Journal of Chromatography, 194 (1980) 43–53
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,728

PRACTICAL ASPECTS OF THE PREPARATION AND CHROMATOGRAPHY OF THE TRIMETHYLSILYL ETHERS OF ECDYSTEROIDS

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(Received January 30th, 1980)

SUMMARY

Some of the difficulties encountered in the silylation of ecdysteroids are described, together with methods for avoiding them. Standard procedures are given for the preparation of trimethylsilyl ethers of ecdysteroids in biological samples and their analysis by gas chromatography with electron capture detection. This is considered to be the most efficient method for ecdysteroid determination in most arthropod tissues.

INTRODUCTION

The analysis of the steroidal insect and crustacean moulting hormones, or ecdysteroids, is currently of great interest to invertebrate physiologists and embryologists. We have reviewed the methods available and made some comparison of their advantages and disadvantages[1]. Excluding the non-specific, though sensitive, radioimmunoassay method, the two techniques of most promise are high-performance liquid chromatography (HPLC) and gas chromatography (GC). Advances in HPLC have improved that method through increased resolution but sensitivity is still limited by the detector (*cf.*, ref. 2). In our hands, the determination by GC using electron capture detection (GC–ECD) of the trimethylsilyl ethers of the ecdysteroids is preferred[3–6]. Although the method requires derivative formation, it has the advantages of great sensitivity, of selectivity, requires the least lengthy preliminary clean-up, and is least subject to losses during handling.

However, the preparation and handling of silyl ethers and the use of the electron capture detector present problems for those not experienced in their use, therefore a description of some of the problems encountered and methods for avoiding or overcoming them are important for those wishing to determine ecdysteroids in this way. Methods for ecdysteroid determination, found necessary and satisfactory in the hands of several workers, are described in this paper.

EXPERIMENTAL

Purification of solvents

Toluene for GC–ECD was purified by shaking twice with small portions of

0021-9673/80/0000–0000/$02.25

conc. sulphuric acid, then washing with distilled water and 5% aqueous sodium hydrogen carbonate to remove all traces of acid. The toluene was dried over anhydrous magnesium sulphate, distilled from phosphorus pentoxide and stored over molecular sieves 4A. Its purity was checked periodically by evaporating a 10-ml portion to 200 μl with a stream of nitrogen and injecting 2 μl onto the gas chromatograph fitted with a ^{63}Ni ECD. The solvent peak should be no more than 1 min in breadth.

Pyridine for silylation reactions was distilled from calcium hydride and stored over molecular sieves. Methanol and diethyl ether were dried with magnesium and sodium respectively in the conventional way.

Cleaning glassware

Glassware was cleaned by soaking in a bath of alkaline detergent overnight and then rinsing thoroughly with water and finally acetone. Reacti-vials used for the silylation reaction were cleaned by soaking in chromic acid overnight, washing with aqueous sodium bicarbonate solution and rinsing repeatedly with distilled water. They were finally rinsed with acetone and baked dry at 140 °C for at least 30 min. Material which was not removed by chromic acid was removed with a commercial powdered pumice abrasive ("Briz") and the Reacti-vials were then washed with water and soaked in chromic acid as above.

Cleaning the electron capture detector

Frequent use of the ECD eventually leads to contamination and build-up of a deposit. Cleaning must depend upon the design and makers instructions. With the Pye 104 detector, the central collecting electrode was best cleaned with metal polish and the barrel was cleaned in an ultrasonic bath for 1–2 h in hexane or toluene. The bath was monitored for radioactivity after the washing but none was ever detected.

Silica for thin-layer chromatography

Commercial silica for thin-layer chromatography (TLC) was purified to remove electron-capturing impurities. Silica gel P F_{254} (Merck, Darmstadt, G.F.R.; 1 kg) was suspended in methanol (2 l) by stirring mechanically for 2–3 h, filtered with vacuum and washed with methanol (1 l) and diethyl ether (0.5 l). The resulting cake was broken up and dried at room temperature.

The glass plates were washed with detergent, rinsed with distilled water, dilute acid and again with water. The purified silica was slurried in distilled water and plates of 0.6 mm thickness prepared in the usual way and dried and activated by heating at 100 °C for 1 h and then stored over saturated sodium chloride solution to produce uniform deactivation.

Preparation of N-trimethylsilylimidazole

All stages of reaction were carried out with as careful an exclusion of moisture as was practical. Imidazole (27.2 g, 0.4 mole) was heated under reflux for 2 h with hexamethyldisilazane (48.4 g, 0.3 mole) and conc. sulphuric acid (two drops). The product was distilled fractionally under reduced pressure to give N-trimethylsilylimidazole (TMSI, 46.8 g, 84% based on imidazole) as a colourless mobile oil, b.p. 91 °C at 12 mmHg. The product was stored by transferring to 1-ml ampoules under

nitrogen and sealing with a flame. Its activity was checked by the rate of silylation of a pure ecdysteroid (see below).

Preparation of trimethylsilyl ethers of pure ecdysteroids

Both ecdysone and 20-hydroxyecdysone (Simes, Milan, Italy) were used. A sample of ecdysteroid (0.2–1.0 mg) was weighed on a microbalance and dissolved in acetone to give (typically) 250 μg ml^{-1}. Several 40-μl aliquots of this were evaporated to dryness in Reacti-vials (Pierce and Warriner, Chester, Great Britain) with a stream of warm nitrogen. Purified pyridine (65 μl) and TMSI (35 μl) were added to each, the vials sealed with screw caps and heated at 120 °C for various periods, from 30 min to 6 h. Each tube in turn was cooled and 10 μl of solution withdrawn and diluted with ECD toluene to give 1–2 ng μl^{-1} and 1 μl of this solution was injected onto the GC. The course of reaction was monitored to find the time required for complete conversion to a single derivative. The derivative, once formed, was stable for several weeks in excess TMSI if the Reacti-vial was kept closed in a refrigerator.

Preparation of biological sample

The biological material to be examined (10–300 g as necessary) was ground in methanol (5 ml g^{-1}) with a high shear stainless-steel grinder (Unishear Mixers, Audnam, Stourbridge, Great Britain) and filtered through sintered glass. The residue was blended twice more with smaller volumes of methanol and filtered. The insoluble residue was discarded.

For smaller samples, such as insect eggs, the sample (0.5–2.0 g) was ground in a glazed mortar with methanol-washed sand and methanol (200 ml). The slurry was filtered as above and the residue extracted twice more with methanol and filtered.

The methanol extracts were reduced to dryness on a rotary evaporator with vacuum at 50 °C. The resulting residue was partitioned between light petroleum (b.p. 40–60 °C) and aqueous methanol (1:4). The light petroleum was extracted twice more with aqueous methanol before being discarded.

The aqueous methanol extracts were reduced to dryness at 50 °C in the same way, the residue partitioned between butanol and water, and the butanol phase was washed twice with water. The combined aqueous phases were washed twice with butanol. The aqueous portion contained any polar conjugates of ecdysteroids, and was evaporated to dryness at 50 °C if these were to be hydrolysed and the ecdysteroids examined, otherwise it was discarded.

The material obtained after evaporation of the butanol was submitted to a third partition system which depended upon the sample material, either equal volumes of ethyl acetate and water, discarding the ethyl acetate, or hexane–2-propanol–water (5:15:36) discarding the hexane. In each case the less polar phase was washed twice with the aqueous phase before being discarded.

The aqueous portion was evaporated to dryness under vacuum at 50 °C. Evaporation can be hastened by addition of 1-butanol and removing a butanol–water azeotrope. The residue was transferred to a centrifuge tube with redistilled methanol (15 ml) and the volume reduced to 2 ml with a stream of warm nitrogen. This volume was transferred in 250-μl portions to a 1-ml Reacti-vial, and evaporated to dryness with warm nitrogen between additions. The residue was dried *in vacuo* at 57 °C for 1 h. The residue, which should amount to 100 mg or less and preferably spread as a

thin film on the walls of the tube, so that drying was efficient, was silylated as described earlier in pyridine (200 μl) and TMSI (100 μl). The time of heating required was about 80% of that required for the pure ecdysteroid and was found by trial and error using a biological sample to which pure ecdysteroid had been added.

Thin-layer chromatography of silyl ethers

The pyridine solutions after silylation of ecdysteroids was reduced in volume while still warm by blowing a jet of nitrogen onto the surface, and the remainder applied as a band to the origin of a TLC plate (20 × 20 cm) prepared as described above, the Reacti-vial was rinsed with toluene, which was also applied to the origin. The plate was immediately developed in ethyl acetate–toluene (3:7) until the solvent front had travelled 15 cm. The plate was removed, dried quickly with a hair dryer and the silica from R_F 0.5 to 0.9 removed and packed into a glass column (15 × 1.0 cm), the lower end of which held a glass wool plug. The silica was eluted with diethyl ether (15 ml). The ether was evaporated, taking care that water did not condense inside the tube. The residue was taken up in a known volume of purified toluene and diluted suitably for GC–ECD.

Gas chromatography columns

Columns used were 1.5 m × 4 mm coiled glass columns packed with 1.5% (w/w) OV-101 silicone phase on Chromosorb Q (100–120 mesh). The material was handled very carefully during coating and packing the column to prevent breaking of the particles and exposure of uncoated surfaces. The column was conditioned at 340 °C for 24 h before use. In repeated use, retention times slowly decreased, and resolutions deteriorated. Column life could be prolonged by replacing the first few centimetres of packing from time to time (approximately every 300 injections) and injecting 10-μl samples of "Silyl-8" (Pierce and Warrener) onto the column at 250 °C ensuring that the detector was disconnected.

Injections were made directly "on column" with an 11-cm needle which reached into the top of the column packing. Injection into a heated injector block with a shorter needle is *not* recommended.

A Pye Series 104 gas chromatograph fitted with flame ionization (FID) and ^{63}Ni ECD detectors was used. Nitrogen, freed of traces of oxygen with an "Oxy-trap" and dried by passing over molecular sieves, was used as carrier gas, flow-rate 50–60 ml min^{-1}, oven temperature 270–280 °C, detector temperature 300 °C. When the carrier gas was switched off, a purge of 15 ml min^{-1} of nitrogen was maintained through the detector.

DISCUSSION

Ecdysteroids have the disadvantages of sensitivity to acid[7] (dehydration at the 14α-OH group), to alkali[7] (the unsaturated ketone) and to heat (non-specific dehydration) and their high polarity conferred by several hydroxyl groups makes them susceptible to irreversible adsorption on activated surfaces such as alumina[8] and silica[9]. They have the advantage of possessing a strongly absorbing ultraviolet chromophore in the unsaturated ketone ($\lambda_{max.}$ 240 nm, $\varepsilon \approx 12{,}000$ l mol^{-1} cm^{-1}). This absorption is

useful for UV detection after HPLC, but a very large number of compounds absorb in the same region.

Where a relatively "clean" material is to be examined, *e.g.*, phytoecdysteroids[10], HPLC is sufficient, but many arthropod tissues require extended "clean-up" before ecdysteroids are freed of co-eluting substances.

Ecdysteroids also possess the advantage of a strongly electron-capturing electrophore[11], which is possessed by a relatively small number of compounds. The method for determining ecdysteroids described here takes advantage of the selective electrophore and attempts to avoid exposure to acids, alkalis, heat and active adsorbents used in chromatography.

The basis of the method is to use several solvent partitions and to resort to chromatography only after conversion to a non-polar derivative. In this way a partially purified concentrate of derivatives of ecdysteroids is prepared and this is clean enough for their selective detection by an electron capture detector.

Determination of any substances in the nanogram range requires care in avoiding contamination and ensuring reproducibility. For the present work it is important to avoid contact with chlorinated solvents since organohalogen compounds are strongly electron capturing. The toluene used for GC and the silica gel for TLC need particular care and must be specially purified. Apparatus must be clean, and traces of acid introduced from chromic acid cleaning must be rigorously removed. In particular, the Reacti-vials used for the silylation reaction must be very clean and free of acid or alkali. The build-up of a silicious deposit on glassware from use of silylating reagents has, in some unknown way, a deleterious effect upon the compounds. Traces of sodium acetate have a catalytic effect upon the rate of the silylation reaction, as do imidazole and other unidentified substances in the biological sample. Since sodium acetate–acetic acid is a common buffer system, acetate can be introduced accidently into ecdysteroid samples where enzymic hydrolysis has been used. This was discovered in hydrolysing ecdysteroid conjugates with the mixture of enzymes from the snail *Helix pomatia*. When an acetate buffer was used for the enzymic step, abnormal results were found in the GC step, and the effect could be reproduced by adding sodium acetate to the silylation mixture. When phosphate buffer was used, the silylated ecdysteroids behaved normally.

The ECD is not as simple to operate as the FID. The price of its greater sensitivity is that it is more easily contaminated. Some instructions in the handling of the detector are therefore included. Detector design differs with the manufacturer and some experimentation must be made using pure ecdysteroids to find the optimum operating conditions, of current, pulse spacing, sensitivity and so on. Because of the nature of the electron-capturing process, the highest practical operating temperature of the detector gives greatest sensitivity[11].

The reactivity of TMSI has been found to vary widely and in an unpredictable way. Some material purchased by us was quite unusable because even prolonged heating in pyridine did not produce satisfactory silylation of ecdysteroids and reactivity seemed to vary widely from batch to batch. We have therefore prepared our own TMSI as described. This material is consistent in its reactivity and separate 1-ml vials sealed for a long time, can be opened and transferred to a Reacti-vial sealed with a rubber serum cap and portions drawn from it to give reproducible reaction times. If the reaction is carried out for 10 min at room temperature, ecdysone tetrakis trimeth-

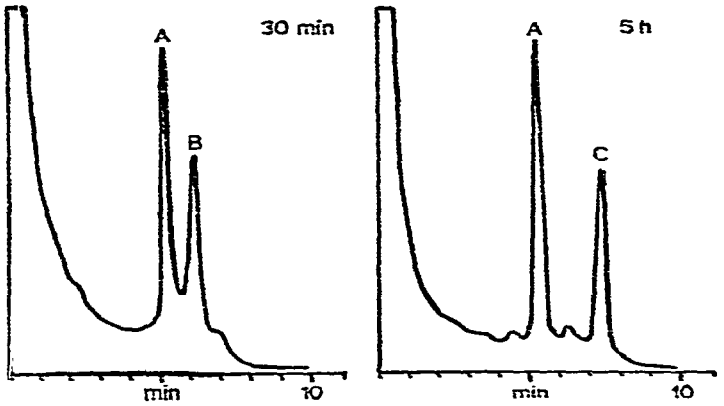

Fig. 1. Examples of gas chromatography traces of the derivatives of ecdysteroids after silylation for 30 min and 5 h. Peaks: A = tetrakis TMS ether of ecdysone; B = tetrakis TMS ether of 20-hydroxyecdysone; C = pentakis TMS ether of 20-hydroxyecdysone. Chromatographic conditions as in text.

ylsilyl ether and 20-hydroxyecdysone tetrakis trimethylsilyl ether are formed. Even when left overnight at room temperature, no further silylation occurs. If the mixture is heated to 110 °C for 5 h, then ecdysone still remains as the tetrakis ether (longer reaction begins to produce the pentakis ether) and 20-hydroxyecdysone is converted to the pentakistrimethylsilyl ether (Fig. 1). The very short reaction time has the advantage of speed of analysis. The longer reaction time with heating, has the advantage that the derivatives formed from ecdysone and 20-hydroxyecdysone are better resolved in GC (Table I).

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES OF TMS ETHERS OF ECDYSTEROIDS ON A 1.5% OV-101 COLUMN OF 1.5 m LENGTH WITH NITROGEN CARRIER GAS AT 60 ml min^{-1}

Parent ecdysteroid	*No. of TMS groups*	*Retention time (min)* **	*Column temperature (°C)*
Ecdysone	4	1.85	280
	5	1.65	280
20-Hydroxyecdysone	4	2.25	280
	5	2.45	280
	6	1.90	280
Inokosterone*	4	2.55	280
	5	2.85	280
	6	2.20	280
2-Deoxy-20-hydroxyecdysone	4	3.9	280
	5	2.4	280
Poststerone	2	0.6 (1.3)	280 (260)
	3	0.9	260
Cyasterone		8.0	280
3-Dehydroecdysone	3	2.15	280
3-Dehydro-20-hydroxyecdysone	4	2.70	280

* Commercial inokosterone apparently consists of two C-25 epimers[15] which are not resolved under these conditions.

** Not the same column as used for the figures.

The user must decide which method, cold reaction or with heating, is preferable. If the reaction is heated, to produce the penta-ether of 20-hydroxyecdysone, then because of the variability of the TMSI reagent, the time necessary for reaction of either ecdysone or 20-hydroxyedysone is first found, using pure materials. Several 10-μg samples of compound are silylated for varying periods at any convenient temperature within the range 100–140 °C and the extent of reaction monitored by GC (Fig. 2). Using one sample and withdrawing aliquots at time intervals is not satisfactory because of the unavoidable exposure to moisture at each opening of the Reacti-vial. Once the reaction time has been decided, practice shows that a slightly shorter time is required for ecdysteroids in a biological sample.

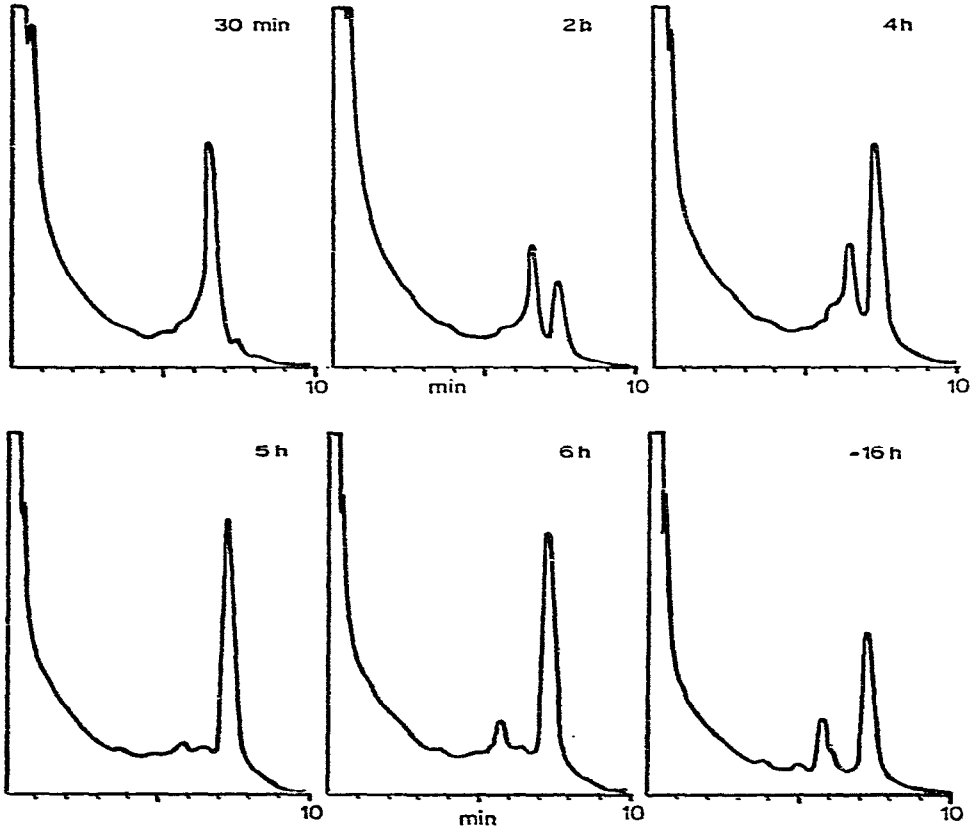

Fig. 2. Gas chromatographic trace of silylation products of 20-hydroxyecdysone after varying periods of time showing the formation of a single derivative (the tetrakis ether) after 30 min and again at 5 h (the pentakis ether). The chromatographic and silylation conditions were as described in the text.

Tailing and non-Gaussian peak shape in GC can be attributed to adsorption on the column walls or uncoated support. It can be corrected by treatment with "Silyl-8" (Fig. 3).

It is advisable to add a known quantity of pure ecdysteroid to a biological sample known to contain little or no ecdysteroid, to give a concentration within the

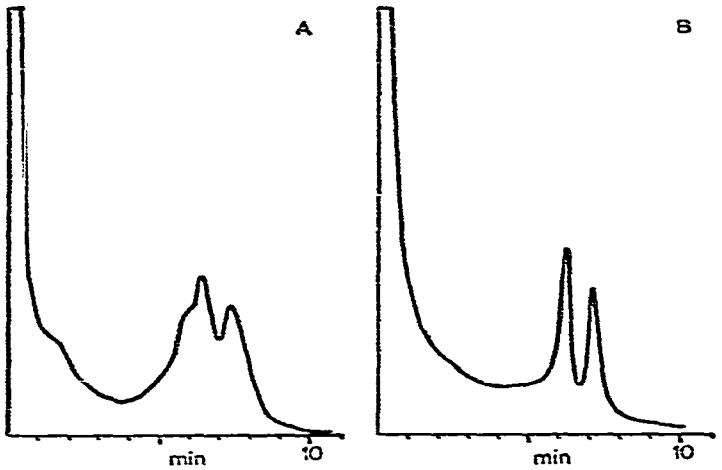

Fig. 3. Effect of condition of the column on peak shape. A sample of 20-hydroxyecdysone was silylated for 2 h to give a mixture of two silyl ethers. This mixture was injected onto the chromatography column at 285 °C, with the carrier gas flowing at 60 m min^{-1}. On a column which has been in use for some time, the result was as in A. The column was then treated with 10 μl of "Silyl-8" (with the detector disconnected) and the sample reinjected, after conditions had returned to equilibrium, to give the result in B.

range of experimental values and to carry out the extraction and derivatization procedures to test the efficiency of recovery. As the limit of detection is approached, manipulation losses increase and a correction factor may have to be found from these results and applied to experimental data. An internal standard provides a useful check on recovery and reproducibility. A substance such as cyasterone or makisterone A can be used. Cyasterone has the disadvantage of having an inconveniently long retention time and consequently a broad peak shape. Makisterone A, which is commercially available has the advantage of retention time closer to ecdysone and 20-hydroxyedcysone, but could conceivably co-elute with a natural ecdysteroid and obscure it.

Using whole adult male *Schistocerca gregaria* as ecdysteroid-free samples, we obtained recoveries for the complete isolation procedure of 95% for 10^{-4} g of added hormone, falling to 85% recovery for 10^{-8} g added hormone.

For the TLC step alone, recoveries were quantitative within the range of 10^{-4} to 10^{-8} g, but recovery fell to $\approx$ 60% when smaller quantities of silylated ecdysteroid were spread on the plate.

Removal of water and methanol from the biological sample in the Reacti-vial before silylation is also important. For large samples ($\approx$ 300 mg) a thick gum may form at the bottom of the tube. This may dry on the surface but retain solvent underneath. A smaller sample should be used if possible or else the material should be spread as a thin film on the walls of the tube before the solvent evaporates. The sample should redissolve completely in the pyridine, if necessary by warming and shaking before silylation.

If a greater proportion of TMSI is used for silylation, to overcome losses of reagent from moisture, then it is important that imidazole should not crystallize from the reaction on cooling. The crystalline imidazole can occlude the silylated ecdysteroids

and lead to completely negative results. If 100 ng of silylated ecdysteroid is co-precipitated with 1 mg of crystalline imidazole, this represents only a 0.01 % contamination of the imidazole.

Once prepared, the trimethylsilyl ethers of ecdysteroids are relatively stable, in the pyridine–TMSI mixture, for several months, and can be heated to 100 °C for several days without decomposition. Trimethylsilyl ethers are subject to hydrolysis, particularly when catalysed by acids. They are therefore of limited stability on TLC plates, and it is advisable to carry out TLC operations as quickly as possible and elute from the silica immediately after chromatography. In a few cases we have found silica gel for TLC caused hydrolysis of the ethers (Fig. 4) but the problem was solved as soon as a new batch of silica was purchased. The R_F values of some ecdysteroid silyl ethers are given in Table II.

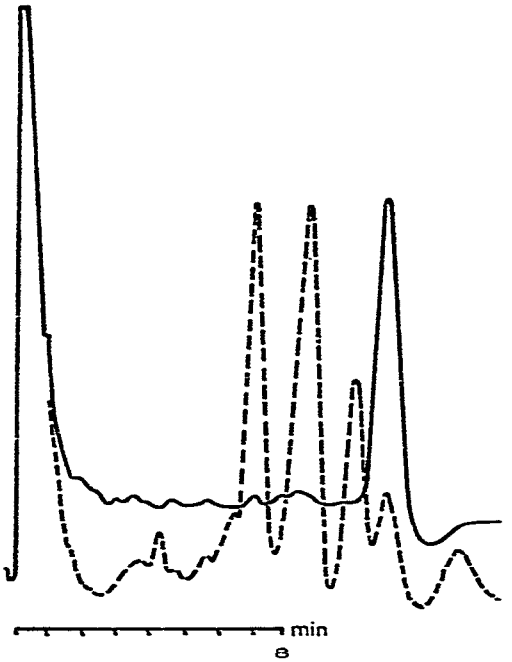

Fig. 4. Effect of thin-layer chromatography on unsatisfactory silica gel. Full line, 20-hydroxyecdysone pentakis silyl ether before thin-layer chromatography. Broken line, same product after TLC. This sample was chromatographed on a different column (longer retention times) than that used for the other figures.

Preparation of biological material

The extent of the preliminary clean-up of a biological sample before formation of the trimethylsilyl ethers and GC must be found by trial and error. In our experience, three solvent partitions is quite sufficient for samples of whole locust bodies, locust haemolymph could be determined after only two solvent partitions, but locust faeces contained much more interfering substances and samples of barnacles (*Balanus balanoides*) are still too impure after three solvent partitions for satisfactory GC. The criterion of purification is whether the large solvent peak elutes before the ecdysteroids,

TABLE II

R_F VALUES OF SOME ECDYSTEROID TMS ETHERS ON SILICA GEL (FROM REF. 1)

Parent compound	*Hydroxyl groups silylated*	*Solvent system*	
		Toluene–ethyl acetate (9:1)	*Toluene–ethyl acetate (7:3)*
2β,3β,14α-Trihydroxy--5β-cholest-7-en-6-one	2β,3β	0.39	—
	2β,3β,14α	0.51	—
Ecdysone	2β,3β,22,25	—	0.69
	2β,3β,14α,22,25	0.58	—
20-Hydroxyecdysone	2β,3β,22,25	—	0.54
	2β,3β,20,22,25	0.22	0.67
	2β,3β,14α,20,22,25	0.69	0.75

so that maximum detector sensitivity is available or whether the ecdysteroid peaks are superimposed on a falling baseline (Fig. 5).

Partition between hexane or light petroleum (b.p. 40–60 °C) and methanol–water (4:1) is best carried out first. This removes a large quantity of non-polar lipids and avoids emulsion formation as far as is possible. Systems such as hexane–water cause formation of very stable emulsions.

The second solvent system is butanol–water, which removes unwanted polar compounds in the aqueous phase, which also contains many polar ecdysteroid conjugates[5,12].

Fig. 5. Comparison of satisfactory purification (full line) and insufficient purification (broken line) for satisfactory quantification. The material was a sample of extract of the barnacle *Balanus balanoides* to which pure ecdysone had been added.

The third solvent partition, if necessary to reduce the volume of the sample or to further purify it, is found by trial and error. Ethyl acetate–water (1:1) or hexane–isopropanol–water (5:15:36) are two useful alternatives. The common ecdysteroids partition into the aqueous phase of both systems.

If further purification is necessary, chromatography on Sephadex, a non-polar reversd-phase material such as Bondapak C_{18} or a weak absorbent such as Floridin earth is helpful, but not alumina or silica.

The most frequently encountered ecdysteroids are easily resolved by the GC conditions. We have experienced no difficulty in distinguishing them by their retention times, some of which are listed in Table I. New ecdysteroids make rather greater demands on techniques and quantities of material for identification[13].

ACKNOWLEDGEMENTS

We wish to thank Overseas Development Administration for support of our work on locusts and the Science Research Council for equipment and studentship support, and the technical staff of the department for their skilfull help and care of equipment.

REFERENCES

1 E. D. Morgan and C. F. Poole, *Advan. Insect Physiol.*, 12 (1976) 17.
2 R. Lafont, G. Somme-Martin, B. Mauchamp, B. F. Maume and J. P. Delbecque, in J. A. Hoffman (Editor), *Progress in Ecdysone Research*, Elsevier, Amsterdam, 1980, p. 45.
3 C. F. Poole, E. D. Morgan and P. M. Bebbington, *J. Chromatogr.*, 104 (1975) 172.
4 E. D. Morgan and C. F. Poole, *J. Insect Physiol.*, 22 (1976) 885.
5 I. D. Wilson and E. D. Morgan, *J. Insect Physiol.*, 24 (1976) 751.
6 D. W. Borst and J. D. O'Connor, *Steroids*, 24 (1974) 637.
7 P. Karlson, H. Hoffmeister, W. Hoppe and R. Huber, *Justus Liebigs Ann. Chem.*, 662 (1963) 1.
8 H. Miyazaki, M. Ishibashi, C. Mori and N. Ikekawa, *Anal. Chem.*, 45 (1973) 1164.
9 M. W. Gilgan and T. E. Farquharson, *Steroids*, 22 (1973) 365.
10 S. Ogawa, A. Yoshida and R. Kato, *Chem. Pharm. Bull.*, 25 (1977) 904.
11 C. F. Poole and E. D. Morgan, *J. Chromatogr.*, 115 (1975) 587.
12 A. R. Gande and E. D. Morgan, *J. Insect Physiol.*, 25 (1979) 289.
13 E. D. Morgan and I. D. Wilson, in J. A. Hoffman (Editor), *Progress in Ecdysone Research*, Elsevier, Amsterdam, 1980, p. 29.