Gossypol Analysis in Cottonseed Oil by HPLC

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Gossypol in cottonseed oil was selectively separated by the extraction of cottonseed oil with a hexane and N,N dimethyl formamide:water (2:1, v/v) solvent mixture. After filtration, the extract was injected into the HPLC with the elution time less than 15 min. The spectrophotometric method showed 2 to 5 times higher values of gossypol content in different types of glanded cottonseed oil than did the HPLC method. This is probably due to the gossypol derivatives and coloring interferences reacting with p-anisidine to develop color and increasing the absorbance reading, whereas gossypol was separated and detected in the HPLC method.

Total gossypol in crude cottonseed oils was first determined gravimetrically with aniline, which required several days to completely precipitate the dianilinogossypol (1). Several spectrophotometric methods have been reported. One method, based on the dianilinogossypol reaction, indicates that the reaction products of gossypol pigments in hydraulic-pressed oil have an absorption spectra different from that of screw-pressed oil and pure gossypol. This was attributed to a slight modification of the molecular structure of the gossypol during preconditioning prior to hydraulic pressing, although no experimental evidence was presented (2). The antimony trichloride reaction applied to alkaline extracts of screw-pressed oils gave reaction products which were not characteristic for gossypol, and no reaction products were obtained in alkaline extracts of hydraulic-pressed oils (3). A colorimetric method based on the reaction with p-anisidine was proposed for the analysis of gossypol pigments in cottonseed oils (4) and was accepted as an official method by the American Oil Chemists' Society (AOCS) (5). Although several spectrophotometric and gravimetric methods involving the use of aniline or p-anisidine have been developed for determination of total gossypol and gossypol-like pigments in cottonseed oils, none of these methods can distinguish the real gossypol from gossypol-like pigments. Berardi and Frampton (1957) developed an analytical method based upon the isolation of gossypol from oil by utilizing the acidic properties of gossypol, its water-insolubility, and its ability to form a water-soluble sodium salt (6). In this method, gossypol was extracted from cottonseed oil with aqueous sodium hydroxide solution, which contains a small quantity of sodium dithionite. The aqueous phase is separated, acidified and extracted with chloroform, and gossypol is determined spectrophotometrically at 366 nm. This method is selective, but lacks sensitivity when compared with the p-anisidine method. Abou-Donia et al. (1981) first developed the HPLC method to quantify gossypol in cottonseed extract using a reversed-phase HPLC with the solvent consisting of 0.1% phosphoric acid in methanol: water (80:20, v/v) as the mobile phase (6). Nomeir and Abou-Donia (1982) later improved this method by increasing the percentage of methanol in the mobile phase from 80 to 90% and eluting the sample at a higher flow rate (7). However, this HPLC method has not been used to determine gossypol in cottonseed oil, and a new mobile phase must be developed to prevent gossypol degradation when methanol is used as the mobile phase (8).

MATERIALS AND METHODS

Apparatus. The liquid chromatograph (Waters Associates, Milford, Massachusetts used consisted of an M-6000 pump, U6-K septumless injector, and a 10-μm Waters u-Bondapak C18 (3.9 mm × 30 cm) reversed-phase column. The sample was eluted isocratically with tetrahydrofuran (THF) and water (60:40, v/v) with 0.001 M phosphate buffer at pH 3.5 and a flow rate of one ml/min. Gossypol was detected by a Waters 440 UV detector at 254 nm with a sensitivity of 0.01–0.02 absorbance unit full scale (AUFS), depending upon the amount of gossypol injected. Chromatograms were recorded on a linear 1101 recorder (Linear Instruments Corp., Reno, Nevada) at a chart speed of 40 cm/hr. Peak heights (mm) at the common sensitivity of 0.01 AUFS were used for quantification.

A DU-6 spectrophotometer (Beckman Instrument, Inc., Irvine, California) was set at 440 nm.

Reagents. Gossypol standard was prepared by dissolving a known amount of standard gossypol (obtained as gossypol acetic acid from Southern Regional Research Center, ARS/USDA, New Orleans, Louisiana) in acetone to give a gossypol concentration range of one μg/ml to 100 μg/ml.

Glanded cottonseed oil was prepared by methylene chloride extraction and screw-press technique. Glandless cottonseed oil was prepared by hexane-extraction and screw-press technique. Refined peanut oil was purchased from a local store.

Solvent Mixture I was 2-propanol:hexane (60:40, v/v), and solvent mixture II was N,N dimethyl formamide:water (70:30, v/v). P-anisidine solution was made by adding purified p-anisidine to 48 ml of solvent mixture I and 2 ml of glacial acetic acid.

Experimental. Four cottonseed oil samples were analyzed for gossypol content by AOCS method Ca 13-56. Oil samples were weighed depending on the gossypol content and diluted with solvent mixture I. An aliquot of each sample was reacted with a p-anisidine solution at 75°C for one hr. The absorbance of p-anisidine derivative was then measured at 440 nm with the spectrophotometer against sample and reagent blanks. The corrected absorbance of the sample aliquot was then used to calculate the percentage of gossypol by using a calibration curve.

Cottonseed oils were also analyzed by two proposed HPLC methods. The first method was called the direct injection method. Glanded cottonseed oil (0.5 g) and glandless cottonseed oil (2.0 g) were weighed accurately and diluted to volume with THF in a 25-ml volumetric flask. The aliquot was then mixed well, filtered through a 0.45-μm filter and injected into the

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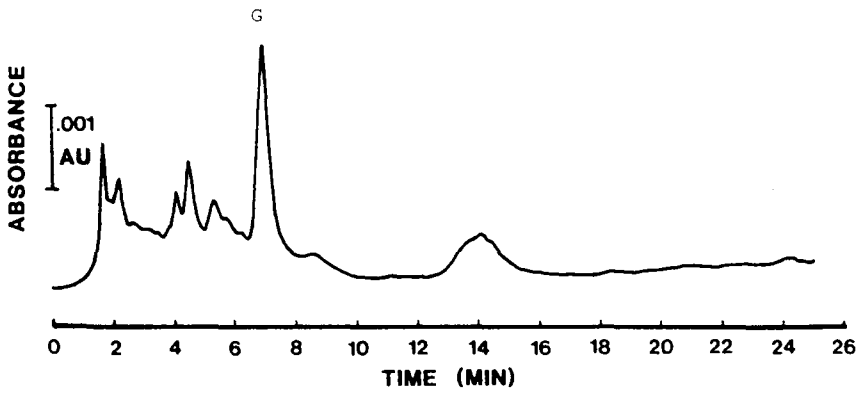

FIG. 1. HPLC chromatogram of screw-pressed glanded cottonseed oil analyzed by direct-injection technique. G, gossypol.

HPLC. To shorten the analysis time and to prevent the accumulation of triglycerides in the analytical column, the second HPLC method was developed. Glanded cottonseed oil (0.5 g) and glandless cottonseed oil (2.0 g) were weighed separately into a 125-ml Nalgene polypropylene screw-cap plastic bottle. The oil samples were mixed with 5.0 ml of hexane and 25.0 ml of solvent mixture II, shaken on a mechanical shaker at high speed for 10 min and then centrifuged at 100 × g for five min. The lower layer consisting of gossypol and solvent mixture was filtered through a 0.45-μm filter and injected into the HPLC.

For the recovery study, standard gossypol in acetone (0.35 mg) was pipetted into 125-ml plastic bottle. Five g of peanut oil was then added and the gossypol was allowed to dissolve. This was repeated for two glanded cottonseed oil (0.5 g) and two glandless cottonseed oil (2.0 g) samples in individual bottles. Each sample was then analyzed for gossypol content by the second HPLC method previously described.

RESULTS AND DISCUSSION

The over-estimation of gossypol in cottonseed oil by the spectrophotometric method was illustrated by Boatner et al. (1947). Crude screw-pressed and hydraulic-pressed cottonseed oils were extracted with aqueous alkaline solution to isolate gossypol from interfering non-acidic compounds. The alkaline extract was then acidified and reacted with antimony trichloride. The results indicated that none of the pigments present in these oils was gossypol. On the other hand, application of the aniline-spectrophotometric method showed the presence of gossypol in both oils. Because no gossypol could be isolated from these samples with aqueous alkali, it would seem that aniline reacted with pigments other than gossypol in the crude oil. In our early experiment, the direct injection technique was used to determine gossypol by the HPLC method. The sample was dissolved in THF and injected into the HPLC. The chromatograms of glanded cottonseed oil indicated that cottonseed oil contained a lot of coloring materials that were extracted along with gossypol during the oil extraction (Fig. 1). These impurities contribute to the color of cottonseed oil and may react with p-anisidine to develop color complex and to increase absorbance reading in the AOAC method.

The direct injection technique can reduce the sample preparation step. With the high efficiency of the analytical column, gossypol was separated from other coloring materials and determined quantitatively. However, due to the high concentration of oil in the sample, the analysis time must be extended to ensure that all nonpolar compounds, particularly triglycerides, are eluted before the next sample can be injected. This requires more than 25 min at 1.0 ml/min. Some samples also contained a high content of impurities and caused base line drift which made it difficult to quantify the peak of interest.

These two disadvantages of the direct injection technique lead to the present technique. By extracting gossypol from cottonseed oil in a hexane layer with the solvent mixture, most triglycerides and other interfering compounds were left in the hexane layer and allowed the gossypol to be determined by the HPLC within 15 min. The chromatograms of glanded cottonseed oil analyzed by two HPLC methods were compared (Fig. 2). Because the absence of very nonpolar compounds in the sample, the run time is shortened and the column life is extended. The advantage of the improved HPLC method can be clearly shown by the analysis of gossypol in glandless cottonseed oil (Fig. 3). With the direct injection technique, glandless cottonseed oil exhibited a complex chromatogram due to the interference; this made it difficult to identify and quantify gossypol. After all the triglycerides were extracted from the sample using the improved method, it was found that none of the pigments remaining in the glandless cottonseed oil was gossypol.

To verify that there are no losses of gossypol during the clean-up procedure, known amounts of standard gossypol were added and the samples were analyzed. The recovery ranged from 93 to 106% (Table 1). The analysis of solvent-extracted and screw-press cottonseed oil by the AOCS method and this improved HPLC method are shown in Table 2. The AOCS method showed two to five times higher gossypol content in solvent-extracted and scew-pressed glanded cottonseed oil than did the HPLC method. Gossypol derivatives including its by-product and coloring interferences may contribute to the color of cottonseed oil and possibly

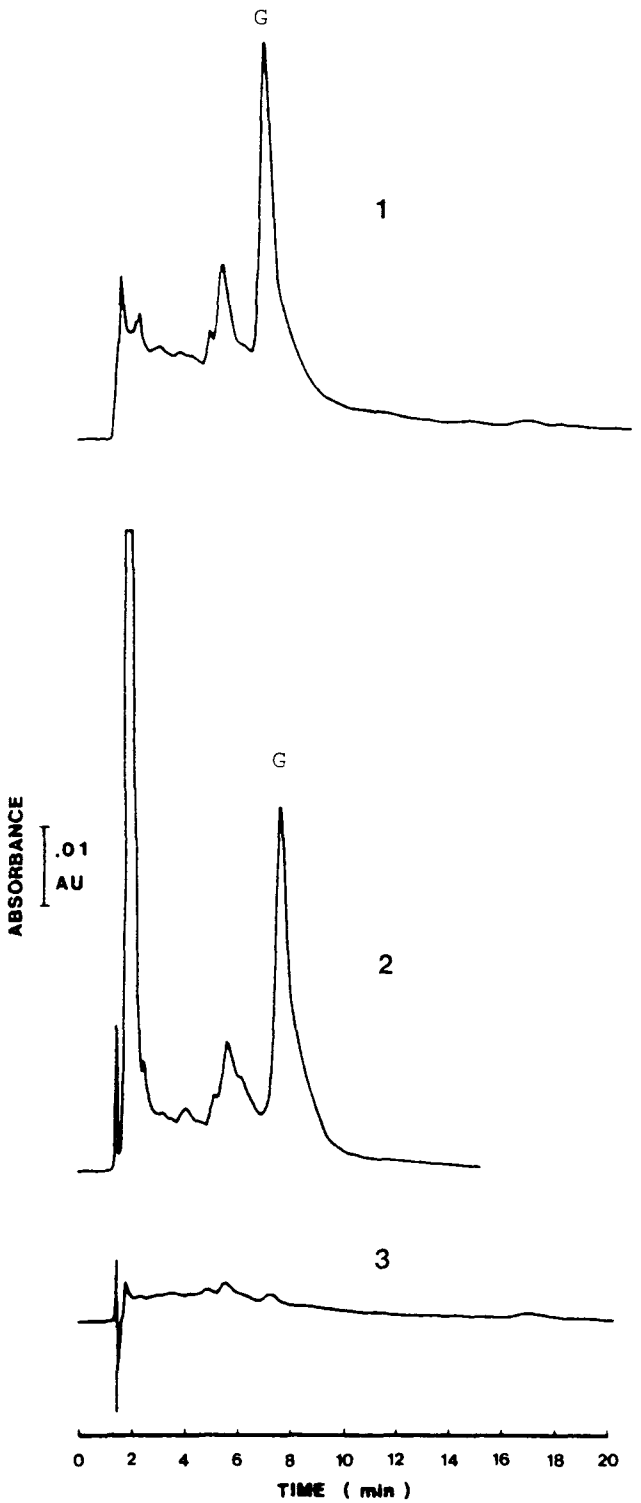

FIG. 2. HPLC chromatograms of 1) solvent-extracted glanded cottonseed oil analyzed by direct-injection technique; 2) solvent-extracted glanded cottonseed oil analyzed by the improved HPLC technique, and 3) hexane portion of G, gossypol.

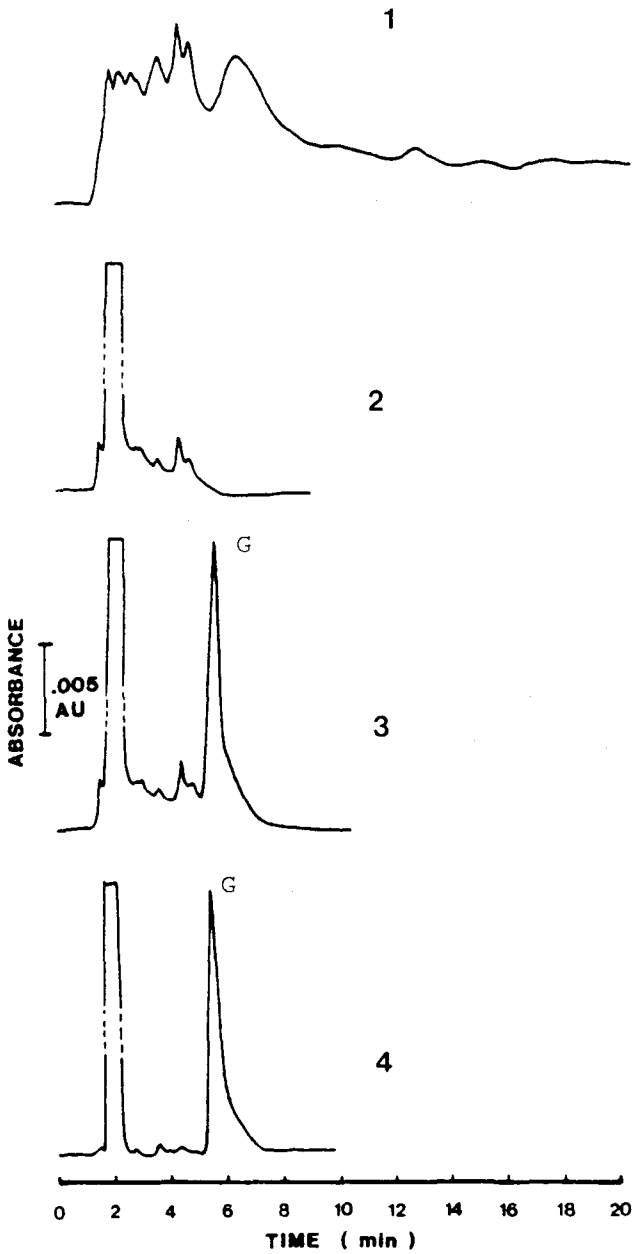

FIG. 3. HPLC chromatograms of 1) solvent-extracted glandless cottonseed oil analyzed by the direct-injection technique; 2) solvent-extracted glandless cottonseed oil analyzed by the improved HPLC technique; 3) gossypol-spiked solvent-extracted glandless cottonseed oil analyzed by the improved HPLC technique, and 4) standard gossypol in acetone analyzed by the direct-injection technique.

react the p-anisidine to increase the absorbance reading in the spectrophotometric method. On the other hand, gossypol was separated before detection in the HPLC method. Gossypol in glandless cottonseed oil is not detectable by either method. The solvent-extracted glanded cottonseed oil used in this experiment was obtained from a pilot plant trial using methylene chloride as the extracting solvent. Methylene chloride is more effective than hexane in extracting gossypol from cottonseed (9). This explains why the gossypol content of this solvent-extracted cottonseed oil was much higher than the screw-pressed sample. Gossypol was not detected in glandless cottonseed samples in this test. This indicates that gossypol is not the major coloring material in cottonseed oil, and gossypol content cannot be estimated by visual inspection.

TABLE 1

Recovery Data of the HPLC Method for Gossypol Determination

Sample	Recovery (%)[a]
Solvent-extracted glanded cottonseed oil	105 ± 2
Screw-pressed glanded cottonseed oil	93 ± 3
Solvent-extracted glandless cottonseed oil	104 ± 2
Screw-pressed glandless cottonseed oil	98 ± 2
Peanut oil	106 ± 2

[a]Average and standard deviation for triplicate analyses.

TABLE 2

Determination of Gossypol in Cottonseed Oil by the AOCS and HPLC Methods

Sample	Method	Replication	Gossypol (%)
Solvent-extracted oil	AOCS	4	1.39 ± .01
	HPLC	9	0.67 ± .01
Screw-pressed oil	AOCS	4	0.25 ± .01
	HPLC	9	0.05 ± .01

Total gossypol content in cottonseed oil can be determined simply by dissolving the oil sample in the THF, filtering and injecting into the HPLC. However, due to the high content of coloring materials other than gossypol and the presence of triglycerides, separation of the gossypol peak from interference was not acceptable. To improve the analytical technique, gossypol was separated from cottonseed oil by liquid-liquid extraction between cottonseed oil in a small amount of hexane and N,N dimethyl formamide:water (2:1, v/v) solvent mixture. Most triglycerides and some coloring materials were left in the hexane layer and gossypol partitioned to the bottom layer of the solvent mixture. After filtration, the extract was injected into the HPLC with a retention time of less than 15 min. The AOCS method showed higher values of gossypol content in glanded cottonseed oil than did the HPLC method. The gossypol derivative or by-products and coloring interference may contribute to the color of cottonseed oil and possibly react with p-anisidine to develop color and increase the absorbance reading, whereas gossypol was separated and detected in the HPLC method.

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