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Factors Interfering in Gossypol Analysis of Okra and Glandless Cottonseed Using Direct Aniline Extraction

Robert D. Stipanovic,* Janet C. Donovan, Alois A. Bell, and Franklin W. Martin

Oxidation products of hydroxylated unsaturated fatty acid triglycerides give a false positive reading for gossypol with aniline and phloroglucinol in okra and cottonseed. Analysis using dianilinogossypol rather than gossypol allows the determination of both free and bound gossypol. Dianilinogossypol could be detected on TLC plates in seed containing 20 ppm of gossypol. When this technique was used, gossypol could not be detected in the nine cultivars of okra seed and one cultivar of glandless cottonseed tested. The hydroxylated fatty acids were readily detected in seed extracts with ^{13}C NMR spectroscopy.

The search for new, plentiful sources of protein has prompted studies on okra seed, *Abelmoschus esculentus* (Karakoltsidis and Constantinides, 1975; Martin and Ruberte, 1979; Martin et al., 1979). Analyses indicate that okra seed has a high-quality protein and an oil with many desirable characteristics.

Okra and cotton are both members of the family Malvaceae. Most commercial cottonseed contains a toxic terpenoid aldehyde, gossypol. Because of gossypol, cottonseed has been used principally as an animal feed supplement rather than as a food supplement for humans. Seed from glandless cotton cultivars are considered to be free of gossypol, and they are suitable for human consumption. The occurrence, chemistry, and toxicology of gossypol has been reviewed (Berardi and Goldblatt, 1980).

Karakoltsidis and Constantinides (1975) reported the presence of small amounts (30 ppm) of gossypol in okra seed. They analyzed for gossypol using the method of Storherr and Holley (1954) in which the seed is extracted with a 2-butanone–water azeotrope containing 0.5% aniline. Gossypol was quantitated colormetrically by reaction with phloroglucinol.

We examined okra seed extracts using thin-layer chromatography (TLC) and failed to locate any gossypol. This observation prompted the present study of okra, cotton, sunflower, and soybean seeds for the presence of compounds that may give a false positive reading with the aniline or phloroglucinol test for gossypol.

MATERIALS AND METHODS

Baker silica gel plates (7GF) (0.5 mm thick), pH 6 or normal, were prepared from 50 g of gel and 100 mL of citrate buffer (pH 6) (Sorensen, 1970) or 100 mL of distilled water, respectively. Okra seed (cultivars: Red Okra, Dwarf Green Long Pod, Clemson Spineless, Evergreen Velvet, Green Velvet, Perkins Spineless, Red River, White Velvet, and Pusa Sawami), cottonseed (glandless, Paymaster 464; glanded, CAM-E), fresh soybean seed, and sunflower seed obtained from a local market were ground in a blender to a fine powder. Seeds from the glandless cotton cultivar Paymaster 464 were cut open and visually examined for glands. All seeds containing glands were discarded. Clemson Spineless and Dwarf Green Long Pod okra seed were cut in half and examined microscopically for glands. Hydroxylated fatty acids were isolated from

National Cotton Pathology Research Laboratory, U.S. Department of Agriculture, Agriculture Research Service, College Station, Texas 77841 (R.D.S., J.C.D., and A.A.B.), and Tropical Agriculture Research Station, U.S. Department of Agriculture, Agricultural Research Service, Mayaguez, Puerto Rico 00709 (F.W.M.).

the okra cultivar Clemson Spinelss or the cotton cultivar Paymaster 464. The okra seed and cottonseed meal (0.5 g) were treated with aniline according to the method of Smith (1958), except that samples testing less than 500 ppm of gossypol were treated with 1 mL rather than 2 mL of aniline. After extraction and filtration, the chloroform was removed by rotoevaporation. The contents of the flask were transferred to a 2-mL volumetric flask and diluted to 2 mL with chloroform. Baker silica gel plates (pH 6) were spotted with 20 μL of this solution and developed with hexane–EtOAc–HOAc (49.75:49.75:0.5). Dianilinogossypol, prepared by the method of Smith (1958), was also spotted as a control. Dianilinogossypol appeared as a visible yellow spot (R_f = 0.68). Hydroxylated fatty acid triglycerides were observed at R_f = 0.75. The silica gel plates were sprayed with a 1:1 solution of 5% phloroglucinol in 95% ETOH and concentrated HCl.

Okra seed and glanded cotton seed, containing known concentrations of gossypol as determined by the aniline method, were mixed in various proportions to simulate gossypol concentrations of 700, 500, 400, 300, 200, 100, 50, 20, and 10 ppm of gossypol. Dianilinogossypol derived from these mixtures and dilutions of these extracts were developed on Baker silica gel plates (pH 6).

The hydroxylated fatty acid triglycerides were extracted from okra or glandless cotton seed meal with CH_3CN. The triglycerides that gave a positive reaction with phloroglucinol were purified by elution from a silica gel (Baker, 40–140 mesh) column with increasing amounts of ether in hexane (2:98–50:50) and submitted to TLC purification [Baker 7GF, $CHCl_3$–$(CH_3)_2CO$–HOAc, 89:10:1, solvent 1]. The resulting mixture of triglycerides were hydrolyzed with 10% KOH in MeOH (60–65 °C/10 min under N_2). The fatty acids were esterified with CH_2N_2 and purified by TLC (Baker, solvent 1). The four hydroxylated fatty acid methyl esters (HFAME) were further purified and quantitiated by HPLC on silica gel (hexane–absolute EtOH, 99.25:0.75) (Chan and Levett, 1977).

RESULTS AND DISCUSSION

Microscopic examination did not reveal glands in the okra seed examined. TLC analysis of the okra and the glandless cottonseed tested failed to show any evidence of dianilinogossypol. Dianilinogossypol was evident in extracts of glanded cottonseed. Dianilinogossypol and its corresponding spot from glanded cottonseed (R_f = 0.68) rapidly turned magenta when sprayed with phloroglucinol. All of the seed meals, including sunflower and soybeans, had a spot with an R_f (0.75) slightly higher than that of dianilinogossypol. This spot gave a false positive reaction when sprayed with phloroglucinol (red color) but did so more slowly than dianilinogossypol. However, if the plates were allowed to stand for several minutes after the solvent

Table I. ^{13}C NMR Chemical Shifts of 13-Hydroxy-cis-9,trans-11-octadecadienoic Acid Methyl Ester[a]

C no.	δ	C no.	δ	C no.	δ
1	174.0	7	29.5	13	72.6
2	33.9	8	27.5	14	37.2
3	24.7	9	132.4	15	25.0
4	28.9	10	125.4	16	31.6
5	28.9	11	127.8	17	22.4
6	29.3	12	135.9	18	13.8
				OCH$_3$	51.2

[a] Reported in ppm downfield from Me$_4$Si with the central peak of CHCl$_3$ (δ 76.9) as an internal standard.

evaporated, both spots changed colors at about the same rate.

The ^{13}C NMR of the compounds comprising the spot at R_f = 0.75 indicated they were a mixture of fatty acid triglycerides. In addition to the two peaks at δ 68.8 and 62.0, indicative of the two sp^3 types of C–O bonds in a triglyceride, a third sp^3 C–O bond at δ 72.7 was also evident. Base hydrolysis and methylation with CH$_2$N$_2$ gave a UV-absorbing spot on TLC analysis that slowly turned red when sprayed with phloroglucinol. The ^{13}C NMR spectrum of this compound indicated a mixture of compounds with an sp^3 C–O bond at δ 72.6. The mass spectrum of the mixture gave a parent peak at m/z 292 indicative of C$_{19}$H$_{32}$O$_2$, formed by the loss of H$_2$O from a mixture of isomeric fatty acid methyl esters containing two carbon–carbon double bonds and an alcoholic group.

Various seed oils, including cottonseed oil, have been reported to contain 13-hydroxy-cis-9,trans-11-octadecadienoic acid, 13-hydroxy-trans-9,trans-11-octadecadienoic acid, 9-hydroxy-trans-10,cis-12-octadecadienoic acid, and 9-hydroxy-trans-10,trans-12-octadecadienoic acid (Chrisholm and Hopkins, 1960, 1965; Morris et al., 1960; Tallent et al., 1966; Powell et al., 1967; Yunusova and Glushenkova, 1976; Kadyrov et al., 1976). HPLC analysis of our compound agreed with previously published chromatograms for these HFAME (Chan and Levett, 1977). Peak 1 from this chromatogram was collected. Its UV and ^{1}H NMR spectra agreed with published spectra (Tallent et al., 1966). Its ^{13}C NMR spectrum is given in Table I. Each of the HFAME gave a delayed red color when sprayed with phloroglucinol.

When the HFAME are carefully purified and reacted with aniline in the same manner as seed meal, a very weak aniline positive test results. However, if HFAME are allowed to stand for several days, a strong positive test results. Thus, it appears that some degradation product(s) of the HFAME, probably oxidative, is (are) the compounds reacting with aniline or phloroglucinol. This is in agreement with the observed induction period when the HFAME are sprayed with phloroglucinol on TLC plates.

Sunflower and soybean seeds contain no gossypol yet they give small positive reactions with aniline (Table II). This is in agreement with earlier reports that other meals (e.g., alfalfa, yellow corn, barley, oats, and wheat) contain substances that either react with the reagent or absorb at the wavelength at which measurements are made (Storherr and Holley, 1954).

TLC analysis of okra seed meal mixed with glanded cottonseed meal containing known concentrations of gossypol, as determined by the aniline method, indicated that 250 ng of dianilinogossypol could be detected on TLC plates. Dianilinogossypol in extracts from 0.5 g of mixed

Table II. Gossypol or Apparent Gossypol in Seed As Determined by the Aniline Test

plant	G, ppm
okra	
Dwarf Green Long Pod	120
Evergreen Velvet	120
Green Velvet	120
Perkins Spineless	120
Red	120
Red River	120
White Velvet	150
Pusa Sawami	250
Clemson Spineless	420
cotton	
Paymaster 464 (glandless)	120
CAM-E (glanded)	9000
sunflower	10
soybean	60

meals containing 50 ppm of gossypol was readily detected, and careful examination of plates could detect concentrations as low as 20 ppm.

The results indicate that low aniline positive readings do not absolutely confirm the presence of gossypol in seed from plants of the Malvaceae family and that degradation products of hydroxylated unsaturated fatty acid triglycerides may be the cause of these false positive readings. Analysis of the okra and the truly "glandless" cottonseed examined in this study indicate a total "gossypol" concentration below 20 ppm. Thus, seed in which gossypol is thought to be present in very low amounts should be analyzed by alternate methods. Since the seed is extracted directly with aniline, the method described here allows the determination of both bound and free gossypol and thus offers an advantage over some other methods. For example, simple solvent extraction of seed allows the determination of only the free gossypol. ^{13}C NMR appears to provide a quick method for analyzing HFAME in seed oil.

Registry No. Gossypol, 303-45-7; dianilinogossypol, 6952-36-9.

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