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Note

High-performance liquid chromatographic analysis of gossypol

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Gossypol is a yellow substance occurring in the pigment glands of the seed, leaf, stem and roots of the cotton plant[1]. Because of the unfavorable toxic effects of gossypol, only limited use has been made of cottonseed meal for monogastric animals. Gossypol is believed to exert its toxicity by uncoupling of respiratory chain-linked phosphorylation[2]. Recent interest in gossypol has been highlighted by a report that it has a contraceptive action for males in humans and other species, *e.g.*, rats, rabbits and rhesus monkeys[3].

A major concern in conducting research with gossypol is the purity of the chemical used. Gossypol is extracted from the seed kernels of the cotton plant and is isolated as gossypol–acetic acid complex[4]. Since other gossypol-related compounds are present in pigment glands, it is essential to develop an analytical procedure that ensures the purity of the product used[1].

Various analytical procedures have been used to determine gossypol: gravimetric[5], titrimetric[6] and spectrophotometric[7]. These methods are not specific since gossypol and other related pigments may react similarly to the same analytical reagents Chromatographic methods, including paper[8], thin-layer[9] and gas–liquid[10], have been developed to analyze gossypol. The paper and the thin-layer chromatographic procedures lack sensitivity, however, and the gas–liquid chromatographic method requires prior conversion to the trimethylsilyl ether derivative of gossypol.

High-performance liquid chromatography (HPLC) is a highly sensitive, selective, and relatively rapid technique to analyze compounds of similar structure. This report describes an HPLC method that was developed for quantitative and qualitative analysis of gossypol. The method was compared with the spectrophotometric procedure that is currently used for gossypol determination.

EXPERIMENTAL

Materials

Gossypol [(2,2′-dinaphthaline)-8,8′-dicarboxaldehyde-1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl] was obtained as gossypol–acetic acid from the

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Southern Utilization Research Development Division, USDA, New Orleans, LA, U.S.A. Stock and standard solutions were freshly made in methanol before use. All reagents and HPLC solvents were obtained from Fisher Scientific (Raleigh, NC, U.S.A.). Cottonseeds were supplied by Cotton (Raleigh, NC, U.S.A.).

High-performance liquid chromatography

A Waters Assoc. liquid chromatograph (Milford, MA, U.S.A.) consisting of two M6000-A pumps, an M660 solvent programmer, an M440 ultraviolet (UV) detector, a U6-K universal injection system and a reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D.) were used. Solvents were filtered through Millipore membrane filters, type HA or FH, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum prior to use. Gossypol was injected in 5–25 μl of methanol and eluted from the column either isocratically or by using gradient elution at room temperature. The solvent used in the isocratic elution consisted of 0.1% phosphoric acid in methanol–water (80:20). The solvents employed in the gradient elution were various mixtures of methanol and water (Table I) which were changed to methanol in 30 min; all solvents contained 0.1% phosphoric acid. The gradient shape employed was Waters No. 6, at solvent flow-rates listed in Table I. Gossypol was detected and quantitated by monitoring the UV absorbance of the column eluates at 254 nm. A reporting integrator (Shimadzu Chromatopak E1A, Shimadzu, Kyoto, Japan) was used to measure peak areas.

Spectrophotometry

Gossypol was determined spectrophotometrically using a Gilford Stasar II spectrophotometer (Gilford Instrument Labs., Columbia, MD, U.S.A.). The following reagents were used, as described previously[7]: (a) Solvent mixture: 715 ml 95% ethanol, 285 ml distilled water, 200 ml diethyl ether (peroxide-free) and 0.2 ml glacial acetic acid. (b) Aniline: Freshly distilled over approximately 1 g of 30 mesh granular zinc. The distillate was colorless. Standard solutions of gossypol in the solvent mixture or aliquots of cottonseed extracts (see below) were converted to dianilinogossypol by adding 1 ml of freshly distilled aniline and placing it into a water bath heated to 75°C for 40 min. After cooling, the solutions were diluted to 25 ml with the solvent mixture and determined at 445 nm using aliquots without added aniline as references.

Percent recovery

The reliability of the two procedures was determined by recovery experiments in which cottonseed samples were fortified with known amounts of standard gossypol.

Gossypol extraction from cottonseeds

Cottonseeds were finely ground using an electric grinder, type SHG (Markson Scientific, CA, U.S.A.). A 1-g sample of the ground cottonseeds was homogenized in 60 ml of the solvent mixture for 3 min using a Polytron ultrasonic homogenizer (Brinkmann, Westbury, NY, U.S.A.). The extract was filtered under reduced pressure through an even layer of Celite (2 g), over an asbestos filter paper disc placed in a 30-ml centered glass funnel. The flask and the sample in the funnel were washed with two successive 5-ml portions of the solvent mixture. The filtrate was diluted to 100 ml with the solvent mixture and mixed in a volumetric flask.

RESULTS AND DISCUSSION

One of the prime objectives of this study was to develop a simple analytical HPLC method that could be used for quantitative determination of gossypol. Gossypol has a complex molecular structure (Fig. 1), with six phenolic groups, two carbonyl groups, two naphthaline rings and two isopropyl and methyl hydrocarbon radicals. Therefore, it is both a hydrophobic and an ionizable compound. Our earlier attempts to analyze gossypol by HPLC resulted in a very broad peak that might have been attributed to its partial ionization. This problem was solved by the addition of 0.1% phosphoric acid to the eluting solvent.

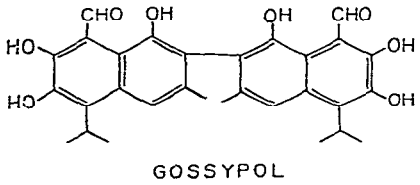

Fig. 1. Chemical structure of gossypol.

The retention times of standard gossypol and gossypol extracted from cottonseeds were in good agreement, *e.g.*, 5.68 and 5.72 min, respectively (Fig. 2). The relationship between the amounts of gossypol injected and the peak areas was linear over at least a 1000-fold range (10 ng–10 μg). Furthermore, detection by UV at 254 nm was very sensitive; the minimum detectable limit was 10 ng. The gossypol content of cottonseeds using this HPLC method was (mean $\pm$ S.E.) 0.64 $\pm$ 0.12% with a recovery of 95.0 $\pm$ 2.1%. It is noteworthy that the chromatogram obtained for the extracted cottonseeds contained an additional unidentified major peak, which accounted for 35% of the total extract, and a minor peak. Since the retention times of these peaks were 1.15 and 2.4 min respectively, they did not affect the analysis of gossypol.

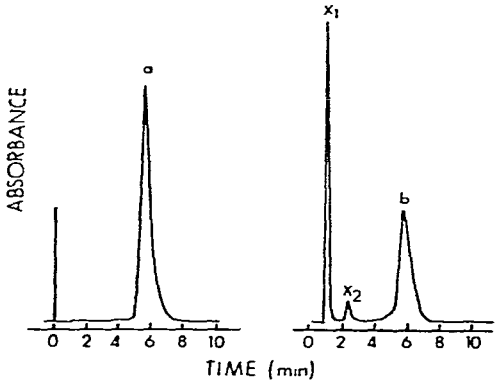

Fig. 2. HPLC chromatograms of standard gossypol (a) and of cottonseed extract showing gossypol peak (b) and two unknowns x_1 and x_2. Gossypol was analyzed on a μBondapak C_{18} column using an isocratic elution with a solvent consisting of 0.1% phosphoric acid in methanol–water (80:20). Quantification was performed by measuring UV absorbance at 254 nm.

Gossypol is unstable and undergoes degradation in biological[9] and non-biological[11] systems to products which are more polar than the parent compound. In order to resolve gossypol successfully from its degradation products in a single chromatographic step, it is necessary to have a longer retention time for gossypol. This could not be accomplished isocratically by decreasing the solvent strength (peak shape was poor and retention time was too long), but was achieved by the use of gradient elution technique (Table I).

TABLE I

CHANGES OF GOSSYPOL RETENTION TIMES AT DIFFERENT CONDITIONS

Column: μBondapak C_{18}.

Sample	*Elution solvent composition (methanol–water)*	*Flow-rate (ml/min)*	*Retention time (min)*
1	70:30	2.0	12.71
2	70:30	2.5	14.38
3	70:30	3.0	16.03
4	60:40	3.0	18.24
5	50:50	3.0	21.40

Experiments using the spectrophotometric method to analyze gossypol indicated that the conversion of gossypol to the dianilino derivative was dependent on the amount of aniline added. Gossypol content of cottonseed was determined spectrophotometrically to be 0.60 $\pm$ 0.1% with a recovery of 94 $\pm$ 4%. The relationship between the gossypol concentration and the optical density was linear over the range 25–200 μg and the minimum detectable limit was 25 μg.

Compared with the published spectrophotometric method, the HPLC method has numerous advantages. (1) It involves fewer steps, thus minimizing any technical errors. (2) It is 2500 times more sensitive, *e.g.*, the minimum detectable limits are 10 ng and 25 μg for HPLC and spectrophotometric methods, respectively. (3) It is specific and can be used for both qualitative and quantitative analysis of gossypol, while the spectrophotometric method may not distinguish between gossypol and some closely related metabolites or cottonseed pigments. (4) It is a non-destructive method, and the eluted gossypol can undergo further uniquivocal identification techniques such as infrared spectroscopy[9] and mass spectrometry[12].

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