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Determination of Saponins in Legumes by Direct Densitometry

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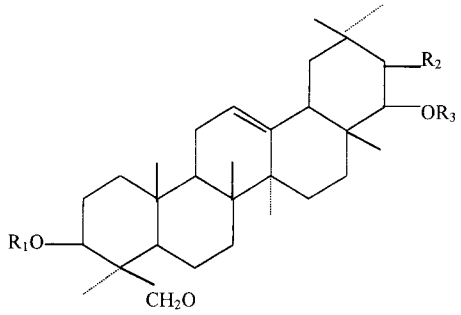
Research has shown that dietary saponins may have health benefits. A simple, rapid method for the determination of saponins in legumes, using densitometry, is described. Saponin preparations, after pretreatment to remove nonsaponin components, are spotted in rows on a thin-layer chromatography plate, along with soyasaponin standards. The plate, without solvent development, is directly treated with sulfuric acid and heated. Violet spots develop which have a density proportional to the amount of saponin present. The standard curve has a correlation coefficient of 0.99 and is linear over the range of 1.25 to 10 μg of soyasaponins applied. The method has a coefficient of variation of less than 3% and compares favorably with quantitative thin-layer chromatography. Using this method the saponin contents of defatted soy flour (0.58%), dried navy beans (0.32%), and dried kidney beans (0.29%) were determined, and these results were found to be consistent with previous reports in the literature.

KEYWORDS: Saponin; soyasaponin; legume; densitometry; thin-layer chromatography

INTRODUCTION

Research has shown that saponins from a variety of sources, such as medicinal plants and foodstuffs, have considerable health benefits (*1*). The major dietary sources of saponins are legumes, and the main saponins present in legumes are the soyasaponins. Soyasaponins are triterpenoid saponins of the oleanene−glucuronide type, i.e., olean-12-ene triterpenes with a C-28 methyl group and a glucuronic acid moiety linked at the C-3 of the triterpene (**Figure 1**). The soyasaponins are divided into two groups: A and B. The group B saponins have the aglycon soyasapogenol B and include the monodesmosides soyasaponin I, II, and III. The group A saponins have the aglycon soyasapogenol A, and include the bidesmosides soyasaponin A_1 and soyasaponin A_2 (*2*). Phaseoside I, a bidesmoside, has also been detected in some legumes (*3*). Studies on the health benefits of the soyasaponins suggest anticarcinogenic, hepatoprotective, and hypocholesterolemic activity, but have been limited to cell culture work and a few animal studies (*4*). The difficulty of readily isolating large quantities of these dietary saponins has hampered efforts to more fully evaluate their biological activity in vivo. In the authors' laboratory, for this reason, preparative methods for the isolation of soyasaponins from defatted soy flour are being studied for use in future animal and human intervention studies. In the course of these investigations the need for a rapid analytical method for determining total saponin content became apparent. This is so that questions such as the following could be readily answered: (a) which legume has the highest concentration of saponins, (b) which extraction/precipitation conditions give the highest saponin yield, (c) what is the relative solubility of saponins in different solvents, and (d) how pure are various saponin preparations?



Soyasapogenol B: $R_1 = H$; $R_2 = H$; $R_3 = H$

Soyasapogenol A: $R_1 = H$; $R_2 = OH$; $R_3 = H$

Soyasaponin I: $R_1 = GlcA^2\text{-}Gal^2\text{-}Rha$; $R_2 = H$; $R_3 = H$

Soyasaponin II: $R_1 = GlcA^2\text{-}Ara^2\text{-}Rha$; $R_2 = H$; $R_3 = H$

Soyasaponin III: $R_1 = GlcA^2\text{-}Gal$; $R_2 = H$; $R_3 = H$

Soyasaponin A_1: $R_1 = GlcA^2\text{-}Gal^2\text{-}Glc$; $R_2 = OH$; $R_3 = Ara^3\text{-}Glc$

Soyasaponin A_2: $R_1 = GlcA^2\text{-}Gal$; $R_2 = OH$; $R_3 = Ara^3\text{-}Glc$

Phaseoside I: $R_1 = GlcA^2\text{-}Gal^2\text{-}Glc$; $R_2 = H$; $R_3 = Ara^2\text{-}Glc$

Figure 1. Saponin chemistry.

A colorimetric method for soybean saponins has been reported, but it was based on sapogenin determination of the

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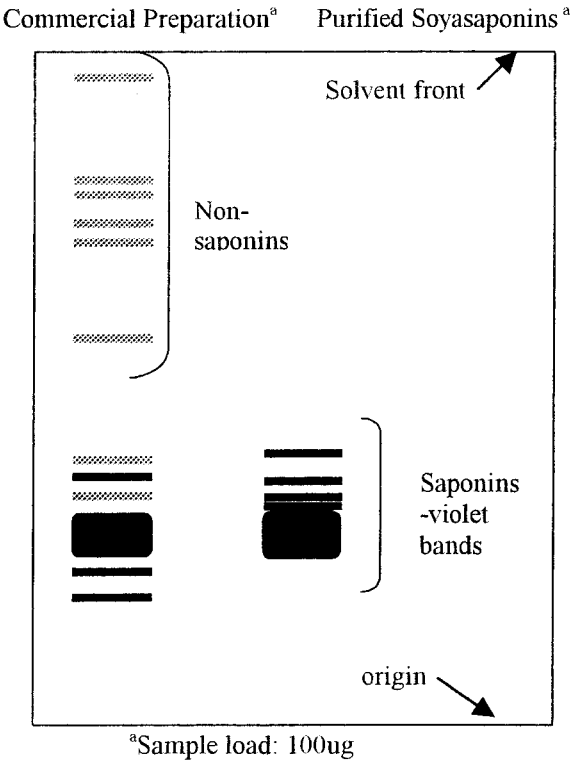

Figure 2. Comparison of commercial saponin preparation, before and after purification.

sample and required time-consuming hydrolysis of soyasaponins (*5*). Hiai and others (*6*, *7*) reported a colorimetric reaction with vanillin−sulfuric acid and unhydrolyzed saponins. But when this procedure was tested in the authors' laboratory, using soyasaponins, no color development was observed. Because of the low hemolytic activity of soyasaponins, methods based on this activity have also been shown to be ineffective (*8*). The combination of thin-layer chromatography and densitometry, i.e., quantitative thin-layer chromatography (QTLC), to determine soyasaponin content in various legumes has been reported and successfully used (*8*). In the present report, however, a simpler method is described that uses densitometry, but it eliminates the need for solvent development of the TLC plates. A saponin preparation, pretreated to remove nonsaponin constituents, is spotted on a TLC plate. The plate is then directly treated with sulfuric acid which results in the development of a violet spot which has a density proportional to the total saponins present. This approach, which will be referred to as direct densitometry (DD), allows for the rapid analysis of many samples.

MATERIALS AND METHODS

Preparation of Standards. Purified soyasaponins were prepared from a commercial preparation of soybean saponins (Wako Chemical), a fine yellow-brown powder (5.5% moisture, 1.7% ash). Nonsaponin constituents were extracted from the preparation by gently agitating the saponins (2.5 g) in acetone (25 mL) at room temperature, and then centrifuging the mixture at 2000*g* for 30 min. This acetone extraction was repeated three times. The residue was then similarly extracted in distilled water three times, and then washed in acetone to facilitate drying. The purified soyasaponin (approximately 250 mg) thus obtained was a white powder, but the acetone and water extracts were yellow in color. Absence of nonsaponin components was confirmed by thin-layer chromatography (100-μg sample load revealed no nonsaponin bands and saponin bands, violet in color and having R_f values of less than 0.5, as shown in **Figure 2**. This sample size was selected to highlight the presence of nonsaponins, but overloads and distorts the saponin bands so they are difficult to interpret. It appears however, that there is a shift in saponin bands from lower R_f values to higher R_f values, i.e., from more polar to less polar compounds. The preparation is water-washed and this may have resulted in the loss of more polar, and hence more soluble, saponins. Also, some hydrolysis of sugars may have occurred). Standard solutions were prepared by dissolving the saponins in 9 parts methanol/1 part water over a concentration range of 0.25 to 2.0 μg/μL.

Thin-Layer Chromatography. Thin-layer chromatography was performed using E. Merck silica gel 60G 20 × 20 cm plates, layer thickness 250 μm, with a preconcentration zone allowing for the formation of saponin bands. The solvent system used was chloroform/methanol/water, 65:35:10 (lower phase). Plates were saturated with 10% v/v sulfuric acid and heated for 15 min at 120 °C. For QTLC 2.5 μL of standards were applied over a concentration range of 0.25 to 1.75 μg/μL. The total density of all saponin bands was determined and used to generate a standard curve.

Sample Application for DD. Standard solution (5 μL) was applied in rows on the silica plate except for the preconcentration zone. A Hamilton syringe with a 90° bevel was used. The tip of the syringe was held close to the plate surface so that as the plunger was pushed down the droplet that formed wicked into the absorbent making a symmetrical, circular spot. It was important, for accuracy and precision, to develop a consistent sample application technique.

Placement of Spots on Thin Layer Plate for DD. The typical application pattern for six samples (e.g., samples A−F) and five standards (2.5−10 μg soyasaponin) was as follows:

Row 1:	A	B	C	2.5	5.0	7.5	8.75	10	D	E	F
Row 2:	A	B	C	2.5	5.0	7.5	8.75	10	D	E	F

To generate a standard curve, the average density of two spots, one from each row, was calculated and plotted for each standard concentration. The saponin concentration of a sample was determined from the average density of two spots, one from row 1 and the second from row 2. Using this arrangement, 8 rows can be easily spotted, allowing the determination of 24 samples per plate.

Visualization of Spots for DD. The spots were visualized by saturating the plate with 10% (v/v) sulfuric acid and heating it at 120 °C for 15 min. The spots that formed were violet in color and approximately 7 mm in diameter. The plates were allowed to cool five minutes and then measured by densitometry. Uniform heating of the plates was essential to the precision and accuracy of the method. The oven was routinely checked for cold spots by spotting a plate with rows of a standard solution and examining for variations in density. In the investigators' laboratory a small gradient in color development was observed from the back of the oven to the front, necessitating the placement of both sample and standard solutions in each row.

Measurement of Densitometry. The model 620 Biorad Video Densitometer was used in reflectance mode. In this mode, quantitation was based on the Kubelka−Munk theorem. Light was reflected off the plate via two mirrors onto a linear array detector. The instrument was calibrated with a white target. A lane on the thin-layer plate free of samples was recorded as background, and this was subtracted from each row of sample spots. Absolute reflectance was measured, and the area under the curve for each spot was plotted against the amount of standard saponins present to generate a standard curve from which the saponin content of unknown samples was calculated.

Investigation of Color Stability. To determine the color stability, a row of spots was applied to a plate and then visualized with acid. The plate was then placed under the densitometry beam and periodically measured. Between measurements, the row of spots was covered to avoid any deleterious effects of light exposure from the beam, but the plate was not moved from its initial position.

Accuracy and Precision Testing of DD. To test the accuracy and precision of the method, solutions containing known amounts of purified soyasaponins were spotted on plates along with the standards. The saponin contents of these solutions were determined and compared with expected results.

Analysis of Legumes. Locally obtained dry navy beans (*Phaseolus vulgaris*) (10 g) and kidney beans (*Phaseolus vulgaris*) (10 g) were finely ground and analyzed for saponins. Commercially defatted soy flour (*Glycine max*) (10 g) was also evaluated. The legumes were extracted in methanol (70 mL) for 4 h at 60 degrees C with gentle agitation. The saponin-containing methanol extract was filtered, and the bean residue was washed with additional methanol, bringing the total volume of methanol extract to 100 mL. A cleanup procedure was then applied to separate the saponin from nonsaponin constituents of the methanol extract. A 5-mL aliquot of the methanol extract was mixed in a 1:1 ratio with 0.4 M ammonium sulfate and left to stand overnight at room temperature. This resulted in the formation of a nonsaponin precipitate. This precipitate was removed by centrifugation (2000*g*, 30 min). The precipitate was washed three times with a 1:1 mixture of methanol and 0.4 M ammonium sulfate to release any entrained saponins. The saponin-containing centrifugate was then slowly passed through an Oasis HLB extraction cartridge (Waters Corporation; 500 mg of adsorbent, 12-mL barrel) which had been previously preconditioned with methanol (10 mL), water (10 mL), and 1:1 methanol/0.4 ammonium sulfate (2 mL). The saponins were retained by the adsorbent. The cartridge was washed with water (10 mL) and the soyasaponins were extracted from the cartridge with methanol (6 mL) (made alkali by the addition of 10 μL of 17% ammonia solution/10 mL of methanol). A 5-μL aliquot was applied directly to a TLC plate for saponin determination by DD and 2.5 μL for QTLC.

Recovery. The recovery of soyasaponins from the cleanup procedure described above was determined in two ways. A solution (5 mL) of purified soyasaponins (2 μg/μL) was processed by the same cleanup procedures and the saponin content of the cleaned-up solution was compared to that of the initial solution. Also, the recovery of purified saponins (1 mL) added to a methanol extract (4 mL) of soy flour (prepared by extracting 1 g of flour in 25 mL of methanol at 60 °C for 3 h) was determined to test for matrix effects.

Evaluation of Purification Methods for Commercial Soyasaponins (Wako Chemicals). The commercial soyasaponin preparation (1 g) was dissolved in methanol (200 mL) and combined with an equal volume of 0.4 M ammonium sulfate. The precipitate that formed was removed by filtration, and the methanol in the filtrate was removed by rotary evaporation. The volume of aqueous solution remaining was adjusted to a concentration of 0.2 M ammonium sulfate (200 mL total volume), and the saponin-containing precipitate that formed was recovered by filtration. The precipitate was dissolved in methanol (200 mL), reprecipitated as above, and redissolved in methanol. This was designated preparation A. Preparation A was washed in water (30 min with gentle agitation). The water-insoluble precipitate was recovered by filtration, redissolved in methanol, and designated preparation B. The amount of solids in each preparation was determined by evaporating an aliquot of methanol solution and weighing the remaining residue. Both DD and QTLC were used to determine the % saponins in each preparation and in the initial sample.

Calculations. Straight lines were fitted to the data using the least-squares method. All calculations were performed using the application Microsoft Excel 2000.

RESULTS AND DISCUSSION

A standard curve for direct densitometry is shown in **Figure 3**. The curve was linear over the range of 1.25 μg to 10 μg soyasaponin applied with a correlation coefficient of 0.99. This range of concentrations is comparable to that in previously reported QTLC (*8*, *9*), gas chromatography (*10*), and liquid chromatography (*11*) methods, and is more sensitive than colorimetric methods (*5*−*7*).

Table 1 indicates the precision and accuracy of the method; the coefficient of variation is less than 3%, and deviation from expected results is less than 4%, based on 3 to 4 determinations per day over 3 days.

Figure 4 demonstrates that there was a decline in spot density with time, but it was less than 5% in the first thirty minutes.

Table 1. Analysis of Known Soyasaponin Solutions

expected (μg saponin)	observed (μg saponin)	n [a]	C. V. (%) [b]	accuracy (%) [c]
7.5	7.77	10	2.2	3.6
12.5	12.68	12	2.8	1.5
17.5	17.35	10	2.4	−0.9

[a] Each determination represents the average density of two spots. [b] (Standard deviation) × 100/mean. [c] (Observed value − expected value) × 100/expected value.

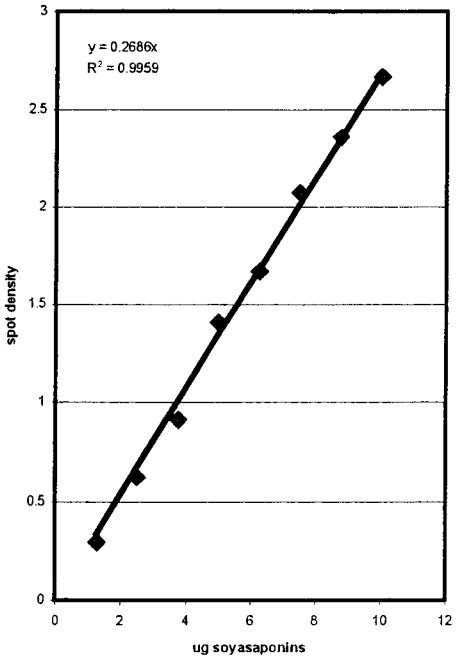

Figure 3. Standard curve for direct densitometry.

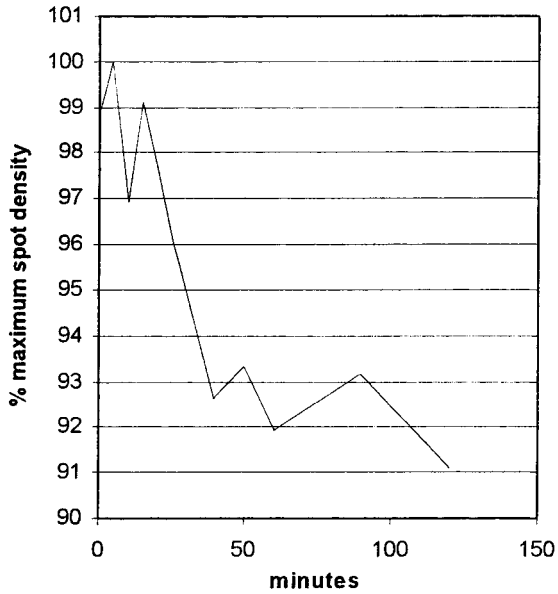

Figure 4. Decline in spot density with time.

As the scanning process was completed within this time frame, color instability was not an important source of error.

Figure 5 shows the standard curve for QTLC which was linear over the range of 0.625 to 5 μg soyasaponins applied and had a correlation coefficient of 0.99. The procedure for QTLC used here differed from that previously reported, in which *n*-butanol−ethanol−ammonia was used as a solvent system and

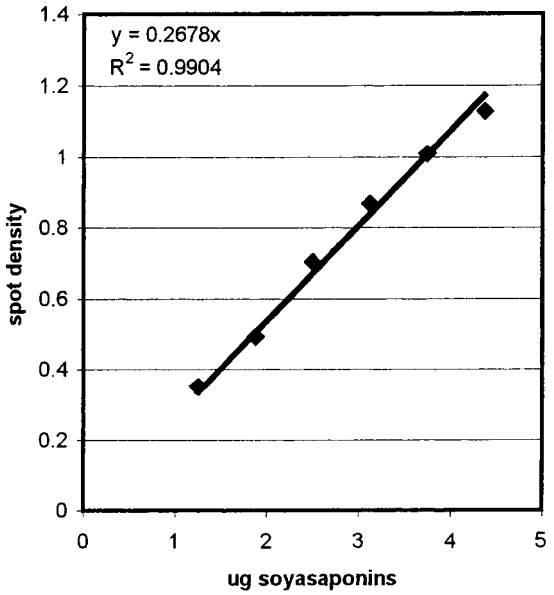

Figure 5. Standard curve for quantitative thin-layer chromatography of soyasaponins.

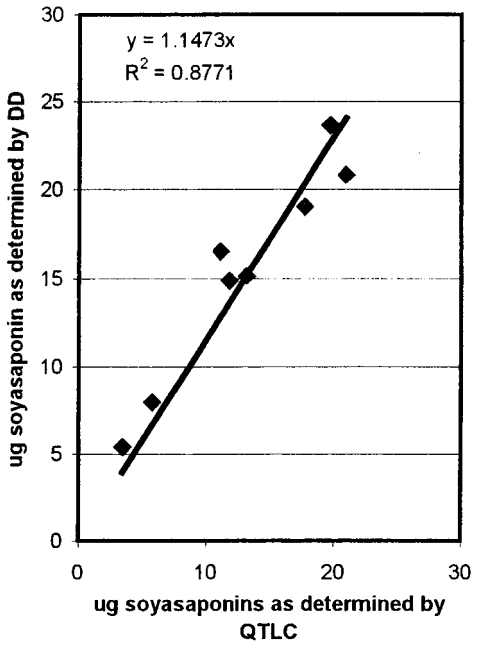

Figure 6. Comparison of quantitative thin-layer chromatography and direct densitometry.

the saponins were visualized with anisaldehyde (*8*). **Figure 6** compares the results obtained from QTLC and DD for several different saponin samples prepared by the methods described in the sections on legume analysis and the evaluation of the purity of commercial soyasaponin. This figure suggests generally good agreement between the two methods, although DD overestimated saponin content relative to QTLC by about 15%. This can be explained by the fact that when QTLC was used some of the minor saponin constituents present may have been separated into bands that were too low in concentration to be readily detected individually, but by DD these minor saponins were detectable in summation. It can also be argued that the discrepancy was due, at least in part, to the presence of nonsaponin contaminants which contribute to the total density measured by DD, but would not interfere with QTLC, which

Table 2. Percent Recovery of Soyasaponins from Cleanup Procedure

saponin standard solution	saponin standard solution in soy flour methanol extract (matrix)
97.3 ± 2.5[a]	102.6 ± 5.7[b]

[a] Four determinations. [b] Three determinations.

Table 3. Determination of Saponin Content of Legumes

legume	% saponin	% saponin literature (*4*)
defatted soy flour	0.58	0.47–0.53
dried navy beans	0.32	0.4–2.1
dried kidney beans	0.29	0.2–1.6

Table 4. Purification of Soyasaponin Preparations

preparation	% saponin[a]
commercial preparation (untreated)	69.8
preparation A	78.5
preparation B	97.5

[a] Mean of two determinations.

can specifically measure only the density of saponins. But thin-layer chromatography revealed no detectable contaminants using sample quantities 30% greater than those applied to the plates in DD, suggesting a negligible contribution from this source of error.

Figure 7 shows how the cleanup procedure applied to the methanol extracts of legumes effectively removes the major contaminants present when extracting saponins from legumes. In the first step, precipitation from a 1:1 methanol/0.4 ammonium sulfate removed contaminants from zone A (Lane 2). These have been previously identified as plant sterols, triglycerides, phospholipids, and flavonoids such as isoflavones (*12*, *13*). When the saponin-containing centrifugate of 1:1 methanol/0.4 ammonium sulfate was loaded onto the OASIS cartridge some contaminants from zone B were unretained (Lane 3). Additional washing with water further removed these and any residual ammonium sulfate (Lane 4). Saponins were then desorbed using basic methanol (Lane 5). Note that isoflavones were also detectable in this lane. The sample size, however, applied to this plate (50 μL) was 10 times that used in DD. Similar TLC of kidney bean and navy bean (not shown) gave almost identical results, except that kidney bean and navy bean contained no isoflavones. **Table 2** indicates that the cleanup procedure did not result in any losses of soyasaponins and there were no matrix effects.

Table 3 shows the analysis of beans and a comparison with the literature showing that results obtained here fell within or close to the range of results reported elsewhere.

Table 4 shows the usefulness of direct densitometry for determining the purity of saponin preparations. As expected, with each processing step the saponin preparation increased in purity.

To conclude, the procedure described here is a simple, rapid method for the determination of saponins from legumes. The approach of eliminating solvent development can be applied to any class of compounds if a very specific visualizing agent is available or a cleanup procedure can be developed that eliminates contaminants or reduces their concentrations below detectable limits.

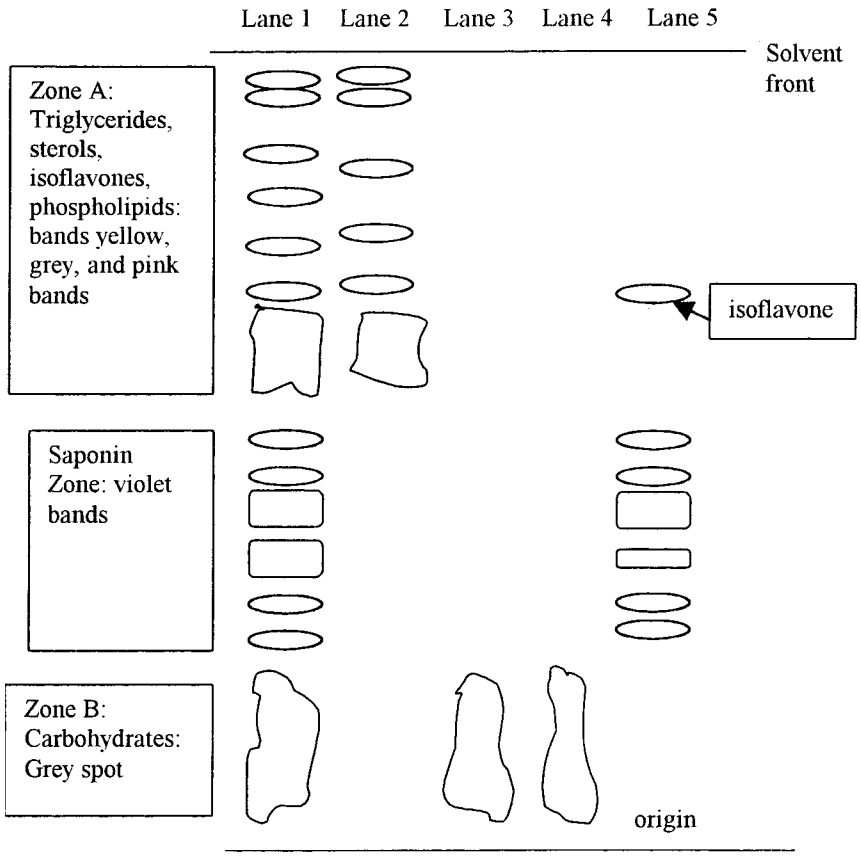

Figure 7. Removal of nonsaponins in each step of cleanup procedure.

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