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A GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF THE RATIO OF GITOGENIN AND DIGITOGENIN IN MIXTURES

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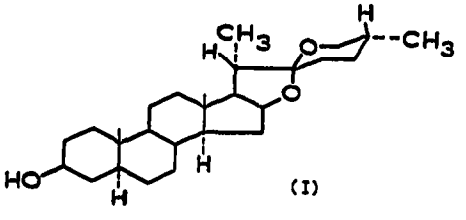
SUMMARY

A quantitative gas–liquid chromatographic method, applicable to microgram amounts, for the determination of the ratio of gitogenin and digitogenin in mixtures is described. The method involves making known mixtures of each sapogenin with tigogenin, preparing the trimethylsilyl ethers and measuring the peak area ratios of these ethers after separation by gas–liquid chromatography. Optimum conditions for the preparation of the ethers is described. The method has been applied to the determination of gitogenin and digitogenin in extracts of *Digitalis purpurea* seeds, it has also been used to determine the purity of commercial digitonin.

INTRODUCTION

The separation of the polyhydroxy sapogenins, gitogenin (II) and digitogenin (III), by chromatographic means has been achieved only with difficulty and the separation factors are low[1–4]. Thin-layer chromatographic (TLC) methods give good separation only after acetate formation[5]. Gas–liquid chromatography (GLC) of several groups of sapogenins has been attempted[6]. This paper describes a quantitative method,



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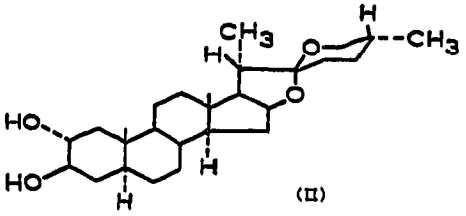

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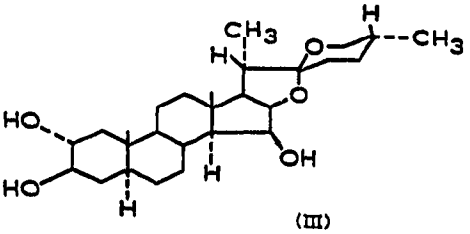

(III)

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applicable to microgram amounts, for the separation of gitogenin and digitogenin. The method is based on the determination of the peak area ratios of the trimethylsilyl (TMS) ethers of these compounds following GLC separation of mixtures with tigogenin (I). Optimum conditions for the preparation of the ethers are described. The method has been applied to the determination of gitogenin and digitogenin in extracts of the seeds of *Digitalis purpurea* L., and the purity of commercial digitonin has also been determined by the method.

EXPERIMENTAL

Reagents

Trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were obtained from Aldrich Chemical Co.; pyridine and tetrahydrofuran from B.D.H. Ltd., these last two were redistilled and stored over sodium wire prior to use. The Silica Gel G was obtained from E.Merck.

Apparatus

A Marryat gas chromatograph was used with a flame ionisation detector and modified in this laboratory to take glass columns with on column injection.

Columns

Four glass columns were used during the development of this method. The packing materials were prepared by the method of HORNING *et al.*[7]. Column 1, 6 ft. long by 1/8 in. diameter, was packed with Chromosorb W (100–120 mesh) AW-DMCS treated obtained from Johns Manville Products, and coated with 1.5 % QF-1 silicone oil from Loenco Inc. Column 2 was of the same size and packed with the same support but coated with 1 % neopentyl glycol succinate from Perkin-Elmer. Column 3 was also similar but coated with 1.2 % SE-30 silicone gum rubber from the General Electric Co. The fourth column was 9 ft. long but otherwise similar to the third column.

Reference sapogenins

Tigogenin was obtained from Koch-Light Ltd., digitonin from Halewood Chemicals Ltd.; the gitogenin and digitogenin were gifts kindly sent by Prof. Reichstein of Basle University. The purity of these compounds was checked by TLC using air-dried Silica Gel G in 250 μ layers. The plates were developed with chloroform–acetone (9:1) for a distance of 10 cm, they were then sprayed with 10 % antimony trichloride in benzene and heated for 10 min at 120°. The digitogenin proved to be pure with an R_F value of 0.44. The tigogenin and gitogenin, with R_F values of 0.85 and 0.45, respectively, both contained small amounts of impurities which showed as faint spots with R_F values of 0.70 (tigogenin) and 0.22 (gitogenin). These compounds were purified by preparative layer chromatography (PLC) on Silica Gel G layers of 500 μ thickness and using the same solvent system as before. The zones corresponding to the tigogenin and gitogenin were removed and the sapogenins eluted with 250 ml of chloroform–methanol (8:2). The solvent was removed by distillation under reduced pressure.

The purity of the digitonin was checked by the following method. A 500 mg sample was refluxed with 40 ml of hydrochloric acid–water–methanol (1:1:1) for 2 h.

After cooling 20 ml of 1 % ammonium sulphate were added and the mixture extracted with 60 ml of chloroform–methanol (8:2) in 10 ml portions. The combined extracts were dried over anhydrous sodium sulphate and then evaporated to dryness by distillation under reduced pressure. The residue was taken up in the minimum quantity of chloroform–methanol (8:2) and a small sample chromatographed on Silica Gel G using dichloromethane–methanol (97:3) as developer. After spraying and heating, as previously described, two spots appeared, the main one corresponding to digitogenin and the other to gitogenin. The remainder of the residue in chloroform–methanol was purified by PLC as described for the tigogenin and gitogenin.

Isolation of the sapogenins from the seeds of D. purpurea

About 10 g of seeds were comminuted in an electric mill and then homogenised with 50 ml of 50 % methanol in a Waring blendor for 10 min. The mixture was filtered and the residue extracted for 2 h in a Soxhlet with 60 ml of methanol. The filtrate and the extract were combined and diluted to 200 ml with 0.5 % ammonium sulphate solution. The lipids were removed from this by extraction with 200 ml of petroleum ether–diethyl ether (3:2), and the saponins were extracted with 200 ml of chloroform–methanol (3:2). This saponin extract was dried over anhydrous sodium sulphate and the solvent removed by distillation under reduced pressure. The residue was hydrolysed and purified by PLC as described for the digitonin, the sapogenins thus obtained were then ready for analysis by GLC.

Silylation methods

About 1.0 to 1.5 mg of the reference compounds and of the mixtures were dissolved in 0.5 ml of one of the silylation mixtures (Table I) in a stoppered bottle. The reaction was allowed to proceed for 2 h at 60° and from 2 to 8 μl were used for the GLC analysis.

TABLE I

SILYLATION REACTION MIXTURES

Mixture	*Composition of mixture*			
	Tetrahydrofuran (ml)	*HMDS (ml)*	*Pyridine (ml)*	*TMCS*
1	0.80	0.40	0.20	20 μl
2	0.80	0.40	0.40	20 μl
3	0.80	0.40	0.80	20 μl
4	0.50	0.40	1.00	0.10 ml
5	1.00	0.40	nil	0.10 ml
6	1.00	0.40	0.05	0.10 ml

The GLC analysis

The retention data for the purified reference compounds and for their TMS ethers, with respect to the four columns, are given in Tables II and III. Column 4 was found to be the best for quantitative work as shown by Fig. 1. The response ratios, using column 4, of gitogenin and digitogenin with respect to tigogenin, all as their TMS ethers, are given in Tables IV and V.

TABLE II

RETENTION DATA ON GLC COLUMN 4

Oven temperature, 260°; nitrogen gas flow, 35 ml/min.

Substance	*Retention time (min)*	*Relative retention time, tigogenin TMS ether = 1*
Tigogenin	19.0	0.76
Tigogenin TMS ether	25.0	1.00
Gitogenin	34.0	1.36
Gitogenin TMS ether	38.5	1.54
Digitogenin	48.0	1.92
Digitogenin TMS ether	46.5	1.86
	54.0	2.16
	61.0	2.44
	64.0	2.56

TABLE III

RETENTION DATA ON COLUMNS 1 AND 2

Column 1: oven temperature, 220°; nitrogen gas flow, 65 ml/min. Column 2: oven temperature, 240°; nitrogen gas flow, 60 ml/min.

Substance	*Retention time (min)*		*Relative retention time, tigogenin TMS ether = 1*	
	Column 1	*Column 2*	*Column 1*	*Column 2*
Tigogenin TMS ether	3.0	8.5	1.0	1.0
Gitogenin TMS ether	6.3	13.26	2.1	1.56
Digitogenin TMS ether	7.02	15.10	2.34	1.78

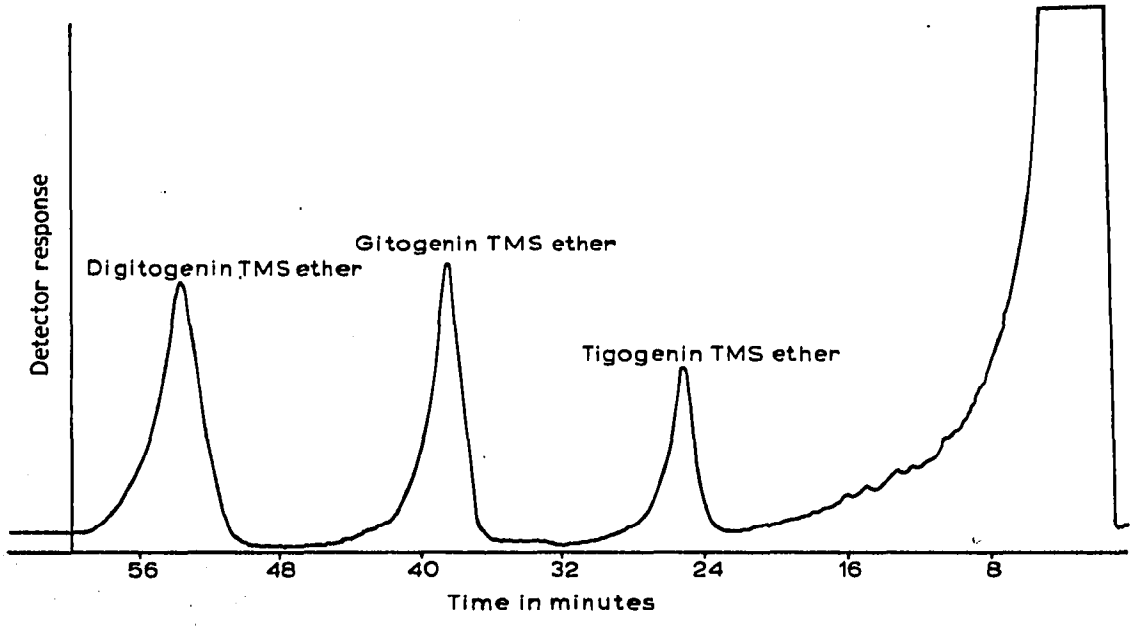

Fig. 1. Gas–liquid chromatogram showing separation of the trimethylsilyl ethers of tigogenin, gitogenin and digitogenin on column 4 at 260° and a nitrogen gas flow of 35 ml/min. Silylation using mixture 6.

TABLE IV

RATIOS OF GITOGENIN TO TIGOGENIN

Weight ratio gitogenin–tigogenin	*Mean area ratio gitogenin TMS ether–tigogenin TMS ether*	*Area ratio/weight ratio*
0.5313	0.5700	1.07284
0.5046	0.5204	1.03139
Mean area ratio/weight ratio	1.052	
N (overall number of determinations)	16	
% Standard error	4.61	

TABLE V

RATIOS OF DIGITOGENIN TO TIGOGENIN

Weight ratio digitogenin–tigogenin	*Mean area ratio digitogenin TMS ether–tigogenin TMS ether*	*Area ratio/weight ratio*
1.4100	1.6091	1.1410
1.6550	1.8157	1.0970
0.3780	0.4120	1.1030
0.5533	0.6319	1.1421
Mean area ratio/weight ratio	1.121	
N (overall number of determinations)	34	
% Standard error	4.74	

RESULTS AND DISCUSSION

Silylation studies

The effect of pyridine concentration and of the time of heating on the formation of the sapogenin TMS ethers at two concentration levels of TMCS have been investigated using GLC column 4. Table I gives the composition of the silylating mixtures used.

It was found that tigogenin and gitogenin, in all the mixtures except number 5, gave only single peaks with retention times of 25.0 and 38.5 min, respectively. When there was no pyridine present, as in mixture 5, neither tigogenin nor gitogenin were fully silylated since they each gave an additional peak with retention times of 19.0 and 34.0 min, respectively, corresponding to the unreacted parent sapogenin. If the time of heating was shortened as found with mixture 6 to less than 1 h, the same two peaks for each compound were produced. From these results it is concluded that full silylation of tigogenin and gitogenin can be facilitated by the presence of a trace of pyridine and with a reaction time of 2 h. It appears that the hydroxy groups are readily available for silylation.

In contrast to this the composition of the silylating mixture and the time of reaction appear to be more critical for the silylation of digitogenin. By increasing the concentration of pyridine, as in mixtures 1 to 3, the number of peaks produced by digitogenin diminished from four, with retention times of 46.5, 54.0, 61.0 and

64.0 min, to two, with retention times of 46.5 and 54.0 min. The relative sizes of the peaks also changed, the peaks at 61.0 and 64.0 min were smaller with mixture 2 than with mixture 1, whilst with mixture 3 the peak at 46.5 min increased in size and the one at 54.0 min decreased. On increasing the amount of TMCS in the silylating fluid, as in mixture 4, only two peaks were obtained, with retention times of 46.5 and 54.0 min. When pyridine was absent, as in mixture 5, peaks at 48.0 and 54.0 min were obtained.

The time of heating also affected the number and the relative sizes of the peaks. If digitogenin was heated for only 10 min in mixture 4 then only the peak at 54.0 min was obtained, however if the time is extended to 45 min an extra peak at 46.5 min appeared and if the time is further extended to several hours only the peak at 46.5 min was present. Prolonged heating in mixture 3 also gave only one peak at 46.5 min, but prolonged heating in mixture 5, pyridine absent, yielded a single peak at 54.0 min.

From a comparison of the relative retention times of the TMS ethers it is suggested that the peak at 54.0 min corresponds to the fully silylated digitogenin, *i.e.* all three hydroxy groups are silylated. It is also suggested that the peak at 46.5 min is the disilyl ether with the 2- and 3-hydroxy groups silylated. These suggestions are supported by comparing the relative retention times of monohydroxy-tigogenin, dihydroxy-gitogenin and trihydroxy-digitogenin. Too high a concentration of pyridine leads to the production of decomposition products[8], as shown by the peaks at 61.0 and 64.0 min. An examination of the stereochemistry of digitogenin shows that the hydroxy groups on carbons 2 and 3 may be silylated comparatively easily compared with the more sterically hindered hydroxy group on carbon 15. This may explain why conditions for the silylation of digitogenin are more critical than for tigogenin and gitogenin.

Response ratios of gitogenin TMS ether and of digitogenin TMS ether to tigogenin TMS ether

The TMS ethers of tigogenin and digitogenin were prepared using mixture 6. Varying amounts of these ethers were injected and the peak areas measured by the product of the height and of the width at half height. The linear dose response of these compounds is shown in Fig. 2. Weights in excess of 10 μg per peak resulted in some tailing with loss of accuracy in measuring.

Mixtures of gitogenin with tigogenin and of digitogenin with tigogenin were prepared and silylated using mixture 6. These reacted mixtures were stable during one week and from 1 to 5 μl were used for the GLC analysis. At least three analyses were performed on each mixture and the peaks areas calculated as before, the results are shown in Tables IV and V. The linearity of the peak area ratio to the weight ratio, in the case of the digitogenin–tigogenin mixtures, is shown in Fig. 3. The peak area ratio/weight ratio for the gitogenin–tigogenin mixtures is 1.052 and for the digitogenin–tigogenin mixtures it is 1.121. The increase in these ratios, namely 0.052 and 0.069, respectively, each represents an increase in molecular weight of a trimethylsilyl group. From the similarity of these two increases we conclude that they are true response ratios in the flame detector.

The method described in this paper enables the ratio of gitogenin and digitogenin to be determined in mixtures. The percentage standard error of the method is about 4 % and it is applicable to microgram quantities. We used the method to analyse

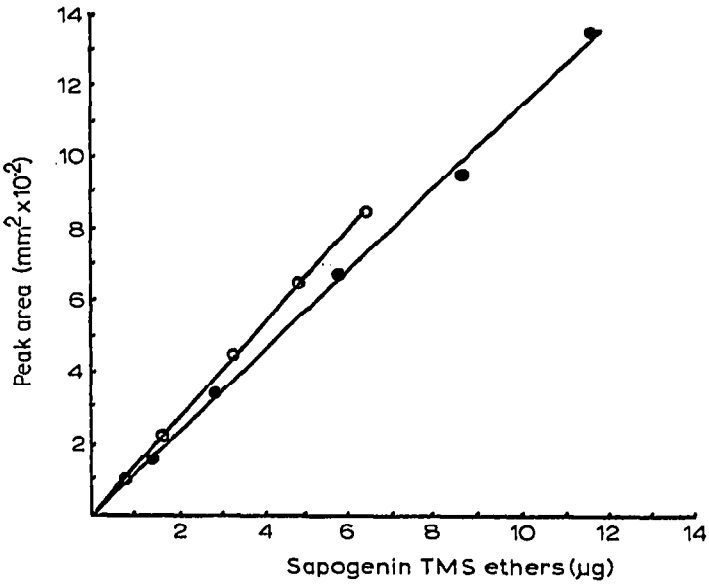

Fig. 2. Response ratio of sapogenin TMS ethers in the flame ionisation detector. ○, Digitogenin TMS ether; ●, tigogenin TMS ether.

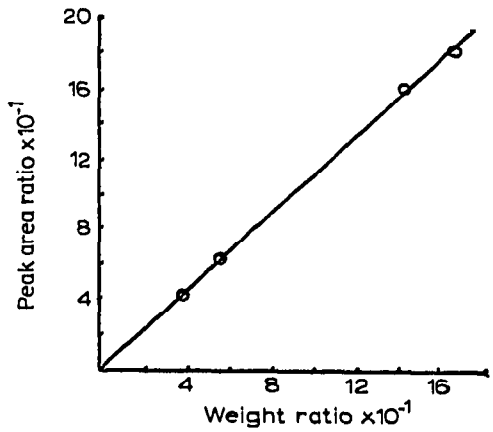

Fig. 3. Response ratio of digitogenin TMS ether to tigogenin TMS ether.

a commercial sample of digitonin and found that it contained 11.1% of gitogenin, showing that commercial digitonin may not be used as a pure source of digitogenin. The method was also used to analyse the ratio of sapogenins extracted from the seeds of *D. purpurea*, this was found to be 74.0% of digitogenin to 26.0% of gitogenin. We did not find tigogenin in the extract although its presence has been reported[9].

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