Microdetermination of Triterpene Sapogenin Content of *Kochia scoparia* Seed Using Gas–Liquid Chromatography

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Saponins were extracted nearly completely (95%) from whole kochia seed without seriously affecting seed germination by washing with 1% aqueous NaOH at room temperature. Hydrolysis of the extracted saponins with 1 *N* sulfuric acid in water–1,4-dioxane (3:1) released a sapogenin (oleanolic acid) which was extracted with ether and methylated with diazomethane. The methylated sapogenin was measured by gas–liquid chromatography on a 1% SE-30 column. Amounts as small as 0.68 μg of oleanolic acid were measured quantitatively by this procedure and considerably smaller amounts were detected. The standard deviation obtained from the analysis of a saponin concentrate was 3.1% at 8 μg.

Kochia scoparia (L.) Schrad. (kochia) has been investigated as a forage crop in Texas (Sherrod, 1971) and as a seed crop in Saskatchewan (Coxworth *et al.*, 1969). One obstacle to its acceptance as a seed crop or as a late season forage crop is the presence of a rather toxic saponin (Souto and Milano, 1966). Methods have been devised in this laboratory to extract the saponin from the seed (Coxworth and Salmon, 1972); however, this would add to the processing cost if kochia were to become a commercial crop. Both chemical treatment (such as washing plant material with water) and selective plant breeding have been investigated for removing toxic saponins from food and feed sources. Saponins have been traditionally removed from the Andean food crop quinua (*Chenopodium quinoa*) by washing whole seed with water, although Bacigalupo (1969) observed that simple water washing did not adequately reduce the saponin content of the seed. Pedersen *et al.* (1967) suggested that the high saponin content in alfalfa may be hereditary and therefore might be reduced by selective breeding. This may also be true for kochia, and a method for rapidly and accurately determining the saponin content of small samples of seed from individual plants or of individual seeds would be valuable in a plant breeding program. This was done indirectly, in the present investigation, by measuring the sapogenin (aglycone) content of seed extracts. The procedure could also be used to determine the sapogenin content of larger amounts of plant material before and after different chemical treatments.

Various methods have been used to determine sapogenins quantitatively in plant material. Sapogenins have been separated by paper chromatography and determined quantitatively by densitometric measurement (Gestetner *et al.*, 1966) and by colorimetry (Souto and Milano, 1966; Wagner and Sternkopf, 1958). Quantitative determinations have also been made using thin-layer chromatography (tlc) in conjunction with various measurements of the spot: area (Mal'chukovskii and Libizov, 1971), fluorescence (Vagujfalvi *et al.*, 1966), densitometry (Brain and Hardman, 1968; Glunden and Hardman, 1968), and colorimetry (Pasich,1963).

Qualitative gas–liquid chromatographic (glc) analyses have been carried out on the methyl esters and trimethylsilyl derivatives of triterpene sapogenins (Ikekawa *et al.*, 1965). After this work was completed, the analogous work of Larry *et al.* (1970, 1972) was encountered. These papers contained a quantitative method for determining the content of the ammoniated triterpene saponin, glycyrrhizin, used as a food additive. The saponin was hydrolyzed in dioxane–sulfuric acid. The trimethylsilyl ether–ester derivative of the sapogenin, glycyrrhetic acid, was prepared instead of the methyl ester as we did for oleanolic acid. The glc analyses were carried out on a glass column filled with 1.5% OV-1 on 60/80 mesh Gas Chrom Q.

Crude kochia saponin consists of one major and several minor components which yield one sapogenin, oleanolic acid, on hydrolysis (Souto and Milano, 1966; Tandon and Agarwal, 1966). The present paper outlines a glc micromethod for determining the sapogenin content of a single kochia seed by measuring oleanolic acid as its methyl ester. The method has also been used to measure the oleanolic acid content of larger samples of whole kochia seed and defatted seed meal.

METHODS AND RESULTS

Apparatus and Materials. *Gas Chromatography.* An F&M Model 810 gas chromatograph equipped with a flame ionization detector and a 3 mm i.d. × 4 ft glass column packed with 1% SE-30 Ultraphase on 100/120 mesh Gas Chrom Q (Chromatographic Specialities, Ltd., Brockville, Ontario, Canada, SPA 293) was used. The column, injector block, and detector temperatures were 235, 270, and 270°, respectively, and the helium flow rate was 65 ml/min. The peak areas were measured by means of an Infotronics Model CRS-104 electronic integrator.

Thin-Layer Chromatography. The saponins were separated on 4 cm × 9 cm glass plates coated with 250 μ of Merck silica gel G prepared with a 0.02 *N* sodium acetate buffer. The plates were developed with 1-butanol–acetic acid–water (4:1:1). The spots were detected by spraying the plates with anhydrous saturated solution of $SbCl_3$ in chloroform and then heating at 110°.

Column Chromatography. The crude saponin was chromatographed on a 4.5 cm × 13 cm column of 80/200 mesh, neutral alumina (Fisher A-950, Brockman activity 1) using 1-butanol–15% aqueous ammonia–95% ethanol (4:4:1.5).

Melting Points. The melting points were determined with an Electrothermal capillary melting point apparatus and are uncorrected.

Determination of Sapogenin Content of Single Seeds. Individual kochia seeds were weighed on a Cahn balance and sealed in a 4-ml vial with a Teflon disk. The seeds were covered with 0.25 ml of 0.25 *N* NaOH and gently rocked back and forth on a shaker for 15 min. The extract was transferred to a 50-ml pointed flask (Downey and Craig, 1964) and the seed in the original vial was covered with 0.25 ml of water and the shaking was repeated. The combined washing and extract in the pointed flask was diluted with 0.25 ml of 1,4-dioxane and 0.25 ml of 4 *N* sulfuric acid, and the mixture was refluxed on the steam bath for 2 hr. [1,4-Dioxane (Fisher Certified ACS, D-111) was refluxed over sodium metal for 6 hr and distilled.] After cooling, approximately 1.5 ml of ether and 100 μl of the internal standard solution were added and the hydrolysate was extracted once with ether, followed by drawing off the

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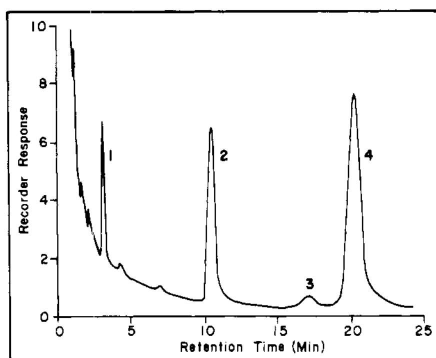

Figure 1. Gas–liquid chromatogram of sapogenin content of single kochia seed. 1. 2,6-di-*tert*-butyl-*p*-cresol (antioxidant); 2. cholesteryl acetate (internal standard); 3. unknown; and 4. methyl oleanolate.

Table I. Sapogenin Content of Single Kochia Seeds

Sample	Seed wt, mg	Sapogenin content[a]	
		μg	%
1	0.911	18.02	2.09
2	1.032	13.39	1.37
3	1.363	12.31	0.95
4	0.843	11.03	1.38
5	1.090	13.97	1.35
Avg	1.048	13.74	1.43
σ (%)			29

[a] Dry basis. The moisture content of whole kochia seed was 5.35%.

aqueous phase with a pipette. [Diethyl ether (Fisher Anhydrous E-138) was treated with $FeSO_4$ (Vogel, 1959) and distilled immediately prior to use. The internal standard solution consisted of 64 μg/ml of cholesteryl acetate in chloroform.] The ethereal extract was washed once with distilled water. Sufficient methanol was added to the ether solution to give a 10% methanolic solution which was treated with diazomethane according to the procedure of Taulli (1969). The excess diazomethane was removed under a gentle stream of nitrogen and 0.5 ml of ether containing a trace of 2,6-di-*tert*-butyl-*p*-cresol was added. The ether solution was washed once with 1 ml of 5% $NaHCO_3$ and once with water. The solvent was evaporated under nitrogen and the flask was rinsed with two 0.3-ml portions of reagent chloroform. The chloroform solution was transferred to a 1-ml Reactivial (Chromatographic Specialties, 13221) and evaporated to dryness under nitrogen. The residue was dissolved in 20 μl of chloroform and 5-μl injections were chromatographed.

Several of the extracted seeds were reextracted with dilute NaOH and washed with water, and these extracts were treated in the same manner.

Figure 1 is a typical glc chromatogram of methyl oleanolate obtained from the extract of a single seed. The results of single kochia seed analyses are recorded in Table I.

The 2,6-di-*tert*-butyl-*p*-cresol (Figure 1) was added to prevent peroxide formation in ether. When concentrated, peroxides appeared to cause decomposition of methyl oleanolate. The area of peak number 3 varies from 1 to 5% of the area of peak number 4, methyl oleanolate. The compound responsible for this peak has not been identified but arises from methyl oleanolate during the evaporation of the methanolic ether under a gentle stream of nitrogen. An attempt will be made to identify this material and to prevent its formation.

No peak for methyl oleanolate was detectable in the chromatogram of the extract obtained by reextracting single kochia seeds. Hence, multiple extractions were considered unnecessary.

Precision of the Sapogenin Analysis. Since a wide variation between the sapogenin contents of individual kochia seeds was observed, the analysis was repeated on a saponin concentrate of comparable concentration to determine the statistical variation involved in the procedure. The saponin concentrate was prepared from crude saponin, which was isolated from hexane-extracted kochia seed meal with hot 80% ethanol by a method (Coxworth and Salmon, 1972) similar to the procedure of Souto and Milano (1966). The crude saponin [major component R_f 0.70; minor components R_f 0.60, 0.53, 0.45 (tlc)] was purified by column chromatography and the residue from the first chromatographic fraction was decolorized by refluxing with Norit-A (Fisher decolorizing charcoal, C-176) in absolute ethanol. Evaporation of the solvent left an off-white residue, mp 215–221°, R_f 0.70.

The precision of this microanalytical method was investigated by analyzing six duplicate samples of a solution of saponin concentrate in dilute NaOH. The 20-μg samples were found to contain 8.75 μg (43.7%) of sapogenin (σ 3.1%). The comparatively high standard deviation (29%) obtained from single seed analyses (Table I) suggests that there is a real difference in the sapogenin content of individual seeds.

Extraction Efficiency. The saponin was hydrolyzed by heating on the steam bath for 5 hr with 1 *N* sulfuric acid in water-1,4-dioxane (3:1) according to the procedure of Gestetner *et al.* (1966). The white precipitate of oleanolic acid was removed by filtration, washed with dilute ethanol and water, dried, and recrystallized twice from methanol to give white needles, mp 296–298° [reported mp, 301–302° (Tandon and Agarwal, 1966); 296–298° (Tschesche *et al.*, 1969)]. In order to determine the efficiency of a single ether extraction, 1-ml aliquots of an approximately 8 μg/ml solution of oleanolic acid in chloroform were placed in several pointed flasks. Some of these aliquots were evaporated to dryness, after which 0.5 ml of water, 0.25 ml of 1,4-dioxane, and 0.25 ml of 4 *N* sulfuric acid were added to each flask. The mixtures were refluxed on the steam bath for 2 hr and cooled, and the sapogenin was extracted, methylated, and chromatographed. A 100-μl aliquot of the internal standard was added to the other flasks and the solvent was evaporated. The residue was dissolved in 1.5 ml of 10% methanolic ether, methylated, and chromatographed in the same manner.

The aliquots of the standard oleanolic acid solution which were only methylated and chromatographed as controls gave a mean concentration of 8.28 μg/ml (σ 1.7%). The aliquots carried through the hydrolysis as well gave a mean concentration of 8.00 μg/ml (σ 3.9%). Considering the controls to be 100%, the recovery of the oleanolic acid from a single ether extraction was 97%.

Determination of the Sapogenin Content of Larger Samples of Kochia Seeds. Kochia seed samples, each containing 100 whole seeds, were treated analogously to the single seed analysis with the following variations. Seed samples were extracted at both room temperature and 10°. The hydrolyzate from a sample was extracted three times with ether and the ethereal extract was washed three times with water and dried over anhydrous $MgSO_4$. The dried ether solution was concentrated to 10 ml, transferred to a pointed flask, and diluted to 25 ml with ether. After 2.5 ml of methanol was added, the solution was methylated, washed with 5% $NaHCO_3$ and water, and concentrated to 2 ml for chromatography.

Two of the seed samples extracted at room temperature were defatted with 25 ml of hexane in Swedish Oil Extraction Tubes (Troeng, 1955). The seed meal was removed by filtration and hydrolyzed in 100 ml of 1 *N* sulfu-

Table II. Sapogenin Content of Samples of 100 Kochia Seeds

Number of samples	Extraction temp	Avg sample wt, mg	Sapogenin content mg	Sapogenin content %
Whole seed				
4	Room temp	86.1	0.961	1.18
2	10°	94.1	1.045	1.18
Seed meal from extracted whole seed				
2	Room temp	86.9	0.0415	0.051

ric acid in water–1,4-dioxane (3:1) by stirring under a reflux for 2 hr. The hydrolyzate was extracted with ether, methylated with diazomethane, and chromatographed.

The results given in Table II indicate that lowering the extraction temperature has no effect on the amount of sapogenin extracted and that a single treatment of whole seed with 1% aqueous NaOH followed by one water wash removed 96% of the sapogenin (and by inference saponin) contained in the seed. This is somewhat analogous to the work of Sosulski *et al.* (1972), who found that glucosinolates present beneath the seed coat could be extracted from whole rapeseed with aqueous ethanolic NaOH, although high temperatures (60°) and long extraction times (4 hr) were required.

Seed Germination. Seeds were placed in Petri dishes containing 2 ml of a nutrient solution (Ellis and Swaney, 1947) and germinated in a low temperature incubator at 24.5°. One set of seeds, extracted at room temperature and at 10°, was germinated immediately. Another similar set was dried between filter papers and refrigerated for 1 week before germination. The results reported in Table III indicate that the NaOH extraction had no significant effect on seed germination immediately following the extraction. Some reduction in viability was observed on seeds refrigerated for 1 week after extraction at room temperature.

Glc Calibration. The response relationship between the internal standard and the methyl oleanolate was determined and found to be linear in the range of 1 to 20 μg of methyl oleanolate. The methyl oleanolate used for the determination of the calibration curve was prepared by treating a solution of oleanolic acid in 10% methanolic ether with diazomethane. After evaporation of the solvent, the residue was recrystallized from methanol to give the ester, mp 199–202° [reported mp, 199–200° (Tandon and Agarwal, 1966)].

DISCUSSION

This procedure describes a precise accurate method of determining the oleanolic acid content of plant material, including whole seeds, without seriously affecting their germination. Since a wide variation in the sapogenin content of individual seeds does exist, there is a possibility that a selective breeding program could develop a variety with a low saponin content. This analytical procedure could be an asset to such a program.

The hydrolysis technique employed was based on the procedure of Gestetner *et al.* (1966). The duration of the hydrolysis was reduced to 2 hr from 4 hr, since equivalent values for sapogenin content were obtained when duplicate saponin samples were hydrolyzed from 1 to 5 hr.

Diethyl ether was found to be superior to benzene for the extraction of the hydrolyzate. However, it was necessary to keep the total volume of ether to a minimum of 8 ml per seed. The increased solvent tail and the extraneous peaks which appeared when larger volumes were used interfered with the integration of the cholesteryl acetate peak.

Table III. Germination Test on Extracted Kochia Seeds

Sample	Extraction temp	Time interval after extraction	% germination 24	48	72 (hr)
Control			68	70	75
1	10°	Nil	60	70	73
2	Room temp	Nil	45	69	73
Control			57	62	66
1	10°	1 week	42	59	63
2	Room temp	1 week	9	39	46

The ether solution of methyl oleanolate was washed with 5% $NaHCO_3$ to remove traces of sulfuric acid retained by the ether. This eliminated the adverse effect of the concentrated acid on both cholesteryl acetate and methyl oleanolate.

The smallest amount of oleanolic acid measured quantitatively by this procedure was 0.68 μg, although considerably smaller amounts were detected. One technician could perform six to eight single seed analyses per day with the facilities available for this study. The analysis is limited to the determination of the sapogenin content only. Further work will be required to relate these results to seed toxicity.

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