Determination of Gossypol in Leaves and Flower Buds of *Gossypium*[1]

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Abstract

A method is described for the determination of free gossypol in cotton leaves with greater accuracy. The improved accuracy results from the elimination of the interference from chlorophyll and from quickly converting the extracted gossypol to the dianilino derivative. Methods for the determination of the free and bound gossypol in cotton flower buds are described.

Introduction

THE PRESENCE OF GOSSYPOL (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′-binaphthalene-8,8′-dicarboxalydehyde) in cottonseed (1,17) has presented problems to both the cottonseed crushing industry (3,4,9) and to the nutritionist (5,6,12, 17). The development of gland-free (and essentially gossypol-free cottonseed) cotton (7,10) was considered as a distinct step towards a solution of the problems associated with gossypol. Genetic studies relating to the development of gland-free cotton and observed susceptibility of glandless cotton to insect and rodent attack (2) indicated the need for more information on the gossypol content of leaves and flower buds. Methods for the determination of the gossypol content of cottonseed, cottonseed meal and cotton roots (8,13,14) were not satisfactory for the determination of gossypol in cotton leaves.

The intense green color of chlorophyll extracted from the leaves interfered with the spectrophotometric readings. In addition, the color intensity of the sample progressively increased after the reaction with aniline should have been complete, becoming a dark yellowish-brown overnight.

Treating the sample with 0.5 ml of concentrated HCl after moistening with 3 ml of 60% ethanol markedly decreased the intensity of the green color but did not prevent the progressive development of the yellowish-brown color believed to result from the oxidation of the excess aniline. This latter difficulty was eliminated by the addition of ascorbic acid.

These studies resulted in the modification of the spectrophotometric method (13) for the determination of free gossypol in cotton leaves and free and bound gossypol in cotton flower buds.

Experimental

Reagents and Equipment

Acetic acid, glacial reagent; aniline, freshly distilled over granular zinc, water-clear; ascorbic acid; ethanol, 95%; ether, peroxide-free; hexane, redistilled; hydrochloric acid, concentrated reagent; solution A (dilute 715 ml of ethanol to 1000 ml with distilled water, add 0.2 ml glacial acetic acid and 200 ml of ether); solution B, (dissolve 3 g of ascorbic acid in 45 ml of solution A); acid-washed Hyflo-Super-Cel (JAOCS *35*, 261, 1958); mechanical shaker, spectrophotometer.

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Procedure for Free Gossypol in Leaves

Air-dry, freeze-dry or oven-dry (in a forced-air oven not above 50C) the leaves and grind them to 40 mesh in a Wiley micro-mill and mix thoroughly. Store the ground samples in a refrigerator in tightly sealed bottles. Allow the samples to come to room temperature before weighing for analysis.

Transfer 0.1000 g of the prepared sample to a 250 ml glass-stoppered Erlenmeyer flask. To the sample add 3 ml of solution B and 0.5 ml of concentrated HCl and mix gently. Place approximately 20 ml of glass beads (6 mm) into the flask. After about 3 min for the decolorization of the chlorophyll, add 30 ml of ethyl ether, swirl the flask in warm water until the ether boils sufficiently to expel the air. Then insert the stopper with a twisting motion. Shake the flask vigorously for 10 min on a mechanical shaker. Filter the extract, under reduced pressure, through a layer of Hyflo-Super-Cel over a filter paper disc placed in a 25 ml Gooch crucible. Wash the flask and the filter with small portions of ethyl ether without pouring the small amount of water layer onto the filter. This procedure prevents the transfer of any anthocyanins into the filtrate. Transfer the filtrate to a 50 ml volumetric flask, (may dilute to 100 ml and use 10 ml aliquots) and make to 50 ml with solution A. Immediately pipet 5 ml aliquots in triplicate to 25 ml volumetric flask containing 5 ml of solution A. Quickly add 0.5 ml of freshly distilled aniline to two of the flasks, reserving the aliquot in the third flask for the reference solution. Place the flasks containing the sample and reference into a water bath heated to 75C for 40 min. Place a cap (vial) over the flask containing the reference solution to prevent contamination with aniline vapor. (The flasks may be heated gently on a steam bath, preferably under a hood, by placing them on the metal top not directly exposed to the steam.) After cooling, dilute reference and samples to 25 ml with solution A. Mix and determine the absorbance at 445 mμ using the aliquot without added aniline as a reference.

The gossypol content of the sample is calculated from a standard absorbance-concentration curve prepared by converting pure gossypol dissolved in solution A to the dianilino derivative.

Prepare the curve by dissolving 25 mg of gossypol in a few ml of ether in a 100 volumetric flask, dilute to 100 ml with solution A and mix. Dilute 10 ml of this solution to 100 ml in a volumetric flask with solution A and mix. From this, pipet aliquots in triplicate into 25 ml volumetric flasks covering a range of 0.025 to 0.200 mg of gossypol and dilute the smaller aliquots to 5 ml with solution A. Dilute one aliquot from each replicate to 25 ml with solution A, mix, and reserve as a reference solution. Convert the gossypol in the remaining aliquots to dianilinogossypol as previously described and read the absorbance at 445 mμ using the appropriate reference.

The gossypol content of the sample may be calculated from the following equation, if cells having a light path of 10 mm and a Beckman DU spectrophotometer are used:

TABLE I

Free Gossypol Content of Cotton Leaves and Percentage Recovery of 1 mg of Added Gossypol

Sample			Gossypol	
Species	Det. No.	Wt.	In leaves	Recovery of added
		g	%	%
G. thurberi (Raleigh)	1	0.1000	1.741	97.8
	2	0.1000	1.733	97.6
	3	0.1000	1.730	97.6
	Average	0.1000	1.735	97.7
G. thurberi (Fish Creek)	4	0.1000	1.430	91.4
	5	0.1000	1.405	96.6
	Average	0.1000	1.418	94.0
G. gossypoides	6	0.1000	0.382	91.1
	7	0.1000	0.340	93.3
	8	0.1000	0.395	90.4
	Average	0.1000	0.372	91.6

% gossypol in sample = absorbance × 1/a × 100/mg of sample in the aliquot used

a = absorbance/mg of gossypol as dianilinogossypol in 25 ml of solution A = 3.064.

Procedure for Cotton Flower Buds

Remove the bracts and calyx and dry the flower buds as described for leaves. Grind the buds to 40 mesh and store in a refrigerator.

Free Gossypol. To 0.1000 g of the ground flower buds placed in a Sorvall Micro Omni-Mixer jar, or similar homogenizer, add 2 ml of solution A, and let stand for 3 min. Then add an additional 20 ml of solution A. Homogenize for 2 min with the jar surrounded by ice water. Rinse the homogenizer blades and shaft with solution A dispensed from a wash bottle. Filter the extract under reduced pressure through a Gooch crucible in which a filter paper disc is covered with a layer of acid washed Hyflo-Super-Cel, and wash with solution A. Transfer the filtrate and washings to a 50 ml volumetric flask, dilute to volume and mix. Transfer 5 ml aliquots to 25 ml volumetric flasks in triplicate, add 0.5 of aniline to two of the flasks and heat them for 40 min as previously described. Dilute the third aliquot to 25 ml with solution A. Mix and reserve as the reference solution. After cooling, dilute the aniline treated aliquots to 25 ml with solution A, mix and read the absorbance at 445 mμ using the corresponding untreated solution as a reference. Calculate the gossypol content as described for leaves.

Bound Gossypol. Transfer the extracted residue from the free gossypol determination to a 250 ml glass-stoppered Erlenmeyer flask, add 2 ml of solution A and 2 ml of freshly distilled aniline and place on the metal top of a steam bath (not directly exposed to the steam) and heat for 45 min to convert the gossypol to dianilinogossypol. Remove the flask from the steam bath, add approximately 20 ml of 6 mm glass beads. Add by pipet 50 ml of redistilled hexane, warm the flask by swirling in hot water (60–70C) to expel the air, then insert the stopper with a twisting motion. Shake vigorously for 30 min on a mechanical shaker. With the funnel covered with a watch glass, filter a portion of the extract through a Whatman No. 4 filter paper into a small narrow-neck flask. Determine the absorbance of the filtrate at 440 mμ using hexane as the reference solution. If the extract is too concentrated to read the absorbance, dilute a suitable aliquot to 25 ml with hexane. Calculate the bound gossypol content of the sample from either a standard absorbance-concentration curve prepared from pure gossypol, or it may be calculated from the following equation (derived from absorbance when the gossypol per 25 ml is expressed in milligrams) if cells having a light path of 10 mm and a Beckman DU spectrophotometer are used:

% gossypol = absorbance × 2 × (1/a) × 100/weight of sample in mg.

a = absorbance/mg of gossypol as dianilinogossypol in 25 ml of hexane = 3.172.

If aliquots are diluted to 25 ml, the equation becomes:

% gossypol = absorbance × 1/a × 100/mg of sample in the aliquot used.

Prepare the standard absorbance curve by dissolving 25 mg of pure gossypol in 25 ml of ethyl ether in a 100 ml volumetric flask, then dilute to volume with hexane and mix. Transfer 10 ml of the solution to a 100 ml volumetric flask, dilute to volume with hexane, mix, and use as the gossypol standard. Take aliquots, convert to dianilinogossypol, and determine absorbance as described for leaves except hexane is used as the solvent and the absorbance is determined at 440 mμ.

Results and Discussion

Free Gossypol in Leaves

Results from the analysis of leaves from wild cotton and for the recovery of 1 mg of pure gossypol added to them before extraction are shown in Table I. The replicates are in reasonably good agreement and the recovery of the added gossypol ranged from 90.4% to 97.8%. A series of 60 samples in duplicate showed a mean difference of 0.031% between duplicates.

It was found that gossypol decreases at a rapid rate in extracts of cotton leaves or when added to leaf-extracts of other plant species. This occurs even after decolorization with hydrochloric acid. This loss is prevented if the gossypol is immediately converted to the dianilino derivative. Consequently, the extraction time is short and the filtration, dilution, taking of aliquots, and conversion to dianilinogossypol is done as rapidly as possible. Aliquots from three different samples taken 5 hr after extraction contained 71.3, 70.1, and 55.4%, respectively, of the amounts found when aliquots were immediately treated with aniline. Only 51.9, 67.6 and 64.0% of 1 mg of pure gossypol added to these samples before extraction was recovered from aliquots taken 5 hr after extraction. Similarly, from another cotton-leaf extract to which 1 mg of pure gossypol was added, aliquots treated with aniline at 0, 15, 30- and 60-min intervals 99.8, 93.6, 85.8 and 73% of the gossypol was recovered, respectively. These data indicate the necessity of rapid operations, which are carried out most efficiently by two analysts, one of whom does the extractions while the other carries out the filtrations, dilutions and converts the gossypol to the dianilino derivative.

Hydrochloric acid probably affects the intensity of the green color by removal of magnesium from the chlorophyll molecule (16). The ascorbic acid apparently prevents the oxidation of aniline by some constituent of the extract (11,15) and thus prevents erroneous high values for gossypol.

Cotton Flower Buds

Typical results for cotton flower buds are shown in Table II.

The removal of the bracts and calyx from the flower bud eliminates the parts that contain the greater part of the chlorophyll; consequently, the hydrochloric acid is not required. During the drying

TABLE II

Gossypol Content of Cotton Flower Buds

Sample No.	Determination No.	Gossypol		
		Free	Bound	Free + bound or total
		%	%	%
1[a]	1	0.010	0.024	0.034
	2	0.007	0.021	0.028
2[a]	1	0.000	0.011	0.011
	2	0.000	0.011	0.011
3	1	0.241	0.300	0.541
	2	0.274	0.273	0.547
4	1	0.281	0.331	0.615
	2	0.294	0.319	0.613
5	1	0.232	0.192	0.424
	2	0.215	0.157	0.372
6	1	0.516	0.536	1.052
	2	0.467	0.593	1.060
7	1	0.366	0.497	0.863
	2	0.323	0.526	0.849

[a] Glandless cotton plants.

of the samples, part of the gossypol apparently combines with some constituent or constituents of the flower bud and the amount varies somewhat with the temperature used in drying the samples. The free or uncombined gossypol is that gossypol which is extractable with the aqueous-ethanol-ether mixture and the bound or combined gossypol is that remaining in the residue after the aqueous-ethanol-ether extraction. The total gossypol values in Table II were obtained by adding the values shown in columns 3 and 4 for the respective samples. The precision of the methods is indicated by mean differences of 0.023, 0.025, and 0.017% between duplicate determinations of 63 samples of flower buds for free, bound and total gossypol (summation of free and bound) respectively. It is possible to determine the total amount of gossypol in flower buds by the method described for bound gossypol.

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