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A Quantitative Method for the Determination of Cyclopropenoid Fatty Acids in Cottonseed, Cottonseed Meal, and Cottonseed Oil (*Gossypium hirsutum*) by High-Performance Liquid Chromatography

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Cyclopropenoid fatty acids (CPFAs), found in cottonseed, have been shown to have detrimental health effects to susceptible livestock. Previous quantitative analytical methods for the determination of CPFAs expressed these acids in terms of their relative abundance with respect to other fatty acids in the oil, necessitating the concurrent analysis of other fatty acids. The proposed analytical method describes the quantitation of three relevant CPFAs for cotton (malvalic acid, sterculic acid, and dihydrosterculic acid) in cottonseed in micrograms per gram fresh weight of sample. The method involves extraction of the oil, saponification, and derivatization of the free fatty acids with 2-bromoacetophenone to give the phenacyl esters. These esters are then separated by dual-column reverse-phase high-performance liquid chromatography and quantitated via external standards. This is the first method to include external calibration standards for CPFAs and, as such, is capable of direct quantification with no further data conversion required. CPFA data generated from the analysis of cottonseed, cottonseed meal, and cottonseed oil produced in the United States in 2002 are presented.

KEYWORDS: Cotton (*Gossypium hirsutum*); cyclopropenoid fatty acid; malvalic acid; sterculic acid; dihydrosterculic acid; composition; HPLC

INTRODUCTION

In cotton, there are three primary cyclopropenoid fatty acids (CPFAs), dihydrosterculic, malvalic, and sterculic acids. It has been suggested that they play both antifungal (*1*) and antifeedant (*2*) roles. The presence of these acids can interfere with fatty acid desaturases in animals that include cottonseed products in their diet (*3*−*5*). In poultry, inclusion of cottonseed products in feed causes discoloration of the egg yolk (*6*, *7*). In rats, CPFA ingestion has produced imbalanced cholesterol ester profiles, resulting in lower blood cholesterol and fecal elimination (*8*). The CPFAs, along with the other cotton antinutrient, gossypol, limit the utility of cottonseed for animal feed even though with its high oil and protein content, cottonseed could otherwise be a valuable food source.

Quantitative data on the CPFA content of cottonseed or cottonseed meal could be used to determine a nondetrimental level of incorporation of these materials into livestock diets. There are methods for determining the total CPFA content in oils (*9*) and methods that provide levels of specific CPFAs based on gas chromatography of methyl esters (*10*), parallel gas−liquid chromatography/high-performance liquid chromatography (HPLC) methodology (*11*), or HPLC following phenacyl derivatization (*12*). The total CPFA method does not provide information on each acid separately. The chromatograhic methods generate data in terms of fatty acid molar percents or fatty acid weight percents based on the relative peak areas of the various fatty acids in the chromatogram. These evaluations require the use of standards encompassing the range of expected fatty acids and concomitant determination of the levels of all individual fatty acids. However, feed formulators utilize databases that provide fatty acid information in terms of grams of fatty acid per grams of cottonseed or cottonseed meal. Previous CPFA methods provide results in relative oil or fatty acid composition terms that are not easily transformed into these dry weight-based measurements. A novel feature of the method presented herein is the utilization of malvalic acid, sterculic acid, and dihydrosterculic acid standards for direct and unambiguous quantification of these analytes. The development of a quantitative analytical method for CPFAs provides the ability to determine the absolute, rather than the relative, concentration of these important antinutrients in cottonseed, cottonseed meal, and cottonseed oil.

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MATERIALS AND METHODS

Analytical Reference Standard. Sterculic (9,10-methylene-9-octadecenoic) and malvalic (8,9-methylene-8-heptadecenoic) acids were synthesized by Gateway Chemical Technology, Inc. (St. Louis, MO) and were 99 and 100% pure, respectively. Dihydrosterculic (9,10-methyl-9-octadecenoic) acid with a purity of 94% was obtained from Matreya, Inc. (Pleasant Gap, PA). The identities of all three standards were confirmed, and their purities were determined by NMR, elemental analysis, moisture analysis, and liquid chromatography.

Cottonseed Materials. The delinted cottonseed used for method assessment was a composite of several varieties grown in the United States in 2001. For determination of CPFA levels, delinted cottonseed, cottonseed meal, and cottonseed oil were produced in 2002 in the United States. Varieties and the states in which the materials were produced are listed in **Tables 2−4**. Cottonseed was processed to cottonseed meal and oil following commercial practices by the Food and Protein Research and Development Center at Texas A&M University. The cottonseed meal was untoasted and did not contain soapstock. The cottonseed oil was refined, bleached, and deodorized.

Analytical Method. *Extraction.* For cottonseed and cottonseed meal samples, 0.5 g of ground material was extracted in a 125 mL flask with 10 mL of Bligh Dyer solvent (100 mL of water, 500 mL of methanol, and chloroform to a final volume of 2000 mL) for 3 min with sonication. The resulting extract was transferred to a separatory funnel, and 0.25 mL of water added. After shaking and separation, the chloroform layer was removed and drained through sodium sulfate into a 100 mL round-bottom flask. The extraction flask was rinsed with 5 mL of chloroform, which was transferred to the separatory funnel containing the aqueous phase from the initial separation. Methanol (1.2 mL) and water (0.25 mL) were added to the separatory funnel, and the liquid/liquid partition was repeated. Again, the chloroform layer was filtered through sodium sulfate and added to that previously collected from the first separation. The chloroform rinse of the extraction flask, the addition of methanol and water, and subsequent separation and pooling of the chloroform layer with that from prior separations were repeated. The combined chloroform phases were then dried by rotary evaporation. Methanol (5 mL) was added to dissolve the residue, and the sample was redried. Additional methanol rinsing and evaporation were performed as needed to remove water from the residue. The sample was then resuspended in 50 mL of chloroform.

Saponification. An amount of the sample in chloroform equivalent to approximately 2 mg of lipid was placed into a 50 mL tube (for cottonseed meal, 2 mL was used) and evaporated to dryness. The sample was then resuspended with 25 mL of 70:30 ethanol:0.01 N potassium hydroxide plus 5 mL of benzene, and the tube was capped tightly (*12*). Saponification was achieved by placing the tube in boiling water for 1 h. After it cooled, the solution was transferred to a separatory flask and the tube was rinsed with 20 mL of 0.013 M HCl that was added to the flask along with 2 mL of 2% HCl. The acidic solution was then extracted three times with 5 mL of 1:1 ethyl ether:hexane. After each extraction, the organic phase was removed and passed through sodium sulfate to dry. The combined organic phases were evaporated to dryness under nitrogen followed by resuspension with small volumes of acetone that was then evaporated to remove the remaining water.

Derivatization. For derivatization, 100 μL of 0.1 M 2-bromoacetophenone in acetone and 0.2 μL of 0.1 M triethylamine in acetone were added to the dried sample in the tube. The tube was tightly capped, vortexed, and heated at 100 °C for 15 min. Then, 280 μL of acetic acid (0.1 g in 50 mL of acetone) was added. The tube was capped, vortexed, and heated at 100 °C for 5 min. After evaporation to dryness under nitrogen, 1.0 mL of acetonitrile was added and the sample was vortexed and sonicated to resuspend the residue. The solution was passed through a 0.2 μm PTFE filter to remove particulates and placed in a tightly capped vial.

High-Performance Liquid Chromatography. The HPLC system included two columns in series. The first was a Nucleosil C18 (3 μM, 50 mm × 4.6 mm), and the second was a Lichrosorb RP-8 (5 μM, 150 mm × 4.6 mm) fitted with 0.5 μM × 4 mm precolumn frits prior to the columns. The columns were maintained at 25 °C with a flow rate of 1.5 mL/min. The mobile phase composition was 2:98 hexane:acetonitrile isocratic for 34 min followed by a 10 min gradient to 20:80 water:acetonitrile, which was then held for 22 min. The return to the starting mobile phase was then achieved with an 8 min gradient. The injection volume was 15 μL. Detection was by UV absorption at a wavelength of 242 nm. Data were captured using Millennium[32] chromatography software.

Calibration Standards. The HPLC calibration standards were generated with each analytical set. A 1.0 mL aliquot of solutions containing 0.5, 2, 5, 10, and 25 μg/mL each of malvalic, sterculic, and dihydrosterculic acids in hexane was placed in a tube and evaporated to dryness under nitrogen. Residues were then taken through the saponification and derivatization steps in an identical manner to the samples.

Calculations. A linear regression curve was generated for each of the three CPFAs based on the UV absorbance of the five calibration standard levels. The amount (μg/mL) of each CPFA detected in the injected HPLC solution was calculated from a standard linear regression curve. This value was converted to the μg/g value for the amount of CPFA in the original sample by dividing the μg/mL value by the weight of HPLC-analyzed sample. For example, an original cottonseed sample weight of 0.5 g was extracted with 50 mL. From this, 1.0 mL was saponified and derivatized. The resulting final sample volume of 1.0 mL resulted in a 0.01 g/mL cottonseed in the final, injected solution (0.5 g/50 mL). Thus, if a cottonseed sample analyzed as described above had a UV response corresponding to a 5 μg/mL malvalic acid concentration in the final solution, the calculated cottonseed concentration was 500 μg/g malvalic acid.

Method Performance. The method was evaluated for precision and accuracy on cottonseed, cottonseed meal, and cottonseed oil. For precision assessments, aliquots of each material were carried through the method eight times on each of two (cottonseed meal and cottonseed oil) or three (cottonseed) days. Means, standard deviations (SDs), and percent relative standard deviations (RSDs) were calculated for each matrix both within each day's results and across the 2 or 3 days of analyses.

To evaluate accuracy, the extracted lipid or oil of each matrix was spiked with levels of each acid both equal to and 2× that found endogenously in the samples during method development. Four cottonseed samples were spiked with 2× the endogenous level of CPFAs prior to extraction to assess the extraction efficiency. For cottonseed meal and cottonseed oil, the CPFAs were added to the extracted lipid or oil prior to saponification. Four fortified samples were analyzed for each matrix on each day of sample analysis. Means, SDs, and percent RSDs were calculated for the recoveries of each acid for both intra- and interday analyses. Dixon outlier tests were also conducted.

Analyses of cottonseed grown in the United States in 2002 at various locations as well as cottonseed meal and oil derived from the cottonseed were conducted in duplicate as described above. For each variety and location of cottonseed, the samples were typically taken from four different plots within a site providing four field replicates. For cottonseed meal and oil, two aliquots of each material were analyzed separately to provide duplicate analyses.

RESULTS AND DISCUSSION

Method Performance. The method describes the extraction of oil from cottonseed and cottonseed meal, saponification of that oil or cottonseed oil, followed by derivatization of the free fatty acids to phenacyl esters. The method provides conditions for separation of the resultant esters by dual-column reverse-phase HPLC and their subsequent quantification using external standards. Calibration curves for the three CPFA standards are presented in **Figure 1**. The method was shown to be quantitative for the determination of malvalic acid, sterculic acid, and dihydrosterculic acid in cottonseed, cottonseed oil, and untoasted cottonseed meal (**Figures 2−4** and **Table 1**). RSDs of 21 analyses across 3 days were below 10% for all three acids in cottonseed. Recoveries of spiked standards for all three acids added to cottonseed were between 90 and 94% over two fortification levels and 12 samples. For cottonseed meal, 16

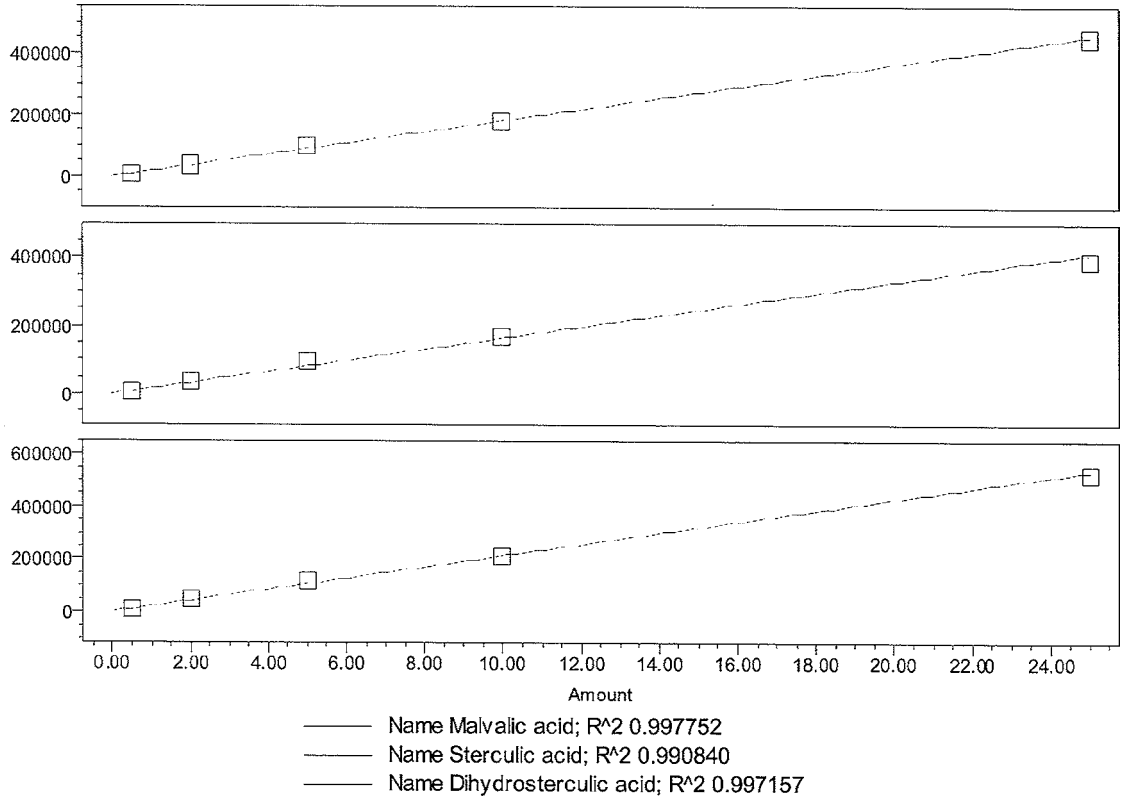

Figure 1. External calibration standard curve.

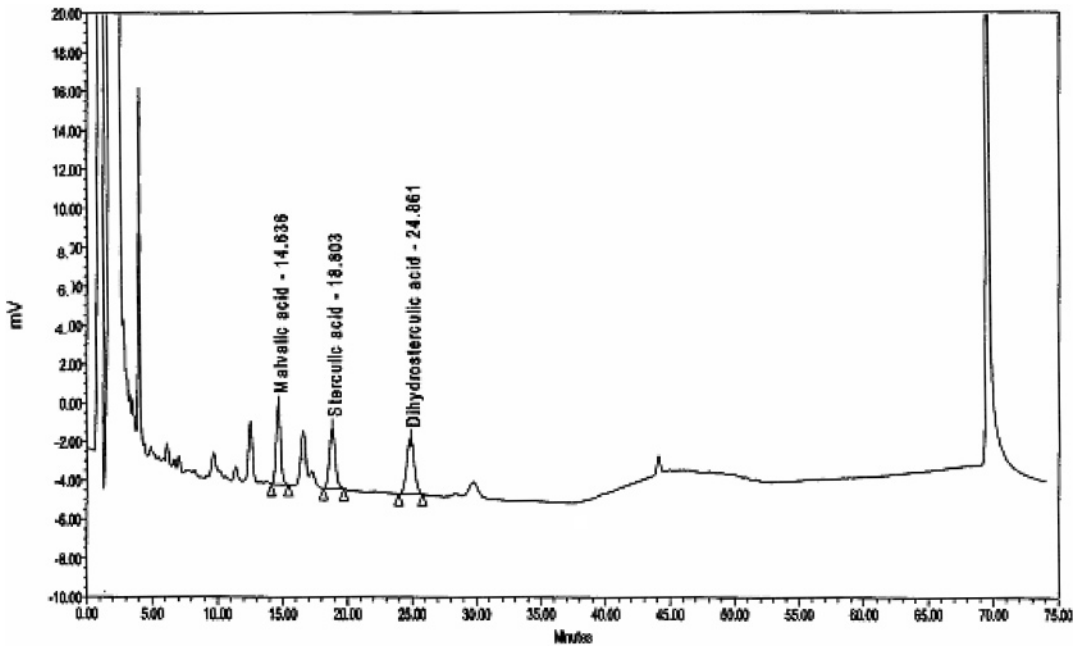

Figure 2. Amount (5.0 μg/mL) of CPFA mixed calibration standard, detected as phenacyl esters.

analyses over 2 days resulted in precision levels of 10−17% RSD for the three acids. The accuracy of the method on cottonseed meal was good with recoveries of fortified standards between 81 and 104% for the three CPFAs. Finally, in cottonseed oil, the precision of the method was comparable, with RSD values between 11 and 14% for the three CPFAs over 16 samples in 2 days. Fortified standards added to cottonseed oil were recovered at between 92 and 99% of the original amount.

Precision levels are adequate with RSD values under 20% overall with the best precision obtained from analyses of cottonseed samples. Likewise, the recovery of the three CPFAs was excellent from all three matrices and fell well within the conventionally accepted guidelines of 80−120% (*13*) with RSD values below 15%.

Given the presence of these fatty acids naturally in cotton, the traditional determination of lower limits of detection and method validation by recovery of known quantities of analyte from a control matrix is not possible. The approach described herein utilized recoveries of the three fatty acids from exogenous fortification at levels equal to and twice that of the endogenous concentrations. The lowest concentration of external standard utilized in this method was 0.5 μg/mL. This corresponds to a tissue concentration of 50 ppm fresh weight, which could be

Table 1. Precision and Accuracy Assessment of Method

			endogenous levels[a]				spiked recovery			
matrix	day	acid	mean	range	SD	RSD (%)	% recovery	range	SD	RSD (%)
cottonseed	1[a]	malvalic[b]	776	706–849	56.1	7.2	75.6	70.5–80.5	4.09	5.4
		sterculic[b]	467	421–482	21.1	4.5	78.4	76.0–84.5	4.06	5.2
		dihydrosterculic	276	248–295	15.7	5.7	83.4	72.6–83.4	4.61	5.8
	2	malvalic[b]	806	727–900	54.5	6.8	97.6	94.0–102	3.35	3.43
		sterculic[b]	514	482–544	22.4	4.4	107	102–111	3.74	3.50
		dihydrosterculic	301	285–323	13.1	4.3	95.6	92.3–98.8	3.01	3.15
	3	malvalic[c]	831	719–952	85.3	10	100[d]	96.1–104[d]	3.95[d]	3.95[d]
		sterculic[c]	519	465–594	50.9	9.8	99.3[d]	95.1–104[d]	4.47[d]	4.50[d]
		dihydrosterculic[c]	294	257–325	29.0	9.9	96.9	91.4–102	4.62	4.77
	3[e]	malvalic[c]					89.2	79.8–97.3	7.26	8.14
		sterculic[c]					93.6	84.3–103	7.66	8.18
		dihydrosterculic[c]					89.9	83.5–101	7.92	8.81
	overall	malvalic	803	706–952	65.6	8.2	90.0	70.5–104	10.8	12.0
		sterculic	500	421–594	39.0	7.8	94.3	76.0–111	12.1	12.8
		dihydrosterculic	290	248–325	22.0	7.6	90.4	72.6–102	8.66	9.58
cottonseed meal	1[a]	malvalic	138	96–150	19.2	14	82.1	80.0–84.7	1.99	2.42
		sterculic	95.1	56–110	18.5	19	103	99.9–107	3.34	3.24
		dihydrosterculic	82.4	65–109	14.5	17	78.6	72.6–82.8	4.29	5.46
	2	malvalic	147	139–157	5.74	3.9	89.9	86.3–94.0	3.17	3.53
		sterculic	101	92–110	6.71	6.6	104	93.3–115	9.19	8.84
		dihydrosterculic	66	61–69	2.76	4.2	83.6	81.3–87.6	2.76	3.30
	overall	malvalic	143	96–157	14.5	10	86.0	80.0–94.0	4.85	5.64
		sterculic	98	56–110	13.8	14	104	93.3–115	6.42	6.17
		dihydrosterculic	74	61–109	12.7	17	81.1	72.6–87.6	4.258	5.28
cottonseed oil	1[a]	malvalic	2820	2590–3090	160	5.7	88.4	83.3–92.3	4.08	4.62
		sterculic	1660	1560–1730	56	3.4	99.1	96.5–102	2.78	2.81
		dihydrosterculic	1400	1330–1490	52	3.7	87.4	81.1–92.3	4.71	5.39
	2	malvalic	3230	2380–3600	390	12	98.2[f]	87.7–104	9.14	9.31
		sterculic	1950	1460–2090	210	11	99.8[f]	99.5–100	0.29	0.29
		dihydrosterculic	1670	1150–1870	232	14	96.4	86.7–102	6.99	7.25
	overall	malvalic	3030	2380–3600	358	12	92.6	83.3–104	7.99	8.63
		sterculic	1800	1460–2090	210	12	99.4	96.5–102	2.01	2.02
		dihydrosterculic	1530	1150–1870	216	14	91.9	81.1–102	7.33	7.98

[a] Eight replicate analyses. [b] One of the eight analyses was identified as a Dixon outlier. [c] Seven replicate analyses; interferences in one sample for malvalic acid and sterculic acid prevented their quantitation. [d] One of the four spike samples not included due to chromatographic interference with malvalic and sterculic acids. [e] These samples were fortified prior to extraction of lipid with 1600 ppm malvalic acid, 700 ppm sterculic acid, and 600 ppm dihydrosterculic acid; $N = 4$. [f] $N = 3$ rather than 4. The fourth had a chromatographic interference with malvalic and sterculic acids.

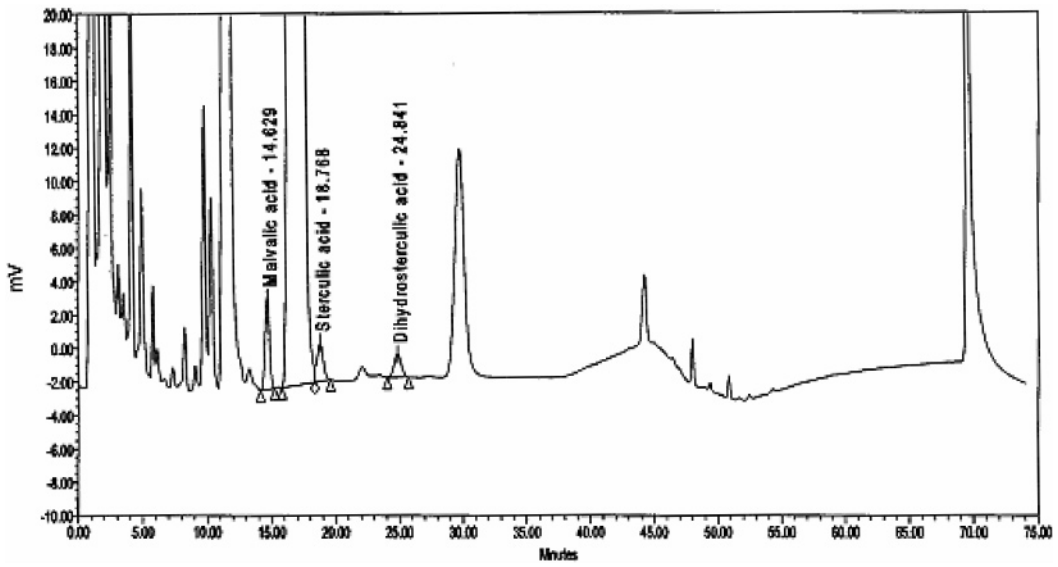

Figure 3. Cottonseed (719 μg/g malvalic acid, 469 μg/g sterculic acid, and 257 μg/g dihydrosterculic acid).

considered a minimum lower limit of detection. Acceptable recoveries from of these fortifications demonstrated linearity of the method over the tested range.

Analysis of CPFAs in Cottonseed and Processed Fractions. The method was used to determine the concentration of CPFAs in cottonseed, cottonseed meal, and cottonseed oil as presented in **Tables 2**−**4**. This method provides an opportunity to determine the absolute concentration of CPFAs in cottonseed matrices rather than a concentration based on their relative abundance to other fatty acids. For cottonseed (**Table 2**), the average concentration of sterculic acid ranged between 517 and 842 μg/g, the average concentration of malvalic acid ranged between 607 and 1278 μg/g, and the average concentration of dihydrosterculic ranged between 311 and 462 μg/g. Levels of

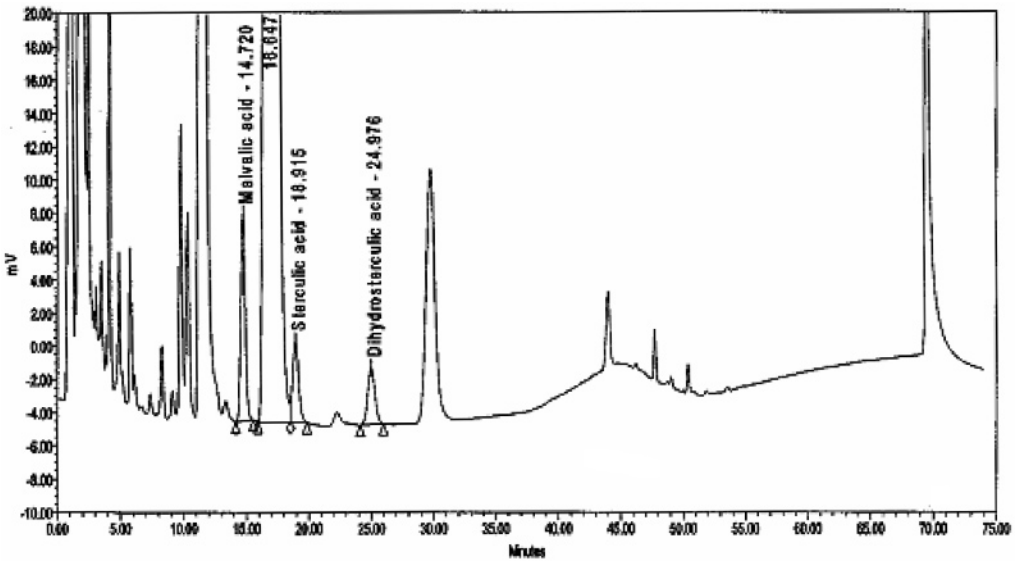

Figure 4. Cottonseed spiked with 800 μg/g malvalic acid, 400 μg/g sterculic acid, and 350 μg/g dihydrosterculic acid.

Table 2. Concentration of CPFAs in Delinted Cottonseed

		acid average[a] (range)		
variety	field location	sterculic	malvalic	dihydrosterculic
Stoneville 474	California	517 (427–616)	607 (438–738)	324 (270–350)
Stoneville 580	California	548 (488–593)	756 (689–817)	287 (258–315)
DP 90	Alabama	650 (555–773)	859 (761–1033)	301 (272–358)
DP 51	Alabama	541 (484–606)	826 (742–924)	222 (194–245)
DP5690	Alabama	634 (540–798)	851 (710–1008)	289 (242–362)
DP5415	Alabama	626 (528–728)	845 (689–949)	275 (236–310)
GTO-MaxxA	California	632 (559–684)	714 (657–806)	388 (358–429)
Phytogen 72	California	709 (629–784)	809 (642–1028)	392 (339–444)
Fibermax 989[b]	Georgia	648 (603–703)	824 (770–892)	347 (293–424)
PSC 355	Georgia	640 (518–705)	787 (710–904)	312 (299–328)
GA 161	Georgia	578 (394–684)	771 (513–986)	229 (157–263)
HS 12	Georgia	488 (392–653)	738 (586–988)	211 (145–307)
Paymaster 330	Texas	603 (586–637)	706 (646–792)	390 (350–412)
Paymaster 2379	Texas	557 (520–621)	613 (586–655)	348 (339–366)
AFD Rocket	Texas	591 (489–686)	709 (533–894)	407 (319–514)
All-Tex Atlas	Texas	594 (526–668)	682 (599–776)	359 (313–405)
PSC 355[c]	Arkansas	842 (826–857)	1201 (1138–1264)	462 (448–475)
SG 125[c]	Arkansas	622 (600–644)	1084 (1057–1110)	363 (360–365)
DP 565[c]	Arizona	564 (553–578)	904 (845–957)	317 (306–327)
ST 580[c]	Arizona	754 (752–755)	1227 (1223–1230)	415 (411–419)
DPL Acala 90[c]	Georgia	822 (796–848)	1191 (1169–1212)	415 (403–426)
HS 12[b]	Georgia	763 (750–776)	1278 (1223–1332)	403 (383–422)

[a] Average and range in μg/g dry weight of four field replicates except as noted in footnotes *b* and *c*. [b] Average and range in μg/g dry weight of three field replicates. [c] Average and range in μg/g dry weight of two analytical replicates.

Table 3. Concentration of CPFAs in Untoasted Cottonseed Meal

		acid average[a] (range)		
variety	field location	sterculic	malvalic	dihydrosterculic
PSC 355	Arkansas	88 (87–89)	131 (128–135)	51 (35–67)
SG 125	Arkansas	55 (46–65)	76 (66–86)	39 (30–48)
DP 565	Arizona	67 (66–68)	81 (80–83)	<46 (<25–46)
ST 580	Arizona	81 (75–87)	109 (108–111)	30 (28–31)
DPL Acala 90	Georgia	78 (74–81)	96 (94–99)	46 (39–54)
HS 12	Georgia	85 (76–93)	111 (103–120)	45 (41–49)

[a] Average and range in μg/g dry weight of two analytical replicates.

CPFAs in cottonseed meal were lower, as expected, with averages ranging between 55 and 88 μg/g for sterculic acid, 76−131 μg/g for malvalic acid, and 30−51 μg/g for dihydrosterculic acid (**Table 3**). In cottonseed oil (**Table 4**), the average concentration ranged between 1619 and 2174 μg/g for sterculic acid, 2582−2912 μg/g for malvalic acid, and 1143−1525 μg/g for dihydrosterculic acid. For oil, these values indicate that roughly 0.5−0.7% of the total cottonseed oil is represented by CPFAs. **Table 5** presents previously reported data for the amount of CPFAs in cottonseed and cottonseed oil. These values are presented as percent oil, and a representative conversion to fresh weight is described.

In conclusion, the results of the validation conducted for this new CPFA method indicate that the described method is capable of providing quantitative data on the CPFA content of cottonseed, cottonseed meal, and cottonseed oil with acceptable precision and accuracy. This method provides data on these nutritionally important compounds without requiring the concurrent analysis of other fatty acids as comparators. It can also provide the data in terms that are easily correlated to the amount of cottonseed, cottonseed meal, or cottonseed oil fed to livestock.

Table 4. Concentration of CPFAs in Cottonseed Oil

variety	field location	acid average[a] (range) sterculic	malvalic	dihydrosterculic
PSC 355	Arkansas	2033 (1019–2047)	2582 (2226–2937)	1470 (1152–1787)
SG 125	Arkansas	2174 (2079–2268)	2881 (2727–3034)	1244 (1062–1425)
DP 565	Arizona	2102 (2015–2189)	2810 (2582–3037)	1143 (1029–1257)
ST 580	Arizona	1619 (1370–1868)	2883 (2347–3419)	1326 (1086–1566)
DPL Acala 90	Georgia	2052 (2036–2067)	2739 (2631–2847)	1525 (1474–1576)
HS 12	Georgia	1747 (1553–1940)	2912 (2773–3051)	1428 (1403–1453)

[a] Average and range in μg/g of two analytical replicates.

Table 5. Previously Reported Values for CPFAS in Cottonseed and Cottonseed Oil

matrix	units	sterculic	malvalic	dihydrosterculic
cottonseed	fatty acid molar %	0.3–0.4[a]	0.3–0.5[a]	0.2–0.4[a]
	μg/g FW	579–772[b]	579–965[b]	386–772[b]
cottonseed	% oil	0.3–0.5[c]	0.7–1.5[c]	
	μg/g FW	579–965[b]	1351–2895[b]	
cottonseed	% FA	0.005[d]–0.126[d]	0.015[d]–0.324[d]	
oil	μg/g FW	50[f]–1260[f]	150[f]–3240[f]	
cottonseed	% FA		0.64[e]–0.98[e]	
oil	μg/g FW		6400[f]–9800[f]	

[a] Ref *12.* [b] μg/g FW = (FA%) × (19.3%/100) [oil content (*14*)] × 10000. [c] Ref *15.* [d] Ref *16.* [e] Ref *17.* [f] μg/g FW = (FA%) × 10000.

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