, the extraction of the

phytohormone proved to be a considerable problem, as the 1–2 g dry mass of sample needed for ABA determination required the harvesting of at least four plants. This constraint seriously limited the number of replicates in the different treatments, as the possible number of plants to be cultured was ultimately controlled by the capacity of the growth chamber. In more complex experiments with a greater number of treatments, successive plant cultures would be necessary in order to achieve a significant number of replicates for the hormone analysis.

Although the GC–ECD and HPLC methods for ABA determination used the same purification process, the need for larger amounts of tissue for HPLC necessitated the use of larger solvent volumes, with a resulting increase in cost per sample extraction. It should also be noted that the added difficulty of handling larger amounts of tissue required a greater time expenditure, and as a result it was possible to process only 4–6 samples per day with this method. Moreover, the higher sensitivity of GC–ECD for ABA permitted its determination in tissues of an individual plant, which was impossible with HPLC. Consequently, the tissue savings provided more replications for the same number of plants and thus offered the possibility of a much better statistical significance. Although the determination of ABA by GC–ECD required an extra step in which the hormone had to be methylated with diazomethane, this added manipulation posed no real extra time expenditure or cost increase when account is taken of the general advantages of GC–ECD over HPLC.

With regard to the purification process used for ABA determination by HPLC and GC–ECD, the recovery of [^{3}H]ABA was 81.1 ± 5.56%. Losses of ABA were highest during the partitioning against ethyl acetate, because despite the solvent's high partition coefficient for ABA at both pH 2.5 and 8 [15], ABA losses still amounted to *ca.* 10% (data not shown).

The sample purification process for ELISA had the advantage that only very small amounts of sample were required, which allowed an increase in the number of replicates per treatment and a high [^{3}H]ABA recovery (92 ± 1%).

ABA quantification

The ABA contents in different bush bean plant tissues treated with 1 or 25 m*M* NaCl, determined by the different methods tested, are given in Table I. Extracts from the 25 m*M* NaCl stressed plants contained up to twice the ABA concentration present in the non-stressed 1.0 m*M* NaCl controls. Other workers have also reported increases in the ABA content of salt-stressed plants [3,4].

A common characteristic observed for the ABA content with all the methods used was the high error values. This may be due at least in

TABLE I

ABA CONCENTRATIONS IN DIFFERENT TISSUES OF BUSH BEAN PLANTS GROWN WITH 1 OR 25 m*M* NaCl AND DETERMINED BY HPLC, GC–ECD, ELISA AND RIA

Values are means ($n = 4$). No significant differences were found among ABA values determined by the different methods tested (analysis of variance).

Method	ABA concentration					
	Leaves (ng g^{-1} dry mass)		Xylem (μmol m^{-3})		Roots (ng g^{-1} dry mass)	
	1 m*M* NaCl	25 m*M* NaCl	1 m*M* NaCl	25 m*M* NaCl	1 m*M* NaCl	25 m*M* NaCl
HPLC	.410 ± 90	770 ± 98	–	–	250 ± 80	310 ± 50
GC–ECD	450 ± 40	830 ± 55	37 ± 3	78 ± 10	320 ± 50	370 ± 30
ELISA	500 ± 10	910 ± 15	42 ± 2	72 ± 7	290 ± 20	330 ± 15
RIA	490 ± 15	850 ± 55	35 ± 5	65 ± 8	278 ± 45	320 ± 20

part to the high phenotypic variability of bean plants. In this respect, analysis by ELISA or RIA would be advantageous, as the analysis of large numbers of samples would be needed to provide a greater statistical significance.

There were no significant differences among ABA values determined by the different methods (analysis of variance).

Fig. 2 shows the typical HPLC (A) ABA standard and bush bean (B) leaf and (C) root samples. ABA determination was achieved with difficulty owing to the large amounts of different products present in the sample, despite the extended purification procedure applied. Root analysis (Fig. 2B) was especially difficult owing to the low ABA content in this tissue and the increase in the amounts of interfering products, as it was necessary to use 2 g dry mass of sample for the extraction. We consider that for ABA determination by HPLC, a more extensively purified sample should be used to increase the ABA resolution, which can be achieved by using a preparative HPLC step prior to quantification [16,17].

Following the same procedure for other tissue samples, the HPLC selectivity was too low to detect the ABA content in the collected samples of xylem sap (Table I), for which purification was necessary. Larger amounts of sap would have be necessary so that interferences from other compounds could be offset by the larger ABA peak areas.

With ABA determination by HPLC, special care should be taken to avoid the accumulation of impurities in the column and solvents, which can seriously affect the ABA resolution. Perhaps owing to a related phenomenon, although proper maintenance and operational procedures were followed, the HPLC columns had a much shorter life span than their GC counterparts.

GC–ECD tracers for ABA are presented in Fig. 3 for (A) standards and (B and C) tissue samples. A superior resolving power was achieved with the capillary GC column compared with HPLC. The electron-capture detector with high sensitivity and selectivity for molecules with high electron affinity [18] proved to be very

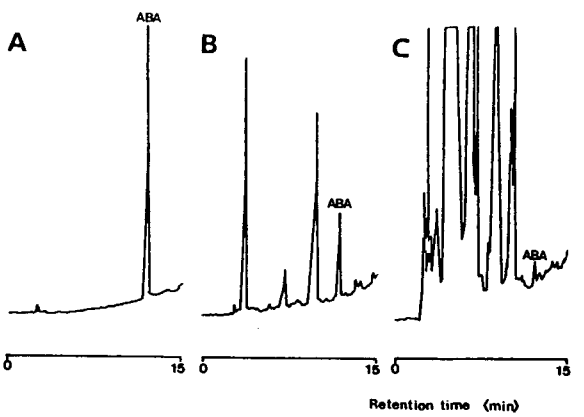

Fig. 2. HPLC of (A) ABA standard (500 ng) and bush bean (B) leaf and (C) root samples.

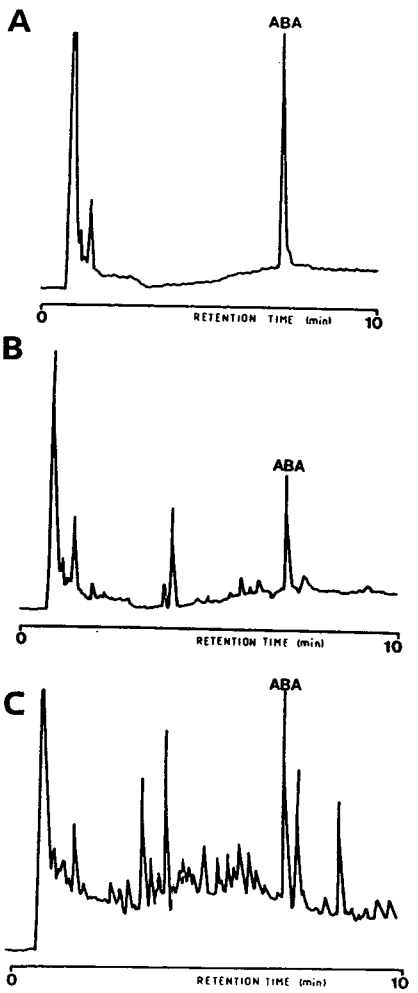

Fig. 3. GC–ECD of (A) ABA standard (500 ng) and bush bean (B) leaf and (C) root samples.

specific for ABA, and hence very few compounds other than the phytohormone were observed.

The standard deviation for ABA determination was not larger in the GC–ECD than the HPLC method. Although higher standard deviations for GC have been reported, these are due to other factors, such as difficulty in achieving reproducible sample injections and the minute injection volume with respect to the sample volume [15]. Concerning sample volume, HPLC had the advantage of using large injection volumes, which made possible the analysis of a greater portion of the total sample for injection. Nevertheless, the standard deviation in this experiment for the HPLC method was larger than that for the GC–ECD method, but this could be due to the heterogeneity of the samples and the smaller number of replicates.

Although the analysis times were similar for the HPLC and the GC–ECD methods, the contamination problem of the HPLC column resulted in an overall greater time expenditure for the HPLC method. Extra flushing and cleaning times were necessary for HPLC, which led to a serious addition of time for the analysis.

Even though the monoclonal antibodies used in the immunoassays are highly specific to the antigen, it is well known that other materials in plant extracts can competitively and non-competitively inhibit the antigen–antibody interaction, given erroneous readings [8,19]. Therefore, there is a need to validate the assay for each type of extract examined. Validation of the ELISA for ABA determination in different tissues of bean plants by comparing the results with those obtained by GC–ECD is shown in Fig. 4A. The good agreement between the ABA values obtained by the ELISA and GC–ECD methods ($r = 0.99$) validated the use ELISA for the determination of ABA in different bush bean plant tissues. The only problem with the extended use of the Idetek kits for this purpose is their high cost.

The RIA assay for ABA was done using a (+)-ABA specific monoclonal that does not bind (−)-ABA or most ABA metabolites and derivatives [13]. For this reason, no purification of the extracts was necessary. The good correlation

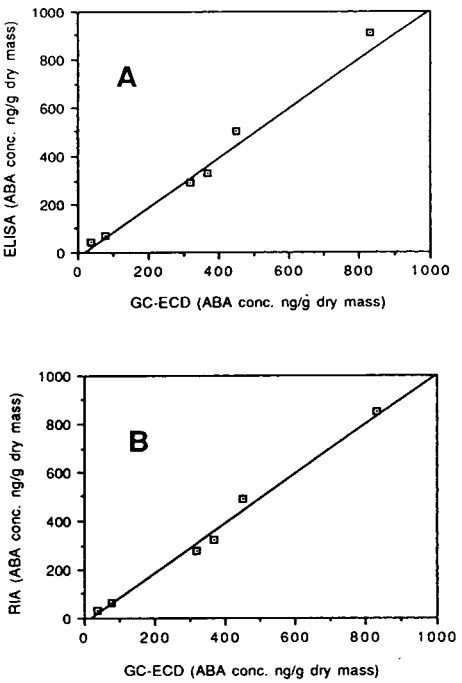

Fig. 4. Comparison of ABA concentrations obtained by (A) RIA and (B) ELISA *versus* GC–ECD. Linear regression estimates are: (A) $y = -26.894 + 1.1057x$; $R^2 = 0.988$; (B) $y = -22.876 + 1.0433x$; $R^2 = 0.988$.

found for ABA contents in the different bean tissues analysed by GC–ECD and RIA validated this method. Because no purification was needed for RIA, this method proved to be the least time consuming, permitting the assay of over 100 samples in duplicate within 1 day.

In conclusion, the determination of ABA in bush bean plants by GC–ECD and RIA was the most suitable with regard to selectivity and cost. Once validated, RIA proved to be the fastest of the four methods. ELISA had the inconvenience of high cost, but was the most sensitive for ABA detection. HPLC was best suited for samples with dry masses greater than 1 g and with high ABA contents, owing to the low selectivity of the UV detector.

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Review

Chromatography of gibberellins

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ABSTRACT

The recent literature on the use of chromatography in the analysis of gibberellins in plants is reviewed. Particular emphasis is placed on the application of solid-phase purification techniques, immunoaffinity chromatography, HPLC, GC–MS and LC–MS.

CONTENTS

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1. INTRODUCTION

Methods of gibberellin (GA) analysis have been recently summarized in reviews by Hedden [1,2], Beale and Willis [3], Barendse [4], Takahashi *et al.* [5] and Crozier and Durley [6]. Gaskin and MacMillan [7] have described in detail the procedures for GC–MS analysis of the GAs and related compounds. In this review the emphasis is on recent developments in the use of chromatography in GA analysis, particularly in the application of solid-phase purification techniques, immunoaffinity chromatography, HPLC, GC–MS and LC–MS. The availability of GC–MS in particular as a sensitive and specific means of detecting and quantifying GAs has led to the increasing adoption of small-scale chromatographic procedures for the purification of GAs from small amounts of plant material.

2. STRUCTURES

The gibberellins are a group of diterpenoids, currently with some 90 members recognized (Fig. 1), and a number of others awaiting the allocation of GA numbers. Their chemistry has been recently reviewed by Mander [8]. A number of structural variations on the *ent*-gibberellane skeleton (Fig. 2) influence their behaviour in chromatography. Two major divisions of structure exist; the C_{19} GAs have a 19-carbon pentacyclic skeleton which includes in most cases a C-19,10 lactone ring (*e.g.*, GA_9), and the C_{20} GAs have a 20-carbon skeleton. In the C_{20} GAs, C-20 is present as either a methyl- (*e.g.*, GA_{12}), carboxy- (*e.g.*, GA_{25}) or aldehyde- (*e.g.*, GA_{24}) function, or is included in a C-19,20 lactone ring (*e.g.*, GA_{15}). All of the GAs are carboxylic acids, at C-7, and may also be carboxylated at C-18 or -19. One, two, three or four hydroxy functions may be present at C-1, -2, -3, -11, -12, -13, -15, -16, -17 or -18. Two GAs have epoxy functions, and two have oxo functions. A number of the C_{19} GAs are dehydrogenated at C-1,2 or C-2,3, and two at C-9,11. Several C-15-ene GAs have been identified, and while such compounds may arise as artefacts of acidic extraction procedures it seems likely that they also occur naturally.

A number of endogenous GA–glucosyl conjugates have been identified. These include esters, which are neutral, and ethers (glucosides), which are acidic (Fig. 3). In addition a small number of other naturally occurring esters have been found [8]. The function of the glucosyl conjugates in plants is uncertain. Their formation may convert GAs to inactive, water-soluble forms suitable for transport or storage. It may also serve in regulation of the pool of active GAs, by removing biosynthetic precursors or inactivating those GAs directly active in growth. The glucosyl ester conjugates may be hydrolyzed to release the free GA [9,10] and thus may serve as a repository of potentially active GAs [9–11]. These recent findings should prompt increased interest in analysis of the GA conjugates.

The immediate precursors of the GAs are kauranoids. Because of the relevance of their physiology to the GAs and since they are sometimes isolated in conjunction with the GAs and GA conjugates, the use of chromatography in their analysis is discussed briefly. Those which are part of the biosynthetic pathway leading to the GAs include compounds with a range of properties, from *ent*-kaurene with no polar functional groups to the acidic and hydroxylated *ent*-7α-hydroxy-kaurenoic acid (Fig. 4).

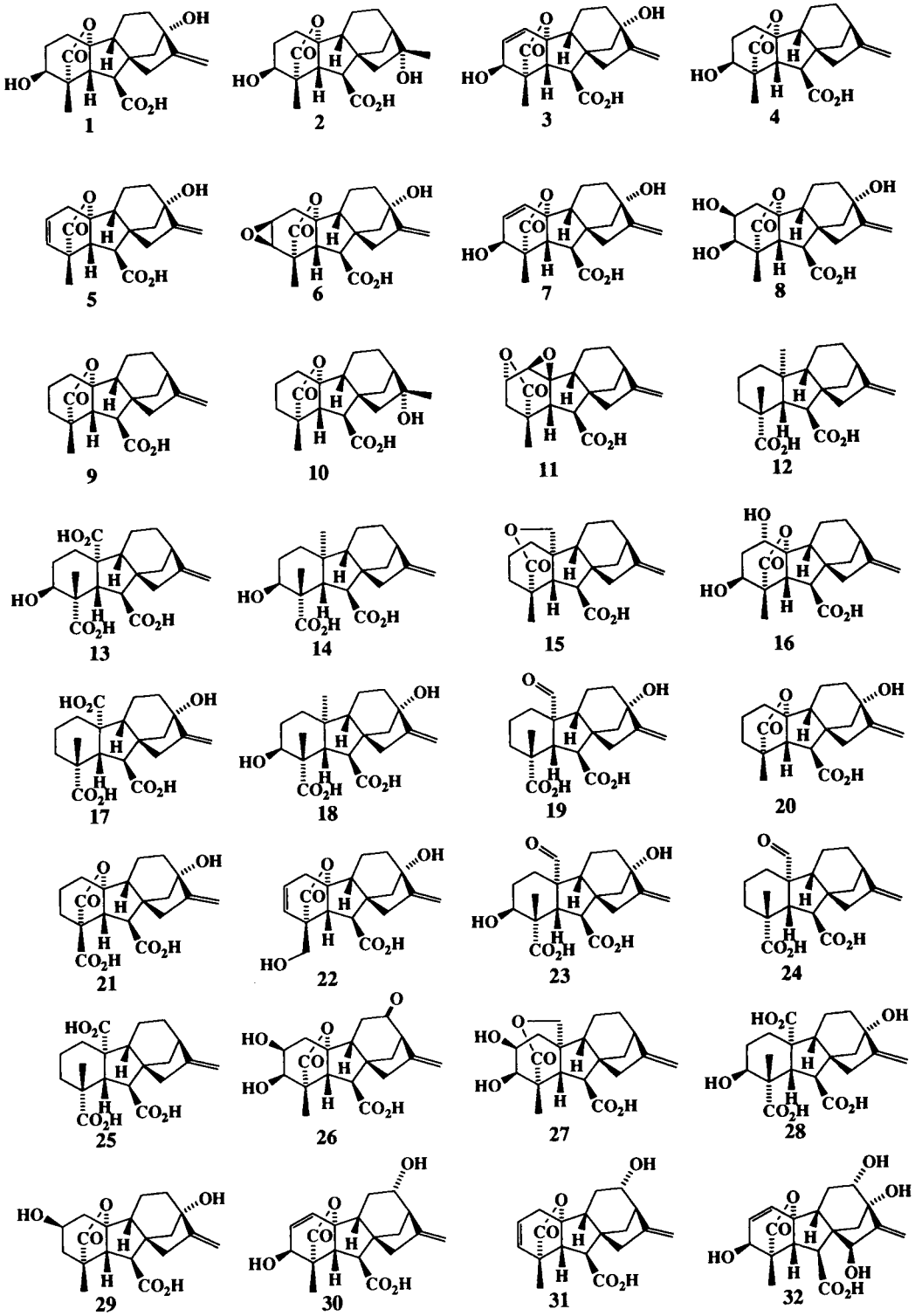

Fig. 1.

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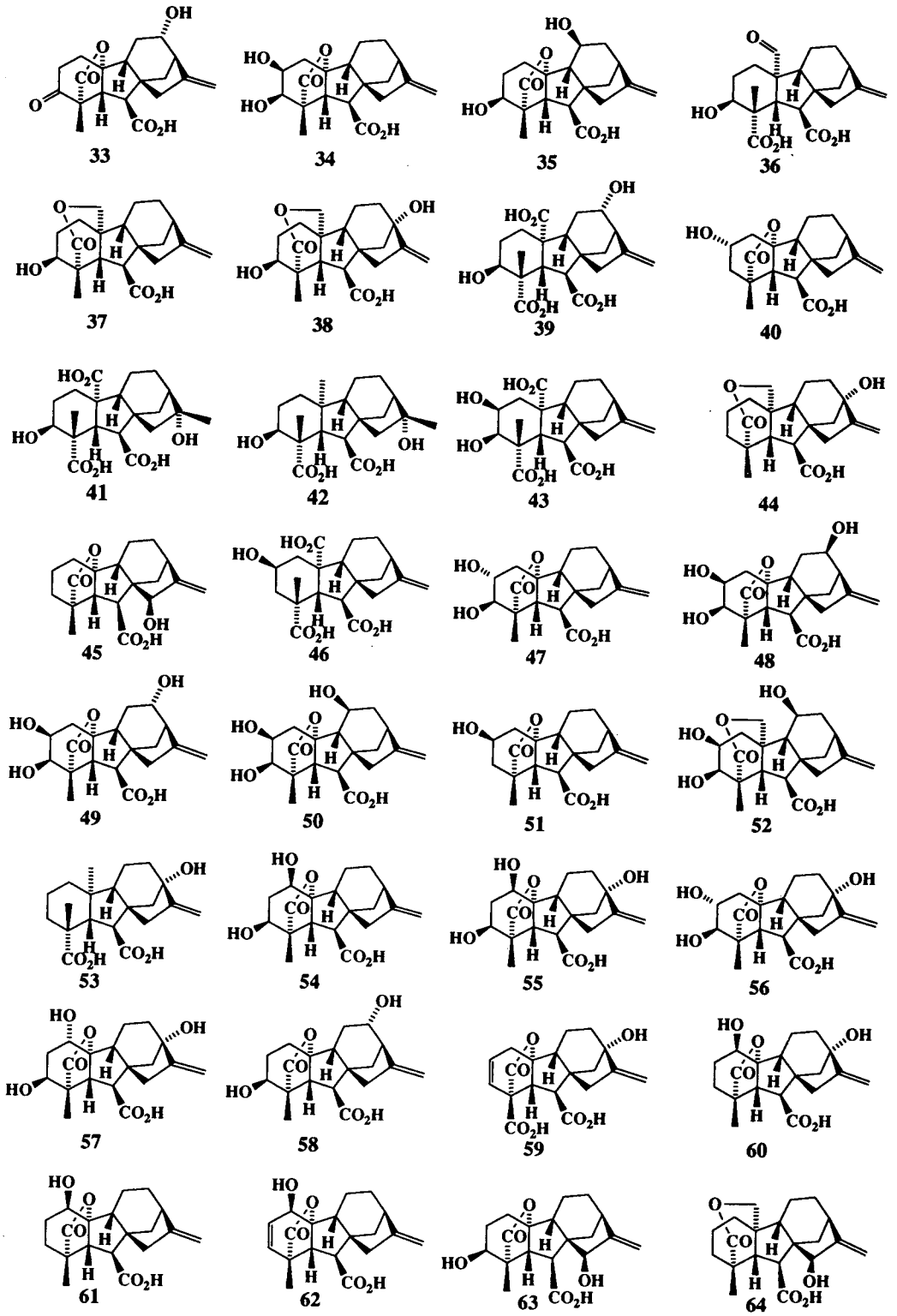

Fig. 1 (*continued*)

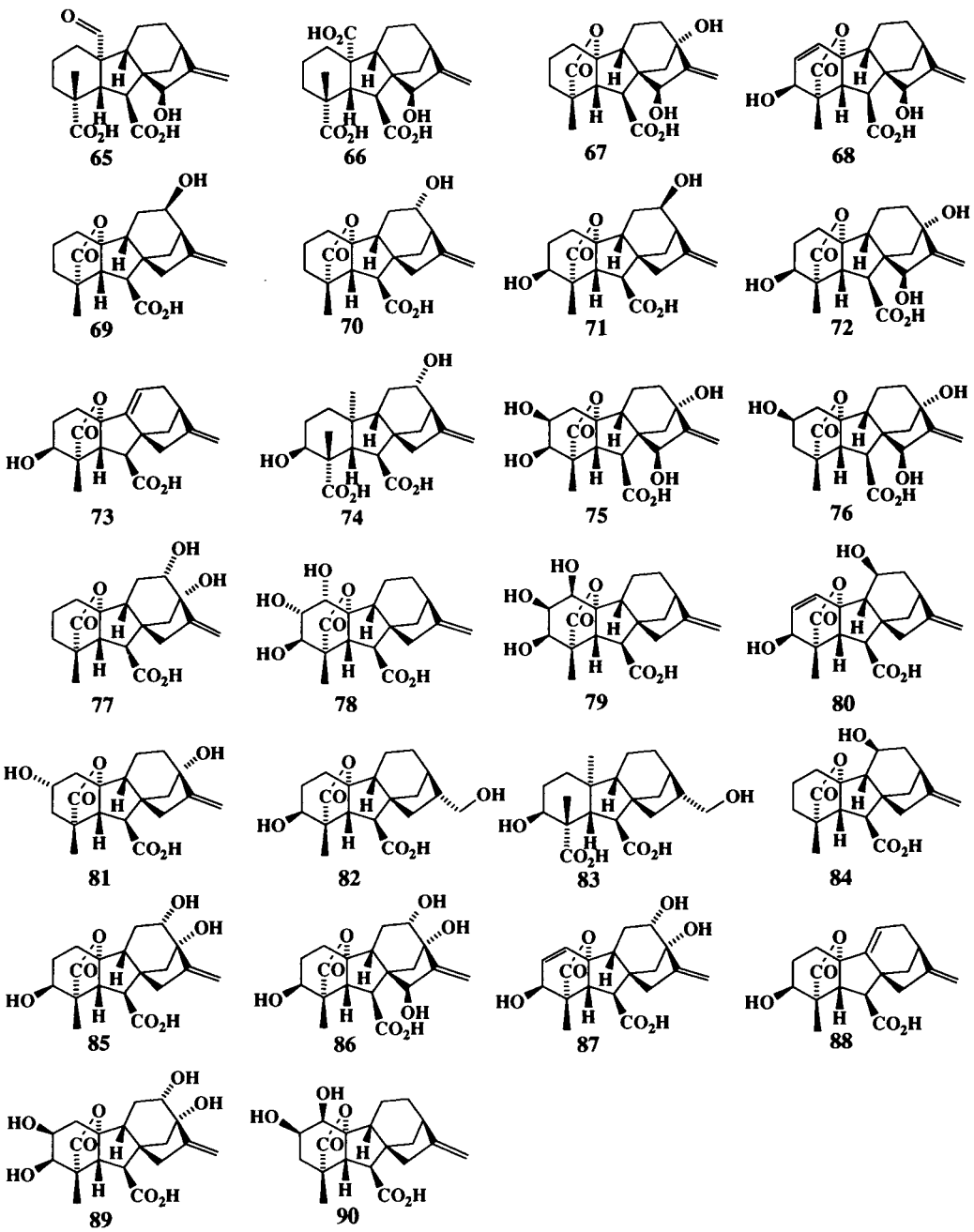

Fig. 1. Structures of the gibberellins. Numbers correspond to n in GA_n.

3. PREPARATIVE CHROMATOGRAPHY

Where samples are destined for GC–MS, the purpose of preparative purification is to concentrate the GAs so that an aliquot containing sufficient mass for identification or quantification (usually in the range ng to pg) can be introduced to GC. At the same time extraneous compounds are reduced so that they do not interfere with chromatography, contribute excessively to the

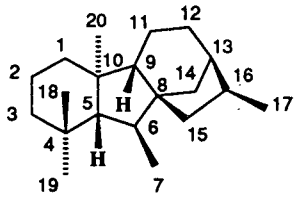

Fig. 2. C-numbering of the *ent*-gibberellane skeleton.

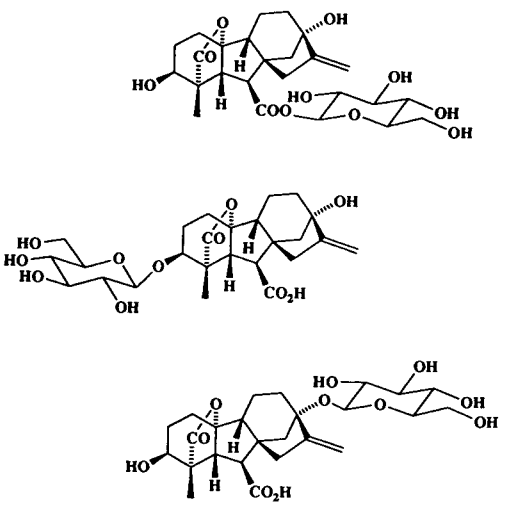

Fig. 3. Structures of GA_1–glucosyl conjugates. Top, GA_1–glucosyl ester; middle, GA_1–3-O-glucoside; lower, GA_1–13-O-glucoside.

background signal, or contaminate the mass spectra or ion chromatograms of the compounds of interest. For convenience in minimizing the number of samples which are submitted to GC–MS analysis, the GAs are fractionated as little as possible during purification. Where immunological methods are used to detect and quantify the GAs, purification is required to eliminate compounds which would otherwise interfere with binding, and often the GAs must be resolved by HPLC to provide additional evidence of identity. Clearly the purification procedures required will vary from sample to sample.

The usual approach in preparative purification of GAs is to retain the free GAs as a group, insofar as possible, separate from the GA conjugates and from the less polar kauranoids. The methods employed have been described in the reviews cited above; that of Hedden [1] is a particularly instructive practical guide to GA analysis. A brief summary of the methods employed in group separation and purification is included here.

3.1. Solvent partitioning

This traditional method of preliminary purification of GAs is still in widespread use. Typical schemes have been summarized in the reviews [1,3,5]. After extraction, usually in 80% aqueous methanol, the methanol is evaporated *in vacuo* and the majority of the GAs are extracted from the aqueous residue by partitioning into ethyl acetate at pH 2.5–3.0. The most polar GAs, such as GA_{32} [12] and other tetrahydroxylated- and some trihydroxylated species (GA_{72}, GA_{75}, GA_{76} [13]) remain to a large extent in the aqueous phase, from which they can be extracted with *n*-butanol. Subsequent partitioning of the initial ethyl acetate phase, against 0.1 *M* phosphate buffer at pH 8.5, leaves neutral compounds in the ethyl acetate. The acidic GAs partition into the aqueous fraction and can be extracted by partitioning into ethyl acetate after the pH is adjusted to 2.5–3. Alternative procedures modify the order of the partition steps and may incorporate early removal of less polar impurities by extraction from the aqueous residue into ethyl acetate or other solvents such as light petroleum, diethyl ether or *n*-hexane at pH 8–9. A significant proportion of the less polar GAs, such as GA_4 and GA_9, can also be expected to partition into ethyl acetate at pH 8, but not into diethyl ether at pH 9 [14].

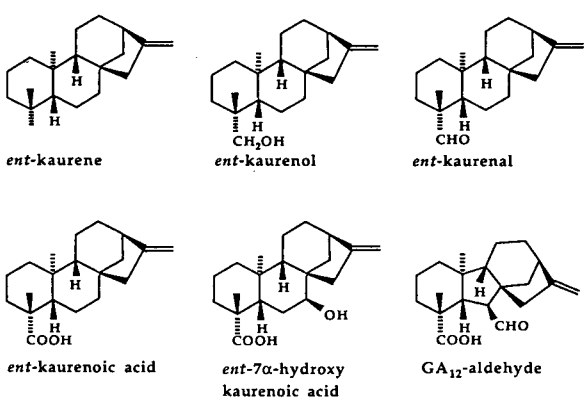

Fig. 4. Structures of GA_{12}-aldehyde and the *ent*-kauranoid precursors of the gibberellins.

The GA conjugates are usually isolated from extracts together with free GAs, with solvent partitioning which results in them being divided amongst neutral ethyl acetate (GA–glucosyl esters) and neutral and acidic *n*-butanol (GA–glucosyl esters and glucosides) fractions [15] (see refs. 1 and 5). Schneider [16] ascribed such partitioning characteristics to the dominating effect on solubility of the hydroxy groups of the glucose moiety, rather than the degree of ionization of the –COOH function of the GA. An alternative approach which avoids this division is to extract the GA-conjugates as a group from aqueous solution into acidic *n*-butanol, to be subsequently divided into neutral and acidic fractions if required [17]. The acidic fraction will also contain the free GAs, and these can later be separated from the conjugates by other methods such as size-exclusion chromatography or on HPLC as the permethyl derivatives.

The kauranoids have also been obtained from the usual extraction in 80% aqueous methanol, or where these are the only class of compounds of interest have been extracted in less polar solvents such as ethyl acetate [18–20] or acetone [21], and then further purified by methods more applicable to their chemistry. *ent*-Kaurene, *ent*-kaurenol and *ent*-kaurenal are neutral, and *ent*-kaurenoic acid is a weak acid which may occur in both neutral and acidic fractions [7].

A number of chromatographic methods have been applied to purify the various fractions from solvent partitioning. Some of these have been successfully used in lieu of solvent partitioning, in keeping with the increasing use of solid-phase extraction techniques in the preparative purification of natural compounds. Other methods, used formerly as analytical procedures, have been supplanted in this rôle by methods which offer improved resolution and are used now mainly as preparative techniques.

3.2. Normal-phase chromatography

Several types of sorbents have been widely used. These include polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP), charcoal, silica and Sephadex in various forms. Small columns of bonded-phase material have become increasingly popular for preparative normal-phase chromatography.

In recent studies the most-used adsorbent for larger-scale purification has been PVPP or PVP, to bind and remove phenolic compounds from aqueous extracts of free GAs [22]. Samples are applied to columns in phosphate buffer at pH 8.0–8.5 and the GAs washed from the columns with buffer; alternatively PVP is simply filtered from slurries. Recent examples of PVP use include the purification of extracts of apple seeds [23], shoots of *Brassica* [24], *Zea* [25] and *Cucumis* [26], and shoots [27] and fruits [28] of *Citrus*. While acidic GAs and their glucosyl conjugates are recovered well from PVP, it seems possible that some might be retained if associated with phenolics [29] which bind to the PVP. GA–glucosyl conjugates could also be purified on PVP [22], but few examples are found. A PVP column was used in the normal manner in the purification of GA_9–glucosyl ester from shoots of *Picea* [30].

Charcoal or charcoal–Celite columns were used routinely in earlier studies to separate salts and polar impurities such as sugars from the GAs. The impurities are eluted with aqueous acetone or sometimes with aqueous methanol, and GAs are eluted subsequently in an increasing proportion of acetone, the most polar first [31]. While providing effective purification, the activity of charcoal can be variable, and acid-induced artefacts may be formed. These include isopropylidene derivatives (acetonides) of GAs with vicinal diols [32,33]. Recoveries may be poor because of irreversible adsorption especially of more polar compounds. These considerations have resulted in recent use being confined to only a few examples, such as the purification of GAs from leaves of *Silene* [34], and shoots of *Citrus* [27] and *Spinacia* [35]. Charcoal columns eluted with aqueous methanol or acetone have been used successfully as a means of purifying GA conjugates from large quantities of seeds in a number of earlier studies [15,36–39].

Normal-phase chromatography on silica also entails risk of poor recovery of strongly adsorbed polar compounds. Nonetheless, silica is often used in preparative purification, either in columns or in TLC. The latter largely replaced

paper chromatography and was originally popular as an analytical technique in GA analysis. It is used now only occasionally, mostly in preparative purification. The behaviour of free GAs and GA methyl esters in a number of acidic and basic solvent systems has been described in a number of studies [40,41]. Analytical use is now confined to situations where only a few compounds need to be resolved, as is often the case in analysis of the products of feeds of various substrates. Thus Ozga *et al.* [42] used silica TLC, with a solvent system of ethyl acetate–chloroform–acetic acid (90:30:1), to separate [^{14}C]GA_{20} and [^{14}C]GA_{19} from extracts of pericarp of *Pisum* fed with [^{14}C]GA_{12}. Such an acidic solvent system is typical of those used. There are a number of recent examples of schemes incorporating similar use of TLC in preparative chromatography of endogenous free GAs [43–45].

Takahashi *et al.* [5] have included tabulations of the R_F values of a range of GA conjugates in TLC obtained in early studies, and Schneider [16] has summarized the early widespread use of TLC in the analysis of GA conjugates formed from the metabolism of applied ^{3}H-, ^{14}C- or unlabelled GAs. Schneider *et al.* [46] have described a more recent use of silica TLC in the purification of permethylated GA–glucosyl conjugates before GC–MS analysis. A solvent system of toluene–ethyl acetate–acetic acid was used to purify the permethylated GA–glucosides as a group. A similar separation was also achieved on a column of silica eluted with a gradient of methanol in toluene [47].

Column chromatography, commonly on deactivated silica, has been more widely used in the purification of free GAs. The procedure pioneered for GAs by Powell and Tautvydas [48] and further developed by Durley *et al.* [49] (see also [6]), continues in routine use in several laboratories. Samples are applied to a column of silica with 0.5 *M* formic acid as the stationary phase and eluted with formate-saturated ethyl acetate in *n*-hexane, in a gradient or steps of increasing proportion of ethyl acetate if some separation of the GAs is required. The GAs elute in order of increasing hydroxylation or increasing carboxylation [49]. A 20×1.3 cm column of Woelm silica gel (deactivated with 20% water) had high capacity (100 mg of plant extract) and recoveries of the GAs exceeded 80%. Smaller columns (5 g, 10×1 cm) have been used in the preparative purification and separation of free GAs [9], or, eluted with 70 ml of ethyl acetate–*n*-hexane (95:5), to separate the GAs as a group from more polar impurities [50]. In these conditions [^{3}H]GA_8 elutes in 20–50 ml [51]. There are a number of other examples of the recent use of these procedures [23,26,45,52]. Less frequently, active silica has been used. Examples include Beale *et al.* [53], who used silica eluted in steps of increasing proportion of ethyl acetate in light petroleum (b.p. 40–60°C) in the purification of GA_{58} from endosperm of *Cucurbita*. Extracts of leaves of *Spinacia* [35] and *Silene* [34] were purified on columns of silica–Celite (1:2, w/w; 12×1.5 cm), with the GAs eluted in chloroform–ethyl acetate to leave adsorbed polar impurities.

Koshioka *et al.* [50] have used the "short-column" deactivated silica method to separate GA conjugates as a group (eluted from the column in methanol), from the free GAs, eluted previously with ethyl acetate–*n*-hexane. Evidence of the separation was based on the analysis of extracts of several [^{3}H]GA-fed plant tissues, with tentative identification of metabolites as being conjugated or otherwise. Subsequently, the procedure has been used routinely to separate conjugated GA-conjugates from free acids in studies of the metabolism of [^{3}H]GAs [9,54–57]. Although the GA conjugates can be expected to be much more polar than their respective aglycones, as seen for example in the separation of GA_9 and GA_9–glucosyl ester in normal-phase HPLC [58], it is now clear that GAs of a wide range of polarity cannot be cleanly separated as a group from their glucosyl esters and glucosides in this manner. Earlier examples of the use of silica in column chromatography for the purification of GA–glucosyl conjugates have been discussed in the reviews [5].

Silica columns have commonly been used to purify kauranoids, to remove impurities such as chloropyll and to provide preparative separation of the various kauranoids. Recent examples include that of Zeevaart and Gage [20], who separated *ent*-kaurene from the more strongly

retained chlorophyll in extracts of *Spinacia* and *Agrostemma* shoots on a 20-ml column of silica eluted with 40 ml of *n*-hexanes. Hazebroek and Metzger [21] purified a more polar kauranoid-containing fraction from shoots of *Thlapsi* on a 10 × 1.5 cm column of silica eluted with 100 ml of ethyl acetate–chloroform, and a less polar fraction on silica eluted in steps of increasing proportion of ethyl acetate in *n*-hexane. Suzuki *et al.* [19] separated kauranoids in the acidic ethyl acetate extract of Zea shoots on a 20-g column of silica eluted with 100 ml volumes of light petroleum (4×); light petroleum–ethyl acetate (95:5), 4×; light petroleum–ethyl acetate–acetic acid (90:10:0.2), 4×; light petroleum–ethyl acetate–acetic acid (85:15:0.2), 2×; and light petroleum–ethyl acetate–acetic acid (80:20:0.2), 2×. *ent*-Kaurene was contained in the first fraction, *ent*-kaurenal in fractions 2–4, *ent*-kaurenol in fractions 7–8, *ent*-kaurenoic acid in fractions 9–13 and *ent*-7α-hydroxy kaurenoic acid and GA_{12}-aldehyde in fractions 11–13. Silica Sep-Pak cartridges have been used to separate fatty acids from *ent*-kaurene in extracts of germinating caryopses of *Hordeum* [59]. The sample was applied in 5 ml of light petroleum (b.p. 60–80°C) and the cartridge washed with a further 5 ml of light petroleum to completely elute the *ent*-kaurene.

Columns of Sephadex G-25, G-50 and LH-20 have been used in normal-phase separations of GAs and kauranoids. MacMillan and Wels [60] described an analytical procedure with capacity for 100–200 mg of plant extract which yielded excellent separation of a range of GAs, but which required 30 h to complete. A 145 × 1.5 cm column was packed with LH-20 which had been equilibrated with the aqueous phase of a solvent mixture of light petroleum–ethyl acetate–acetic acid–methanol–water (100:80:5:40:7). The column was eluted with the organic phase of the mixture. Another column used with the solvents described above but in the proportions 50:15:10:10:2 provided better separation of the less polar GAs and kauranoids. A "non-polar" column used with light petroleum–acetic acid–methanol (100:1:40) separated *ent*-kaurene, *ent*-kaurenol and *ent*-kaurenoic acid. While partition chromatography clearly predominated in these cases, the mechanism of separation operating with this type of support may also include a component of size-exclusion chromatography. The use by Yamaguchi *et al.* [61] of LH-20 eluted with methanol–acetone, in which seven different GAs eluted in a narrow range of volumes, was described by those authors as "rough gel permeation". This procedure has been used subsequently in several studies as a means of preparative group purification of the free GAs [62–64]. Sephadex G-50 [15,36,39] and Sephadex LH-20 [17,38] have also been used for the normal-phase purification of GA conjugates.

Poling and Maier [27] described the use of bonded-NH_2 Sep-Pak cartridges for the purification of samples in preparation for HPLC on $N(CH_3)_2$ columns. Samples were loaded in methanol, the cartridge eluted with 15 ml of methanol, and the GAs eluted with 10-ml volumes of each of methanol–acetic acid (99:1) and methanol–acetic acid (98:2). From extracts of shoots of *Citrus* and of seeds of *Pisum*, GA_9, GA_{20}, GA_{29}, GA_{51} and GA_{29} catabolite eluted mainly in the methanol–acetic acid (99:1) fraction, with 15–20% of GA_{20} and GA_{29} eluting in the methanol, and 20–25% of the GA_{29} catabolite eluting in the methanol–acetic acid (98:2) fraction. With 20 mg loading, the sample mass was reduced by 90%. We have purified various samples using a similar procedure with NH_2-PrepSep cartridges and found that the retention of GAs on loading, and also when the cartridge was eluted with non-acidic solvent, was not always predictable [65]. As might be expected, the GAs were better retained initially if loaded in ethyl acetate–methanol (4:1) instead of methanol. After the cartridge was eluted with 1-ml volumes of ethyl acetate–methanol (4:1 and then 1:1), [^{3}H]GA_3, [^{3}H]GA_8, [$^2H_2$]GA_{19}, [^{3}H]GA_{20} and [^{14}C]GA_{53} were eluted in 1 ml of methanol–acetic acid (99:1) and 3 ml of methanol–acetic acid (98:2). The method could be further modified by adjusting the pH of the sample to ensure that the GAs were ionized and so better retained on loading.

A number of Japanese workers in particular have reported use of a similar procedure, entailing chromatography on cartridges such as Bond Elut DEA, as a routine preparative step in GA

analysis. Samples are applied in methanol, impurities eluted with methanol, and the GAs eluted with methanol containing a small percentage of acetic acid. While the behaviour of standard GAs in this system does not seem to have been described, GAs representing a range of structures have been identified in samples purified by this method [28,66–69].

3.3. Reversed-phase chromatography

Small cartridges of reversed-phase material, such as C_{18} Sep-Pak, are now often used in preparative purification schemes. An example of the use of the method has been described by Hedden [1]. After washing the cartridges with methanol and then with 5% acetic acid, samples are loaded 0.1 *M* phosphate buffer pH 2.5. Polar impurities are eluted with 5 ml of 5% aq. acetic acid, and then with 5 ml of water, and the GAs subsequently eluted with 5 ml of 80% aq. methanol, leaving less polar impurities on the cartridge. Very polar GAs may elute from the cartridge in the wash with 5% acetic acid and water, and this step can be omitted. There are many recent examples of the use of C_{18} cartridges, which include various minor variations on the procedure described above [23,24,26,70–72].

Commercial C_{18} cartridges have limited capacity and are thus suitable for use later in purification schemes or for small samples. For larger samples, small columns packed with C_{18} material can be used in the same fashion as described for cartridges, with the volumes of eluting solvents increased accordingly. Such columns have also been used in lieu of solvent partitioning of aqueous extracts (against light petroleum, diethyl ether or hexane) to effectively separate chlorophyll, carotenoids and other non-polar impurities from the GAs in aqueous methanolic extracts [50].

These procedures are also suitable for separating non-polar impurities from the GA conjugates, which elute from reversed-phase chromatography before their aglycones (see section 4.1.3). Similarly, some of the more polar kauranoids can be purified in this manner [19].

3.4. Size-exclusion chromatography

The use of size-exclusion chromatography (SEC) for GA purification was first described by Reeve and Crozier [73,74]. Bio-Beads SX-4 (porous polystyrene, exclusion limit M_r 1500) was packed into two 100×2.5 cm columns connected in series which were eluted with tetrahydrofuran at 2 ml min^{-1}. Solutes elute in order of decreasing size, and the free GAs were obtained in an elution volume of 450–570 ml. This method has high capacity and recoveries are high, but is time-consuming. Faster, small-scale HPLC-based methods were subsequently developed [75]. In chromatography on a 300×8 mm column of PL gel (cross-linked polystyrene–divinylbenzene copolymer) in tetrahydrofuran–acetic acid (99.5:0.05), free GAs (defined by [^{3}H]GA_{43}, M_r 394, and [^{3}H]GA_9, M_r 316) were eluted in 6.8–7.8 ml. Acetic acid was added to suppress ionization of the GAs and its use resulted in improved chromatography. The capacity of this column was 100 mg of plant extract. In similar fashion a variety of C_{19} and C_{20} GAs ranging in molecular mass from 316 (GA_9) to 364 (GA_8) were eluted from a 500×8 mm column of Shodex A-801 (polystyrene–divinylbenzene polymer, with an exclusion limit of about M_r 1000) in terahydrofuran at 1 ml min^{-1}, with retention times between 11.9 and 13.7 min [61]. The method has been used recently for preparative purification of GAs in a number of studies [63,71,76–78]. SEC may be of general practical value when used early in a purification procedure to reduce sample mass for subsequent chromatography [75]. Its usefulness later in purification will depend on the nature of the particular sample. Thus when used after preparative RP-HPLC in the purification of GA_9 from extracts of *Picea*, HPSEC yielded little useful additional purification of the GA_9-containing fractions [79].

The large difference in mass between free GAs and GA conjugates has been exploited to separate these two groups of compounds by SEC. Thus ^{3}H-labelled GA-glucosides and glucosyl esters, produced in shoots of *Phaseolus* seedlings fed with [^{3}H]GA_4, have been separated as a group from [^{3}H]GAs [80] with the

open column SEC procedure of Reeve and Crozier [73]. The ^{3}H-labelled high-molecular-mass compounds eluted in 340–365 ml, and the ^{3}H-labelled low-molecular-mass compounds eluted in 370–440 ml.

SEC has also proved useful in the purification of kauranoids. From an extract of shoots of *Thlapsi*, several of the kauranoids were separated as a group from impurities of higher-molecular-mass (notably chlorophyll), on a 500 × 6 mm column of Bio-Beads SX-8, exclusion limit M_r 1000 [18]. About 90% of the sample mass eluted in the first 90 ml of ethyl acetate, and the next 30 ml contained *ent*-kaurene, *ent*-kaurenoic acid and *ent*-kaurenol. This method is a valuable alternative to chromatography on silica in such purifications.

3.5. Anion-exchange chromatography

Anion-exchange chromatography on DEAE-Sephadex A-25 was used by Gräbner *et al.* [81] to separate GA_3, GA_7 and GA_3–3-O-glucoside. Adaptations of this procedure, including chromatography on other anion-exchange materials, have become increasingly popular as a means of separating neutral and weakly acidic impurities from the more acidic GAs, or for separating GA–glucosyl esters from the free GAs. The DEAE-Sephadex is prepared in the acetate form, neutral compounds are eluted with methanol, and increasingly acidic compounds eluted with increasing amounts of acetic acid in methanol, as described, for example, by Fujioka *et al.* [25]. In this study a 100-ml column was eluted with successive 100-ml volumes of methanol, 0.25 *M* acetic acid in methanol, 0.5 *M* acetic acid in methanol, 0.75 *M* acetic acid in methanol, 1 *M* acetic acid in methanol and 3 *M* acetic acid in methanol. Free GAs apparently eluted in all of the acidic fractions. This procedure yields some resolution of the acidic compounds, but if this is not required a simple two-step elution with methanol or weakly acidic methanol and then with more acidic methanol should suffice [6].

The stronger anion-exchanger QAE-Sephadex A-25 has also been used in a number of studies. Aqueous extracts at pH 7.5–8.0 were applied to a 5-ml column with had been equilibrated with sodium formate, neutral impurities were eluted with 15 ml of water, and the GAs eluted with 20 ml of 0.2 *M* formic acid [23,24]. QAE-Sephadex A-25 in the acetate form has been used in a similar fashion [34,35]. Other materials which have been used in recent studies include DEAE-cellulose (DE-52) in the hydroxylated form [45].

DEAE-Sephadex A-25 has been widely used in preparative chromatography of GA conjugates, to separate the neutral GA–glucosyl esters from the acidic GA–glucosides [10,46,80,81]. The method is the same as described above for the free GAs. QAE-Sephadex A-25 has been used to purify GA glucosyl esters from shoots of *Picea* [30]. The sample was applied to a 10 × 1 cm column in 5 m*M* sodium phosphate pH 8 and the GA–glucosyl esters eluted with 25 ml of the same buffer.

ent-Kaurenoic acid, *ent*-7α-hydroxy kaurenoic acid and GA_{12}-aldehyde from extracts of *Zea* shoots have also been purified on DEAE-Sephadex A-25 [19]. On a 25-ml column eluted with 10-ml volumes of methanol (4×), 0.25 *M* acetic acid in methanol (4×), and 0.5 *M* acetic acid in methanol (4×), these compounds eluted with the last of the methanol fractions and in the first three weakly acidic fractions.

3.6. Immunoaffinity chromatography

Immunoaffinity chromatography (IAC) is a valuable technique for selectively purifying small quantities of GAs before identification or quantification, with a minimum of other preparative steps. Antibodies for use in immunoassays for GAs have been developed in a number of laboratories, and used in studies which include those of Fuchs and Fuchs [82], Weiler and Wieczorek [83], Atzorn and Weiler [84,85], Eberle *et al.* [86], Knox *et al.* [87,88], Odén *et al.* [79], Yamaguchi and co-workers [78,89,90], Durley *et al.* [91] and Nakajima and co-workers [92,93]. Such antibodies were first used in IAC by Fuchs and Gertman [94]. However, not until recently has the application been revived, and reports of use are confined to only a few examples. The principles of IAC have been described in one of these [70]. Briefly, anti-GA antibodies (monoclonal or polyclonal) are raised against

GAs linked to a carrier protein [bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH)]. The position of attachment of the protein to the GA-hapten determines the portion of the GA molecule which is presented for immune recognition, and thus determines the specificity of the antibodies. The antibodies are subsequently bound to a support, usually Sepharose, which is used to prepare a column. Partially purified samples are added to the column, and antigenic GAs form ligands with the antibodies. After non-antigenic impurities are eluted from the column, the bound GAs can be eluted with water, aqueous methanol or methanol, in order of increasing affinity for the antibody [95]. Depending on the specificity of the antibody or antibodies which are used, IAC may be used to selectively bind a single GA, a certain class of GAs (*e.g.*, C-3- or C-13-hydroxy), or a wider range of GAs. In common with immunological techniques, the binding of the primary antigen(s) to the antibody in IAC is subject to interference by inhibitory components in plant extracts, and by cross-reactive compounds which bind with similar affinity as the GA(s) of interest. Various preparative purification steps may be required to remove such compounds, as illustrated in the examples which follow. Results of these studies emphasize the need for verification of identities and quantities by a less equivocal method such as GC–MS.

Polyclonal antibodies, which might be expected to offer a range of specificities and thus be most useful in binding a wide range of GAs in IAC, were used in IAC by Durley *et al.* [91]. Antibodies were prepared against a mixture of GAs (GA_1, GA_3, GA_4, GA_7 and GA_9) each conjugated to BSA via *p*-aminohippuric acid at the carboxy function. These five GAs include common structural features found in C_{19} GAs (C-3 and C-13 hydroxylation, and C-1,2 and C-2,3 double bonds). The anti-GA antibodies, immobilized on matrices of Affi-Gel 10 or Sepharose CL-4B, retained C_{19} GA methyl esters, but did not retain free GAs or any of the C_{20} GA methyl esters which were examined (GA_{13}, GA_{14}, GA_{18}, GA_{23} and GA_{27}). Spikes of the methyl esters of the GAs used for antibody preparation, and in addition the methyl esters of GA_{20}, GA_{29}, GA_{34}, GA_{54} and GA_{57}, were recovered from a soybean leaf extract with greater than 90% yield. In order to avoid non-specific binding of substances such as phenolics to the column, extracts were first purified by anion-exchange chromatography on DEAE-cellulose (DE-52), and then by chromatography on a small (1.5–2 ml) C_{18} column. The methylated samples were taken up in methanol, diluted with phosphate-buffered saline (PBS), and applied to the column. Impurities were washed from the column with PBS and water, and the bound GA methyl esters eluted with methanol, which was found to be the most effective of a variety of chaotropic agents and changes in pH. A 3-ml column of Affi-Gel 10 loaded with 35 mg ml^{-1} of immunoglobulin G (IgG) could retain 390 ng ml^{-1} of GA_1 methyl ester, 1090 ng ml^{-1} of GA_4 methyl ester and 1280 ng ml^{-1} of GA_9 methyl ester. Columns could be reused as many as 500 times. Durley *et al.* noted that high IgG loading is an advantage in immunoaffinity chromatography of small molecules such as GAs, since the column capacity is maximized, the amount of immunosorbent is minimized, the flow-rate is maximized, and the amount of methanol required to elute the GAs is minimized. A high column capacity (10- to 100-fold greater than the content of the sample) is necessary to ensure complete recovery of the GAs of interest in the face of competition from cross-reacting GAs and interference from other components in plant extracts.

Knox *et al.* [87,88] and Nester-Hudson *et al.* [96] have prepared a number of monoclonal antibodies raised against GAs coupled to KLH at either C-3 or C-17. These antibodies recognize free acids, and have been used in IAC in a number of studies for the purification of GAs from a variety of materials.

The antibody MAC 183 has a high affinity for GA_4 and discriminates between GA_4 and GA_9, while MAC 136 binds C-13-hydroxy GAs. These were tested for use in IAC by Smith and MacMillan [95]. An aqueous extract of *Pisum* cotyledons was effectively purified by MAC 136, and yielded GA_{29} by GC–MS in similar quantity to that found in an extract purified by other methods. In a test of the possible effect of

interference by components in extracts, the binding of labelled GA_{20} to the matrix was found to be constant in the presence of increasing amounts of an extract of internodes of *Pisum*. Results with MAC 183, however, illustrated some of the potential problems with IAC, particularly if immunoassay is used subsequently to identify and quantify GAs. The MAC 183 column bound a small proportion of the large amount of GA_{43} and GA_{46} present in *Marah* endosperm, and these GAs were subsequently also bound by MAC 183 in radioimmunoassay (RIA). The amount of GA_4, the primary antigen, was negligible. The method thus erroneously indicated the presence of GA_4. Given these problems, Smith and MacMillan concluded (of the successful results with MAC 136) that "... confidence in the reliability of these results was entirely dependent on the information provided by GC–MS."

Smith *et al.* [70] used MAC 136, and MAC 213 (which binds C-3-hydroxy GAs), in IAC to purify GAs from stems and leaves of *Cucumis*. The aqueous methanolic extracts were first partitioned against light petroleum (b.p. 60–80°C), and passed through a C_{18} Sep-Pak, to remove components which interfered with antigen–antibody binding. It was also necessary to pass the extract through foetal calf serum (FCS)–Sepharose to remove components which would bind non-specifically and irreversibly to FCS in the IAC matrix, and thus also interfere with binding. FCS was the major non-antibody protein present in the culture medium of the antibody-producing cells and as such was the major bound protein in the IAC matrix. The purified extracts were then passed through MAC 136–Sepharose and the GAs which were bound to the immunosorbent were eluted with water. The non-bound component was applied to MAC 213–Sepharose and the bound GAs were then eluted with 30% aq. methanol. Gibberellins $A_{1,3,4,8,20,29 \text{ and } 34}$ were subsequently identified and quantified by GC–MS.

The C-13-hydroxy GAs from shoots of *Pisum* [97] and *Lactuca* [98] have also been purified on MAC 136–Sepharose. Preparative purification entailed adsorption chromatography on PVPP, the usual sequence of solvent partitioning steps, and chromatography on DEAE-Sephadex A-25. A final partitioning step was necessary to remove lipids which remained after earlier extraction with hexanes and which prevented binding of GAs to the MAC 136 matrix. Immunoaffinity chromatography effectively purified the samples for GC–MS, whereas RP-HPLC did not provide sufficient purification.

Nakajima *et al.* [93] raised monoclonal antibodies against GA4 coupled to BSA at C-16. One antibody, 1-A8(1)/B8, with a relatively low affinity constant for GA_1 and broad cross-reactivity with a number of free acid C_{19} and C_{20} GAs, was selected for IAC. After preparative chromatography on a Bond Elut DEA cartridge, GA-containing fractions from extracts of small amounts of anthers of *Oryza* and immature seeds of *Phaseolus* (27 and 50 mg fresh mass, respectively) were purified on a column of 1-A8(1)/B8-Sepharose. The GAs which were bound to the matrix were eluted with 40% methanol in 0.1 *M* acetic acid buffer pH 4.0 containing 0.5 *M* potassium thiocyanate. After RP-HPLC, fractions were analyzed by RIA. The amount of GA_1 in the *Phaseolus* seeds as determined by RIA was in good agreement with that obtained by GC–MS.

4. ANALYTICAL CHROMATOGRAPHY

The methods used in the identification and quantification of GAs include HPLC, GC and GC–MS, and LC–MS. Each method has valuable applications. For analysis of endogenous GAs, GC–MS and LC–MS yield positive identification and are the methods of choice, given the large number of GAs which may be present in any sample. More than 20 of the 90 known GAs, and other as yet unnumbered GAs, have been identified in extracts from single source [23,35,99]. Given expected precursors for the several biosynthetic pathways, and logical metabolites, presumably a number of other GAs would also be present in small amounts. Gradient-elution HPLC is not adequate to resolve all of these compounds in admixture, and with the exception of mass spectrometers or certain bioassays, HPLC detectors lack either the specificity or the sensitivity to enable unequivocal identifi-

cation of the GAs. A smaller number of GAs, metabolites of feeds of various substrates for example, can be separated either by gradient HPLC or isocratic HPLC, and provided that the system is well characterized can be identified with reasonable certainty.

Detectors for HPLC which have been used for the GAs include UV, bioassay, immunoassay and radiocounters. The GAs absorb only weakly in the UV at 200–210 nm in common with other carboxy acids, with the limit of detection about 50 ng in optimal conditions [6]. Certain GA derivatives such as benzyl esters [74], *p*-nitrobenzyl esters [100] and *p*-bromophenylacyl esters [101] absorb at wavelengths more amenable to analysis (256, 265 and 256 nm, respectively). The limit of detection for GA-monobenzyl esters, however, is only about 300 ng. The limit for the latter two derivatives, as the mono esters, is about 10 ng [6] and 5 ng, respectively. Greater sensitivity can be obtained with methoxycoumaryl esters [102] which fluoresce strongly (excitation at 320 nm, emission at 400 nm) and can be detected at about 1 pg for the GA3 mono ester [102]. While these derivatives can be detected in smaller amounts than the free GAs, the method is not selective for the GAs; other carboxy acids in natural samples will form derivatives which absorb in a similar fashion. Crozier and Durley [6] have provided a detailed discussion of the use of such GA derivatives in HPLC.

Sensitive bioassays are available to detect those GAs active in promoting elongation. The dwarf rice microdrop assay [103] is a widely used method for detection of a range of active GAs. The sensitivity of this assay can be improved by treating the seeds during imbibition with the GA biosynthesis inhibitor uniconazole [104] or with both uniconazole and the inhibitor prohexadione calcium [105]. Normal rice can be substituted for the less-easily obtained dwarf varieties [105,106]. The minimum detectable dose of GA_1, GA_3, GA_4 and GA_7 is 1–3 fmol (*ca.* 0.3–1 pg) when applied to the dwarf cultivar Waito C, and 3–30 fmol when applied to the cultivar Tan-ginbozu or the normal variety Koshihikari [105]. This remarkable sensitivity compares favourably with that of immunoassay. Other bioassays in use include lettuce hypocotyl, cucumber hypocotyl, barley aleurone, dwarf pea and dwarf maize [6]. Bioassays can be used for quantitative analysis, but are limited to detection of active GAs and are subject to interference from other components in plant extracts. A comparison of results from bioassay, radioimmunoassay, and GC–MS in the analysis of GA_9 in shoots of *Picea* [79] illustrates this point.

RIA and enzyme-linked immunoassay (EIA) are sensitive methods for the detection of GAs with limits of detection ranging to the fmol level. For example, in the EIA of Atzorn and Weiler [85], the limits were 0.5 fmol for GA_3, 1.0 fmol for GA_4 and 1.5 fmol for GA_7. Antibodies to the GAs have been raised in a number of laboratories (see section 3.6). Their use in immunoassay has been outlined in Hedden [1] and Beale and Willis [3]. The specificity of the immunoassays varies, depending on the cross-reactivity of the antibodies. In common with bioassays, immunoassays are subject to interference from other components in plant extracts.

Radiocounters have been used in many studies to detect metabolities from feeds of radio-labelled substrates. Where the substrate was ^{14}C-labelled, or also labelled with a stable isotope, or accompanied by a stable isotope-labelled analogue, the fractions containing the heavy isotope-labelled metabolites can be identified for subsequent analysis by GC–MS [19,21,72,76,107–109]. If used on-line [110,111], radiocounters provide accurate measurements of retention time, but on this basis alone enable only tentative identification of potential metabolites. Where fractions are collected from HPLC and analysed for radioactivity [50,54] confidence in identifications is less certain, unless the system has been well characterized [112,113]. In other cases identification may be confounded not only by other GAs with properties similar to those of expected metabolites but also by the formation of labile associations of GAs with compounds such as phenolics [29] with unpredictable chromatographic behaviour. Confidence in the identification can be improved by chromatography on more than one type of column [114].

For the endogenous GAs the use of HPLC in analysis has been confined largely to the prepara-

tive separation and purification of GAs before definitive analysis by GC–MS. Compilations of retention times can be useful in aiding identification in such cases, and also in guiding the choice of fractions for pooling before GC–MS, often in conjunction with information from bioassay and the elution of radioactive internal standards. They also serve to indicate possible candidates for search by GC–(selected ion monitoring (SIM), if there is not a sufficient amount of the GA present for detection by GC–MS.

With the use of immunological methods as a means of identifying and quantifying GAs, HPLC retention times of immuno-reactive fractions provide additional evidence of identity. More recently, however, with the introduction of LC–MS has come the beginning of use of HPLC as an integral part of the analytical method by which GAs and GA conjugates may be identified.

4.1. Reversed-phase HPLC

4.1.1. Free GAs

Ion-suppressed reversed-phase HPLC has been the most favoured means of separating the GAs. The established practice is to separate GAs of a wide range of polarity on columns of bonded octadecylsilica (ODS, C_{18}) with a polar mobile phase consisting of a gradient of increasing methanol in acidic water (*e.g.*, refs. 115 and 116). Aqueous acetonitrile has also been used in a few studies [42,92,117]. Acetic acid is widely used as an ionic suppressor in concentrations ranging to 1 ml l^{-1}, although 50 μl l^{-1} is sufficient [1]. It can be readily evaporated from the fractions after HPLC with little danger to the GAs. It is less suitable for use where the elution of GA standards is being monitored by UV absorption since it yields a high background. Phosphoric acid has also been used [97,118] though it is not recommended in preparative purification for risk of degrading the GAs [1]. Formic acid was used by Lin and Heftmann [119].

In such conditions the GAs elute in order of decreasing polarity, first described in Jones *et al.* [120], who reported the separation of 20 GAs in gradient elution. Barendse and Van den Werken [121] reported the development of methods for the separation of isomers such as GA_1 and GA_3, GA_5 and GA_{20}, and GA_4 and GA_7 in isocratic and gradient-elution HPLC, and Lin and Heftmann [119] described the separation of ten GAs in isocratic HPLC.

Koshioka *et al.* [115] extended the earlier results [120,121] to include the retention times in gradient-elution HPLC of three additional GAs and a range of derivatives, isomers and related compounds. The most extensive original list, with precise retention times in isocratic HPLC, is that of Jensen *et al.* [118], which includes 41 of the first 53 numbered GAs plus GA_{12}-aldehyde. A list of the retention times in gradient-elution HPLC of 24 GAs, including three of those (GA_{32}, GA_{54} and GA_{55}) not described previously [122] was later extended by Lin *et al.* [116] to include GA_2, GA_{61}, GA_{62}, GA_{63} and GA_{68}. With interpolation of retention times from Jensen *et al.* [118] and Koshioka *et al.* [115] 66 compounds were represented, including 51 of the numbered GAs. This list is reproduced in Table 1, with retention times of several other compounds added, estimated from recent reports. Koshioka *et al.* [115] and Jensen *et al.* [123] provided analyses of the effects of the various structural features on elution of the GAs, and these were extended by Lin *et al.* [116]. The other of elution of the non-hydroxylated GAs reflects the relative polarities conferred on the molecules by the substituent at C-20 and the C-19 carboxy function, for the C_{20} GAs (GA_{24}, GA_{25}, GA_{12} and GA_{15}), or by the C-19,10-lactone, for GA_9. Hydroxylation of these GAs reduces their retention. The effect of hydroxylation at different positions on the polarity of the C_{19} GAs is $12\alpha > 13 > 11\beta > 16\beta > 1\alpha > 2\alpha > 15\beta > 1\beta > 2\beta > 3\alpha > 3\beta$ [116,123]. Local interactions, such as that of the C-13 hydroxyl with the exocyclic methylene at C-16, may modify the effect that individual hydroxyl functions might have otherwise [123]. With the exception of hydroxylation at C-15, hydroxylation in the C- and D-rings has greater effect in reducing retention time than does hydroxylation in the A-ring, possibly a consequence of the already hydrophilic nature of the A/B region of the molecule [123]. This generalization does not hold

TABLE 1

RETENTION TIMES OF GIBBERELLINS, GIBBERELLIN METHYL ESTERS AND RELATED COMPOUNDS IN ION-SUPPRESSED REVERSED-PHASE HPLC

Adapted from Lin *et al.* [116] and Lin and Stafford [122]. Unless otherwise indicated, analysis on Ultrasphere ODS (5 μm, 250 × 4.6 mm); gradient: A = linear from 35% aqueous methanol containing 0.05% acetic acid to 100% methanol with 0.05% acetic acid in 40 min, B = linear from 40% methanol to 100% methanol in 30 min; 1 ml min^{-1}.

Compound	Retention time (min)		Compound	Retention time (min)	
	A	B (methyl ester)		A	B (methyl ester)
GA_{89}	2–5[a]		GA_{10}	17.8–18.4[e]	
12α-Hydroxy GA_{43}	2–5[b]		GA_{16}	18.42	
GA_{55}	5.03	6.64	GA_{20}	18.97	19.41
GA_8	5.08	7.36	GA_{46}	19.0–19.5[b]	
GA_{85}	5.1–6[c]		GA_{27}	19.61	
GA_{81}	5.0–6.5[d]		GA_{47}	19.6–20.4[e]	
GA_{29}	6.0–6.5[e]		GA_{36}	20.44	20.44
GA_{39}	6.0–6.5[e]		GA_{13}	20.47	26.10
Iso-GA_3	6.36		GA_{68}	20.5[i]	
GA_{32}	6.50		GA_{40}	20.5–22.0[e]	
GA_{33}	6.98		Allogibberic acid	20.5–22.5[e]	
Gibberellenic acid	7.42		12α-Hydroxy GA_{25}	20.5–21.4[b]	
GA_{30}	7.46		GA_{44}	21.4	
GA_{23}	7.7–8.2[e]		GA_{63}	21.5[i]	
GA_{28}	8.0–8.4[e]		GA_{19}	22.41	23.54
GA_{38}	8.3–8.6[e]		GA_{54}	22.58	22.85
GA_{41}	9.1–9.3[e]		GA_{34}	22.83	22.80
GA_{26}	9.1–9.3[e]		GA_{62}	23.5[i]	
GA_3	9.38	10.32	GA_{17}	23.74	25.35
C-3-*epi* GA_1	9.41	11.51	*epi*-Allogibberic acid	23.5–24.0[e]	
GA_1	10.51	11.41	GA_{37}-15-ene	24–26[f]	
GA_{29}-catabolite	10.5–11.0[e]		C-3-*epi*-GA_{37}	24–26[f]	
$\Delta^{1,10}$ GA_1-counterpart	11.32		GA_{51}	24.5[i]	
GA_6	11.5–12.3[e]		GA_{37}	24.07	22.94
GA_{18}	12.0–12.5[e]		GA_{61}	24.5[i]	
GA_{77}	12–14[g]		GA_{88}	24.5[h,i]	
GA_{35}	12.89		C-3-*epi*-GA_4	24.42	
GA_1 methyl ester	13.40		GA_7	24.92	23.69
$\Delta^{1,10}$ GA_1-counterpart-7-methyl ester	13.2–13.6[e]		Iso-GA_7	25.0–26.0[e]	
GA_{22}	13.61		12α-Hydroxy GA_{12}	24–26[b]	
GA_{21}	14.25		GA_4	26.07	24.60
GA_2	16.07		GA_{53}	27.81	27.34
GA_{31}	16.07		GA_{14}	28.17	28.64
GA_{43}	14.5–17.5[e]		GA_{24}	28.86	29.91
GA_5	17.86	18.67	GA_9	29.36	27.79
			GA_{25}	29.54	31.21
			GA_{15}	29.76	27.87
			GA_4 methyl ester	29.0–31.0[e]	
			GA_{12}	32.0–38.0[e]	
			GA_{12}-aldehyde	38.0–44.0[e]	
			ent-Kaurenoic acid	44.82	
			ent-Kaurene	60.72	

[a] Estimated from ref. 126.
[b] Estimated from ref. 72.
[c] Estimated from ref. 127.
[d] Estimated from ref. 128.
[e] Estimated from refs. 115 and 118.
[f] Estimated from ref. 129.
[g] Estimated from ref. 68.
[h] From ref. 130.
[i] ±0.5 min.

for the C_{20} GAs, where GA_{17} and GA_{19} elute from RP-HPLC later than their C-3 hydroxy counterparts, GA_{13} and GA_{36}. Those GAs which are dehydrogenated in the A-ring at C-1,2 or C-2,3, or in the C-ring at C-9,11, elute before their saturated counterparts. Precise retention times for other GAs not included in Table 1 are not readily available but the approximate elution volumes of GAs of particular structure can be confidently predicted. Description of the behaviour of these other GAs in RP-HPLC have not been published or are limited to large elution volumes for the natural isolates.

Heavy isotope-labelled GAs are frequently included in extracts for quantification of their endogenous counterparts. Since ^2H-labelled GAs elute from RP-HPLC slightly before their endogenous counterparts, as observed for similarly labelled standards of other hormones [124,125], care must be taken not to separate the two.

4.1.2. GA derivatives

Lin and Stafford [122] listed the retention times of 23 GA methyl esters in gradient-eluted RP-HPLC (Table 1), and described the method as having the highest separation efficiency in comparison with RP-HPLC of the free GAs and silica HPLC of free GAs and GA methyl esters. The GA methyl esters elute from RP-HPLC in approximately the same order as the free acids, with exceptions expected of C_{20} GAs which have more than one carboxy function (which, of those included in Table 1, are GA_{13}, GA_{14}, GA_{17}, GA_{19}, GA_{24}, GA_{25} and GA_{36}). Gibberellins in methylated plant extracts may be better separated by RP-HPLC from certain impurities than they would otherwise, but biologically active free GAs are virtually inactive if methylated, and so the opportunity to detect these subsequently by bioassay is lost. The method is relevant in studies where the GAs have been methylated for recognition in certain immunoassays, and also where radio-labelled products of feeds of radiolabelled substrates are to be purified, since these compounds can be easily detected by HPLC–radiocounting. There are only a few reports of the purification of GAs from methylated plant extracts [42,131,132].

p-Bromophenylacyl esters have been analyzed on RP-HPLC by Morris and Zaerr [101]. Separations of the highly fluorescent methoxycoumaryl esters on RP-HPLC [102] are generally similar to those obtained for the free GAs (as shown, for example, in Table 1). Thus GA_1, GA_4 and GA_{20} are easily distinguished from their more polar A-ring dehydro analogues (GA_3, GA_7 and GA_5, respectively); increasing hydroxylation increases polarity; and an increasing number of methoxycoumaryl groups decreases polarity. The derivatives are not sufficiently volatile for analysis by GC–MS, and so their use has been confined to identification of GAs in only few studies [80,114].

RP-HPLC has also been used to introduce GA methyl esters and free GAs to MS. This is discussed in section 4.4.

4.1.3. GA–glucosyl conjugates

RP-HPLC has been popular for the purification and separation of the GA conjugates, polar compounds that are strongly adsorbed and often not well recovered from other types of chromatography. Comprehensive descriptions of the behaviour of GA conjugates in isocratic RP-HPLC are found in Yamaguchi *et al.* [39], Schneider [16], Jensen *et al.* [118] and Sembdner and Schneider [133]. Koshioka *et al.* [115] have described the behaviour of GA conjugates in gradient-elution RP-HPLC.

All of the GAs whose conjugates are represented in these studies can be separated from their glucosyl esters or glucosides, and all are less polar in RP-HPLC than their conjugates (Table 2). The glucosyl esters of GA_1, GA_3, GA_4 and GA_7 elute after their 3-O-glucoside counterparts. Sembdner and Schneider [133], however, have shown GA_4- and GA_7-glucosyl esters to elute before the 3-O-glucosides. For GA_1 and GA_3, the 3-O-glucosides are less polar than the 13-O-glucosides. Jensen *et al.* [123] suggested that the order of elution of the GA_1–glucosyl conjugates (GA_1–13-O-glucoside > GA_1–3-O-glucoside > GA_1–glucosyl ester) reflects (a) the polarity conferred by the highly polar carboxyl group, and (b) the orientation, primarily, and also the position, of the glucose moiety. In GA_1–3-O-glucoside the glucose is

TABLE 2

RETENTION TIMES OF GIBBERELLIN CONJUGATES AND THEIR AGLYCONES IN ISOCRATIC REVERSED-PHASE HPLC

Adapted from Jensen *et al.* [118]. Supelcosil LC C18 (5 μm, 250 × 4.6 mm), isocratic methanol in aqueous phosphoric acid pH 3.0, 1 ml min^{-1}.

Compound	Methanol (%)									
	10	15	20	25	30	35	40	45	50	55
GA_{29}–2-O-glucoside	19.9	11.2	7.4							
GA_8–2-O-glucoside	23.8	12.6	8.0							
GA_8			11.5	8.2						
Gibberellenic acid-2-O-glucoside		25.7	13.6	8.9						
GA_3–13-O-glucoside		26.1	13.9	8.9						
GA_{29}			14.8	10.3	8.2					
GA_1–13-O-glucoside		30.0	15.9	10.0						
GA_3–3-O-glucoside			22.1	12.8	8.4					
GA_1–3-O-glucoside			23.3	13.5	8.7					
GA_{38}–glucosyl ester			23.9	14.5	9.2					
GA_{26}–2-O-glucoside			25.1	15.6	9.6					
GA_3–glucosyl ester			26.4	16.0	9.8					
GA_1–glucosyl ester			31.1	17.8	11.2					
GA_{38}				19.1	12.4	8.2				
GA_{35}–11-O-glucoside				19.6	11.3	8.0				
GA_{26}				22.3	14.0	9.1				
GA_3				23.0	14.0	9.2				
GA_1				26.5	16.5	10.8				
GA_{35}					24.2	15.8	10.5			
GA_5–13-O-glucoside					24.4	14.5	9.2			
GA_{20}–13-O-glucoside					25.2	15.2	9.8			
GA_5–glucosyl ester					44.9	23.1	13.1			
GA_{20}–glucosyl ester						24.3	14.0	9.4		
GA_{37}–glucosyl ester						25.3	17.3	11.2		
GA_7–3-O-glucoside							20.1	12.6	8.4	
GA_5							24.3	14.6	9.6	
GA_4–glucosyl ester							24.5	15.3	9.8	
GA_{20}							28.0	17.2	11.2	8.4
GA_7–glucosyl ester								22.6	13.6	8.9
GA_{37}									24.2	16.8
GA_7									30.0	20.6
GA_4									36.8	25.0

axial, while in GA_1–13-O-glucoside it is equatorial.

Endogenous GA conjugates have been identified in recent studies from a variety of sources by MS after final isolation on RP-HPLC (see section 4.5). In a recent novel use of RP-HPLC which culminated in the identification of GA–glucosides from *Pisum*, *Hordeum* and *Zea*, the permethylated GA–glucosides were separated as a group on RP-HPLC from the GA permethyl derivatives [11,47]. The retention times are shown in Table 3.

Radiolabelled GA conjugates, produced from feeds of a labelled free GA substrate to various systems, have been tentatively identified by comparison of retention times on analytical RP-HPLC with the retention times of authentic standards of GA conjugates [9,50,54,55,80]. Further evidence of identity was provided by hydrolysis of the presumed [^{3}H]conjugates to yield products which eluted at the retention times of the expected ^{3}H-free GAs on HPLC and/or GC. The hydrolysis of fractions containing presumed GA conjugates, to yield free GAs which can be further purified on RP-HPLC and/or identified by GC–MS, has been a common technique in studies where definitive methods of identifying conjugates have not been available.

TABLE 3

RETENTION TIMES OF PERMETHYLATED (PME) GIBBERELLINS AND GIBBERELLIN-O-GLUCOSIDES IN REVERSED-PHASE HPLC

Adapted from Schneider *et al.* [11]. LiChrospher 100 RP-18 (5 μm, 125 × 4 mm); isocratic methanol–water (70:30), 1 ml min^{-1}.

Compound	Retention time (min)
GA_1-PME	2.87
GA_{29}-PME	3.32
GA_5-PME	3.40
GA_3-PME	3.45
GA_8-PME	3.60
GA_{20}-PME	3.78
GA_3–13-O-glucoside-PME	5.27
GA_{29}–13-O-glucoside-PME	5.27 [134]
GA_5–13-O-glucoside-PME	5.50 [134]
GA_{29}–2-O-glucoside-PME	5.67
GA_{20}–13-O-glucoside-PME	6.23
GA_8–2-O-glucoside-PME	6.35
GA_1–13-O-glucoside-PME	6.67
GA_3–3-O-glucoside-PME	6.67
GA_1–3-O-glucoside-PME	7.33

RP-HPLC has also been used to introduced GA conjugates to MS, as described in section 4.4.

4.1.4. Kauranoids

If included with fractions that also contain GAs, these compounds, most of which are less polar than the GAs, can be separated by RP-HPLC in 100% methanol at the end of the usual gradient of aqueous methanol (Table 1). Where the kauranoids have been examined as a separate group of compounds, solvent programs more appropriate to their polarity have been used. Examples include that of Turnbull *et al.* [135] (Table 4). The behaviour in RP-HPLC of a number of other kauranoids which are not intermediates in the pathway to the GAs and which are not included in Table 4, including kaurenolides, and mono-, di- and trihydroxylated kaurenoic acid, has been described by Metzger and Hazebroek [18]. Suzuki *et al.* [19] have described examples of solvent programs

TABLE 4

RETENTION TIMES OF KAURANOIDS IN REVERSED-PHASE HPLC

Retention times have been estimated from Turnbull *et al.* (ref. 135, Fig. 1). ODS Hypersil (5 μm, 250 × 5 mm); aqueous methanol in dilute acetic acid (pH 3): 0–15 min, 50–80% methanol; 15–19 min, 80–100% methanol; 19–44 min, 100% methanol; 1 ml min^{-1}.

Compound	Retention time (min)
ent-6α,7α-Hydroxy kaurenoic acid	19.1
ent-7α-Hydroxy kaurenoic acid	19.8
GA_{12}	20.8
GA_{12}-aldehyde	21.9
ent-Kaurenoic acid	24.7
ent-Kaurenol	24.7
ent-Kaurenal	25.9
ent-Kaurene	32.4

suited for the purification of *ent*-kaurene alone, and for groups of kauranoids of similar polarity (*ent*-kaurenal, *ent*-kaurenol, *ent*-kaurenoic acid; and *ent*-kaurenoic acid, GA_{12}-aldehyde and *ent*-7α-hydroxy kaurenoic acid).

4.2. Normal-phase HPLC

Normal-phase HPLC on unmodified silica has been little-used in GA separation. This is a reflection of the likelihood of poor recoveries especially of the more polar compounds because of irreversible adsorption. Also, resolution of such compounds is likely to be poor (*cf.* ref. 122). Lin and Heftmann [119] defined the behaviour of ten GAs in silica HPLC, eluted with *n*-hexane–ethanol–acetic acid (93:7:0.05). This work was extended by Lin and Stafford [122], who determined the retention times of 23 GAs and their methyl esters eluted from silica with *n*-hexane–ethanol (90:10) containing 0.05% acetic acid (free GAs) or *n*-hexane–ethanol (92:8; methyl esters) (Table 5). As expected, most efficient separation was achieved with the methyl esters. The C_{19} GAs elute more or less in the reverse of their order from RP-HPLC. Notably, C-3-*epi*-GA_1 is better retained than expected from its behaviour in RP-HPLC and thus

TABLE 5

RETENTION TIMES OF FREE GIBBERELLINS AND THEIR METHYL ESTERS IN SILICA HPLC

Adapted from ref. 122. Conditions: free GAs: Spherisorb S5W (5 μm, 250 × 4.6 mm), isocratic *n*-hexane–ethanol (90:10) containing 0.05% acetic acid, 2 ml min^{-1}. GA methyl esters: isocratic *n*-hexane–ethanol (92:8).

Gibberellin	Retention time (min)	
	Free GA	GA methyl ester
GA_9	2.77	2.08
GA_{24}	2.90	1.84
GA_{15}	3.20	2.38
GA_{25}	3.31	1.74
GA_4	4.00	3.36
GA_7	4.03	3.50
GA_{14}	4.36	2.74
GA_{36}	4.73	3.31
GA_{37}	4.84	4.38
GA_{34}	4.93	4.30
GA_{53}	4.96	3.13
GA_{54}	5.32	4.45
GA_{20}	5.73	4.70
GA_5	5.75	4.87
GA_{19}	5.81	3.93
GA_{17}	6.67	3.89
GA_{13}	6.74	3.05
GA_1	8.99	9.24
GA_3	9.34	9.73
GA_8	11.69	12.49
GA_{55}	12.21	12.57
GA_{32}	12.61	12.21
C-3-*epi*-GA_1	12.68	12.31

well separated from GA_1. The order of elution of the free C_{20} GAs is determined by the substituent at C-20 and the position of hydroxylation. The polar –COOH group at C-20 dominates behaviour. Thus GA_{25} is the best retained of the non-hydroxylated C_{20} GAs, eluting after GA_{15} (δ-lactone) and GA_{24} (CHO). This order is maintained for the representatives of the C-3- and C-13-hydroxylated C_{20} GAs. Amongst these hydroxylation at C-13 increases retention more than does hydroxylation at C-3. Examples of the use of silica HPLC in preparative purification schemes include Birnberg *et al.* [131] and Maki *et al.* [136]. Other uses include the separation of GA benzyl esters [74] and GA *p*-nitrobenzyl esters [100].

Normal-phase chromatography of GAs on open columns of deactivated silica, with a mobile phase of formate-saturated ethyl acetate in *n*-hexane, has been described in section 3.2. The early use of this system in HPLC for the separation of free GAs by Reeve *et al.* [110] has been superseded by chromatography on bonded phases. However, few applications are reported. Methoxycoumaryl esters of GAs were separated on bonded cyanopropyl (CPS Hypersil) eluted with 3% ethanol in *n*-hexane–dichloromethane (88:12 or 80:20) [102] and more recently on CN nitrile eluted isocratically with dichloromethane–*n*-hexane or ethyl acetate–*n*-hexane [114]. Gibberellin-4-bromophenacyl esters have also been separated on cyanopropyl silica [101]. Odén *et al.* [58] used a column of bonded NO_2, eluted with a gradient of *n*-heptane (half-saturated with 1 *M* formic acid) to ethyl acetate containing 1% water and 0.5% formic acid, to separate 12 GAs, GA_{12}-aldehyde, and several kauranoids (Table 6). The water and formic acid

TABLE 6

RETENTION TIMES OF GIBBERELLINS AND KAURANOIDS IN NORMAL PHASE HPLC

Retention times estimated from Odén *et al.* (ref. 58, Fig. 1). Nucleosil NO_2 (5 μm, 125 × 4.6 mm), gradient of *n*-heptane (half-saturated with 1 *M* formic acid) to ethyl acetate containing 1% water and 0.5% formic acid over 60 min; 2 ml min^{-1}.

Compound	Retention time (min)
ent-Kaurene	1.8
ent-Kaurenal	2.4
ent-Kaurenol	10.3
ent-Kaurenoic acid	11.5
GA_{12}-aldehyde	18.2
GA_9	19.4
GA_{15}	22.7
GA_{12}	23.6
GA_4	27.9
GA_{20}	31.8
GA_{44}	36.4
GA_{53}	37.3
GA_1	42.4
GA_3	43.9
GA_{19}	44.8
GA_{29}	50.3
GA_8	57.9

were added as polar modifiers and ion suppressors. The compounds elute in the order expected from the earlier work [60,110]. The C_{19} GAs elute in order of increasing hydroxylation, the reverse of that observed in RP-HPLC (section 4.1.1). The C-13 hydroxylated C_{20} GAs (GA_{19}, GA_{44} and GA_{53}) elute after the non-hydroxylated C_{20} GAs (GA_{12} and GA_{15}). The method has proved effective in the semi-preparative purification of GAs from *Picea*, in combination with subsequent analytical RP-HPLC [58]. From similar material Odén *et al.* [79] purified GA_9 on μBondapak NH_2 eluted with *n*-hexane–ethanol–acetic acid (96:2:2).

Normal-phase HPLC has not been routinely applied in the analysis of GA conjugates. Odén *et al.* [58] tentatively identified GA_9–glucosyl ester from extracts of *Picea* after chromatography on a column of Polygosil NO_2 with a mobile phase of *n*-heptane (half-saturated with 1 *M* formic acid) to ethyl acetate containing 1% water and 0.5% formic acid. The GA conjugate eluted at 350 ml, much later than GA_9 (110 ml) and GA_1 and GA_3 (250 ml), reflecting the influence of the glucose moiety on polarity. In similar fashion Moritz [30] purified GA_9–glucosyl ester on a column of Nucleosil NO_2.

4.3. Dimethylamino Nucleosil HPLC

Yamaguchi *et al.* [61] introduced the use of HPLC on columns of dimethylamino [$N(CH_3)_2$] Nucleosil to GA analysis. The application, in which the GAs are eluted in a mobile phase of isocratic methanol with 0.05% acetic acid, has gained considerable acceptance because of the unusual separations afforded. Retention of the C_{20} GAs is determined largely by the degree of oxidation at C-20 (Table 7); thus the GAs with $-CH_3$ are least retained, eluting ahead of -δ-lactone, –COOH and –CHO. Separation of the C_{19} GAs is determined by the position and orientation of hydroxyl functions, which can increase (C-3β-hydroxy) or decrease (C-2α, -2β or -13-hydroxy) retention. Hydrogenation in the A-ring increases retention. Thus $N(CH_3)_2$ HPLC is particularly useful for the separation of GA_1, GA_4 and GA_{20} from their A-ring dehydro counterparts (respectively GA_3, GA_7 and GA_5).

TABLE 7

RETENTION TIMES OF GIBBERELLINS IN DIMETHYLAMINO NUCLEOSIL HPLC

Adapted from Yamaguchi *et al.* [61]. Nucleosil $N(CH_3)_2$ (10 μm, 250 × 6 mm), isocratic methanol containing 0.05% acetic acid, 2 ml min^{-1}, 50°C.

Gibberellin	Retention time (min)
GA_{12}	10.7
GA_{14}	11.4
GA_{18}	11.7
GA_{37}	13.8
GA_{27}	15.6
GA_4	20.0
GA_{17}	21.4
GA_1	22.0
GA_{35}	22.5
GA_{16}	25.6
GA_8	26.0
GA_9	27.0
GA_7	27.8
GA_{13}	28.0
GA_{40}	28.0
GA_{30}	28.6
GA_3	28.9
GA_{20}	30.0
GA_{51}	30.2
GA_{36}	31.4
GA_{24}	32.4
GA_{31}	37.6
GA_5	38.2
GA_{19}	41.6
GA_{26}	48.2
GA_{22}	49.0

The isomers GA_1 and C-3-*epi*-GA_1 are also well separated [137]. These pairs of GAs are often not well resolved in gradient-eluted RP-HPLC. The method has been frequently used as an additional purification step after separation on RP-HPLC [28,64,68], or as a single HPLC step before GC–MS [25,76,138].

4.4. LC–MS

The recent introduction of LC–MS has permitted GAs and their conjugates to be introduced to MS for analysis usually at low temperature without converting them to a derivative. This is an advantage for analysis of the GA–glucosyl

ester conjugates in particular. Since LC–MS is not yet widely available, and improved methods for transferring the solutes from LC to MS are still being sought, techniques for analysis of GAs and GA conjugates by LC–MS are in their infancy.

Two of the available LC–MS interfaces have been used in GA-conjugate analysis. Murofushi *et al.* [139] employed an atmospheric pressure ionization interface to introduce GA–glucosides and GA–glucosyl esters from RP-HPLC to MS. The GA conjugates were separated in gradients of 10 m*M* ammonium acetate–methanol, at 1 ml min^{-1}. In the interface, the eluate from HPLC is nebulized (at 380°C) and then vaporized (at 380°C) and the solutes subsequently ionized by atmospheric pressure chemical ionization (APCI). A 1-ng amount of GA_3–3-O-glucoside could be detected by single ion monitoring. The method can accommodate various flow rates (0.1 to 2 ml min^{-1}) without the need for splitting.

In the second approach Moritz [30] and Moritz *et al.* [140] have used capillary RP-HPLC–frit fast atom bombardment (FAB)-MS for the analysis of GA conjugates. In this method the eluate from capillary HPLC, at a flow-rate of about 5 μl min^{-1}, is introduced through a frit into the ion source, and is then ionized by accelerated xenon atoms. Moritz *et al.* [140] first separated GA–glucosyl esters in HPLC in a solvent program of methanol–water–acetic acid–glycerol, and found negative ion mass spectra to be the more useful in identification. The GA–glucosides were introduced from the capillary column without any attempt to provide separation, and positive ion spectra were found to be the more useful. Full mass spectra were obtained from injections of 10 to 100 ng of compound; in the identification of GA_9–glucosyl ester from shoots of *Picea* by this method 5 ng of GA_9–glucosyl ester were considered sufficient to obtain a spectrum [30]. Capillary HPLC offers the advantage that there is no need to split the effluent before the detector; the disadvantage is that only a small volume can be injected. The FAB mass spectra of other GA conjugates have been described by Voigt and Dube [141].

Free GAs and their methyl esters have also been analyzed by LC–APCI-MS [139]. The compounds were introduced to MS from RP-HPLC with a mobile phase of 10 m*M* ammonium acetate–methanol (20:80). A 10-ng amount was required to obtain spectra, and several hundred pg was required for detection by single ion monitoring. A thermospray interface was used by Hansen *et al.* [142] in the LC–MS analysis of free GAs and GA methyl esters. The free GAs were separated with a mobile phase of increasing acetonitrile in aqueous 0.1 *M* ammonium acetate. The minimum amount needed to obtain the very simple positive ion thermospray mass spectra was about 100 ng of GA_3 methyl ester and about 250–500 ng of GA_3. Negative ion spectra were obtained at 10× less sensitivity. The isomers GA_3 and GA_{30} were not separated on HPLC and could not be distinguished by their positive ion spectra, but tandem mass spectra obtained from the collisionally activated dissociation of the $[M + NH_4]^+$ ions did show differences.

Provided that the limited resolution of HPLC is not an obstacle in analysis, likely developments in LC–MS interfaces which result in improved sensitivity should lead to increased application of LC–MS for the direct analysis of both free GAs and GA–glucosyl conjugates from plant extracts.

4.5. GC and GC–MS

The use of GC in conjunction with MS in GA analysis was introduced in 1967 [143] and developed since then by MacMillan and co-workers, in particular. A useful practical summary of methodology is found in Hedden [1], and Gaskin and MacMillan [7] have provided an authoritative reference to current methods. GC–MS is now the most widely used method for identifying known GAs. It is the method of choice for establishing the characteristics of new GAs isolated in small amounts from plant extracts, with identity confirmed through comparison of Kováts retention index (*I*) and mass spectrum with that of the synthetic equivalent of known structure. This is illustrated in the identification of most of the GAs recently assigned A-numbers (see refs. 64,126–128 and 144, and the refs. cited in ref. 7). GC–MS is also a sensitive and specific method

for quantifying GAs, as discussed briefly in section 4.5.4.

The usual approach in analysis of GAs from plant extracts is to prepare derivatives of purified samples (groups of fractions after HPLC, for example) which are more suited for GC–MS analysis than are the parent compounds. These include methyl- and trimethylsilyl esters and ethers. These are then introduced onto capillary columns for GC and separated in a temperature gradient, with the volatile compounds emerging directly into the ion source of a mass spectrometer. The mass spectra, and retention indices, provide sufficient evidence to distinguish all of the known GAs.

Detectors other than mass spectrometers have also been used in GA analysis. These include flame ionization and electron-capture (which can be used for GA derivatives which have electron-capturing properties) detectors, and radiocounters. None of these is specific for the GAs; the only evidence of identity obtained is the retention time. Packed-column GC–radioactivity counting has been used to provide tentative identifications of metabolites from the application of radiolabelled substrates in a number of studies [145]. In such circumstances where the identity of the metabolites was predictable and where chromatography was carried out on several different columns, the identifications have proved reliable [6]. However, for unequivocal evidence of identity GC–MS provides not only retention times but also mass spectra. Compounds entering into the ion source of the mass spectrometer are ionized, usually by electron impact (EI) at 70 eV, and characteristic fragmentation of the molecule results. The positive ions produced are detected by scanning of the mass range of interest to yield a mass spectrum. With a cycle time of about one second, a peak from GC can be sampled about 5–10 times. The total ion current (TIC) at each scan yields a chromatogram which can be integrated for estimates of the relative amounts of the various compounds. From the spectra, chromatograms of individual ion currents can be reconstructed. These are often useful in pointing out the location of GAs present in small amounts or hidden in co-eluting impurities [1,7]. The mass spectrometer can also be used to sample only certain ions of those produced in the fragmention process. In GC–SIM sensitivity is improved about 100-fold over GC–MS, since each ion can be sampled more frequently. The method is used to provide ion chromatograms which can be integrated for quantitative analysis [1], and to provide evidence of identity of GAs present in amounts too small to yield full mass spectra. In our experience the limit of detection of the GAs in analysis on commonly used benchtop gas chromatograph–mass spectrometers is of the order of 3 pmol (*ca.* 1 ng) in GC–MS and 30 fmol (*ca.* 10 pg) in GC–SIM. Greater sensitivity can be achieved on other instruments; Gaskin and MacMillan [7] have reported 50–100 pg in GC–MS and 0.1–1 pg in GC–SIM. The use of GC–SIM in particular often allows for GAs to be identified or quantified from samples which have not been highly purified. This is particularly the case for the more hydroxylated GAs, with derivatives which yield molecular ions of high molecular mass which are unlikely to be contaminated by ions from the fragmentation of co-eluting compounds.

4.5.1. Derivatives and spectra

Hedden [1] and Gaskin and MacMillan [7] have provided details of the methods of preparation of the various derivatives for GC–MS. The best suited for the hydroxylated free GAs and kauranoids is the methyl ester trimethylsilyl ether (MeTMSi). It is sufficiently volatile and stable for GC and yields MS spectra which provide useful diagnostic information on structure. Free GAs are usually methylated with ethereal diazomethane which can be synthesized using a convenient small-scale procedure described by Cohen [146]. Hydroxylated GAs are then converted to trimethylsilyl ethers with (N,O - bis - trimethylsilyltrifluoroacetamide (BSTFA), N-methyl-O-trimethylsilyltrifluoroacetamide (MSTFA) or hexamethyldisilazane–trimethylsilyl chloride–pyridine (3:1:9) (Sweeleys reagent). The compendium of Gaskin and MacMillan [7] includes the spectra of the methyl esters of non-hydroxylated GAs, and the MeTMSi derivatives of hydroxylated GAs, for GAs up to GA_{86}. In addition the spectra of a

host of other GAs, kauranoids and related compounds are shown. They have also provided a comprehensive analysis of the fragmentation of GA methyl and MeTMSi derivatives. Binks *et al.* [147] have provided the only other compilation of the full spectra of MeTMSi and methyl ester derivatives, of GAs up to GA_{24}. Takahashi *et al.* [148] have also described and discussed the fragmention of GA methyl esters. Tabulations of major ions in the spectra of GA methyl- or MeTMSi derivatives have been given by Beale and Willis [3], Hedden [1], Takahashi *et al.* [5] and Crozier and Durley [6]. Otherwise full spectra or tabulations of major ions can be gleaned from the literature, with references to the identification of all GAs up to GA_{86} found in Mander [8]. For the hydroxylated free GAs, other derivatives occasionally used include methyl esters, but these are not as amenable to chromatography as the MeTMSi derivatives [7]. Also occasionally used are TMSi esters, and TMSi ester TMSi ethers [76], and since the ester and ether bonds are readily hydrolyzed in water these offer the opportunity to recover the free GA. A limited number of spectra of these derivatives have been recorded by Gaskin and MacMillan [7] and their fragmentation discussed.

There has been very limited use of permethyl derivatives of the free GAs [149,150]. The usual permethylation procedure with sodium hydride and methyl iodide in dimethylformamide [149] is somewhat involved and much less suitable for routine use than methylation and silylation. However, the use of an alternative simple procedure for permethylation has recently been reported [150]. MethElute (0.2 *M* trimethylanilinium hydroxide in methanol) is added to the sample which is then injected onto GC, with the derivative being formed on-column. Fang and Rappaport [150] have described the spectra and fragmentation of 23 permethylated GAs; the spectra of a number of permethyl GAs have also been recorded by Gaskin and MacMillan [7].

Unequivocal identification of small amounts of GA conjugates requires their analysis either by GC–MS, as permethyl or silyl derivatives, or by LC–MS, where they may be introduced to MS without modification. Neither of the derivatives for GC–MS is ideal, since they yield weak or non-existent M^+ and the EI mass spectra are dominated by fragmentation of the glucosyl moiety [151,152]. However, permethyl derivatives are preferred because their molecular masses are within the range of commonly used gas chromatographs–mass spectrometers (often 10–800 u), and they yield fragmentation by EI which is of more value in indicating structure [149,152]. Schmidt *et al.* [152] have described the spectra of 14 permethylated GA–glucosides and provided a detailed discussion of their fragmentation. The spectra of the permethyl derivatives of 15 GA–glucosides have been recorded by Gaskin and MacMillan [7]. A number of GA–glucosides have been successfully identified as permethyl derivatives either as endogenous constituents or as products of metabolism of feeds [10,46,47,149,153]. Rivier *et al.* [149] have reported the identification of two glucosyl esters (of GA_1 and GA_4) as the permethyl ethers, but it seems that in general GA-glucosyl esters cannot be easily permethylated since transesterification occurs to produce the permethylated aglycone [154].

The spectra and fragmentation of the methyl ester TMSi ether derivatives of six GA–glucosides, and of the TMSi ethers of five GA–glucosyl esters, have been described by Yokota *et al.* [151]. However, these derivatives are less suitable for routine analysis by GC–MS since they have high molecular masses and may decompose at the high temperatures required for GC analysis [155]. Both GA–glucosides and glucosyl esters have been identified from natural samples by GC–MS as the TMSi derivatives [17,39], but their use has been very limited.

4.5.2. Columns, injection techniques and temperature programmes

Packed-column GC is little used now in GA analysis. While the capacity is high, resolution is low compared with that which can be obtained in capillary GC. Crozier and Durley [6] detail the conditions for chromatography, and list the retention times, of MeTMSi derivatives of 43 GAs and other related compounds on columns of 2% QF-1, 2% SE-30 and 1% XE-60.

In most laboratories analyses have been standardized on fused-silica capillary columns with

non-polar dimethylpolysiloxane silicone coatings such as OV-1 and equivalents (*e.g.*, DB-1, BP-1, CPSil-5, HP-1). Such practise permits ready comparison of *I* values obtained in different laboratories. Columns of 12 or 15 m are suitable for most routine analyses, but for complex separations or resolution of GAs with similar *I* a longer 25-m [7] or 30-m column is preferred. Columns used have included those with internal diameters which range from 0.18 to 0.32 mm. The capacity for individual GA components is about 50–100 ng, quite sufficient to obtain spectra of high quality.

GAs have also been successfully analyzed on columns with slightly more polar coatings such as DB-5 (5% diphenyl 95% dimethyl polysiloxane). Separations for most of the GAs examined are similar to those obtained in analyses on DB-1 [156].

Analyses of permethylated GA conjugates have been made on the same type of capillary columns used for analysis of the free GAs [11,149,152], and also on packed columns of SP-2100 or QF-1 [46]. The packed-column GC of TMSi and MeTMSi derivatives of a number of GA conjugates has been described by Hiraga *et al.* [157] and Schneider *et al.* [158]. Since the derivatives are volatile at relatively high temperatures, analysis time in capillary GC can be shortened by increasing the oven temperature rapidly after injection to the maximum required, as illustrated in Table 9 [152].

Samples are introduced onto the column usually by splitless injection into a heated injection port. Details of common methods have been given by Hedden [1] and Gaskin and MacMillan [7]. Briefly, the sample is vaporized on injection (at 280°C) and refocussed at the head of the column which is held at low temperature (30–35°C). The oven temperature is increased rapidly (to 150°C) and then slowly at 3°C min^{-1} to 300°C [7]. This type of programme has been used in most GA analyses in various laboratories. Alternatively, cool on-column injection can be used. Here, the sample is introduced directly onto the column at a temperature below the boiling point of the solvent, and the temperature is increased from there in similar fashion to that described above. In order to extend column life the injection should be made into a precolumn which is connected to the main column. The precolumn is inert (uncoated, deactivated fused silica), 0.32 or 0.53 mm I.D. to accommodate the syringe, and 0.5 to 1 m long. It can be readily and cheaply replaced when it becomes contaminated with involatile residues. If required, a longer precolumn will also serve as a retention gap to permit greater volumes than the usual (*ca.* 1 μl) to be injected. This might be of particular advantage in analyses where the GA content of the sample was approaching the lower limit for quantification or identification, but requires that the sample be well purified. A clear discussion of these sample introduction techniques is found in Poole and Poole [159]. The cool on-column method is useful for the quantitative analysis of compounds of varying volatility, since there is little of the discrimination in transfer of solutes from syringe to the column which can occur in splitless injection. Also, there is less likelihood that thermally labile compounds will decompose. We have successfully used this method in all of our GC–MS analyses [126,160].

4.5.3. Retention indices

Mass spectra alone are sometimes not sufficient evidence of identity, since some isomers, such as GA_1 and C-3-*epi*-GA_1, have spectra which are very similar. Identity can be confirmed by retention time, or preferably, by *I* [161]. The *I* value is calculated from the retention times of *n*-alkanes co-injected with the sample. A suitable range of *n*-alkanes, from $C_{21}H_{44}$ to $C_{36}H_{74}$, can be conveniently extracted from Parafilm [162]. The retention index is calculated from a calibration curve of carbon number of the *n*-alkanes plotted against retention times, or from the formula below as $I = 100\ [(t_{R(x)} - t_{R(C_n)})/(t_{R(C_{n+1})} - t_{R(C_n)})] + 100n$, where $t_{R(x)}$ is the retention time of the compound of interest, $t_{R(C_n)}$ is the retention time of the *n*-alkane of carbon number *n* which elutes immediately before the unknown, and $t_{R(C_{n+1})}$ is the retention time of the *n*-alkane of carbon number $n+1$ which elutes immediately after the compound of interest.

Absolute *I* values will differ from system to system, dependent on temperature program, gas

TABLE 8

KOVÁTS RETENTION INDICES OF GIBBERELLINS AND KAURANOIDS

Calculated from analyses of methyl esters or MeTMSi derivatives in capillary GC-MS on DB1-15N (15 m × 0.25 mm I.D., 0.25 μm dimethylpolysiloxane film); 60°C (0.1 min) to 200°C at 20°C min^{-1}, to 300°C at 5°C min^{-1}. Values are also included for a number of common isomers, and also for some GAs (synthetized by Professor L.N. Mander) which have not been identified in natural samples.

Type	-OH groups	Gibberellin	*I*	M^+
C_{20} Gibberellins				
C-20 CH_3				
C-7 CHO	–	GA_{12}-aldehyde	2356	330
	–	GA_{12}	2345	360
	3β	GA_{14}	2496	448
	13	GA_{53}	2508	448
	3β,13	GA_{18}	2646	536
	3β,12α	GA_{74}	2682[a]	536
C-16,17-dihydro	3β,16α	GA_{42}	2718[a]	538
	3β,17	GA_{83}	2778	538
δ-Lactone	–	GA_{15}	2628	344
	15β	GA_{64}	2754[a]	432
	3β	GA_{37}	2773	432
	13	GA_{44}	2800	432
	2β,3β	GA_{27}	2897	520
	3β,13	GA_{38}	2940	520
	2β,3β,11β	GA_{52}	3076[a]	608
C-20 CHO	–	GA_{24}	2469	374
	15β	GA_{65}	2580[a]	462
	3β	GA_{36}	2606	462
	13	GA_{19}	2608	462
	3β,13	GA_{23}	2747	550
C-20 COOH	–	GA_{25}	2460	404
	15β	GA_{66}	2563[a]	492
	13	GA_{17}	2585	492
	3β	GA_{13}	2597	492
	2β	GA_{46}	2616[a]	492
	3β,13	GA_{28}	2720	580
	2β,3β	GA_{43}	2725[a]	580
	3β,12α	GA_{39}	2776[a]	580
C-16,17-dihydro	3β,16α	GA_{41}	2817	582
C_{19} Gibberellins				
Non-hydroxy				
C-2,3-dehydro	–	$\Delta^{2,3}GA_9$	2333	328
C-9,11-dehydro	–	GA_{73}	2344	328
C-15,16-dehydro	–	Iso-GA_9	2292	328
	–	GA_9	2332	330
C-1,10-epoxy	–	GA_{11}	2402[a]	344
Monohydroxy				
C-1,2-dehydro	1β	GA_{62}	2439	416
	13	$\Delta^{1,2}$-GA_{20}	2503	416

TABLE 8 (*continued*)

Type	–OH groups	Gibberellin	*I*	M^+
	3β	GA_7	2541	416
	12α	GA_{31}	2564	416
C-2,3-dehydro	13	GA_5	2495	416
C-9,11-dehydro	3β	GA_{88}	2512	416
C-1,10-dehydro, 19,2-lactone	3β	Iso-GA_7	2509	416
	1β	GA_{61}	2405	418
	11β	GA_{84}	2488	418
	13	GA_{20}	2499	418
	15β	GA_{45}	2499	418
	12β	GA_{69}	2512	418
	3β	GA_4	2519	418
	2β	GA_{51}	2534	418
	2α	GA_{40}	2543	418
	12α	GA_{70}	2561	418
	3α	*epi*-GA_4	2639	418
C-16,17-dihydro	16α	GA_{10}	2591	420
C-2,3-epoxy	13	GA_6	2590	432
C-2,3-dehydro, C-18 COOH	13	GA_{59}	2727	460
C-18 COOH	13	GA_{21}	2729	462
Dihydroxy				
C-1,2-dehydro	3β,11β	GA_{80}	2672	504
	3β,15β	GA_{68}	2695	504
	3β,13	GA_3	2700	504
	3β,12β	12β-OH-GA_7	2719	504
	3β,12α	GA_{30}	2771	504
C-1,10-dehydro, 19,2-lactone	3β,13	Iso-GA_3	2642	504
C-2,3-dehydro	1β,13	1β-OH-GA_5	2592	504
	12β,13	12β-OH-GA_5	2635	504
	12α,13	12α-OH-GA_5	2641	504
	1α,13	1α-OH-GA_5	2675	504
	13,18	GA_{22}	2700	504
	1β,13	GA_{60}	2583	506
	1β,3β	GA_{54}	2609	506
	13,15β	GA_{67}	2625	506
	12β,13	12β-OH-GA_{20}	2637	506
	2α,3β	GA_{47}	2638	506
	1α,3β	GA_{16}	2641	506
	12α,13	GA_{77}	2643	506
	3β,11β	GA_{35}	2644	506
	2β,3β	GA_{34}	2669	506
	3β,13	GA_1	2675	506
	2α,13	GA_{81}	2681	506
	2β,13	GA_{29}	2690	506
	1α,13	1α-OH-GA_{20}	2694	506
	3β,12β	GA_{71}	2695[a]	506

(Continued on p. 118)

TABLE 8 (*continued*)

Type	–OH groups	Gibberellin	*I*	M^+
	3β,15β	GA_{63}	2712	506
	3β,12α	GA_{58}	2742[a]	506
	3α,13	*epi*-GA_1	2794	506
C-16,17-dihydro	3β,16α	GA_2	2761	508
	3β,17	GA_{82}	2818[a]	508
C-3-oxo	1β,12α	GA_{33}	2695	520
C-12-oxo	2β,3β	GA_{26}	2844	520
Trihydroxy				
C-1,2-dehydro	3β,13,15β	15β-OH-GA_3	2814	592
	3β,12β,13	12β-OH-GA_3	2843	592
	3β,12α,13	GA_{87}	2854	592
	1α,2α,3β	GA_{78}	2740[a]	594
	1β,2β,3β	GA_{79}	2753[a]	594
	1β,3β,13	GA_{55}	2764	594
	2α,3β,13	GA_{56}	2769	594
	2β,13,15β	GA_{76}	2776[a]	594
	1α,3β,13	GA_{57}	2791	594
	2β,3β,12β	GA_{48}	2796[a]	594
	3β,12β,13	12β-OH-GA_1	2803	594
	3β,12α,13	GA_{85}	2817	594
	2β,3β,13	GA_8	2821	594
	3β,13,15β	GA_{72}	2830	594
	2β,3β,12α	GA_{49}	2837[a]	594
	2β,3β,11β	GA_{50}	2847	594
	1α,3α,13	*epi*-GA_{57}	2898	594
Tetrahydroxy				
C-1,2-dehydro	3β,12α,13,15β	GA_{32}	2973	680
	2β,3β,12α,13	GA_{89}	2910	682
	2β,3β,13,15β	GA_{75}	2947[a]	682
	3β,12α,13,15β	GA_{86}	2975	682
Kauranoids				
ent-kaurene			2044	272
ent-kaurenol			2302	260
ent-kaurenal			2249	286
ent-kaurenoic acid			2270	316
ent-7α-hydroxy-kaurenoic acid			2430[a]	404

[a] Adapted from Gaskin and MacMillan [7] based on a comparision of *I* values of GAs of similar structure.

flow, column coating and condition, and other components in the sample [7], but relative retention times can usually be predicted with reasonable accuracy.

The reader is referred to Gaskin and MacMillan [7] for the most comprehensive published list of *I* values which includes those for all GAs up to GA_{86} as well as a host of isomers and related compounds. Beale and Willis [3] and Hedden [1] have provided less extensive compilations. Table 8 is a list of *I* values from our analyses of GA methyl ester or MeTMSi derivatives on DB1-15N columns, complemented with additional values estimated from the data of Gaskin and MacMillan [7].

The most extensive list of *I* values of a number

TABLE 9

RETENTION TIMES (t_R) AND KOVÁTS RETENTION INDICES (I) OF PERMETHYLATED GA–GLUCOSIDES IN GC

Adapted from Schmidt *et al.* [152]. HP1, 25 m × 0.31 mm i.d., 0.17 μm methylsilicone film; injection at 275°C, GC temperature programme 60°C (1 min) to 260°C (25°C min^{-1}).

Compound	t_R	KRI
GA_1–3-O-glucoside PME	24.369	3602
GA_1–13-O-glucoside PME	22.042	3532
C-3-*epi*-GA_1–3-O-glucoside PME	25.361	3625
C-3-*epi*-GA_1–13-O-glucoside PME	24.208	3597
GA_3–3-O-glucoside PME	21.460	3514
GA_3–13-O-glucoside PME	21.480	3515
GA_4–3-O-glucoside PME	19.419	3440
GA_5–13-O-glucoside PME	18.196	3392
GA_7–3-O-glucoside PME	18.168	3391
GA_8–2-O-glucoside PME	26.014	3640
GA_8–13-O-glucoside PME	24.484	3604
GA_{20}–13-O-glucoside PME	18.320	3398
GA_{29}–2-O-glucoside PME	23.287	3569
GA_{29}–13-O-glucoside PME		3502[a]
GA_{35}–11-O-glucoside PME	18.755	3415

[a] Estimated from Schneider *et al.* [10].

of permethyl GA–glucosides in such conditions has been provided by Schmidt *et al.* [152] (Table 9). These values together with the mass spectra were considered sufficiently distinctive for positive identification of the 14 GA-glucosides examined in that study.

4.5.4. Quantitative analysis

GC–MS is the method of choice for quantitative analysis of endogenous GAs, and results obtained by other methods (immunoassay, for example) should be verified by GC–MS. The principle of the method is well known. A known amount of an internal standard (IS), ideally the GA of interest labelled with a heavy isotope, is added to the sample after extraction. GAs labelled with atoms of ^2H, ^{13}C and ^{14}C have been used [2,19,25,76]. [17,17-$^2H_2$]-Labelled standards of a variety of GAs have been synthesized by Professor L. Mander (Research School of Chemistry, Australian National University, Canberra, Australia) and made available for purchase. These have been used in numerous studies [24,34,160,163]. If such standards are not available, another GA of similar structure to the endogenous GAs of interest can be used. Care must be taken to ensure that such an internal standard is carried through the various chromatographic steps with the endogenous GAs. The relative quantities of the IS and the endogenous GA are calculated finally after analysis by GC–MS or GC–SIM. The ion chromatograms are integrated and the ratio of areas of the signals for the IS and endogenous GA entered into a calibration curve to determine the ratio of the amounts of the two, as described, for example, by Hedden [1,2]. Alternatively, the distribution of masses in the molecular ion clusters can be used to determine isotope dilution [7,25]. Hence the absolute amount of endogenous GA in the sample can be found.

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Chromatographic analysis of G_1–G_3 natural cyclic peroxides and compounds obtained from Ru(II)-catalysed reaction of G_3

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ABSTRACT

Compounds obtained in the Ru(II)-catalysed reaction of endoperoxide G_3 were analysed by means of liquid and capillary gas chromatography. The chromatographic data obtained by analytical liquid and capillary gas chromatography of the natural endoperoxides G_1, G_2, G_3 are discussed.

INTRODUCTION

Natural peroxides constitute important biological mediators in various biochemical processes. For example, prostaglandin endoperoxides [1] are involved in the arachidonic cascade and antitumoral activities have been claimed for cyclic peroxyacetals isolated from marine organisms [2]. More recently, great attention has been devoted to the analysis and synthesis of arteannuin [3,4], a sesquiterpene lactone containing a peroxide linkage; this compound, common referred to as qinghaosu, extracted from *Artemisia annua L.*, is an effective antimalarial agent.

We were interested in other cyclic 1,4-epiperoxides (endoperoxides) that are the plant growth regulators designated as G factors G_1, G_2, G_3. They have been identified in leaves of *Eucalyptus grandis* and other myrtaceous plants and shown to be of general molecular structure **I** [5,6].

Their presence has been associated with frost resistance by controlling the active electron transport properties of membranes [7] and with stomatal conductance, photosynthesis and water loss reduction [8,9].

Based on the physiological importance of G factors, we developed a programme for the flexible chemical synthesis of the precursor syncarpic acid **II** [10] of G peroxides and their extraction from *E. grandis* [11]. We have also reported the chemical behaviour of G_3 in the presence of a Ru(II) catalyst [12], a transition metal complex that has the capability of inducing a reaction to occur via a one-electron exchange mechanism [13]. Ru(II) is a member of the iron triad and, as Fe peroxidases could be involved in the metabolism of the G peroxides, it can simulate a possible biosynthetic mechanism.

This paper reports chromatographic data for compounds obtained through the Ru(II)-catalysed reaction and a comparison between ana-

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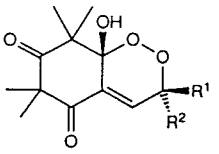

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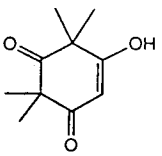


II

	R^1	R^2
G_1	Et	Me
G_2	Me	Et
G_3	Me	Me

G_1, G_2 : 4-ethyl-1-hydroxy-4,8,8,10,10-pentamethyl-7,9-dioxo-2,3-dioxabicyclo[4.4.0]decene-5

G_3 : 1-hydroxy-4,4,8,8,10,10-hexamethyl-7,9-dioxo-2,3-dioxabicyclo[4.4.0]decene-5

lytical liquid and capillary gas chromatography (GC) in the analysis of G factors.

EXPERIMENTAL

Instrumentation

Medium-pressure liquid chromatography was performed on an Axxial apparatus (Axxial Moduloprep, Martigues, France) equipped with a refractometric detector (Jobin–Yvon R.I. Iota). Analytical liquid chromatography was performed on a Waters Model 600E apparatus equipped with a Rheodyne injector (20 μl) and a Waters 990 UV Spectroflow detector. The column used was Novapak silical gel (150 mm × 3.9 mm I.D.; particle size 4 μm) purchased from Waters. Capillary gas chromatography was performed on a Hewlett-Packard HP-5890 apparatus equipped with an HP-1 column (25 m × 0.2 mm I.D.; 0.33 μm).

Gas chromatography–mass spectrometry was performed on an HP-S 971A apparatus equipped with an HP-1 column (25 m × 0.2 mm I.D.; 0.33 μm). NMR spectra were recorded in deuterochloroform with the tetramethylsilane as reference on a Bruker AC-200 apparatus and IR spectra were recorded on a Perkin-Elmer Model 883 spectrophotometer. Melting points were measured on a Kofler bench apparatus.

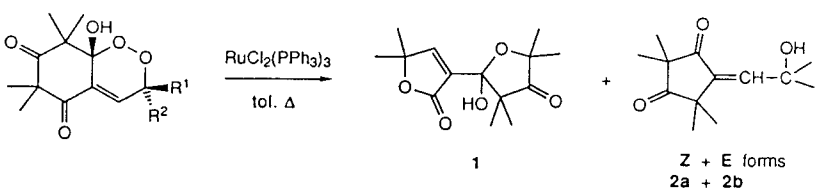
Chemicals

The three G factors (G_1, G_2, G_3) were synthesized according to the literature [11].

Ru(II)-catalysed reaction

In a round bottom two-neck flask equipped with a reflux condenser and a $CaCl_2$ tube we introduced under argon a solution of G_3 (0.375 mmol, 100 mg) in anhydrous toluene (2 ml) and $RuCl_2(PPh_3)_3$ (0.024 mmol, 100 mg) in toluene (2 ml). The mixture was stirred under reflux and the reaction was followed by TLC until complete consumption of the G_3 substrate. Thermal activation seemed necessary as no reaction occurred at room temperature. TLC on a silica gel plate with diethyl ether–light petroleum (4:6) as eluent showed the appearance of two products; one ($R_F = 0.10$) was revealed with a 10% ethanolic solution of phosphomolybdic acid and the other ($R_F = 0.22$) absorbed at a UV wavelength of 254 nm. The reaction mixture was dissolved in toluene (5 ml), passed through Celite, concentrated and purified by medium-pressure liquid chromatography [20 g of 6–35 μm silica gel (Amicon), column I.D. = 20 mm]. We obtained 68 mg (yield 68%) of compound **1** ($R_F = 0.10$) and 25 mg (yield 29%) of compound **2** ($R_F =$ 0.22).

Compound **1**: m.p. = 142°C; IR ($CHCl_3$, ν cm^{-1}), 3500 (OH), 1750 $(C{=}O)_{lac.}$, 1740



$(C{=}O)_{ket.}$, 1600 (C=C); ^1H NMR (200 MHz, $CDCl_3$, δ ppm), 0.14, 1.28, 1.38, 1.44 (4s, 4 × 3H), 1.51 (s, 6H), 4.00 (s, 1H, OH), 7.46 (s, 1H, HC=C); ^{13}C NMR (50.32 MHz, $CDCl_3$, δ ppm), 220, 161.1, 157.7, 131.1, 103.0, 85.3, 81.3, 52.4, 26.5, 25.8, 25.1, 24.1, 27.6; MS (EI, m/z), 268 (M^+, 2%), 251 (5%), 164 (14%), 139 (10%), 112 (100%), 97 (20%), 84 (79%), 69 (78%), 43 (55%). For the X-ray structure, see ref. 11.

Compounds **2a** and **2b**: IR ($CHCl_3$, ν cm^{-1}), 3383 (OH), 1752 (C=O), 1699 (C=0), 1613 (C=C); ^{13}C NMR (50.32 MHz, $CDCl_3$, δ ppm), 219.6, 212.4, 153.2, 141.2, 123.7, 70.4, 53.4, 50.4, 53.1, 50.9, 29.5, 27.3, 21.9.

RESULTS AND DISCUSSION

The crude mixture from the Ru(II)-catalysed reaction was analysed by using capillary GC. The chromatogram obtained under optimum conditions is presented in Fig. 1a. We observe three main peaks at $t_{R1} = 2.41$ min, $t_{R2} = 4.16$ min and $t_{R3} = 8.70$ min in a ratio 29.2:8.3:62.5, respectively. After medium-pressure liquid chromatography we obtained the two fractions mentioned above corresponding to the spots observed on the TLC plate.

Capillary GC of these fractions (Fig. 1b and c) showed that the first (TLC, $R_F = 0.10$, compound **1**) corresponds to the peak at $t_{R3} = 8.70$ min and the second (TLC, $R_F = 0.22$) corresponds at two major products at $t_{R1} = 2.28$ min and $t_{R2} = 4.14$ min in a 77:23 ratio inseparable under liquid chromatographic conditions. NMR spectroscopy ($CDCl_3$ as solvent) of compounds **2a** and **2b** showed major trends in their structure (*e.g.*, ethylenic protons at $\delta = 6.31$ and 6.82 ppm and six major peaks corresponding to the methyl groups at $\delta = 1.42$, 1.29, 1.20 and 1.52, 1.47, 1.19 ppm) and their identification was performed by GC–MS. The GC–MS analysis (Fig. 2) showed that the two compounds have the same molecular mass ($m/z = 224$) and identical fragmentation patterns, in agreement with an ethylenic β-diketone structure **2**. It is noteworthy that the transformation of the cyclic G peroxides in a probably one-electron reaction leads to the major product **1**, which shows a *gem* dimethyl

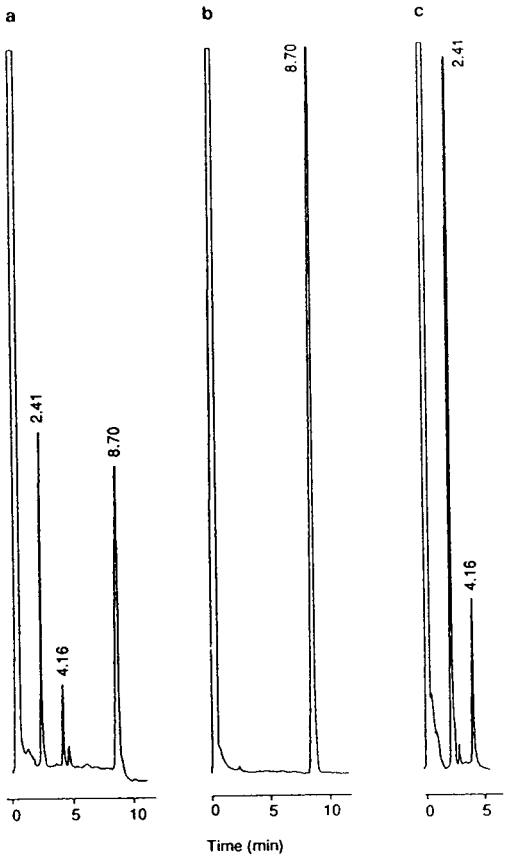


Fig. 1. Capillary GC of the Ru(II)-catalysed reaction of G_3. (a) GC of reaction mixture; (b) GC of compound **1** after purification; (c) GC of compound **2** after purification. Column, HP-1 (25 m × 0.2 mm I.D.; 0.33 μm); $T_{oven} = 150°C$; $T_{inj.} = 180°C$; $T_{det.} = 220°C$; flow-rate = 1 ml/min.

butenolide structure framework that is commonly found in various natural products extracted from plants [14–16]. It would therefore be interesting to examine further whether compounds **1** and **2** can be identified in different *Eucalyptus* species, especially those where G factors have not been identified.

As capillary GC was very efficient in the identification of the catalytic degradation products of G3, we were also interested in the comparison of the two chromatographic techniques (GC and LC) in the analysis of G_1, G_2 and G_3 peroxides. Concerning the G_1, G_2 and G_3 growth factors, the data obtained by analytical chromatography showed a clear and rapid

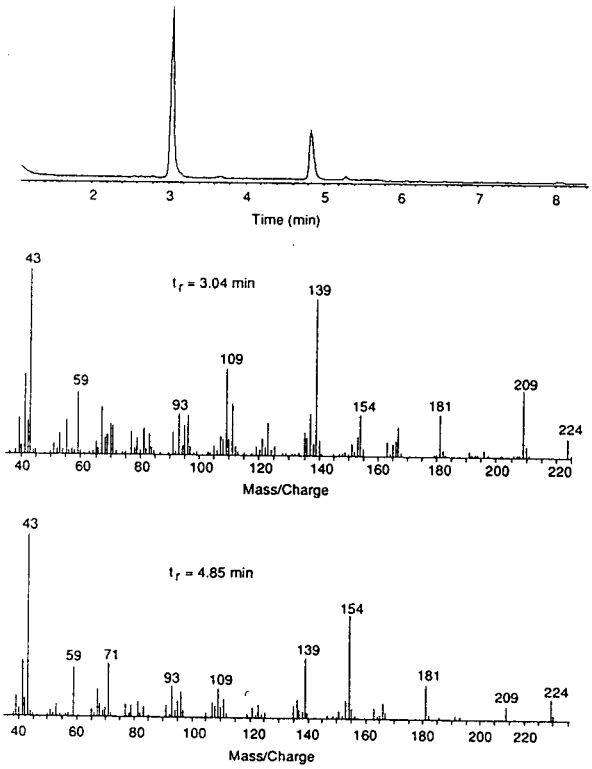


Fig. 2. GC–MS analysis of compound **2**. Column, HP-1 (25 m × 0.2 mm I.D.; 0.33 μm); $T_{oven} = 150°C$; $T_{inj.} = 180°C$; $T_{det.} = 220°C$.

differentiation of the three compounds under the experimental conditions described previously [11]. On the other hand, capillary GC of these compounds seems to be more difficult because of thermal modification of the products.

Capillary GC was performed on a BP-1 column (12 m × 0.33 mm I.D.; 0.5 μm). For G_3 ($T_{oven} = 145°C$, $T_{inj.} = 190°C$, $T_{det.} = 200°C$) a peak with retention time of 11.42 min was observed. A glass liner was added to increase the heat capacity as G_3 has a higher melting point than G_1 and G_2 (170 *versus* 100 and 127°C, respectively) [11]. Under such conditions we observed a degradation of the compound at $T_{inj.} > 190°C$.

A well defined mixture of G_1 and G_2 compounds (8:92) determined by analytical LC (Fig. 3a) was examined by capillary GC (Fig. 3b). It appears from the latter chromatogram (form of the peaks, G_1:G_2 ratio) that a good differentiation between the two compounds cannot be achieved and that G_1 and G_2 tend to equilibrate in the column. It was verified that this was due to heating; when a solid mixture of G_1 and G_2 (8:92) was heated and then measured by analytical LC, the same equilibration was observed. A correct measure of G_1 and G_2 was therefore not possible using capillary GC.

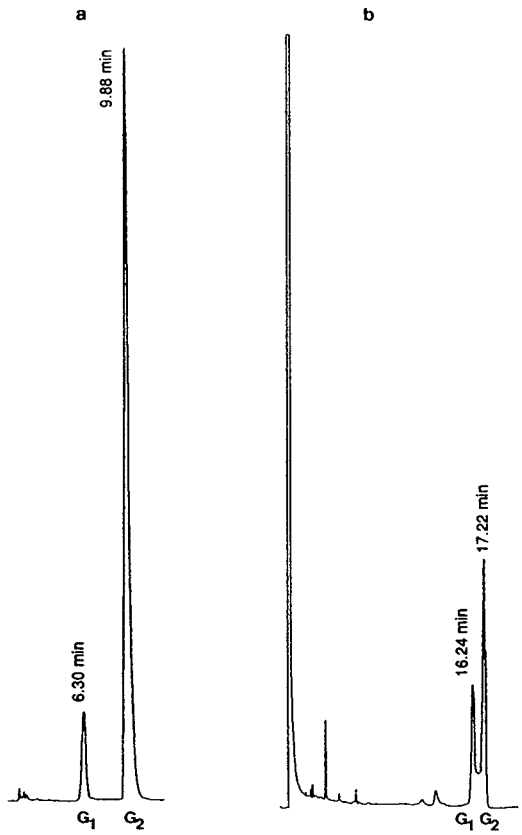


Fig. 3. Analytical HPLC and GC of an 8:92 mixture of G_1 and G_2. (a) HPLC: Novapak silica gel column (150 mm × 3.9 mm I.D.; particle size 4 μm); eluent, isooctane–methylene chloride (40:60); flow-rate, 1.5 ml/min; detection wavelength, 254 nm. (b) GC: column, BP-1 (12 m × 0.33 mm I.D.; 0.5 μm); $T_{oven} = 145°C$; $T_{inj.} = 200°C$; $T_{det.} = 200°C$; flow-rate = 1 ml/min.

CONCLUSIONS

We have described the analysis and identification by chromatographic methods of the growth factors G_1, G_2 and G_3 and the products of the Ru(II)-catalysed reaction of G_3. Whereas ana-

lytical LC can be used with success for the analysis of the G factors, capillary GC is preferred for studying and identificying the compounds obtained from the Ru(II)-catalysed reaction.

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Review

Separation of a new type of plant growth regulator, jasmonates, by chromatographic procedures

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ABSTRACT

Jasmonic acid and its related compounds are short-chain alkylcyclopentanone or alkylcyclopentane carboxylic acids and their derivatives, and have been recognized as a new type of plant growth regulator because of their wide occurrence in the plant kingdom together with abscisic acid-like physiological activities at low concentrations. These compounds each have two enantiomeric and diastereomeric forms due to the presence of the two chiral centres in the cyclopentanone or cyclopentane ring. For this reason, the separation of jasmonates is relatively difficult. This review surveys the experimental conditions for the separation of jasmonates using column chromatography, thin-layer chromatography, gas chromatography and high-performance liquid chromatography and some other techniques in the purification procedure based on their chemical properties. Qualitative and quantitative analyses of jasmonates using combined gas chromatography–mass spectrometry and with selected-ion monitoring are also described.

CONTENTS

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1. INTRODUCTION. DISCOVERY OF JASMONATES AND THEIR OCCURRENCE IN THE PLANT KINGDOM

Jasmonic acid and its related compounds are widely distributed in the plant kingdom [1,2] and have been recognized as a new type of plant hormone-like growth regulator. The methyl ester form of jasmonic acid, methyl jasmonate, was first isolated from the essential oil of jasmine (*Jasminum gradiflorum* L.) in 1962 [3] and from Tunisian rosemary (*Rosmarinus officinalis* L.) in 1967 [4] as an odoriferous compound. The structure of this compound was elucidated but no biological activity was found at that time. In 1971, jasmonic acid was first isolated from the culture filtrates of *Lasiodiplodia theobromae* (the synonym of *Botryodiplodia theobromae* Pat.) as a plant growth inhibitor [5]. This was the first report that jasmonate has plant growth regulating activity. In 1980, methyl jasmonate was isolated and identified as a senescence-promoting factor of plant tissues [6], indicating that jasmonates have distinctive biological activities in plant growth and development. Since then there have been numerous reports on the physiological effects of jasmonates, including the inhibition of seed [7] and pollen [8] germination, stem growth [9], cell expansion and multiplication [10] and the promotion of senescence [6,11,12], abscission [13,14] and tuber formation [15,16]. Further, it has been found that jasmonates affect plant gene expression and regulation [17–19]. Judging from this evidence together with their wide distribution in the plant kingdom, jasmonates may act as significant plant hormones in almost all aspects of physiological phenomena in the life cycle of plants in a similar manner to abscisic acid (ABA).

In this paper, we review chromatographic procedures for the separation of jasmonic acid and related compounds, as used for purification during their isolation.

2. CHEMICAL PROPERTIES OF JASMONATES

The structures of naturally occurring jasmonic acid and related compounds are shown in Fig. 1. Jasmonates belong to cyclopentane compounds with a keto or hydroxyl group at the C-6 position (C-3 in the numbering of the cyclopentane ring), a carboxylic acid moiety at the C-3 (C-1) position and 2′-*cis*-pentenyl or other alkyl substituents at the C-7 (C-2) position. Jasmonic acid is chemically similar to ABA in molecular mass, solubility properties and p*K*.

As shown in Fig. 2, conjugate forms of jasmonates have also been isolated and their structures have been elucidated. They conjugate with

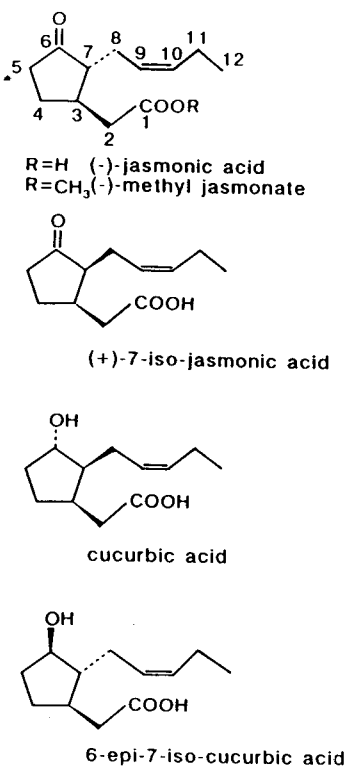
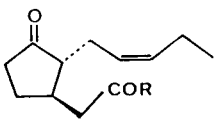
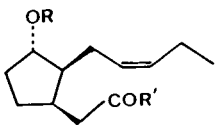


Fig. 1. Structural formulae of jasmonic acid and related compounds.



R= tyrosine N-[(-)-jasmonoyl]-S-tyrosine

R= tryptophan N-[(-)-jasmonoyl]-S-tryptophan



R= ß-D-glucose
R'= OH O-ß-D-glucopyranosylcucurbic acid

R= H
R'= tryptophan N-[(+)-cucurbinoyl]-S-tryptophan

Fig. 2. Structural formulae of conjugated jasmonates.

amino acid or glucose moieties. 3-O-β-D-Glucopyranosylcucurbic acid and its methyl ester, which have a reduced type of keto function at the C-6 (C-3) position, have been found in seeds of *Cucurbita pepo* L. [20–22]. 3-Oxo-2-(5′-β-D-glucopyranosyloxy- 2′ -pentenyl)cyclopentane-1-acetic acid (tuberonic acid O-β-D-glucoside) has been isolated from potato leaves as a specific tuber-inducing stimulus [16,23]. It has also been found that (±)-9,10-dihydrojasmonic acid is mainly converted into the O(11)-β-D-glucopyranoside of the 11-hydroxy derivative in a feeding experiment using barley seedlings [24]. Jasmonic acid conjugated with an amino acid moiety has been found in plant tissues [25–27] and fungi [28], whereas its glucosyl ether and glucosyl ester have not, although their existence is to be expected. The β-D-glucopyranosyl ester of jasmonic acid has been synthesized and was very unstable under acidic and alkaline conditions [29].

Jasmonic acid is biosynthesized from linolenic acid via the formation of 12-oxo-10(*Z*),15(*Z*)-phytodienoic acid in *Vicia faba* L. pericarp and other plant tissues [30,31]. Therefore, jasmonic acid and its related compounds each have two enantiomeric and diastereomeric forms due to the presence of the two chiral centres in the cyclopentanone or cyclopentane ring. The naturally occurring *cis* forms (3*R*,7*R*-forms) of jasmonic acid and its related compounds are known to be rapidly transformed into the *trans* forms by 7-epimerization (2-epimerization) during isolation procedures and other treatments, resulting in an equilibrium molar ratio of *trans* to *cis* forms of 9:1.

3. DETECTION OF JASMONATES

Detection of jasmonates has been based on their inhibitory effects in bioassays or their chemical properties.

A seed germination test on lettuce seeds [7], growth inhibition test on rice or lettuce seedlings [7] or chlorophyll degradation test on oat leaf segments [6] is recommended as a bioassay for the detection of jasmonates in extracts of plant tissues.

One can also detect jasmonates on thin-layer plates by spraying with anisaldehyde reagent, which consists of acetic acid–sulphuric acid–anisaldehyde (100:2:1) [32], with a 5% solution of vanillin in concentrated sulphuric acid [20] or with a 1% potassium permanganate solution [20] or by exposure to iodine vapour [33] (see Table 5). These compounds are also detected under UV radiation as fluorescent spots after spraying with 5% H_2SO_4–EtOH followed by heating at 130°C for 5 min [20].

4. EXTRACTION AND SOLVENT FRACTIONATION OF JASMONATES

In general, the concentrations of jasmonic acid and related compounds in plant tissues are low, as are those of plant hormones. Therefore, if one wants to isolate or identify these substances in a certain plant, the separation of the plant tissues from other parts is necessary prior to solvent extraction.

Plant materials containing jasmonates should be homogenized in a blender using a water-miscible organic solvent such as ethanol, methanol or acetone several times and then solvent extraction should be carried out in the usual way [34]. After the evaporation of the organic solvent at low temperature *in vacuo*, the aqueous solution should be partitioned with an organic solvent based on the distribution coefficient between the organic and aqueous phases. Free

forms of jasmonates are easily extracted with organic solvents because they are non-polar. As the acidic form of jasmonates is a short-chain alkylcyclopentanone or alkylcyclopentane carboxylic acid of $pK_a = 4–5$, the aqueous solution is adjusted to pH 2–3 with HCl and partitioned with ethyl acetate [7,35], diethyl ether [32,36], benzene [8] or chloroform [37,38]. If one wants to isolate jasmonates conjugated with amino acid moieties, chloroform can be recommended as the organic solvent for solvent fractionation [25,26].

5. CHROMATOGRAPHIC SEPARATION OF JASMONATES

5.1. Column chromatography

Column chromatography is a powerful purification technique for the isolation and the identification of jasmonates. In the purification of the acidic form of jasmonates, adsorption column chromatography on charcoal [7,33] or silica gel [5,36,39,40], partition column chromatography on silicic acid impregnated with formic acid [7,35,41] and ion-exchange column chromatography on DEAE-Sephadex A-25 [32,40] have been used. On the other hand, the neutral form can easily be purified by adsorption column chromatography on charcoal [6] or silicic acid [6]. DEAE-Sephadex A-25 column chromatography has frequently been applied to the purification of conjugate jasmonates [25,26], as also has adsorption column chromatography on silicic acid and Celite [20–22,28]. Supports and solvent systems used in column chromatography for the purification of jasmonates are given in Tables 1–3.

5.2. Thin-layer chromatography (TLC)

TLC has frequently been used for the purification and identification of jasmonates. In Table 4,

TABLE 1

ADSORPTION COLUMN CHROMATOGRAPHY OF JASMONATES

Compound	Adsorbent	Solvent system	Ref.
Jasmonic acid	Charcoal	35–65% aqueous acetone	7
	Charcoal	80% aqueous acetone	35
	Silica gel	Benzene–$CHCl_3$ (1:1)	5
	Silica gel L	$CHCl_3$–EtOAc (9:1)	36
	Silica gel	$CHCl_3$–MeOH (95:5)	39
	Silica gel	EtOAc–$CHCl_3$ (3:7–7:3)	40
Methyl jasmonate	Charcoal	70–80% aqueous acetone	6
	Silica gel (Wako-gel C 100)	Benzene–EtOAc (95:5)	6
	Silica gel (Wako-gel C 100)	*n*-Hexane–EtOAc (90:10)	6
Cucurbic acid	Silicic acid–Celite	Benzene–EtOAc (1:1)	20–22
	Charcoal–Celite	40–45% aqueous acetone	20–22
6-Epicucurbic acid	Silica gel	EtOAc–$CHCl_3$ (3:7–7:3)	40
7-Isocucurbic acid	Silica gel	EtOAc–$CHCl_3$ (3:7–7:3)	40
6-Epi-7-isocucurbic acid	Silica gel	EtOAc–$CHCl_3$ (3:7–7:3)	40
N-Jasmonoylisoleucine	Silica gel–Celite (1:2)	EtOAc–light petroleum (3:2)	27
N-Dihydrojasmonoylisoleucine	Silica gel–Celite (1:2)	EtOAc–light petroleum (3:2)	27
Cucurbic acid glucoside	Silicic acid–Celite	EtOAc–MeOH (90:10)	20–22
Cubcurbic acid glucoside methyl ester	Silicic acid–Celite	EtOAc–MeOH (90:10)	20–22

TABLE 2

PARTITION COLUMN CHROMATOGRAPHY OF JASMONATES

Compound	Support	Stationary phase	Mobile phase	Ref.
Jasmonic acid	Silicic acid	0.5 *M* formic acid	*n*-Hexane–EtOAc (99:1)	7, 35, 41
Cucurbic acid	Celite	1 *M* phosphate buffer (pH 5.4)	Benzene–*n*-butanol (97.5:2.5–95:5)	20–22
	Sephadex LH-20–Celite	1 *M* phosphate buffer (pH 5.4)	Benzene–*n*-butanol (97:3)	20–22
Cucurbic acid glucoside	Celite	1 *M* phosphate buffer (pH 5.4)	Benzene–*n*-butanol (80:20–70:30)	20–22
Cucurbic acid glucoside methyl ester	Celite	1 *M* phosphate buffer (pH 5.4)	Benzene–*n*-butanol (90:10–85:15)	20–22

TABLE 3

ION-EXCHANGE, GEL PERMEATION AND REVERSED-PHASE COLUMN CHROMATOGRAPHY OF JASMONATES

Compound	Support	Solvent system	Ref.
Jasmonic acid	DEAE-Sephadex A-25	80% aqueous MeOH–AcOH (98.5:1.5)	32, 36, 40
	Sephadex LH-20	MeOH	39
	Silica gel ODS-SQ_3	MeOH	33
	Silanized silica gel RP-2	$CHCl_3$–EtOAc (9:1)	37
(+)-7-Isojasmonic acid	Silanized silica gel RP-2	$CHCl_3$–EtOAc (9:1)	37, 42
(−)-9,10-Dihydrojasmonic acid	Silanized silica gel RP-2	$CHCl_3$–EtOAc (9:1)	37
3,7-Didehydrojasmonic acid	Silanized silica gel RP-2	$CHCl_3$–EtOAc (4:1)	37
4,5-Didehydrojasmonic acid	DEAE-Sephadex A-25	AcOH in 80% aqueous MeOH[a]	32
(+)-6-Epi-7-isocucurbic acid	DEAE-Sephadex A-25	AcOH in 80% aqueous MeOH[a]	32
	Silanized silica gel RP-2	$CHCl_3$–EtOAc (4:1)	37
N-[(−)-Jasmonoyl]-S-tyrosine	DEAE-Sephadex A-25	0.85 *M* AcOH in MeOH	25, 26
N-[(−)-Jasmonoyl]-S-tryptophan	DEAE-Sephadex A-25	0.85 *M* AcOH in MeOH	25, 26
N-[(+)-Cucurbinoyl]-S-tryptophan	DEAE-Sephadex A-25	0.85 *M* AcOH in MeOH	25, 26
(−)-9,10-Dihydro-11-hydroxyjasmonic acid O-β-D-glucopyranoside	DEAE-Sephadex A-25	0.5 *M* AcOH in MeOH	24
(+)-Jasmonic acid-β-D-glucosyl ester	Silanized silica gel	$CHCl_3$–EtOAc with increasing EtOH	29
Tuberonic acid O-β-D-glucopyranoside	Dowex 1 × 4 (CH_3COO^- form)	2 M AcOH	23
	Sephadex LH-20	10% or 30% MeOH	23
	LiChroprep RP-8	50% MeOH	23

[a] Discontinuous gradient.

adsorbents, developing solvent systems and R_F values are presented. Silica gel is the most commonly used support; in some instances other materials such as alumina have been used [36].

Jasmonates can be detected on thin-layer plates by spraying with reagents (Table 5). However, jasmonates cannot be detected as a quenching spot against a green fluorescent background because they do not show significant UV absorption.

TABLE 4

THIN-LAYER CHROMATOGRAPHY OF JASMONATES

Compound	Support	Solvent system	R_F [a]	Ref.
Jasmonic acid	Kieselgel GF_{254}	EtOAc–*n*-hexane–$CHCl_3$–AcOH (20:15:8:1)	–	7
	Kieselgel $60F_{254}$, 0.5 mm	*n*-hexane–EtOAc–AcOH (24:6:1)	–	8
	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.56	32
	Silica gel $60F_{254}$	Toluene–EtOAc–AcOH (80:10:4)	0.34	33
	Silica gel $60F_{254}$	2-Propyl alcohol–ammonia–water (10:1:1)	0.56	33
	Silica gel $60F_{254}$	Benzene–EtOAc–AcOH (10:1:1)	0.43–0.50	35
	Silica gel $60F_{254}$	*n*-Hexane–EtOAc–AcOH (10:1:1)	0.34–0.48	35
	Silica gel GF_{254}	Benzene–EtOAc–acetone–AcOH (40:10:5:1)	0.50–0.70	36
	Silica gel	Benzene–EtOAc (1:1)	0.35	39
	Silica gel	$CHCl_3$–MeOH (9:1)	0.39	39
	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.70	40
	Silica gel $60F_{254}$, 0.25 mm	*n*-Hexane–EtOAc–AcOH (10:1:1)	0.28–0.36	41
7-Isojasmonic acid	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.70	40
	Silica gel	$CHCl_3$–MeOH–AcOH (70:10:0.5)	0.22	43
4,5-Didehydro-jasmonic acid	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.56	32
4,5-Didehydro-7-isojasmonic acid	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5.1)	0.56	32
	Silica gel GF_{254}	*n*-Hexane–EtOAc–AcOH (60:40:1)	0.42	38
	Silica gel GF_{254}	$CHCl_3$–MeOH–AcOH (140:20:1)	0.20	38
(+)-11,12-Didehydro-7-isojasmonic acid	Silica gel	$CHCl_3$–MeOH–AcOH (70:10:0.5)	0.20	43
(−)-9,10-Dihydro-jasmonic acid	Silica gel, 1 mm	*n*-Hexane–EtOAc–AcOH (60:40:1)	0.45	37
(+)-9,10-Dihydro-7-isojasmonic acid	Silica gel	$CHCl_3$–MeOH–AcOH (70:10:0.5)	0.23	43
3,7-Didehydro-jasmonic acid	Silica gel, 1 mm	*n*-Hexane–EtOAc–AcOH (60:40:1)	0.47	37
Cucurbic acid	Silica gel G, 0.25 mm	Isopropyl ether–AcOH (95:5)	0.52	20–22
	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.36	32
	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.45	40
6-Epicucurbic acid	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.64	40
7-Isocucurbic acid	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.59	40
6-Epi-7-iso-cucurbic acid	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.42	32
	Silica gel, 1 mm	*n*-Hexane–EtOAc–AcOH (60:40:1)	0.32	37
	Silica gel, 1 mm	$CHCl_3$–MeOH–water (140:20:1)	0.17	37
	Silica gel GF_{254}, 0.3 mm	$CHCl_3$–EtOAc–acetone–AcOH (40:10:5:1)	0.52	40
	Silica gel	$CHCl_3$–MeOH–AcOH (70:10:0.5)	0.13	43
(+)-3-Oxo-2-(pentenyl)cyclopent-1-yl-propionic acid	Silica gel GF_{254}	$CHCl_3$–MeOH–AcOH (140:20:1)	0.25	38
	Silica gel GF_{254}	*n*-Hexane-EtOAc–AcOH (60:40:1)	0.50	38
(+)-3-Oxo-2-(pentenyl)cyclopent-1-yl-butyric acid	Silica gel GF_{254}	$CHCl_3$–MeOH–AcOH (140:20:1)	0.27	38
	Silica gel GF_{254}	*n*-Hexane–EtOAc–AcOH (60:40:1)	0.68	38
Methyl jasmonate	Kieselgel $60F_{254}$, 0.5 mm	Benzene–EtOAc (9:1)	–	8
	Silica gel PF_{254}, 0.5 mm	*n*-Hexane–EtOAc (5:1)	0.27–0.37	6
	Silica gel PF_{254}, 0.5 mm	Benzene–EtOAc (10:1, multiple)	0.30–0.34	6
	Al_2O_3	Benzene–EtOAc (3:2)	0.72	36
	Silica gel G	Benzene–EtOAc (3:2)	0.59	36
	Silica gel G	Diethyl ether	0.65	36
	Silica gel $60F_{254}$, 0.25 mm	*n*-Hexane–EtOAc (5:1)	0.59–0.72	41
Ethyl (+)-7-iso-jasmonate	Silica gel GF_{254}	$CHCl_3$–MeOH–AcOH (140:20:1)	0.91	38
	Silica gel GF_{254}	*n*-Hexane–EtOAc–AcOH (60:40:1)	0.68	38
Cucurbic acid glucoside	Silica gel G, 0.25 mm	$CHCl_3$–MeOH (85:15)	0.51	20–22
Cucurbic acid glucoside methyl ester	Silica gel G, 0.25 mm	$CHCl_3$–MeOH–AcOH (75:20:5)	0.60	20–22

[a] Dashes indicate data not given.

TABLE 5
COLOUR REACTIONS OF JASMONATES ON THIN-LAYER CHROMATOGRAPHY

Compound	Support	Reagent	Colour of spot	Ref.
Jasmonic acid	Silica gel GF_{254}, 0.3 mm	Anisaldehyde: heat[a]	Reddish brown	32
Cucurbic acid	Silica gel G, 0.25 mm	EtOH–H_2SO_4: heat[b] (UV)	Orange (pale yellow)	20
	Silica gel G, 0.25 mm	Vanillin–H_2SO_4: heat[c]	Blue green	20
6-Epi-7-iso-cucurbic acid	Silica gel GF_{254}, 0.3 mm	Anisaldehyde: heat	Greyish blue	32
Cucurbic acid glucoside	Silica gel G, 0.25 mm	EtOH–H_2SO_4: heat (UV)	Brown (pale yellow-green)	20
	Silica gel G, 0.25 mm	Vanillin–H_2SO_4: heat	Blue-green	20
Cucurbic acid glucoside methyl ester	Silica gel G, 0.25 mm	EtOH–H_2SO_4: heat (UV)	Brown (pale yellow-green)	20
	Silica gel G, 0.25 mm	Vanillin–H_2SO_4: heat	Blue green	20

[a] AcOH–H_2SO_4–anisaldehyde (100:2:1).
[b] 5% H_2SO_4–EtOH.
[c] 5% solution of vanillin in H_2SO_4.

5.3. High-performance liquid chromatography (HPLC)

HPLC is suitable for the analysis and separation of plant hormones. HPLC has also been investigated intensively for the separation of jasmonates. In HPLC, UV detection is commonly used because of its sensitivity. However, jasmonates and gibberellins [42] show no significant UV absorption. For these compounds, UV detection is carried out by using an end absorption of 205–230 nm. Reversed-phase columns of medium polarity such as LiChrosorb RP-8 and Polyol RP-8 or non-polar such as LiChrosorb RP-18 and Polyol RP-18 have been used extensively [38,41,43].

5.3.1. Free acid and methyl ester forms of jasmonates

As shown in Tables 6 and 7, solvent systems consisting of aqueous methanol with or without a low concentration of AcOH or H_3PO_4 are commonly used for the separation of jasmonic acid [33,37,40] and its derivatives [33,37,38,40] and their methyl esters [32,37,39]. In some instances using 60% aqueous methanol containing H_3PO_4 as a solvent system, retention times longer than 100 min have been obtained [43].

5.3.2. Conjugate jasmonates

As shown in Table 8, jasmonates conjugated with an amino acid moiety were successfully separated by using HPLC on LiChrosorb RP-8 or Hypersil RP-8. The β-D-glucopyranosyl ester of jasmonic acid, which has not been found in the plant kingdom but has been synthesized, has also been successfully separated by using HPLC on Polyol RP-8 with the solvent system methanol–water–H_3PO_4 (40:60:0.1) [29].

5.3.3. Enantiomers of jasmonates using diastereomeric derivatives on an achiral stationary phase

As described above, methyl jasmonate isolated from natural sources consists of an equilibrium mixture of the 7-epimers (2-epimers) in a ratio of *ca.* 9:1. Attempts to separate the enantiomers have been made using HPLC. Previous attempts have involved conversion to the diastereomeric ketal with (−)-2,3-butanediol [44] or esters of (−)-borneol [45]. Nucleosil 10 CN and Nucleosil 100-5 columns, respectively, were used for separation. In each separation, the (+)-enantiomer eluted faster than the (−)-enantiomer with a solvent system consisting of *n*-hexane or *n*-hexane containing 13–15% of ethyl acetate (Figs. 3 and 4).

A clear separation of the isomers of the methyl ester forms of jasmonic acid and its derivatives has recently been achieved by HPLC on LiChrosorb RP-18 (7 μm) with a solvent system of methanol–water (11:9) [40]. However, free acid forms, jasmonic acid and 6-epicucurbic acid, were incompletely resolved by HPLC on

TABLE 6

HPLC OF FREE ACID FORMS OF JASMONATES

Compound	Column (length × I.D.)	Solvent system	Flow-rate (ml/min)[a]	Retention time (min)[a]	Ref.
Jasmonic acid	Zorbax ODS (150 × 4.6 mm) (40°C)	MeOH–0.01% AcOH (3:7)	–	–	33
	LiChrosorb RP-18 (250 × 10 mm)	MeOH–0.2% AcOH (11:9)	4	22.7	40
	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (11:9)	1	9.5	37
(+)-7-Iso-jasmonic acid	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (11:9)	1	9.5	37
	Polyol RP-8 (310 × 25 mm)	MeOH–H_2O–H_3PO_4 (60:40:1)	2	135–158	43
(−)-9,10-Dihydro-jasmonic acid	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (11:9)	1	13.5	37
(+)-9,10-Dihydro-7-isojasmonic acid	Polyol RP-8 (310 × 25 mm)	MeOH–H_2O–H_3PO_4 (60:40:1)	2	135–158	43
3,7-Didehydro-jasmonic acid	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (11:9)	1	7.2	37
(+)-4,5-Didehydro-7-isojasmonic acid	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (1:1)	1	9.2	38
(+)-11,12-Didehydro-7-isojasmonic acid	Polyol RP-8 (310 × 25 mm)	MeOH–H_2O–H_3PO_4 (60:40:1)	2	100–130	43
Cucurbic acid	LiChrosorb RP-18 (250 × 10 mm)	MeOH–0.2% AcOH (11:9)	4	17.3	40
6-Epicucurbic acid	LiChrosorb RP-18 (250 × 10 mm)	MeOH–0.2% AcOH (11:9)	4	22.5	40
7-Isocucurbic acid	LiChrosorb RP-18 (250 × 10 mm)	MeOH–0.2% AcOH (11:9)	4	20.5	40
6-Epi-7-iso-cucurbic acid	LiChrosorb RP-18 (250 × 10 mm)	MeOH–0.2% AcOH (11:9)	4	19.5	40
(+)-3-Oxo-2-(pentenyl)-cyclopent-1-yl-propionic acid	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (1:1)	1	11.7	36
(+)-3-Oxo-2-(pentenyl)-cyclopent-1-yl-butyric acid	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (1:1)	1	12.5	36

[a] Dashes indicate data not given.

TABLE 7

HPLC OF ESTER FORMS OF JASMONATES

Compound	Column (length × I.D.)	Solvent system	Flow-rate (ml/min)[a]	Retention time (min)[a]	Ref.
Methyl jasmonate	Nucleosil 50-5 (250 × 4.6 mm)	EtOAc–*n*-hexane (5:95)	–	–	33
	LiChrosorb RP-18 (250 × 10 mm)	MeOH–H_2O (11:9)	4	16.6	40
Ethyl (+)-7-iso-jasmonate	Polyol RP-18 (250 × 4.6 mm)	MeOH–0.1% aqueous H_3PO_4 (1:1)	1	14.5	40
Methyl cucurbate	LiChrosorb RP-18 (250 × 10 mm)	MeOH–H_2O (11:9)	4	19.5	40
Methyl 6-epicucurbate	LiChrosorb RP-18 (250 × 10 mm)	MeOH–H_2O (11.9)	4	23.0	40
Methyl 7-isocucurbate	LiChrosorb RP-18 (250 × 10 mm)	MeOH–H_2O (11:9)	4	20.3	40
Methyl 6-epi-7-iso-cucurbate	LiChrosorb RP-18 (250 × 10 mm)	MeOH–H_2O (11:9)	4	17.9	40

[a] Dashes indicate data not given.

TABLE 8

HPLC OF CONJUGATED FORMS OF JASMONATES

Compound	Column (length × I.D.)	Solvent system	Flow-rate (ml/min)	Retention time (min)[a]	Ref.
N-[(−)-Jasmonoyl]-S-tyrosine	LiChrosorb RP-8 (250 × 4.6 mm)	MeOH–0.2% aqueous AcOH (55:45)	1	–	25
	LiChrosorb RP-8 (250 × 4.6 mm)	MeOH–0.2% aqueous AcOH (45:55)	1	–	25
N-[(−)-Jasmonoyl]-S-tryptophan	LiChrosorb RP-8 (250 × 4.6 mm)	MeOH–0.2% aqueous AcOH (55:45)	1	–	26
N-[(−)-Jasmonoyl]-S-tyrosine methyl ester	LiChrosorb RP-8 (250 × 4.6 mm)	MeOH–0.2% aqueous AcOH (60:40)	1	–	25
N-[(−)-Jasmonoyl]-S-tryptophan methyl ester	LiChrosorb RP-8 (250 × 4.6 mm)	MeOH–0.2% aqueous AcOH (65:35)	1	–	25
	Hypersil RP-8 (200 × 4.6 mm)	MeOH–0.2% aqueous AcOH (40:60)	1	–	26
(−)-9,10-Dihydro-11-hydroxyjasmonic acid O-β-D-glucopyranoside	LiChrosorb RP-18 (250 × 4.6 mm)	MeOH–H_2O–AcOH (200:300:1)	0.6	7.4	24
(+)-Jasmonic acid-β-D-glucosyl ester	Polyol RP-8 (250 × 4.6 mm)	MeOH–H_2O–H_3PO_4 (40:60:0.1)	1	8.6	29
Tuberonic acid O-β-D-glucopyranoside	μBondapak (150 × 19 mm)	10% CH_3CN	9.9	–	23
	Aminex HPX-87 (300 × 7.8 mm)	0.01 *M* H_2SO_4	1	–	23
	Resolve C_{18} (150 × 3.9 mm)	30% MeOH containing 0.1% AcOH	0.45	–	23
	Novapak C_{18} (100 × 8 mm)	10% CH_3CN containing 0.1% AcOH	1	–	23
	Novapak C_{18} (100 × 8 mm)	3% THF containing 0.1% AcOH	1	–	23

[a] Dashes indicate data not given.

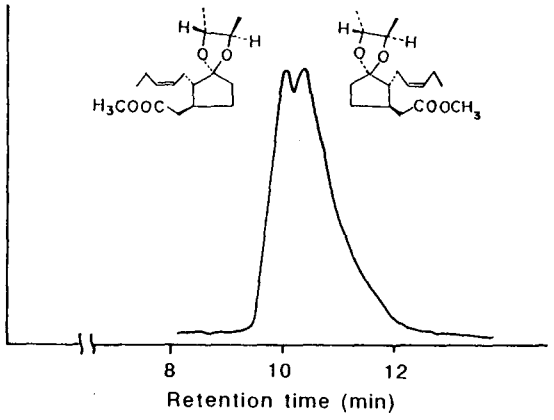


Fig. 3. HPLC of diastereomeric ketals on an analytical scale. Column, Nucleosil 10 CN (25 cm × 4 mm I.D.); mobile phase, *n*-hexane; flow-rate, 90 ml/h. Reproduced from *Agric. Biol. Chem.*, 45 (1981) 1709, by permission of the Japan Society for Bioscience, Biotechnology and Agrochemistry. Absorbance measured at 205 nm.

the same column with methanol–0.2% acetic acid (11:9) as the eluent (Tables 6 and 7) [40].

5.3.4. Enantiomers of jasmonates on a chiral stationary phase

Direct resolution using a column with a chiral stationary phase rather than the resolution of diastereomeric derivatives may be recommended for convenience. The direct separation of the enantiomers of methyl jasmonate has recently been demonstrated using a Chiralpak AS column (chiral stationary phase) [46]. As shown in Fig. 5, the optical resolution of methyl jasmonate or methyl epijasmonate (methyl 7-isojasmonate) was completely achieved by using the solvent system *n*-hexane–2-propanol (9:1) [46]. On the other hand, (±)-methyl cucurbate was not resolved by using a Chiralpak AS column but was by using a Chiralcel OF column with *n*-hexane–2-propanol as the eluent [46].

5.4. Gas chromatography (GC)

The GC of jasmonates has been extensively investigated, especially in combination with mass spectrometry for identifications. Many kinds of jasmonates can be analysed by GC after conversion into their methyl ester forms with ethereal

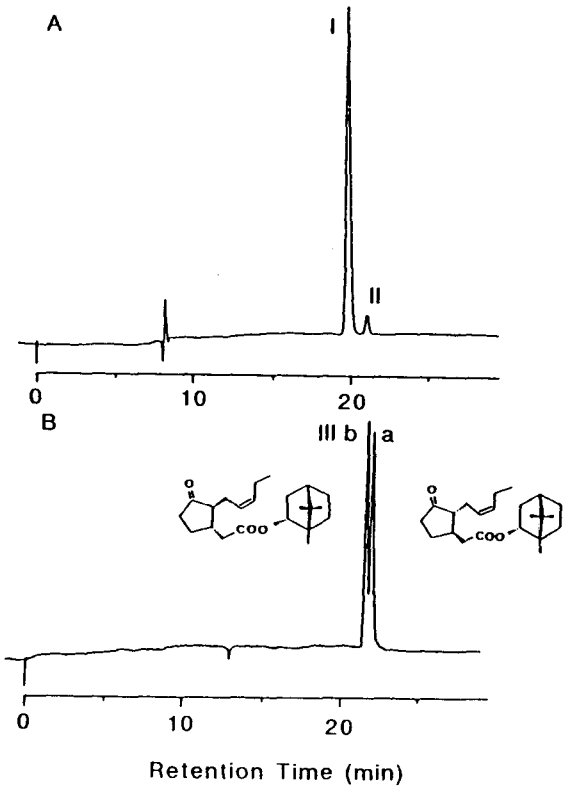


Fig. 4. HPLC of jasmonic esters (refractive index detection). (A) Methyl jasmonate (I) + methyl epijasmonate (II) (95:5). Two Nucleosil 100-5 columns (300 mm × 8 mm I.D.); mobile phase, EtOAc–hexane (15:85); flow-rate, 3 ml/min. (B) Bornyl jasmonates (IIIa:IIIb = 1:1). Three Nucleosil 100-5 columns (300 mm × 8 mm I.D.); mobile phase, EtOAc–hexane (13:87); flow-rate, 3 ml/min. The eluates were monitored with a Waters R 401 differential refractometer. Reproduced from *Agric. Biol. Chem.*, 49 (1985) 769, by permission of the Japan Society for Bioscience, Biotechnology and Agrochemistry.

diazomethane (Table 9). Because of the presence of some impurities, there is a possibility that not only may different compounds show the same retention time, but also same compound may show different retention times. Using packed columns such as SE-30 and OV-1, the retention time of methyl jasmonate differs owing to the difference in the amount of the compound injected. If a smaller amount is injected, the retention time is longer. This serious problem may be solved by using a fused-silica capillary column such as DB-1.

TABLE 9

GC OF JASMONATES

The carrier gas was nitrogen in all instances.

Compound	Column (length × I.D.)	Support	Column temperture (°C)	Flow-rate (ml/min)	Retention time (min)	Ref.
Methyl jasmonate	SE-30 (5%) (2 m × 3 mm)	Chromosorb W (80–100 mesh)	180	30	3.8	6
	EG SS-X (10%) (3 m × 4 mm)	Gas Chrom P (125–150 μm)	190	93	24.6	32
	XE-60 (3%)	–[a]	135	38	5.7	36
	OV-1 (1%) (2 m × 3 mm)	Chromosorb W (80–100 mesh)	170, 180	40	–[a]	41
	EG SS-X (10%) (4 m × 3 mm)	Gas Chrom P (125–150 μm)	170	50	38.5	43
	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	15	43
Methyl (+)-7-iso-jasmonate	EG SS-X (10%) (4 m × 3 mm)	Gas Chrom Q (100–120 mesh)	170	50	47	43
	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	17.8	43
Methyl (+)-9,10-dihydro-7-iso-jasmonate	EG SS-X (10%) (4 m × 3 mm)	Gas Chrom P (125–150 μm)	170	50	36	43
	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	15.9	43
Methyl (−)-9,10-dihydrojasmonate	EG SS-X (10%) (4 m × 3 mm)	Gas Chrom P (125–150 μm)	170	50	30.5	43
	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	13.6	43
Methyl (+)-11,12-didehydro-7-iso-jasmonate	EG SS-X (10%) (4 m × 3 mm)	Gas Chrom P (125–150 μm)	170	50	69	43
Methyl (−)-11,12-didehydrojasmonate	EG SS-X (10%) (4 m × 3 mm)	Gas Chrom P (125–150 μm)	170	50	97.5	43
Methyl (+)-cucurbate	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	21.1	43
Methyl (+)-6-epi-cucurbate	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	18.3	43
Methyl 6-epi-7-isocucurate	EG SS-X (10%) (3 m × 4 mm)	Gas Chrom P (125–150 μm)	190	93	28.2	32
Methyl 9,10-dihydrocucurbate	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	19.2	43
Methyl 9,10-dihydro-6-epi-cucurbate	OV-225 (3%) (2 m × 4 mm)	Gas Chrom Q (100–230 mesh)	150	110	16.5	43

[a] Not given.

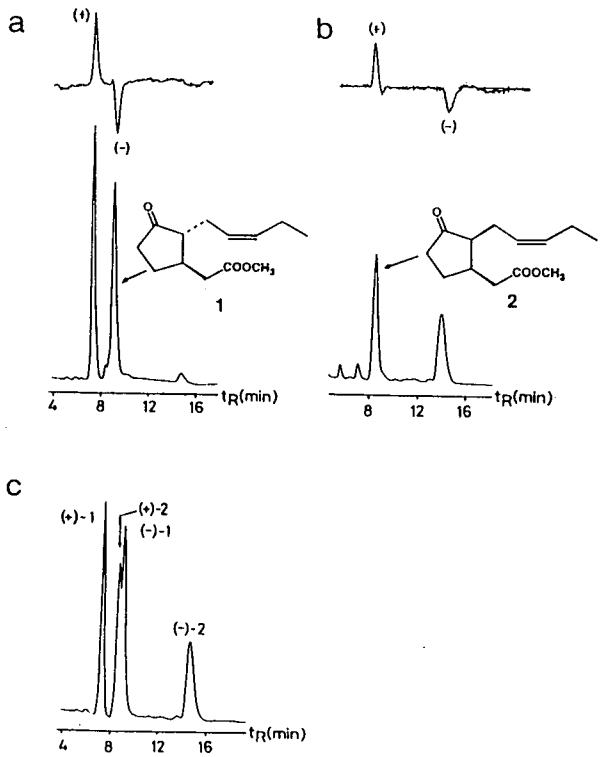


Fig. 5. (a) Chromatographic resolution of (±)-methyl jasmonate on a Chiralpak AS column (250 × 4.6 mm I.D.) (Daicel). Mobile phase, *n*-hexane–2-propanol (9:1); flow-rate, 1.0 ml/min; temperature, ambient; injection volume, 10 μl (5 μg). Upper trace, polarimetric detection (Showa Denko OR-1 employing a near-infrared LED of 780 nm); lower trace, photometric detection at 230 nm. (b) Chromatogram obtained for (±)-methyl epijasmonate. Conditions as in (a). (c) Chromatogram resulting from injection of 10 μl (5 μg each) of a mixture of (±)-methyl jasmonate and (±)-methyl epijasmonate. UV detection at 230 nm; other conditions as in (a). Reproduced from *Agric. Biol. Chem.*, 56 (1992) 1172, by permission of the Japan Society for Bioscience, Biotechnology and Agrochemistry.

The column temperature in analyses using packed columns is around 180°C, as in the analysis of fatty acid methyl esters [47]. The separation of the isomers of the methyl esters was performed by using a cross-linked methylsilicone fused-silica column with a temperature programme such as 50°C isothermal for 1 min, increased to 140°C at 25°C/min, held at 140°C for 1 min and then increased to 160°C at 2.5°C/min.

6. QUALITATIVE AND QUANTITATIVE ANALYSES OF JASMONATES BY USING COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC–MS) AND WITH SELECTED-ION MONITORING (GC–SIM)

Methyl esters of jasmonates can be easily separated and analysed by GC, and the identification of jasmonates can be successfully performed by using combined GC–MS. This method is convenient and reliable for the identification of jasmonates. In this method, a fused-silica capillary column is commonly used because of its good separation properties. Mass spectral data for methyl ester forms of jasmonates are summarized in Table 10.

Combined GC–MS with selected-ion monitoring (GC–SIM) is used not only as an identification method but also in quantitative analysis. The GC–SIM profile of methyl jasmonate is shown in Fig. 6. For the determination of methyl jasmonate using [9,10-^{2}H] jasmonic acid as an internal standard, prominent peaks at *m/z* 224 and 226, which are the molecular ion peaks of methyl jasmonate and the deuterium-labelled compound, respectively, are monitored [48]. The level of jasmonic acid in the sample was de-

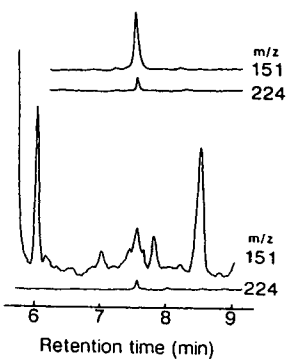


Fig. 6. GC–SIM profile of an authentic sample of methyl jasmonate (top) and the methyl ester form of an unknown material in the jasmonic acid fraction of *Euglena gracilis* Z (bottom). JEOL DX-300 mass spectrometer combined with a gas chromatograph (160°C isothermal, 70 eV, 300 μA). A DB-1 fused-silica capillary column (30 m × 0.254 mm I.D.) was used. The carrier gas was helium and the splitting ratio was 12:1. Reproduced from *Agric. Biol. Chem.*, 55 (1991) 275, by permission of the Japan Society for Bioscience, Biotechnology and Agrochemistry.

TABLE 10

MASS SPECTRAL DATA FOR THE METHYL ESTERS OF JASMONATES

Compound	m/z (relative intensity, %)	Ref.
Jasmonic acid	224(M^+, 37), 206(13), 193(17), 177(14), 156(24), 151(50), 135(20), 133(20), 121(17), 109(34), 95(44), 83(100)	6
(−)-9,10-Dihydro-jasmonic acid	226(M^+, 3), 195(3), 156(38), 153(34), 83(100)	49
(+)-9,10-Dihydro-7-isojasmonic acid	226(M^+, 3), 195(3), 156(38), 153(34), 83(100)	49
4,5-Didehydro-7-iso-jasmonic acid	222(M^+, 21), 193(17), 191(11), 167(15), 154(81), 149(18), 133(21), 119(16), 107(21), 95(100)	38
(+)-3-Oxo-2-(2-pentenyl)cyclopent-1-yl-propionic acid	238(M^+, 24), 220(16), 207(12), 191(18), 170(21), 164(8), 165(7), 151(75), 133(13), 121(9), 109(40), 97(67), 83(100)	38
(+)-3-Oxo-2--(2-pentenyl)cyclopent--1-yl-butylric acid	252(M^+, 15), 234(12), 221(5), 196(10), 184(12), 151(61), 133(27), 124(13), 109(27), 95(37), 83(100)	38
(+)-7-Isojasmonic acid[a]	238(M^+, 23), 220(6), 209(4), 193(17), 191(12), 170(16), 151(43), 133(23), 109(34), 95(43), 93(39), 83(100), 79(35)	38
(−)-9,10-Dihydro-11-hydroxyjasmonic acid[b]	242(M^+, 5), 224(1), 211(4), 209(5), 198(3), 182(3), 169(20), 156(81), 151(35), 137(10), 125(22), 109(29), 96(27), 83(100)	24
Cucurbic acid	226(M^+, 1), 208(9), 195(2), 165(7), 156(13), 153(73), 152(32), 139(11), 134(33), 119(16), 83(100), 79(66), 74(26)	40
6-Epicucurbic acid	226(M^+, 3), 208(12), 195(16), 165(17), 156(2), 153(33), 152(63), 139(42), 134(96), 119(61), 83(48), 79(100), 74(28)	40
6-Epi-7-isocucurbic acid	226(M^+, 2), 208(12), 165(7), 153(7), 152(19), 139(24), 134(100), 119(30), 83(38), 79(59), 74(22)	40
N-[(−)-Jasmonoyl]-S-tryptophan	410(M^+, 4), 378(4), 201(41), 170(4), 159(4), 143(5), 130(100), 117(3)	26
N-[(−)-Jasmonoyl]-S-tyrosine	378(M^+, 21), 369(3), 355(26), 249(19), 236(12), 220(5), 210(17), 196(13), 192(11), 178(95), 151(19), 147(24), 142(12), 136(22), 107(100)	25
N-[(+)-Cucurbinoyl]-S-tryptophan	412(M^+, 6), 394(2), 201(74), 170(6), 159(7), 143(8), 130(100), 117(3)	26

[a] Ethyl ester form.

[b] Mixture with (−)-9,10-dihydro-12-hydroxyjasmonic acid.

termined from the ratio of the m/z 224 and 226 peak areas.

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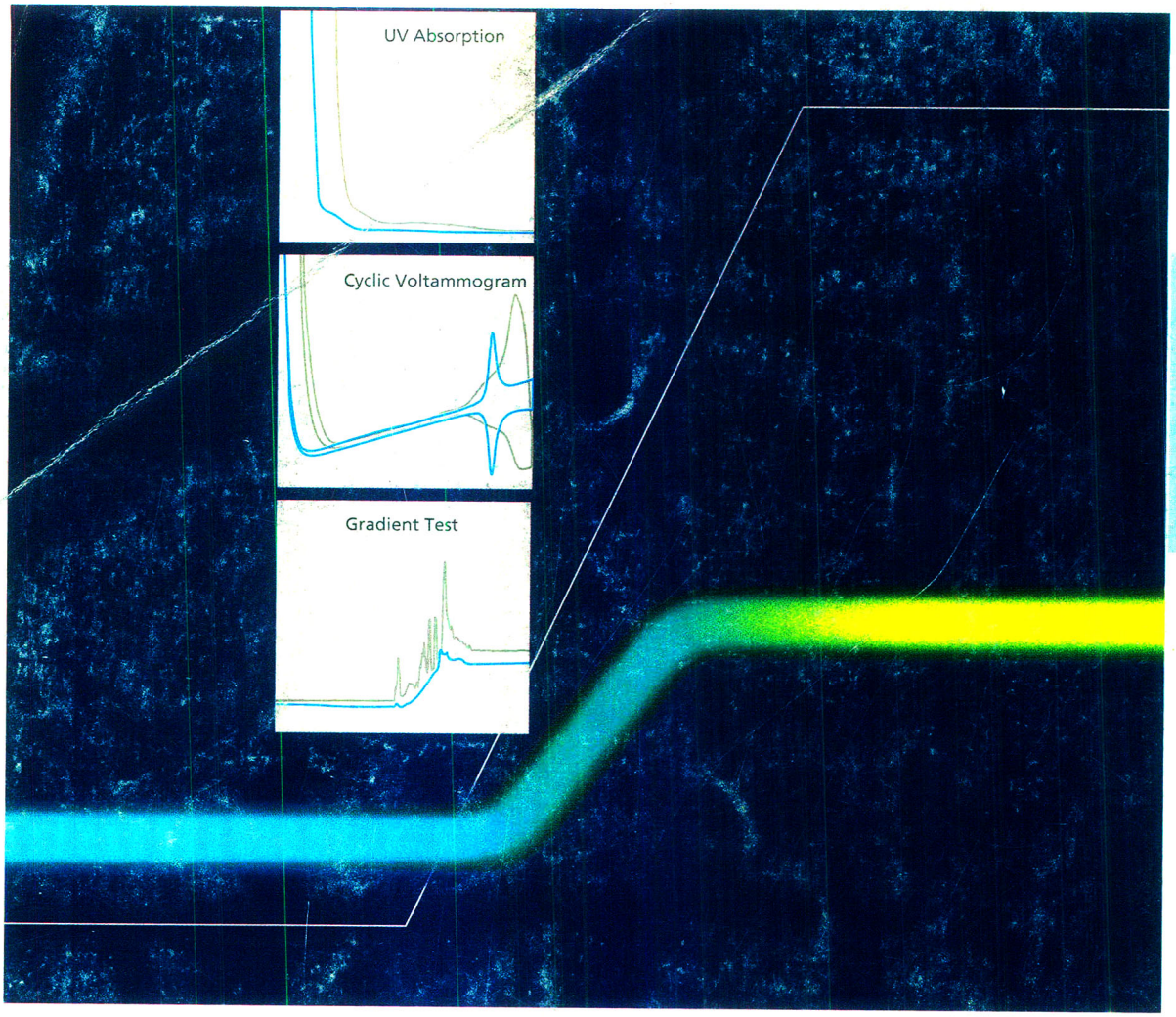

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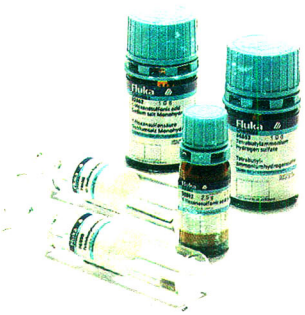

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