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ION-EXCHANGE CHROMATOGRAPHY OF SOYBEAN SAPONINS*

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SUMMARY

Resolution of soybean saponins on Dowex-1 is influenced by resin particle size, flow rate, temperature and eluting agent. Separations were good when columns of −400 mesh resin were operated at 50° and eluted at slow flow rates with either acetic or propionic acid. A linear gradient of 1.5 to 3 *N* propionic acid separated the saponins into twelve fractions, many of which consisted of two or more components when analyzed by thin-layer chromatography. Glucuronic acid, glucose, galactose, rhamnose, xylose and arabinose were found in acid hydrolyzates of all fractions, but relative amounts of the neutral sugars varied in most fractions. On analysis of the aglycones, three column fractions contained only soyasapogenol A, one contained only soyasapogenol E, while four consisted of soyasapogenol B plus small amounts of soyasapogenols C and D. Another fraction yielded a previously unreported aglycone, which appears related to soyasapogenol D.

INTRODUCTION

Recent studies indicate that soybeans contain about 0.5% saponins[1]. These surface active compounds are hemolytic when tested *in vitro*[2] but are innocuous when ingested by mice, rats and chicks[3]. On hydrolysis soybean saponins yield five sapogenins—soyasapogenols A, B, C, D and E—and six sugars—glucose; galactose, rhamnose, xylose, arabinose and glucuronic acid[4]. The saponin mixture is very complex and difficult to fractionate. After attempting to separate the saponins by alumina column and paper chromatography, GESTETNER *et al.*[4] concluded that differences in properties between individual saponins were too small to resolve them by available chromatographic techniques.

We recently found that multiple-development thin-layer chromatography (TLC) resolves the saponin mixture into ten or more fractions[5]. We also described preliminary results obtained by chromatography of the saponins on Dowex-1***

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columns. Our studies show that the saponins can be separated into seven fractions but that most of the fractions are still mixtures. We have examined the ion-exchange behavior of soybean saponins in greater detail and now describe conditions that improve separations of this complex mixture.

EXPERIMENTAL

Preparation of soybean saponins

Saponins were prepared by the procedure of BIRK *et al.*[2] with minor modifications as described earlier[5].

Ion-exchange chromatography

High pressure-type chromatographic columns (0.9 × 70 cm) with water jackets for temperature control supplied by Phoenix Precision Instrument Co., Philadelphia, Pa., were packed with a resin bed of 60–65 cm of —400 mesh AG 1-X4 resin (analytical grade Dowex-1 from Bio-Rad Laboratories, Richmond, Calif.). The resin was converted into the appropriate form by treatment with a molar solution of the sodium salt of the acid used for elution. Saponins were dissolved with the aid of ethylenediaminetetraacetic acid (EDTA) as described earlier[5]. Parameters influencing ion-exchange separations were evaluated with column loads of 20 mg of saponins. For preparative separations, column loads were up to 150 mg. Linear elution gradients were generated with a Beckman Model 131 gradient pump (Beckman Instruments, Inc., Fullerton, Calif.) as previously reported[5]. The elution gradients were determined by titrating portions of the effluents with 0.1 *N* sodium hydroxide. Pumping rates were varied, but all experiments described here were conducted at 7.5 ml/h; 2-ml fractions were collected.

Formic, acetic, propionic, butyric and isobutyric acids used for elution were reagent grade. Saponins in the column effluents were estimated by determining sugar content as glucose with phenol–sulfuric acid[6]. Samples containing formic acid were treated with phenol–sulfuric acid in 250-ml erlenmeyer flasks in a hood because of vigorous foaming resulting from formation of carbon monoxide. After the elution diagrams were determined, saponins were recovered by pooling tubes for each fraction and then freeze-dried. The columns were re-used after washing with water until the effluents were free of eluting acid.

Polystyrene beads (Bio-Beads S-X4, 200–400 mesh) used to evaluate adsorption effects during ion-exchange chromatography came from Bio-Rad Laboratories, Richmond, Calif. The beads were slurried in water for packing the columns.

Diethylaminoethyl (DEAE)-Sephadex A-25 was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J.

Thin-layer chromatography of saponins

Saponins and saponin fractions were analyzed on precoated analytical silica gel plates (Brinkmann Instruments Inc., Westbury, N.Y.), developed six times with chloroform–methanol–water (65:25:4)[5]. The saponins were detected by spraying the plates with 50% sulfuric acid and then heating to 90–95°.

Hydrolysis of saponins

Samples of 2–4 mg of saponins were heated for 4 h at 100° in 5 ml of anhydrous 1.7 *N* methanolic hydrochloric acid in a sealed tube. After the tubes had been cooled and opened, the methanol was carefully evaporated on a steam bath. The dried residue was then extracted with 0.5-ml portions of benzene until the extracts were colorless. The benzene extracts contained the sapogenins. The residue remaining after extraction with benzene was dissolved in 2 ml of 1 *N* hydrochloric acid and heated on a steam bath for 1.5 h to convert the methyl glycosides to free sugars. The solutions were then neutralized with silver carbonate, treated with hydrogen sulfide to remove silver ion and either centrifuged or filtered. Next, the neutralized solution was passed through a 5 × 60 mm column of Bio-Rad AG 1-X4 200–400 mesh (acetate form) and washed with a few milliliters of water to yield the neutral sugars. Elution of the column with 5 ml of 8 *N* acetic acid followed by freeze-drying of the effluent yielded the acidic sugar fraction.

Chromatography and identification of sapogenins

Sapogenins were identified by TLC on silica gel plates developed with petroleum ether–chloroform–acetic acid[7]. Sapogenins were detected with 50% sulfuric acid plus heat or with antimony trichloride reagent[8]. Sapogenins were also analyzed by circular paper chromatography as outlined by GESTETNER[9]. Infrared spectra were determined in carbon tetrachloride with a Perkin-Elmer Model 621 infrared spectrophotometer.

Paper chromatography of sugars

Neutral sugars were separated on 589 Blue Ribbon-C paper (Schleicher and Schuell) with two descents of butanol–pyridine–water (6:4:3) while the acidic sugar fractions were chromatographed with ethyl acetate–acetic acid–water (3:1:3). The sugars were detected with an alkaline silver nitrate dip[10].

Gas–liquid chromatography of sugars

Neutral sugars were analyzed by gas–liquid chromatography (GLC) as alditol acetates as described by SLONEKER[11]. The saponins were hydrolyzed in two steps[12]. For the primary hydrolysis, 2–3 mg of saponins were weighed into a screw-capped (Teflon-lined) culture tube, carefully dissolved in 0.3 ml of 72% sulfuric acid and heated at 30° for 1 h. The acid was then diluted by adding 8.4 ml water and heated for 4.5 h at 100° for the secondary hydrolysis. After cooling the mixture, 0.50 ml of 2-deoxy-D-glucose solution (1 mg/ml) was added as an internal standard. The hydrolyzate was mixed thoroughly, neutralized with lead carbonate and centrifuged. The precipitate was washed once with water. Next, the neutralized hydrolyzate and washings were combined and extracted with diethyl ether to remove residual sapogenins. The ether-extracted solution was then made alkaline by adding 0.1 ml of 1 *M* sodium carbonate to saponify any glucuronolactone present and thus prevent its reduction to glucitol. After a 20-min saponification, 10 mg of sodium borohydride was added and 3 h later, the mixture was acidified by adding 1 *N* acetic acid to destroy excess sodium borohydride. Sodium ions were removed by passing the solution through a small column (~4 ml resin bed) of Dowex 50-X4 resin (H^+ form). The sample was then taken to dryness on a rotary evaporator and transferred to a 13 × 100 mm

screw-capped (teflon-lined) culture tube with methanol. After the methanol had been removed on a rotary evaporator, three 1-ml portions of methanol were added and the sample was taken to dryness after each addition to remove borate ions. After traces of methanol had been removed, 0.1 ml of pyridine–acetic anhydride (1:1) was added. The tubes were then tightly capped and heated at 100° for 3 h.

Equimolar mixtures of rhamnose, arabinose, xylose, glucose, galactose and potassium D-glucuronate were treated as described for the saponins to provide a standard mixture of the alditol acetates obtained from the saponins. Standard mixtures of sugars were reduced and acetylated without and with the sulfuric acid hydrolysis step; to the latter standard mixture, 2-deoxyglucose was added after the hydrolysis step.

The alditol acetates were analyzed with a Packard Model 7409 gas chromatograph equipped with a Model 804 oven. Samples were injected directly onto glass columns (6 ft. long and 1.5 mm I.D.) packed with 3% ECNSS-M coated on 100–120 mesh Gas-Chrom Q. The oven was operated isothermally at 185° with inlet and detector (hydrogen flame ionization) temperatures of 210°. Helium carrier gas flow was 25–28 ml/min. Peak areas were measured by making Xerox copies of the recorder charts, cutting out the peaks and weighing them on an analytical balance.

Data for GLC analyses were treated statistically by analysis of variance, and significance of differences between means was determined by DUNCAN's method[13].

RESULTS AND DISCUSSION

Effects of flow rate and mesh size

Preliminary studies with acetic acid as the eluting agent showed that slow flow rates are essential to resolution of soybean saponins on Dowex resin. Variation of flow rates from 60 to 7.5 ml/h revealed that resolution improved markedly as flow rate was decreased to 7.5 ml/h at 25°. At high flow rates elution peaks were broad and overlapped badly. Mesh size likewise was important in separating the saponin mixture. Comparison of 200–400 mesh resin with −400 mesh resin showed that the smaller particle size gave the better resolution. Because of these results we adopted a flow rate of 7.5 ml/h with −400 mesh resin for most of our studies.

Influence of temperature

Varying flow rate and mesh size indicated that incomplete equilibration occurred at high flow rates and with large resin particle sizes. Since equilibration during ion exchange can be hastened by increasing temperature[14], we compared the effects of chromatographing the saponins at 25° and 50° (Fig. 1). Resolution at 25° was slightly better with a column 0.9 cm in diameter than earlier results with a narrower (0.6 cm) column[5]. At 50° the peaks eluted earlier and sharper and resolved more completely than at 25°.

As previously noted[5], one of the fractions gave a pinkish yellow with phenol–sulfuric acid instead of yellowish orange that is typical of sugars. The region of elution of the compound(s) giving the atypical color is indicated in Fig. 1 by shading. This pink color served as a useful indicator of resolution in later experiments.

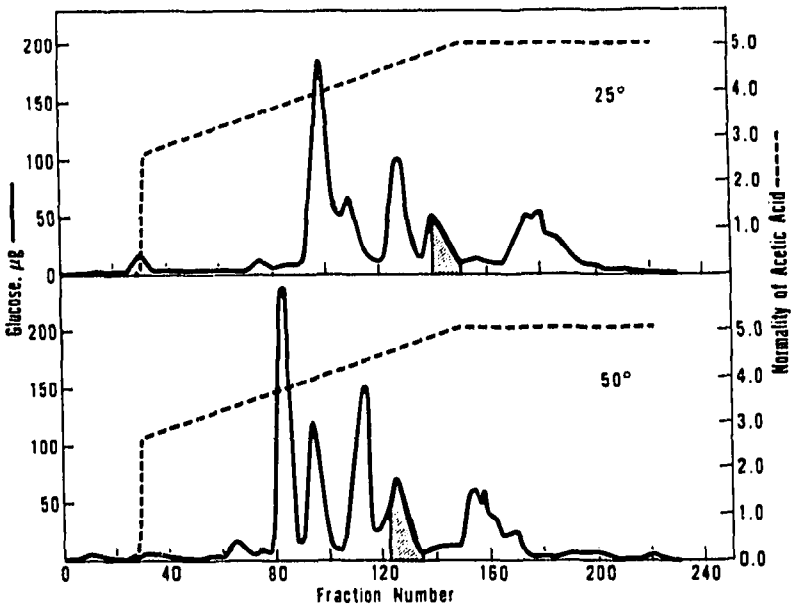

Fig. 1. Elution of 20-mg samples of soybean saponins with a 2.5–5 *N* acetic acid gradient at 25° and 50°. The dashed curve is the acetic acid gradient, the solid curve is the glucose content and the shaded region indicates the atypical color with phenol–sulfuric acid. Flow rate, 7.5 ml/h.

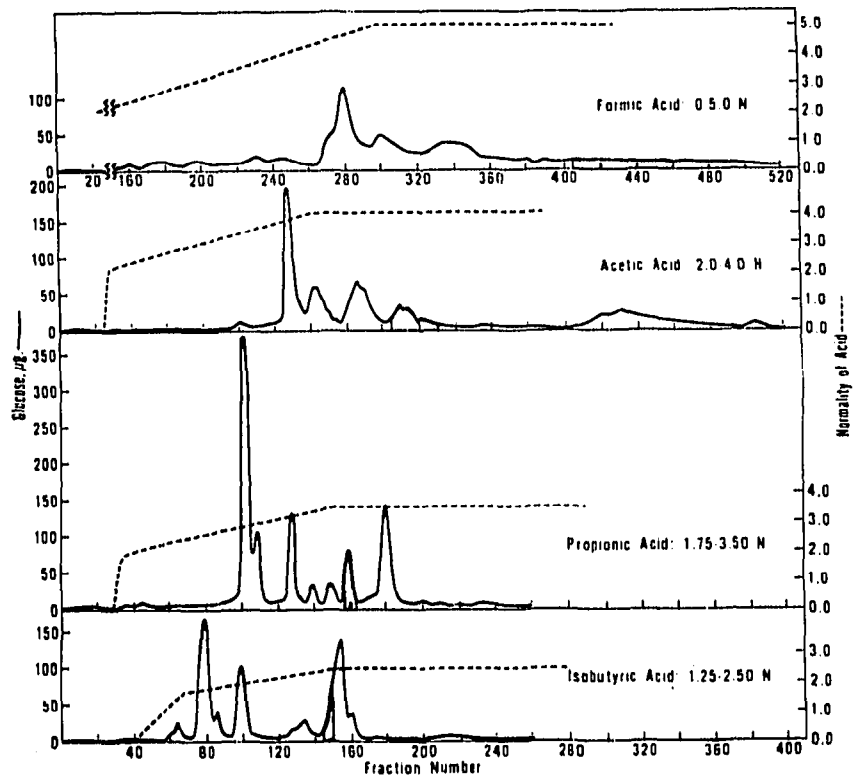

Fig. 2. Elution of soybean saponins at 50° with formic, acetic, propionic and isobutyric acids. The dashed curves are acid gradients, the solid curves are glucose contents and the shaded regions indicate the atypical color with phenol–sulfuric acid. Column loads, 20 mg; flow rate, 7.5 ml/h.

Elution with acids of varying chain length

The effect of different organic acids on elution of saponins from Dowex-1 was studied. Gradient elution was carried out with formic, acetic, propionic, butyric and isobutyric acids (Fig. 2). Since butyric and isobutyric acid gave nearly identical results, only elution with isobutyric acid is shown in the figure. Because the concentration of acid required to achieve elution decreased as carbon chain length of the acid increased, comparative studies were impossible with a constant elution gradient. Elution with formic acid required a concentration up to 5 N, whereas isobutyric acid eluted the saponins effectively with a final concentration of 2.5 N. Resolution with formic acid was poor when compared with the other three acids. Degradation of the saponins during chromatography with formic acid is not ruled out but seems unlikely on the basis of TLC analysis of the column fractions. In all fractions, the spots corresponded in R_F and color with spots in the unfractionated saponins that had not been treated with formic acid.

Use of formic acid also had the disadvantage of carbon monoxide formation during reaction with sulfuric acid. The compound giving the pink color with phenol–sulfuric acid eluted slowly and as a broad zone with formic acid. The color coincided more nearly with elution peaks when acetic or propionic acids were used. The position of the pink in the isobutyric acid elution diagram clearly showed that resolution of the slowly eluting saponins was poorer than with acetic or propionic acids.

Influence of adsorption

Results with the different acids indicated that the chromatographic process is more complex than simple ion exchange. Since formic acid is more completely ionized (pK 3.7) than the other acids (pK 4.8), it should be the most efficient eluting acid used. Results, however, did not agree with this expectation (Fig. 2). In another experiment, 20 mg of saponins were placed on a column in the acetate form and a 0–1 M sodium acetate gradient was pumped through the column. No saponins appeared in the effluent under the influence of the gradient (~500 ml of effluent) or on continued elution with 500 ml of 1 M sodium acetate. Nonetheless, the saponins were readily eluted with ~40 ml of 5 N acetic acid following attempted elution with sodium acetate.

The role of adsorption during ion exchange was evaluated by using a column of polystyrene beads (Bio-Beads S-X4, 200–400 mesh) to duplicate the hydrophobic polymer backbone of the ion-exchange resin. With a 2.5–5 N acetic acid gradient at 25°, the bulk of the saponins emerged with the holdup volume of the column (~20 ml) and the remainder eluted with trailing in the region of the acetic acid gradient. That adsorption is a factor during the ion-exchange process was demonstrated more clearly by repeating the experiment and pumping 200 ml of water through the column before starting the acetic acid gradient. Under these conditions (Fig. 3) about 20% of the saponins (estimated from areas under peaks) was adsorbed to the polystyrene beads and eluted under the influence of the acetic acid gradient. Under comparable conditions on Dowex-1 columns (25° elution in Fig. 1), no significant amounts of saponins were eluted until the gradient reached ~3.5 N. Thus fractions II–IV are eluted later from Dowex-1 than from Bio-Beads. Analysis of fractions I–IV by TLC on silica gel with six developments of chloroform–methanol–water (65:25:4) showed that fraction I was similar to the unfractionated saponins while fractions II–IV separated

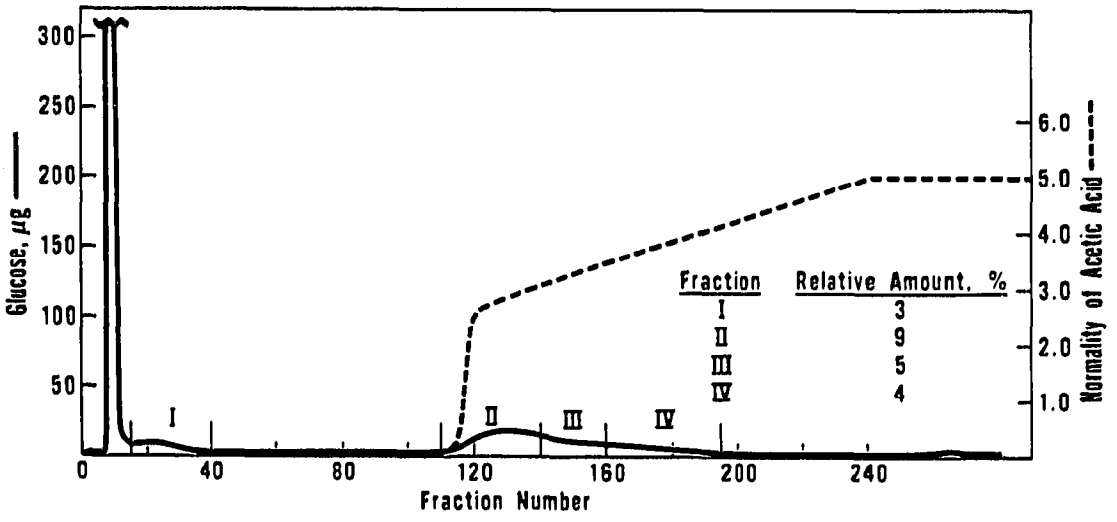

Fig. 3. Chromatography of 20 mg of soybean saponins on Bio-Beads S-X4 at 25°. The dashed curve is the acetic acid gradient and the solid curve is the glucose content.

into three spots corresponding to the spots of highest R_F in the starting mixture. No differences among fractions II, III and IV were apparent.

Ion-exchange materials with a hydrophilic backbone were also evaluated. DEAE-Sephadex A-25 in the acetate form was tried at 50° with acetic acid gradients ranging from 2.0–4.0 N to 3.0–6.0 N. Resolution, however, was poor as compared to that with acetic and propionic acid on Dowex-1.

Variation of elution gradient

An additional factor influencing separation of the saponins on Dowex-1 was

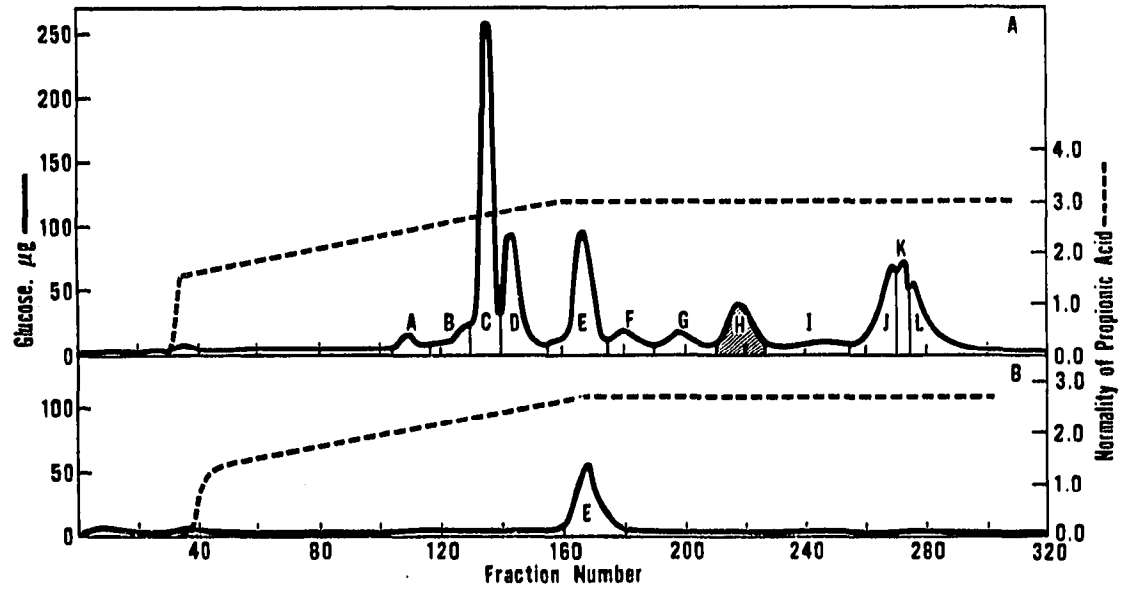

Fig. 4. Elution of (A) 20 mg of saponins and (B) 13 mg of fraction E with 1.5–3.0 N propionic acid at 50°. The dashed curves are the propionic acid gradients, the solid curves are the glucose contents and the shaded region designates the atypical color with phenol–sulfuric acid. Flow rate, 7.5 ml/h.

the slope of the acid gradient. The 50° curve in Fig. 1 and the acetic acid curve in Fig. 2 show the effects of varying the slope of the gradient. The 2.5–5.0 N gradient (Fig. 1) gave sharper peaks and elution was completed earlier than with the 2.0–4.0 N gradient (Fig. 2).

A 1.5–3.0 N gradient of propionic acid (Fig. 4A) instead of a 1.75–3.50 N gradient (Fig. 2) gave broader peaks, but some fractions appeared to separate more completely. The less steep gradient also partially separated the last major peak into several fractions (fractions J–L of Fig. 4A).

The conditions described in Fig. 4A were adopted for preparative work to obtain sufficient amounts of the fractions for characterization. Column loads were increased to 150 mg with little effect on the separations. A total of twelve fractions (A–L) were obtained as compared to only seven fractions in earlier studies[5]. Fraction E (portion of pooled samples from three preparative runs) rechromatographed in a single peak (Fig. 4B) in the same position as originally observed. This duplication indicates that the saponins are not modified by acid conditions of the chromatographic procedure.

TLC of saponin fractions

Saponin fractions A–L separated by elution with propionic acid (Fig. 4) were analyzed by TLC (Fig. 5). Many fractions gave more than one spot, but definite fractionation of the original mixture was indicated. The fractions show an overall trend of increasing in R_F with order of elution from the column as noted when an acetic acid gradient was used[5]. Eighteen major spots were observed on the TLC plate, but incomplete separation is likely between some fractions, *e.g.* B–C and J–K–L–. Fractions J–K–L, however, apparently consist of at least three saponins on the basis

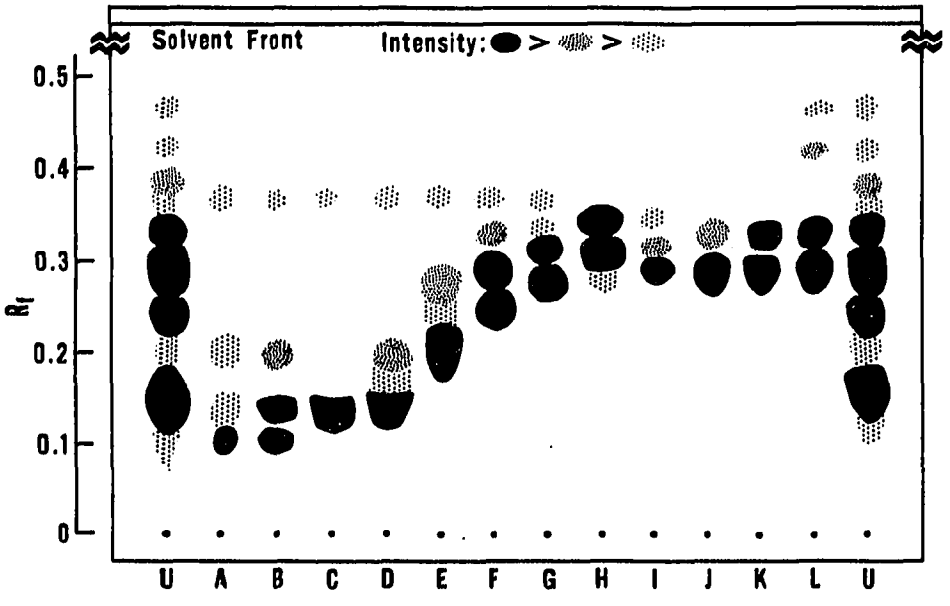

Fig. 5. Diagram of thin-layer chromatogram of unfractionated (U) saponins and saponin fractions A–L obtained by gradient elution of saponins with propionic acid as shown in Fig. 4. The plate was developed six times with chloroform–methanol–water. The R_F scale was arbitrarily set at 0.0 at the origin and 1.0 at the solvent front.

of the elution diagram (Fig. 4). After allowing for overlapping of fractions there are still fifteen major spots and thus presumably as many or more different saponins in the unfractionated mixture.

Fraction H, which formed the pink color with phenol–sulfuric acid, gave distinctive bluish-purple spots with 50% sulfuric acid on the TLC plate. Only traces of blue spots were detected in fractions G and I; thus overlapping of fractions appeared to be minimal.

Analysis of hydrolyzates of saponin fractions

The saponin fractions from elution on Dowex-1 were hydrolyzed with methanolic hydrochloric acid and separated into sapogenins and sugars. The sapogenin fractions were analyzed by TLC according to the solvent system reported by SHANY

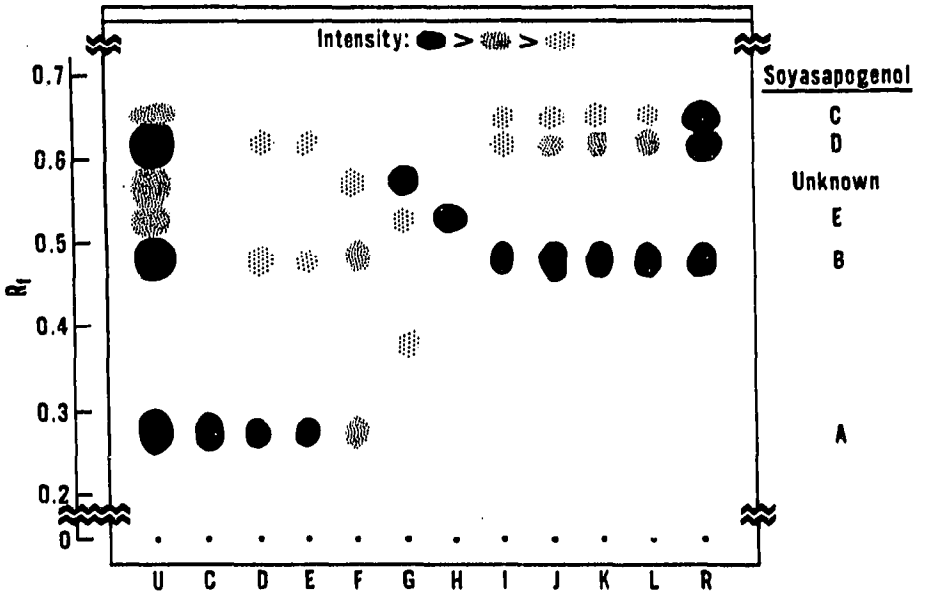

Fig. 6. Diagram of thin-layer chromatogram of sapogenins from unfractionated (U) saponins and Dowex-1 column fractions C–L. Column R is a mixture of soyasapogenols B, C and D used as reference compounds.

et al.[7] (Fig. 6). Sapogenins from unfractionated saponins (sample U) and reference samples of soyasapogenols B, C and D (sample R) are included in the chromatogram. Sapogenins in sample U separated into three major and three minor spots. The spot of R_F 0.28 is tentatively identified as soyasapogenol A based on results of SHANY *et al.*[7] The next major spot (R_F 0.48) and the two uppermost spots (R_F 0.62 and 0.65) were identified as soyasapogenols B, D and C, respectively, by comparison with the reference compounds. The spot of R_F 0.53 was identified as soyasapogenol E as described later, while the remaining spot (R_F 0.57) is an unknown. All spots in sample U gave brown to magenta colors when the TLC plate was sprayed with 50% sulfuric acid except soyasapogenol E that turned blue.

When the TLC plate was sprayed with antimony trichloride as the detecting reagent, fraction G gave a green spot while fraction H initially was yellow and slowly turned green. The spots in sample U that corresponded to the R_F values of fractions

G and H gave the same color changes with antimony trichloride. All other sapogenins gave brown or violet spots[9]. Formation of a green color with antimony trichloride on circular paper chromatograms is reported as a characteristic of soyasapogenol E[15]. When the sapogenins of fractions G and H were chromatographed by the circular technique[9], H gave a green color with an R_F corresponding to the green color observed in a hydrolyzate of the unfractionated saponins. Fraction G gave a pinkish violet spot with an R_F slightly higher than fraction H. In our hands this technique was not satisfactory since the spots were diffuse and R_F values varied. Tentative identification of the sapogenin in fraction H as soyasapogenol E was therefore confirmed by infrared spectroscopy. The spectrum for the sapogenin in carbon tetrachloride showed an absorption band at 1708 cm^{-1} in good agreement with a band reported at 1706 cm^{-1} for the six-membered ring ketone of soyasapogenol E (ref. 15).

The spectrum for the aglycone from fraction G showed a small shoulder at 1710 cm^{-1}, probably because of a trace of soyasapogenol E as indicated by TLC (Fig. 6). An absorption band was also observed at 1098 cm^{-1}. Absorption at 1100 cm^{-1} is characteristic of soyasapogenol D presumably because of its ether linkage. Saponin fraction G thus appears to contain a previously unreported aglycone whose structure is related to soyasapogenol D.

Saponin fractions A and B (Fig. 4) were not obtained in sufficient amounts to study their hydrolysis products. TLC analysis of the remaining fractions (Fig. 6) indicated that separations on the Dowex-1 column were primarily on the basis of the sapogenin constituent. Fractions C–E contained soyasapogenol A while F consisted of soyasapogenols A and B plus a trace of the unknown aglycone. The unknown aglycone was concentrated in fraction G with a trace of soyasapogenol E while fraction H was comprised only of soyasapogenol E. Fractions I–L contained primarily soyasapogenol B with small amounts of soyasapogenol D and traces of soyasapogenol C.

Neutral sugars from hydrolysis of saponin fractions C–L were chromatographed on paper with butanol–pyridine–water (6:4:3). Rhamnose, glucose, galactose and arabinose plus small amounts of xylose occurred in all fractions. The chromatograms, however, indicated that variations in sugar ratios occurred in different fractions. When the acidic sugars were chromatographed on paper with ethyl acetate–acetic acid–water (3:1:3), glucuronic acid was detected in all saponin fractions. Although the saponins were extensively fractionated, all fractions contained the six sugars found in the starting saponin mixture. Many fractions were mixtures of two or more saponins (Fig. 5); consequently, we cannot rule out the possibility that certain saponins may contain less than six sugars. GESTETNER *et al.*[4] isolated a saponin fraction consisting only of soyasapogenol A and glucose. Because this fraction is neutral it should elute with the holdup volume of the Dowex-1 columns (~20 ml), but we did not observe significant amounts of materials eluting under these conditions (Figs. 1, 2 and 4). If present in our starting mixture, this saponin must be adsorbed under conditions similar to those demonstrated in Fig. 3 and eluted in one of the first saponin fractions, such as A or B.

GLC of sugars in saponin fractions

Variations of sugar ratios in different saponin fractions suggested by paper chromatography were confirmed by GLC analysis. The procedure described by SLONEKER[11] was followed with minor modifications. Isothermal operation of the

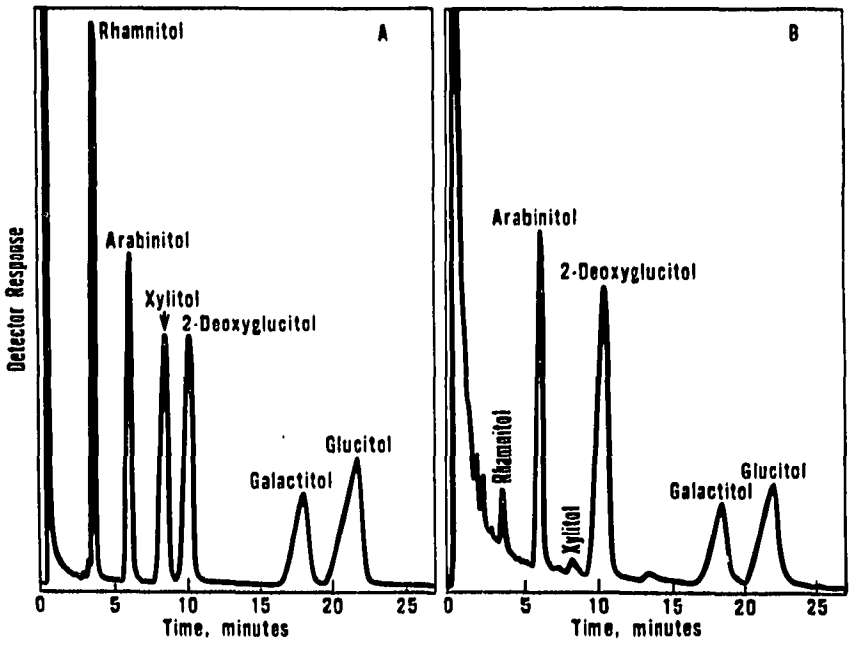

Fig. 7. Gas–liquid chromatograms of (A) a standard mixture of alditol acetates and (B) alditol acetates from the hydrolyzate of saponin fraction E (Fig. 4).

column gave excellent separations of the five neutral sugars found in soybean saponins, plus 2-deoxyglucose, which was used as an internal standard (Fig. 7A).

In preliminary studies, purified saponins were hydrolyzed for 4 h with 1 *N* sulfuric acid in dioxane–water[1,4]. Analysis of the sugars by GLC after reduction and acetylation, however, showed two unidentified peaks between 2-deoxyglucitol and galactitol plus a peak after glucitol. Evidently incomplete hydrolysis had occurred. The procedure of SAEMAN *et al.*[12] for hydrolysis of cellulosic materials gave only minor extraneous peaks; hence, it was used instead. A typical GLC pattern for a saponin hydrolyzate is seen in Fig. 7B.

Molar response factors were measured for the alditol acetates obtained by reduction and acetylation of an equimolar mixture of the six sugars found in soybean saponins, plus 2-deoxyglucose. Response factors were calculated relative to 2-deoxyglucitol by dividing the GLC peak area for a given alditol acetate by the area for 2-deoxyglucitol acetate. In a control experiment with glucuronic acid, extensive degradation of this sugar during acetylation produced a dark red reaction mixture. A small peak with a retention time slightly less than the retention time for galactitol acetate occurred on GLC of the glucuronic acid reaction products. Nonetheless, this degradation product was not observed in the standard sugar mixture (Fig. 7A) and did not occur in significant amounts in most of the saponin hydrolyzates.

Molar response factors for the sugars before and after hydrolysis are listed in Table I. Of the unhydrolyzed sugars only glucose gave a response factor differing significantly from unity. On hydrolysis the response factors decreased for all sugars, but the decreases were statistically significant only for rhamnose, xylose and glucose. The response factors of the hydrolyzed sugars were used to calculate values for the hydrolyzates from the saponin fractions.

Results of GLC analysis of the saponin fractions are shown in Table II. In-

TABLE I

MOLAR RESPONSE FACTORS FOR GLC ANALYSIS OF ALDITOL ACETATES

Parent sugar	*Molar response factor*[a]		
	Unhydrolyzed (U)	*Hydrolyzed (H)*	*U/H*[b]
Rhamnose	1.073 bc[c]	0.785 a	1.37 b
Arabinose	1.005 ab	0.881 b	1.14 a
Xylose	0.956 a	0.803 a	1.19 a
Galactose	1.064 bc	0.956 bc	1.11 a
Glucose	1.122 c	0.929 bc	1.21 a

[a] Relative to 2-deoxyglucose which was arbitrarily assigned a value of 1.00. Each value is the mean obtained with two standard sugar mixtures analyzed in duplicate. Two means in a column with no letter in common differ significantly at the 5% level.
[b] A ratio of U/H exceeding 1.17 is significantly different from 1.0.
[c] Variations of ± 8% from 1.00 are significantly different from 1.00.

cluded in Table II are the results of the TLC analysis of the fractions and their sapogenins. Although not determined quantitatively, glucuronic acid occurred in each fraction when examined by paper chromatography as discussed earlier. All fractions differed from each other in the content of one or more sugars except fractions H, K and L. Fraction H, however, is readily differentiated from fractions K and L because it contains soyasapogenol E. Fraction J differed significantly from fractions K and L only in its arabinose content, but the overall similarity of these three fractions is not surprising because they are subfractions of the last major peak eluted from Dowex-1 (Fig. 4A).

A characteristic of all fractions is the low content of xylose as also noted for

TABLE II

NEUTRAL SUGAR CONTENT OF SOYBEAN SAPONIN FRACTIONS BY GLC ANALYSIS[a]

Fraction	*No. of TLC components*	*Soyasapogenol*[b]	*μmoles sugar/mg*[c]				
			Rhamnose	*Arabinose*	*Xylose*	*Galactose*	*Glucose*
C	1	A	0.119 a	0.737 fg	0.034 a	0.650 de	1.463 d
D	2	A (B, D)	0.465 b	0.604 f	0.072 c	0.487 bc	0.839 c
E	2	A (B, D)	0.146 a	0.844 g	0.052 b	0.584 cde	0.855 c
F	3	A, B (unknown)	0.437 b	0.239 bc	0.077 c	0.447 ab	0.324 a
G	2	Unknown (E)	0.945 c	0.398 e	0.190 f	0.752 e	0.559 b
H	2	E	0.866 c	0.287 cd	0.125 e	0.502 bcd	0.707 bc
I	2	B (C, D)	0.458 b	0.155 a	0.055 b	0.380 a	0.685 bc
J	2	B (C, D)	0.992 c	0.209 b	0.091 cd	0.605 cde	0.715 bc
K	2	B (C, D)	1.002 c	0.316 de	0.113 de	0.604 cde	0.783 c
L	2	B (C, D)	0.895 c	0.304 cd	0.106 de	0.521 bcd	0.708 bc
Unfractionated	>10	A, B, C, D, E, unknown	0.475 b	0.605 f	0.030 a	0.581 cde	0.695 bc

[a] Each value is the mean obtained from two hydrolyzates except for samples G and I, where insufficient sample prevented duplicate analysis.
[b] Compounds in parentheses present in small or only trace amounts.
[c] Any two means in a column with no letter in common differ significantly at the 5% level with the exception of fractions G and I.

three fractions isolated by GESTETNER *et al.*[4]. Molar ratios of the other four sugars to xylose are high. For example, fraction C has a glucose to xylose ratio of 43:1. Based on the sugar and sapogenin content of soybean saponins, GESTETNER *et al.*[1,4] concluded that an average of only three sugar residues are attached to each sapogenin. High sugar to xylose ratios suggest that the Dowex-1 column fractions are still very heterogeneous. Alternatively, the consistently low xylose values for all the fractions may be the result of incomplete hydrolysis of an acid-stable structure such as an aldobiuronic acid of xylose and glucuronic acid. Aldobiuronic acids of this type have been isolated from a number of plant materials and are known for their resistance to acid hydrolysis[16].

Although we have isolated what appears to be a new sapogenin, the diversity of soybean saponins cannot be explained on the basis of the six different sapogenins now known to be present. Differences in the structure of the carbohydrate moieties clearly account for some of the multiplicity of saponins we have observed. For example, separation of fractions C, D and E on Dowex-1 (Fig. 4) obviously depends on the variations in the carbohydrate structure of these saponins since soyasapogenol A with only traces of other sapogenins occurs in all three fractions. Fractions G and H likewise contain mainly a single sapogenin, but each fraction contains at least two saponins by TLC analysis (Fig. 5). In these two fractions, variations in the carbohydrate structures do not affect properties of the saponins sufficiently to permit resolution on Dowex-1.

Our results demonstrate that chromatography on Dowex-1 separates soybean saponins largely on the basis of their sapogenin components, but TLC and carbohydrate analyses indicate that additional fractionation is needed to secure homogeneous saponins.

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