Improved Method for the Rapid Determination of Terpenoid Aldehydes in Cotton

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A simple, rapid method for the extraction of cotton terpenoid aldehydes from green tissues and seed is described. Samples were treated by ultrasonification with acidified acetonitrile/water followed by centrifugation. The resulting extract was injected directly onto a C_{18} HPLC column and no sample concentration or further cleanup steps were required. The level of gossypol, the most labile of the target analytes, decreased by only 2% after 12 h of storage at room temperature, thus enabling automated analysis of individual terpenoid aldehydes by HPLC. The method gives excellent reproducibility and enables large numbers of samples to be screened quickly and accurately.

Keywords: *Terpenoid aldehydes; gossypol stability; cotton; Gossypium; HPLC*

INTRODUCTION

Gossypol and a suite of related terpenoid aldehydes (TAs) are uniquely found in the lysigenous glands of cotton (*Gossypium* spp.) and other plants of the Malvaceae tribe Gossypieae Alefeld. The importance of these compounds in conferring resistance of cotton to insects, nematodes, and fungal diseases is well documented (*1*). Gossypol is also of interest as a male antifertility agent (*2*).

Many methods have been developed for the analysis of gossypol, including gravimetry (*3*), titration (*4*), paper chromatography (*5*), spectrophotometry (*6*, *7*), and thin-layer chromatography (TLC) (*8*). None of these methods will quantitatively resolve all of the TAs found in cotton. Bell and Stipanovic (*9*) devised a TLC method that enables rapid screening of cotton lines for TAs, but this method is at best semiquantitative. Satisfactory separation and quantification of individual cotton TAs has been achieved only with the use of high-performance liquid chromatography (HPLC) (*10*−*12*), gas chromatography (GC) (*13*), or nuclear magnetic resonance (NMR) instrumentation (*14, 15*). Of these, HPLC is the most applicable, because, unlike GC methods, it does not require derivatization, and it offers greater sensitivity and specificity than NMR. However, the published HPLC workup procedures still require much sample manipulation, with steps that include extract concentration and cleanup (*10*−*12*). In situations requiring a high throughput of samples, such as in the screening of lines for host-plant resistance potential, these procedures are time-consuming, must be carried out with considerable care to ensure good recoveries, and can entail substantial material costs. More importantly, as gossypol is very sensitive to air oxidation and readily forms acetals in alcoholic solutions, minimal exposure to air, catalytic surfaces such as silica, or alcoholic solvents is necessary to achieve accurate and reproducible results. Extracts must be analyzed promptly to avoid degradation of gossypol, precluding the use of an autosampler.

The objective of the current study was to develop a new extraction method for cotton TAs that is fast and reproducible, while yielding a more stable extract amenable to automated analysis.

MATERIALS AND METHODS

Chemicals. Ethanol (commercial grade) was purchased from CSR Ltd., Queensland, and filtered through a 0.5-μm membrane filter. All other organic solvents were HPLC grade from Mallinckrodt. MilliQ ultrapure purified water was used throughout. Phosphoric acid was AR grade from BDH Ltd., UK. Gossypol acetic acid and *tert*-butylanthraquinone were purchased from Sigma (NSW, Australia). Silica cartridges were Millipore Sep-Paks from Waters Associates.

Plant Materials. Samples of leaves (the first fully opened − usually node 3), squares (1/3−1/2 grown buds), and boll coats (carpel wall from 1/3−1/2 grown bolls) were taken from cotton (*G. hirsutum* and *G. barbadense*) plants grown in the field at the Cooperative Research Centre for Cotton Research, Myall Vale, NSW, Australia.

Preparation of External Calibration Standards. Hemigossypolone (HGQ), 7-methoxyhemigossyplone (MHGQ), and heliocides H_1 and B_1 were isolated from plant sources, as described by Bell et al. (*16*) and Stipanovic et al. (*17*, *18*). These compounds and commercially prepared gossypol (G) (as gossypol acetic acid) were dissolved in acetonitrile/water/phosphoric acid (80:20:0.1; solvent 1). Standard curves were obtained for G, HGQ, MHGQ, H_1, and B_1 with concentrations in the range of 3−113 mg L^{-1} in 6 increments. The standard curves for H_1 and B_1 were used for H_1−H_4 and B_1−B_4, respectively.

Internal Standard. *tert*-Butylanthraquinone was evaluated as an internal standard. Each of the TA standards (2−3 mg) and *tert*-butylanthraquinone (2 mg) was dissolved in solvent 1 (10 mL). A series of dilutions was made to give internal standard concentrations from 5 to 200 mg L^{-1}, to test the reproducibility of response factors over this range. For quantification by internal standard, a solution containing 25 mg L^{-1} of *tert*-butylanthraquinone in solvent 1 was used in

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Table 1. Comparison of the Conventional Extraction Methodology with a New Improved Rapid Extraction Method

	levels of TAs in lyophilised cotton powder[a] (μg/g of dry wt)								
	HGQ	MHGQ	G	H4	H1	H3	H2	B4	B1
Siokra 1-4 leaf									
method 1	2560	nd[b]	846	1240	2020	360	913	nd	nd
method 2	3010	ND	929	1450	2170	390	1090	ND	ND
Siokra 1-4 square									
method 1	438	nd	2780	940	1530	271	705	nd	nd
method 2	585	nd	3620	1210	1850	351	989	nd	nd
Siokra 1-4 boll coat									
method 1	265	nd	582	2880	4600	355	781	nd	nd
method 2	331	nd	701	3450	5210	396	952	nd	nd
Pima S-7 boll coat									
method 1	261	1590	94.6	1020	1670	trace	trace	1980	3000
method 2	404	1930	114	1160	1840	trace	trace	2080	3130

[a] Data are means of 3 to 10 replicates. [b] Not detected.

place of solvent alone to extract lyophilised cotton samples as per method 2.

Sample Extraction. *Method 1.* This method, which was modified from those of Mahoney and Chan (*11*) and Stipanovic et al. (*12*), has been used for several years at CSIRO Division of Entomology, Narrabri, NSW. The finely ground, lyophilised cotton samples (100 mg) were weighed into centrifuge tubes and extracted by ultrasonification (3 min) with solvent 2 (12 mL) (hexanes/ethyl acetate/acetic acid (500:500:1). The samples were centrifuged (3 min at 2800*g*) and an aliquot of each (10 mL) was evaporated under vacuum. The residue was redissolved in solvent 2 (1 mL) and transferred to a silica Sep-Pak. The Sep-Pak was dried with N_2 gas and washed with 5 mL (leaf and square) or 7.5 mL (boll coat) 2-propanol/acetonitrile/water/ethyl acetate (35:21:39:5; solvent 3). An aliquot of the supernatant was transferred to an autosampler vial.

Method 2. The ground, lyophilised cotton samples (100 mg) were weighed into centrifuge tubes and extracted by ultrasonification (3 min) in solvent 1 (10 mL). The samples were centrifuged (3 min at 2800*g*), and an aliquot of the supernatant was transferred directly into an autosampler vial.

Recovery of Gossypol. Gossypol acetic acid was dissolved in solvent 1 to give 38 mg L^{-1} gossypol. Lyophilised cotton square samples (100 mg) were spiked with this solution (200 μL) so as to approximately double the gossypol concentration, and the samples were extracted by method 1 or 2.

HPLC Analysis. All samples were analyzed on a Hewlett-Packard 1090 high-performance liquid chromatograph equipped with diode array detector and autoinjector (fitted with a 20-μL loop). Samples were isocratically eluted from a 150 × 3.9 mm i.d. Waters (4 μm) C_{18} Novapak column maintained at 40 °C. The mobile phase was the same as that used by Stipanovic et al. (*12*) and was helium purged. Solvent flow rate was 1.0 mL min^{-1} and total run time was 30 min. The signal was monitored at 272 nm. Data collection and integration were performed on Hewlett-Packard Chemstation software revision A.03.01.

RESULTS AND DISCUSSION

Earlier work at this laboratory had established that solvent 1 was ideal for extracting gossypol from cotton seeds (*19*). The objective of the current study was to evaluate its suitability as an extracting solvent for the green tissues in cotton, which contain a range of TAs in addition to gossypol. A variety of lyophilised green cotton tissues were selected for the comparison, to provide a range of sample matrixes and TA profiles. Leaves of Upland cotton (*G. hirsutum*) are richest in hemigossypolone, squares are richest in gossypol, and boll coats are richest in heliocides H_1 and H_4 (*12, 20*). *G. barbadense* boll coats have low gossypol levels and are high in methoxylated TAs, which are absent from *G. hirsutum* glands (*16*). Leaf, square, and boll coat samples from a commercial Australian *G. hirsutum* variety (Siokra 1-4/649) and boll coat samples from *G. barbadense* (Pima S-7) were extracted using both methods.

Table 2. Method 2 Reproducibility for TA Determinations

	levels of TAs in lyophilised cotton powder (μg/g of dry wt)					
Siokra 1-4 Square	HGQ	G	H4	H1	H3	H2
1	614	3720	1310	1890	369	1010
2	619	3850	1320	1960	392	1060
3	577	3690	1260	1880	374	1050
4	577	3600	1150	1800	316	959
5	550	3540	1230	1860	384	965
6	567	3590	1160	1790	332	933
7	538	3440	1130	1760	312	960
8	588	3650	1180	1840	336	1000
9	586	3640	1200	1840	368	984
10	631	3520	1160	1830	326	971
mean	585	3620	1210	1850	351	989
CV	5.09	3.18	5.58	3.08	8.43	4.15

Pima Boll Coat	HGQ	MHGQ	G	H4	H1	B4	B1
1	413	2020	127	1250	1930	2220	3310
2	362	1950	108	1130	1810	2090	3180
3	378	1820	121	1100	1750	1980	2960
4	408	1880	116	1130	1760	2000	2970
5	415	1960	96.7	1210	1880	2130	3220
6	445	1960	114	1170	1870	2090	3140
mean	404	1930	114	1170	1830	2090	3130
CV	7.30	3.65	9.30	4.83	3.92	4.20	4.47

The results obtained are shown in Table 1. For each type of substrate, and every TA detected, method 2 always extracted higher concentrations (4−55%) than those obtained with method 1. In part, this is due to the elimination of sample transfer, evaporation, and cleanup steps, but it is primarily a function of the higher extraction efficiency of the more polar solvent used. This is borne out by the observation that the discrepancy between the two methods is most marked with respect to levels of the most polar compound, hemigossypolone, and least marked with respect to the relatively nonpolar methoxyheliocides B_1 and B_4.

The reproducibility of method 2 was evaluated using Siokra 1-4/649 squares and Pima S-7 boll coats, as demonstrated in Table 2. Coefficients of variation were uniformly lower than those obtained using method 1 (data not shown), reflecting the minimal sample manipulation and hence lower experimental error inherent in method 2. Gossypol recoveries by both extraction procedures were in excess of 90%, with method 2 giving recoveries above 95% (Table 3).

The most marked difference between the two extraction procedures was in the stability of gossypol in the extracts (Table 4; Figure 1). Extraction of cotton square

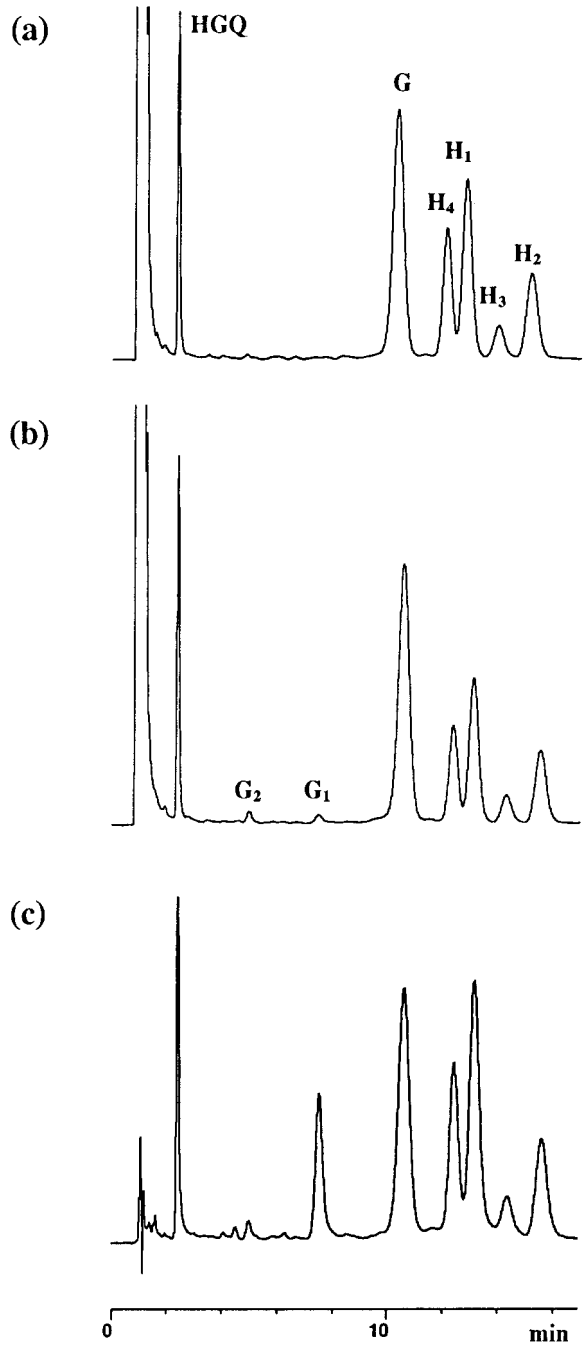

Figure 1. HPLC chromatograms of (a) cotton square sample freshly extracted by method 2; (b) cotton square sample extracted by method 2, after 12 h at 23 °C; (c) cotton square sample extracted by method 1, after 2 h at 23 °C (HGQ = hemigossypolone; G = gossypol; H1, H2, H3, and H4 = heliocides 1, 2, 3, and 4 respectively; G1 and G2 = gossypol breakdown products).

powder using method 1 resulted in observable gossypol breakdown within 15 min of preparation at room temperature. Wang (*2*) made similar observations when extracting with a solvent mixture that included ethanol. The rate of gossypol breakdown varied widely between samples, with between 18% and 57% remaining at 18 h post extraction.

Samples extracted by method 2 did not show a significant decline in gossypol levels, with up to 97% remaining at 18 h and low variance (Table 4). Nomeir and Abou-Donia (*21*) found that gossypol was more stable in acetonitrile and other nonalcoholic solvents than in either methanol or ethanol, but did not attempt to identify the degradation products. Experiments at this laboratory have shown that gossypol reacts with alcohols to form mono- and di-acetal structures initially, later degrading to more polar compounds (G_1 and G_2 in Figure 1b,c), and that this occurs far more rapidly in cotton extracts than in pure gossypol solutions. Acetonitrile extracts may be more stable because reactive hemiacetals are not formed. Whereas the mobile phase used here contained methanol and ethanol, gossypol is not retained on the column long enough to show adverse effects.

Table 3. Recovery of Gossypol Added to Cotton Square Samples

	% recovery determination				
	1	2	3	4	mean ± SD
method 1	93.1	95.2	92.0		93.4 ± 1.6
method 2	97.6	99.8	98.6	95.7	97.9 ± 1.7

Table 4. Stability of Gossypol in Cotton Square Extracts at 23 °C

hours post extraction	% gossypol remaining (mean ± SD)	
	method 1	method 2
1	95.6 ± 6.0	99.8 ± 1.2
4	78.3 ± 6.1	99.4 ± 2.8
8	60.5 ± 13	98.6 ± 1.3
12	48.4 ± 14	97.6 ± 1.7
18	34.8 ± 18	96.2 ± 1.3

Table 5. Response Factors of Terpenoid Aldehyde Standards

terpenoid aldehyde	response factor	standard deviation
HGQ	0.82	0.02
MHGQ	0.92	0.03
G	1.54	0.04
H_1	1.21	0.01
B_1	1.43	0.04

The elimination of sample transfer, concentration, and cleanup steps, all of which contribute to loss of sample, lessens the need for an internal standard. However, where analyses involve large numbers of samples of different chemical composition and matrix characteristics, an internal standard has clear utility, although most HPLC protocols employ external calibration. Mahoney and Chan (*11*) describe the synthesis and use of *p*-bromophenacyl undecanoate as an internal standard. This compound is not ideal, as it is not commercially available and must be synthesized, it elutes long after the heliocides on a C_{18} column, and it is chemically unrelated to the analytes. In the present study, a number of compounds containing naphthaquinone structures were evaluated for use as internal standards. *tert*-Butylanthraquinone was selected as a suitable internal standard because it is readily available at high purity, is cheap, elutes conveniently between MHGQ and G, and responds similarly to the TAs. Acetonitrile solutions of *tert*-butylanthraquinone did not degrade after 12 months at room temperature. Table 5 shows response factors (together with their standard deviations) for each TA standard relative to *tert*-butylanthraquinone.

CONCLUSION

The use of acetonitrile/water for extracting cotton TAs provides a fast, simple alternative method for sample preparation for HPLC. Sample cleanup is unnecessary

and a substantial number of samples can be prepared in a day. The method is reproducible and yields extracts that are stable for overnight runs with an autosampler.

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