J Am Oil Chem Soc (2015) 92:947–956
DOI 10.1007/s11746-015-2669-5

ORIGINAL PAPER

Determination of Nutritional and Cyclopropenoid Fatty Acids in Cottonseed by a Single GC Analysis

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Received: 15 September 2014 / Revised: 26 May 2015 / Accepted: 27 May 2015 / Published online: 21 June 2015

Abstract Historically, a complete analysis of cottonseed fatty acids required two separate analyses: gas chromatography (GC) for nutritional fatty acids and a separate analysis for cyclopropenoid fatty acids (CPFA). Using base esterification and optimized GC conditions, the method presented combines both analyses into a single GC procedure that improves analytical processes and streamlines workflow. While there were challenges, the resolution of critical pairs malvalic/stearic and dihydrosterculic/alpha linolenic methyl esters were adequately separated, allowing for accurate quantitation. Single lab reproducibility measurements (RSD) for major nutritional fatty acids ranged from 0.7 to 2.0 %. For CPFA the RSD ranged from 1.1 to 5.4 %, with the higher variability seen in the extracted cottonseed. In oils, the precision was similar between nutritional fatty acids and CPFA at equivalent concentrations, indicating the variation comes from the extraction process. Average spiked recovery results ranged from 93.3 to 106.5 % for selected fatty acids. In addition, complete fatty acid profile results compare favorably with other methodologies and historical data, demonstrating that it is possible to combine two legacy methods into one.

Keywords Cottonseed · Fatty acids · Cyclopropenoid fatty acids · GC · Malvalic · Sterculic · Dihydrosterculic

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Introduction

Based on the Organization for Economic Co-operation and Development (OECD) recommendations, the determination of fatty acid profiles in cottonseed is of importance to nutritional composition studies. Not only is it important for human and animal nutritional aspects, but the anti-nutrient properties of cyclopropenoid fatty acids (CPFA) make it necessary [1]. In the past, CPFA (sterculic (C18:1 cpe), malvalic (C19:1 cpe), and dihydrosterculic (C19:0 cpa) acids), required different methodology than the nutritional fatty acids (NFA). Methods for total CPFA included the qualitative Halphen test [2], hydrogen bromide titration [3], and a spectrophotometric method based on the Halphen test [4]. HPLC methods [5, 6] require multiple steps to convert the triacylglycerols, first to free acids, then to phenacyl derivatives. Currently, methods for CPFA utilize gas chromatography (GC). However, these methods are inherently different from those used for NFA, which employ acid catalyzed esterification, and often a different GC column stationary phase [7]. Using base esterification as a starting point [8], a method was developed that analyzes both CPFA and NFA in a single GC procedure. The method optimizes the chromatographic conditions using a polyethylene glycol (PEG) stationary phase for the resolution of all fatty acids of interest. While the elution of NFA and CPFA using a 100 % biscyanopropyl GC stationary phase works very well [9, 10], the PEG stationary phase was chosen as this has the least impact on existing procedures for laboratories running crop studies. The method presented here is applicable to routine analysis of cottonseed and cottonseed oil with the focus being on analysis of those fatty acids suggested by the OECD consensus documents. Other fatty acids present could be analyzed with the method if needed.

Materials and Methods

Samples

Acid delinted cottonseed, crude cottonseed oil, and refined cottonseed oil were used in the study. The cottonseeds (three different lots) were obtained from Dow AgroSciences (Indianapolis, IN) and Monsanto Company (St. Louis, MO). The crude cottonseed oil (Proflo oil) was obtained from Archer Daniels Midland Company (Chicago, IL). The refined cottonseed oil (Nut-ola cottonseed oil) was obtained from CBS Food Products (Brooklyn, NY). The cottonseed samples were ground to a fine powder under liquid nitrogen prior to analysis, and stored at −70 °C. The oils were stored at room temperature.

Reagents

Toluene, heptane, pentane and chloroform, all HPLC grade, were from Sigma-Aldrich (St. Louis, MO). Sodium methoxide (0.5 N) was from Supelco (Bellefonte, PA). Glacial acetic acid, anhydrous sodium sulfate, and sodium chloride, all ACS grade, were from Fisher Scientific (Pittsburgh, PA).

Standards

Fatty acid methyl ester (FAME) standards were obtained from Nu-Chek Prep, Inc. (Elysian, MN). The quantitation standards were prepared in heptane using special mixtures from Nu-Chek Prep. Portions of the standard mixtures were diluted in heptane to create five working standards ranging between 0.01 and 3.5 mg/mL, depending on the specific fatty acid. Dihydrosterculic methyl ester was obtained from Matreya LLC (Pleasant Gap, PA), Cat. No. 1823. A stock solution was prepared at approximately 0.80 mg/mL in heptane. Aliquots of the stock solution were diluted to create 5 additional standard levels ranging from 0.008 through 0.4 mg/mL. All quantitation standards contain tridecanoic methyl ester at a concentration of 1.0 mg/mL.

Tritridecanoin obtained from Nu-Chek prep was diluted in chloroform at 5.0 mg/mL and used as the internal standard for sample preparation. Trioctadecanoin (C18:0) and α-trioctadecatrienoin (C18:3n3) were obtained from Nu-Chek prep and used for spiking.

Additional standards used for identification of chromatographic peaks were palmitic (C16:0), stearic (C18:0), oleic (C18:1n9) and linoleic (C18:2n6) acids, and ricinoleic (C12-OH 18:2) and vaccenic (C18:1n7) methyl esters obtained from Nu-Chek Prep. Vernolic (C18:1 ep) methyl ester was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Lipid Extraction

Approximately 3 g of ground cottonseed was transferred into a cellulose thimble, mixed with anhydrous sodium sulfate, and extracted using a Soxhlet apparatus with pentane for 16 h. The pentane extract was dried over steam under a gentle stream of nitrogen and the lipid content of the seed calculated from the net weight of the extracted oil [11]. Pentane, rather than petroleum ether, was used as the extraction solvent. Pentane is a less expensive option and is similar in both polarity and boiling point.

Sample Preparation and Derivatization

The transesterification procedure was performed in 4-dram glass vials with PTFE lined screw caps, to which 1 mL of 5.0 mg/mL tritridecanoin internal standard solution was added. The solvent was evaporated under nitrogen. Approximately 0.1 g of oil was weighed into the prepared vial, the weight recorded to the nearest 0.1 mg, and dissolved in 1 mL of toluene. To this solution, 2 mL of 0.5 N sodium methoxide was added, and the mixture was allowed to stand, capped, at room temperature. After at least 10 min, 0.1 mL of glacial acetic acid was added, followed by 5 mL of heptane and 5 mL of saturated sodium chloride solution. The vials were shaken for 1 min, and the layers were allowed to separate. A portion of the heptane layer was dried over sodium sulfate, and an aliquot transferred to a GC vial for analysis.

Chromatography

All analyses were performed on either Agilent 6890N or 7890A gas chromatographs equipped with split/splitless injection ports, electronic pressure control, and flame ionization detectors (FID). GC columns used were PEG: 30 m × 0.25 mm × 0.25 micron film thickness (specifically Restek Rtx-Wax or Phenomenex ZB-Wax). Other manufacturer's columns were not investigated as part of this validation. Inlet and detector temperatures were set at 250 °C. The carrier gas was hydrogen, at a flow rate of 1.2 mL/min, split ratio 1:100. FID gases were hydrogen at 40 mL/min, air at 350 mL/min, and nitrogen make-up gas at 30 mL/min. The oven temperature program used was: 170 °C hold 1.5 min, 1 °C/min to 182 °C, 5 °C/min to 210 °C hold 17.5 min, 20 °C/min to 245 °C hold 1.5 min. Injection volume was 0.2 µL. Adjustments to the carrier flow rate can be made to ensure adequate resolution between critical peak pairs. Resolution factors were calculated according to the United States Pharmacopeia (USP) [12]. Minimum resolution requirements are ≥1.0 for C18:1 cpe and C18:0, and ≥0.9 for C19:0 cpa and alpha linolenic

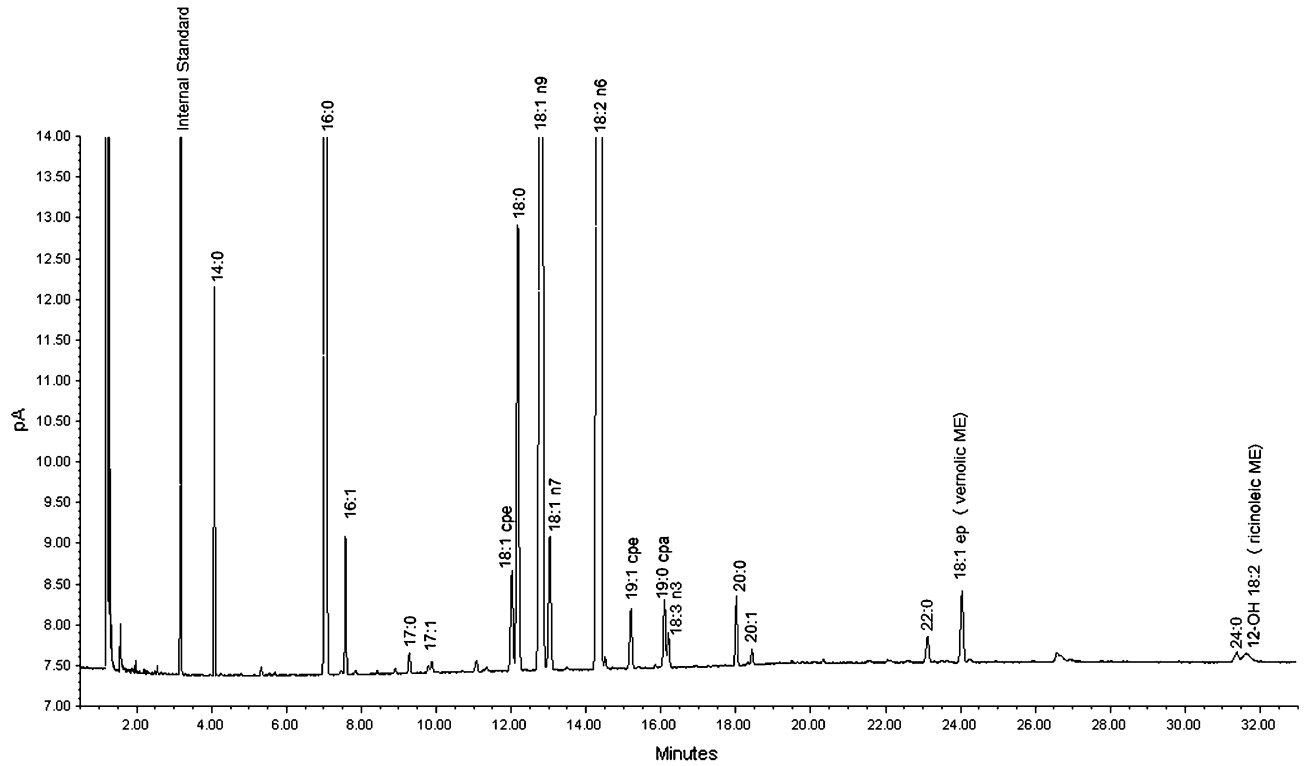

Fig. 1 Chromatogram of typical cottonseed sample using a PEG capillary column, 30 m × 0.25 mm × 0.25 μm

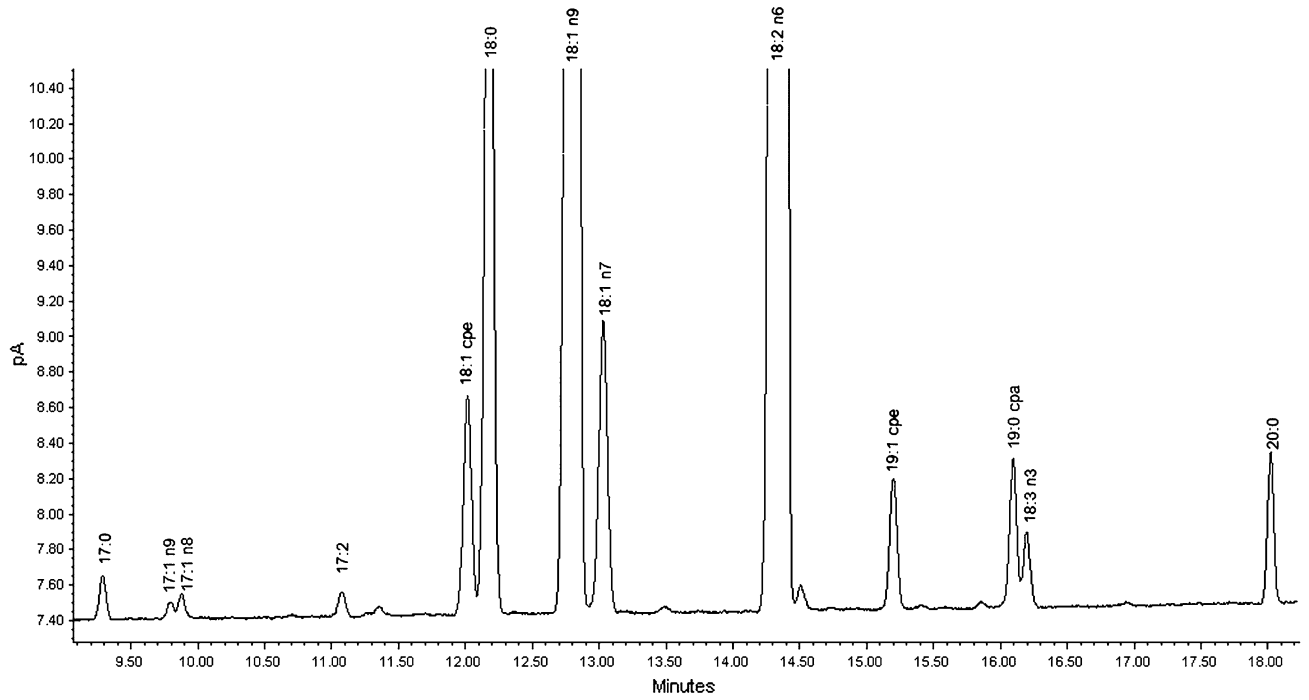

Fig. 2 Expanded view of chromatogram showing the region where CPFA elute

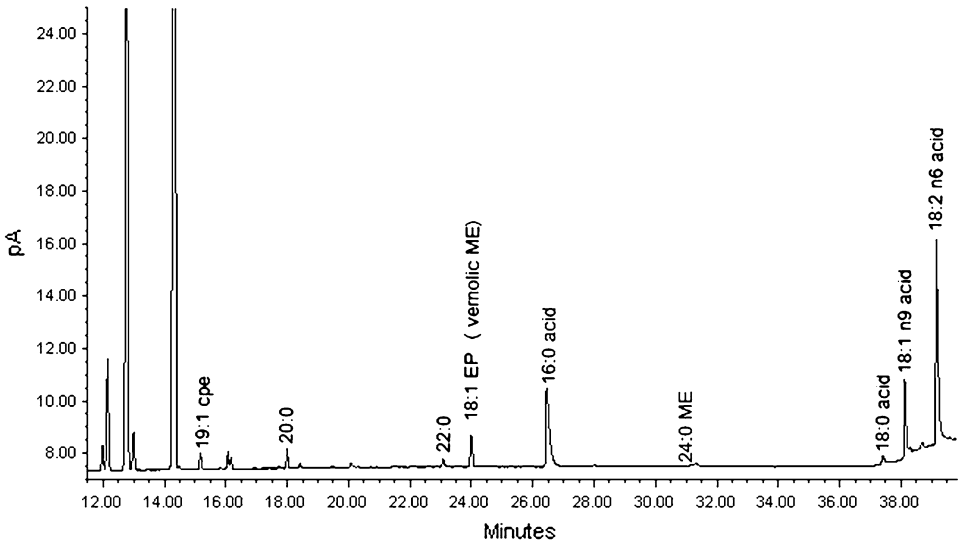

Fig. 3 Chromatogram of cottonseed containing high levels of free fatty acids (11 % on a lipid basis). The free fatty acids elute later than FAME, 16:0 elutes at 26.5 min, and 18:0, 18:1 and 18:2 acids elute near the end of the chromatogram

methyl ester (C18:3n3). Typical chromatograms are shown in Figs. 1 and 2.

Additional temperature programs used to demonstrate the effect of temperature on resolution and the effect of resolution on the results were: 150 °C hold 1.5 min, 1 °C/min to 182 °C, 5 °C/min to 210 °C hold 22.9 min, 25 °C/min to 250 °C hold 5 min; 175 °C hold 1.5 min, 1 °C/min to 182 °C, 5 °C/min to 210 °C hold 17.5 min, 20 °C/min to 245 °C hold 1.5 min; 180 °C hold 1.5 min, 1 °C/min to 182 °C, 5 °C/min to 210 °C hold 22.9 min, 25 °C/min to 250 °C hold 5 min; and 185 °C hold 1.5 min, 5 °C/min to 210 °C hold 22.9 min, 25 °C/min to 250 °C hold 5 min.

Supporting Methods

Mass spectroscopy confirmation of peak identification was performed on an HP 6890 gas chromatograph equipped with an HP 5973 mass-selective detector (MSD). The same chromatographic conditions, as described above, were applied, except helium was used as the carrier gas at a flow rate of 1.0 mL/min. The transfer line to the MSD was set at 250 °C, ionization EI 70 eV, full-scan mode *m*/*z* 20–550.

A 5 % diphenyl 95 % dimethyl polysiloxane (HP-5) column, 30 m × 0.32 mm × 0.25 micron film thickness was used to evaluate selectivity. Inlet and detector temperatures were set at 250 °C. The carrier gas was hydrogen at a flow rate of 2.0 mL/min, and the split ratio was 1:50. FID gases were hydrogen at 40 mL/min, air at 350 mL/min, and nitrogen make-up gas at 30 mL/min. The oven temperature program was: 150 °C hold 4.0 min, 3 °C/min to 230 °C, hold 10.0 min.

For comparison, CPFA were also analyzed by HPLC using the method reported by Obert, et al. [5]. Likewise, NFA results were compared to those from the traditional esterification methods using boron trifluoride as described in AOCS method Ce 2-66 [13], and quantitated using the same GC conditions and standards as described in the chromatography and standard sections. Total free fatty acids were determined by titration, according to the USP method for Acid Value, Method 1 [14]. For all three tests, the lipid was extracted by the Soxhlet method described in the "Lipid Extraction" above.

Results and Discussion

Chromatography and Resolution

Identities of FAME and free acids were determined by comparing retention times of standards and/or by mass spectroscopy (MS). Underivatized free fatty acids (FFA) elute late in the run (Fig. 3). To avoid interference in subsequent injections, the GC oven program must be long enough to ensure the FFA elute prior to the next injection. Vernolic ME elutes after behenic ME (22:0) at ~24 min, ricinoleic ME elutes just after lignoceric ME (24:0). As these two fatty acids are not part of the OECD guidelines, they were not quantitated as part of this method. They do not interfere with the analysis of the other fatty acids and could be quantitated in future studies. Heptadecadienoic acid (C17:2) has been reported to be present in cottonseed at low concentrations, 0.06–0.1 % [15, 16]. The small peak eluting at ~11 min was identified by GCMS as C17:2 by comparison to library spectra [17], although further investigation would be needed to confirm. In the samples analyzed for this study, the C17:2 concentrations are approximately 0.01/100 g in the seed and 0.05/100 g in the crude oil. This is based on a data set of $n = 5$ for each matrix, and quantitated using the C17:1 standard. Historically, 17:2 has not been reported in composition studies.

Table 1 Comparison of results at different USP resolution factors

FAME	Resolution 1.9 150 °C[a]		Resolution 1.3 170 °C[a]		*T* test result
	Mean (mg/g)	% RSD	Mean (mg/g)	% RSD	*P* value*
C18:1 cpe	4.94	1.21	4.89	0.74	0.18
C18:0	23.12	0.12	23.10	0.14	0.69
FAME	Resolution 1.4 180 °C[a]		Resolution 1.1 170 °C[a,b]		*T* test result
	Mean (mg/g)	% RSD	Mean (mg/g)	% RSD	*P* value*
C19:0 cpe	3.10	0.46	3.07	0.72	0.05
C18:3n3	1.49	1.58	1.48	1.25	0.21
FAME	Resolution 1.4 180 °C[a]		Resolution 0.8 175 °C[a,c]		*T* test result
	Mean (mg/g)	% RSD	Mean (mg/g)	% RSD	*P* value*
C19:0 cpe	3.10	0.46	3.09	0.82	0.32
C18:3n3	1.49	1.58	1.45	0.92	0.01

* *P* value >0.05 results equivalent at a 95 % confidence level

[a] Initial GC oven temperature, see "Chromatography" for complete oven program

[b] GC column: ZB Wax

[c] GC column: RTx-Wax

Table 2 Calculation of LOQ (on column concentration in mg/mL) using the equation LOQ = 10 σ/S

FAME	Slope (*S*)		*y*-intercept		LOQ (mg/mL)
	Mean	% RSD	Mean	σ (SD)	
C8:0	0.89	1.67	8.28E−05	2.83E−04	0.003
C10:0	0.94	1.13	6.80E−04	3.02E−04	0.003
C12:0	0.99	0.33	1.56E−03	1.74E−04	0.002
C14:0	1.02	0.52	4.65E−04	1.40E−04	0.001
C14:1	1.01	0.92	6.38E−04	3.14E−04	0.003
C15:0	1.04	0.84	5.57E−04	1.78E−04	0.002
C15:1	1.01	1.00	4.30E−05	2.46E−04	0.002
C17:0	1.08	1.13	−7.34E−05	2.01E−04	0.002
C17:1	1.07	1.08	−2.14E−04	3.78E−04	0.004
C18:0	1.09	1.36	−1.22E−03	7.56E−04	0.007
C18:1n9	1.09	1.38	−1.41E−03	1.35E−03	0.012
C18:2n6	1.08	1.45	−2.27E−03	6.82E−04	0.006
C18:3n3	1.08	1.57	3.16E−04	2.43E−04	0.002
C20:0	1.11	1.83	9.13E−06	4.61E−04	0.004
C20:1	1.11	1.83	−1.56E−04	2.92E−04	0.003
C20:2	1.11	1.84	−4.84E−04	2.46E−04	0.002
C20:3	1.08	1.73	2.41E−04	6.65E−04	0.006
C20:4	1.09	1.87	1.89E−04	4.48E−04	0.004
C22:0	1.13	1.76	8.30E−04	1.22E−03	0.010
C24:0	1.15	2.57	−2.53E−03	1.13E−03	0.010
C18:1 cpe	0.98	1.91	7.64E−04	7.64E−04	0.008
C19:1 cpe	0.95	1.82	7.87E−04	7.87E−04	0.008
C19:0 cpa	1.06	1.15	−1.82E−04	6.63E−04	0.006

PEG columns allow for excellent resolution of FAME. However in cottonseed, resolution between the methyl esters (ME) of C18:1 cpe/C18:0 and C19:0 cpa/C18:3 n3 can be inadequate. By adjusting the oven temperature, adequate resolution between these critical pairs of methyl esters can be achieved. Lower initial oven temperatures (e.g. 150 °C) increase the resolution between C18:1 cpe and C18:0, but have an adverse effect on the resolution between C19:0 cpa and C18:3n3. Increasing the initial oven temperature (e.g. 185 °C) increases the resolution between C19:0 cpa and C18:3n3, but eliminates the resolution between C18:1 cpe and C18:0. The oven program used in this method is a compromise between the two, with slightly better resolution between C18:1 cpe and C18:0 due to the difference in concentration between the two compounds. To confirm that resolution below 1.5 does not impact results, methylated crude cottonseed oil was injected in quintuplicate using different GC conditions to produce different resolutions (Table 1). *T* test results show that C18:1 cpe and C18:0 results obtained with a resolution of 1.8 are statistically equivalent to those with a resolution of 1.3. For C19:0 cpa and C18:3n3, resolution of 1.4 and 1.1 are equivalent, but 1.4 and 0.8 are not equivalent. Helium was not evaluated as a carrier gas as hydrogen is more widely used today, readily available, and allows for better resolution at higher linear velocity.

It should be noted that C19:1 cpe and γ linolenic methyl ester (C18:3n6) are not sufficiently resolved. This is not problematic in cottonseed or cottonseed oil as C18:3n6 is not present. An alternate GC stationary phase, 5 % diphenyl 95 % dimethyl polysiloxane, was used to confirm the absence of C18:3n6. This stationary phase allows for better resolution of the CPFA from NFA, compared to PEG columns [7]. In addition to evaluating retention times, the relative proportions of the CPFA injected on each column were calculated by area % and found to be equivalent, indicating an absence of co-eluting peaks.

Quantitation and Linearity

Quantitation was performed using a linear regression curve, plotting the standard concentration as *X* and the ratio of the peak area to the internal standard peak area multiplied by the internal standard concentration as *Y*. A weighting factor of 1/*X* was used for each FAME to ensure a tighter fit to the lower standards. The concentration of the standard curves varied from 0.3 to 3.0 mg/mL for major fatty acids, 0.01 to 1.0 mg/mL for minor fatty acids, and 0.008–0.8 mg/mL for C19:0 cpa. Correlation coefficients (*r*) for all analytes were ≥0.999.

Table 3 Precision: $n = 5$ for each day, overall $n = 20$

Fatty acid	Mean (g/100 g)					RSD (%)				
	Overall	A	B	C	D	Overall	A	B	C	D
Cottonseed										
C14:0	0.188	0.189	0.186	0.190	0.187	1.0	0.2	0.6	0.4	0.2
C16:0	4.66	4.67	4.60	4.74	4.63	1.2	0.4	0.6	0.3	0.2
C16:1	0.105	0.106	0.104	0.107	0.103	1.3	0.6	0.7	0.4	0.2
C17:0	0.0157	0.0157	0.0155	0.0162	0.0156	5.1	2.8	2.3	9.2	0.7
C17:1	0.0142	0.0141	0.0139	0.0142	0.0144	4.5	3.0	7.4	4.2	2.2
C18:1cpe	0.0859	0.0881	0.0846	0.0862	0.0846	4.2	6.0	3.4	3.0	3.7
C18:0	0.421	0.422	0.419	0.428	0.416	1.2	0.5	0.7	0.7	0.2
C18:1n9	3.00	3.01	2.97	3.05	2.98	1.2	0.4	0.5	0.5	0.3
C18:1n7	0.158	0.158	0.158	0.163	0.156	1.8	0.5	0.4	0.7	0.3
C18:2	10.0	10.0	9.85	10.2	9.95	1.4	0.5	0.6	0.3	0.4
C19:1 cpe	0.0452	0.0457	0.0450	0.0455	0.0445	4.0	5.4	4.1	3.8	2.8
C19:0 cpa	0.0457	0.0459	0.0444	0.0477	0.0447	5.4	5.5	5.8	4.5	3.4
C18:3n3	0.0239	0.0237	0.0237	0.0243	0.0237	2.8	2.2	4.6	2.1	1.5
C20:0	0.0477	0.0479	0.0474	0.0484	0.0472	1.2	0.9	0.3	0.8	0.5
C20:1	0.0117	0.0114	0.0122	0.0117	0.0114	4.0	3.3	3.4	3.2	2.3
C22:0	0.0229	0.0226	0.0226	0.0240	0.0224	3.6	1.9	2.7	3.1	1.4
C24:0	0.0178	0.0180	0.0185	0.0182	0.0167	7.0	6.2	5.2	7.0	7.2
Total fatty acids	18.9	18.9	18.6	19.2	18.8	1.3	0.4	0.6	0.3	0.3
Crude fat	20.0	20.1	19.8	20.0	20.1	1.2	0.6	1.7	0.6	1.1
Crude cottonseed oil										
C14:0	0.833	0.837	0.821	0.841	0.834	1.0	0.4	0.3	0.3	0.3
C16:0	21.4	21.4	20.9	21.7	21.4	1.4	0.4	0.4	0.3	0.3
C16:1	0.496	0.499	0.489	0.505	0.493	1.3	0.5	0.6	0.8	0.2
C17:0	0.0874	0.0867	0.0866	0.0883	0.0879	2.7	0.9	3.6	3.7	2.1
C17:1	0.0846	0.0814	0.0840	0.0875	0.0853	5.2	3.9	6.6	6.0	0.6
C18:1cpe	0.545	0.548	0.536	0.547	0.548	1.1	0.5	1.0	0.7	0.3
C18:0	2.35	2.36	2.32	2.40	2.34	1.3	0.5	0.5	0.3	0.4
C18:1n9	16.5	16.5	16.2	16.8	16.5	1.4	0.4	0.4	0.3	0.4
C18:1n7	0.765	0.758	0.756	0.790	0.757	2.0	0.8	0.5	0.7	0.4
C18:2	49.2	49.3	48.2	50.2	49.4	1.5	0.4	0.4	0.3	0.3
C19:1 cpe	0.304	0.305	0.300	0.307	0.305	1.3	0.8	1.1	1.2	0.6
C19:0 cpa	0.315	0.317	0.308	0.320	0.315	1.6	1.2	0.9	0.5	0.8
C18:3n3	0.153	0.152	0.152	0.153	0.153	1.3	1.4	1.1	1.4	1.4
C20:0	0.276	0.277	0.268	0.281	0.276	2.0	0.6	1.3	1.4	0.1
C20:1	0.0612	0.0583	0.0621	0.0638	0.0603	4.4	3.0	1.7	4.8	0.8
C22:0	0.140	0.139	0.137	0.145	0.139	3.0	3.5	1.8	1.4	0.9
C24:0	0.0854	0.0882	0.0761	0.0950	0.0822	12.5	9.0	8.4	12.2	9.1
Total fatty acids	93.6	93.7	91.7	95.4	93.8	1.5	0.4	0.4	0.3	0.3

A: analyst 1, RTx-Wax SN 0603617: USP resolution between 18:1cpe/18:0 = 1.4; between 19:0 cpa/α 18:3 = 1.0

B: analyst 2, ZB-Wax SN 119407: USP resolution between 18:1cpe/18:0 = 1.4; between 19:0 cpa/α 18:3 = 1.1

C: analyst 3, ZB-Wax SN 118102: USP resolution between 18:1cpe/18:0 = 1.4; between 19:0 cpa/α 18:3 = 1.0

D: analyst 1, ZB-Wax SN 209302: USP resolution between 18:1cpe/18:0 = 1.3; between 19:0 cpa/α 18:3 = 0.9

Table 4 Evaluation of the effect of Soxhlet extraction procedure on the repeatability of CPFA results: $n = 20$, units = g/100 g

Fatty acid	Non-extracted oil		Extracted oil		*F* test
	Mean	% RSD	Mean	% RSD	*P* value*
18:1 cpfe	0.545	1.1	0.474	3.0	0.000276
19:1 cpfe	0.304	1.3	0.263	3.1	0.00114
19:0 cpfe	0.315	1.6	0.314	2.7	0.0227
16:1	0.496	1.3	0.491	1.2	0.338
18:1	16.5	1.4	16.5	1.2	0.202
18:3	0.153	1.3	0.147	1.8	0.341

* *P* value >0.05 results equivalent at a 95 % confidence level

Due to the lack of readily available standards, it is widely accepted to use theoretical FID correction factors (TCF) for quantitation of CPFA [10, 18]. For this study, C19:0 cpa was used for quantitation of C18:1 cpe and C19:1 cpe. The difference between the TCF for these FAME, calculated according to AOCS method Ce 1i-07, is less than 1 %. As deviations between empirical and theoretical factors of 3 % or less are considered acceptable [19], it was decided to apply the linear regression curve of C19:0 cpa to C18:1 cpe and C19:1 cpe.

Limit of Quantitation (LOQ) and Reporting

The lowest level quantitation standard (~0.01 mg/mL for most analytes) was used to determine the limit of reporting: 0.05/100 g in oil, 0.01/100 g in cottonseed. Actual LOQ was calculated using the following equation [20]:

$$\mathrm{LOQ} = 10\sigma/S$$

where σ = the standard deviation of the *y*-intercept, S = the mean slope of the calibration curve.

Six different columns and three instruments were used to determine a robust LOQ that was not limited to one instrument/column. The quantitation standards were injected individually on each column, and a linear regression curve was calculated. For C18:1 cpe and C19:1 cpe, different sample weights of the crude oil were used to create a curve, using the mean concentration from precision to calculate the concentrations. The calculated LOQs (Table 2) are at or below the lowest quantitation standard concentrations.

Precision

Repeatability was performed by analyzing ground cottonseed and crude cottonseed oil in quintuplicate. Intra-laboratory reproducibility was determined by comparing quintuplicate results by different analysts over 4 days for a total of 20 replicates (Table 3). Different GC columns were used each day to confirm that the resolution between critical pairs of peaks could be maintained. The % RSD for the CPFA in cottonseed oil is similar to that of NFA at the same concentration levels; however, in cottonseed samples, the % RSD is higher. It is possible this difference is due to the effect of the extraction process on the ring structure of

Table 5 Refined cottonseed oil precision, $n = 5$ for each day, overall $n = 10$, one analyst, one column

Fatty acid	Mean (g/100 g)			RSD (%)		
	Overall	Day 1	Day 2	Overall	Day 1	Day 2
C14:0	0.888	0.891	0.884	0.6	0.4	0.3
C16:0	18.8	18.9	18.7	0.7	0.4	0.4
C16:1	0.588	0.594	0.600	1.2	0.6	0.5
C17:0	0.0872	0.0878	0.0866	2.8	3.6	1.8
C17:1	0.0922	0.0924	0.0921	3.5	4.0	23.3
C18:1 cpe	<0.05	<0.05	<0.05	–	–	–
C18:0	2.51	2.52	2.49	0.7	0.5	0.4
C18:1n9	17.7	17.8	17.6	0.9	0.5	0.4
C18:1n7	0.865	0.869	0.861	0.7	0.6	0.5
C18:2	51.4	51.8	51.1	0.8	0.5	0.5
C19:1 cpe	<0.05	<0.05	<0.05	–	–	–
C19:0 cpa	0.311	0.312	0.310	1.4	1.5	1.4
C18:3n3	0.126	0.126	0.126	1.2	1.1	1.4
C20:0	0.293	0.297	0.289	1.9	0.8	1.8
C20:1	0.0733	0.0720	0.0747	4.0	3.5	3.8
C22:0	0.145	0.143	0.146	2.3	2.3	2.1
C24:0	0.0858	0.0874	0.0842	8.1	9.2	7.3
Total fatty acids	94.0	94.6	93.4	0.8	0.4	0.5

Table 6 Average spiked recoveries: Day 2 and Day 3 cottonseed spikes added prior to lipid extraction

FAME	Cottonseed							
	Day 1		Day 2		Day 3		Overall	
	Mean ($n = 3$)	% RSD	Mean ($n = 3$)	% RSD	Mean ($n = 3$)	% RSD	Mean ($n = 9$)	% RSD
C18:0[a]	99.2	0.3	96.2	0.6	93.3	7.5	97.3	1.7
C18:3 n3[b]	106.5	0.7	96.1	1.6	99.0	6.0	100.2	5.2
C19:0 cpa[c]	100.3	2.6	95.2	1.7	97.0	5.8	96.3	3.4
FAME	Crude cottonseed oil							
	Day 1		Day 2		Day 3		Overall	
	Mean ($n = 3$)	% RSD	Mean ($n = 3$)	% RSD	Mean ($n = 3$)	% RSD	Mean ($n = 9$)	% RSD
C18:0[a]	98.6	0.7	100.2	0.4	98.5	0.6	99.1	1.0
C18:3 n3[d]	105.5	0.2	99.4	0.1	97.7	0.5	100.9	3.5
C19:0 cpa[c]	96.5	1.6	102.5	0.8	100.0	1.3	99.7	2.8

[a] 50 % of innate value (Day 1), 100 % of innate value (Day 2, 3)

[b] 200 % of innate value (Day 1), 100 % of innate value (Day 2, 3)

[c] 100 % of innate value

[d] 200 % of innate value

Table 7 Comparison of CPFA, by GC and HPLC analysis, as % of total fatty acids

Fatty acid	Mean (%)		RSD (%)		*P* value*
	HPLC[a]	GC[b]	HPLC[a]	GC[b]	
Cottonseed					
C18:1 cpe	0.497	0.516	3.5	4.7	1.1E−03
C19:1 cpe	0.241	0.276	12.8	4.1	1.3E−10
C19:0 cpa	0.182	0.250	20.5	4.4	2.5E−14
Crude cottonseed oil					
C18:1 cpe	0.567	0.582	4.9	1.0	3.7E−02
C19:1 cpe	0.264	0.325	9.2	0.9	6.6E−10
C19:0 cpa	0.302	0.336	11.3	0.8	2.1E−04

* *P* value >5.0E−02 results equivalent at a 95 % confidence level

[a] $n = 10$

[b] $n = 20$

the CPFA. To evaluate the effect of the extraction procedure on the CPFA, the crude oil was treated with the same conditions as the ground seed prior to analysis. Twenty replicate extractions of the crude oil were run over 4 days, and the results were compared to the results from the non-extracted oil. For the extracted oil, the % RSD of the NFA was similar to the non-extracted oil; however, the CPFA showed higher % RSD for the extracted oil compared with the non-extracted oil. Representative results are shown in Table 4. *F* test results confirmed the CPFA extracted oil and non-extracted oil variances are unequal. Since the only difference between the NFA and the CPFA is the ring structure in the CPFA, the higher variability for this compound class is attributable to the effect of the extraction on the ring structure.

A refined cottonseed oil also was analyzed over 2 days by one analyst (Table 5). The concentration of both C18:1 cpe and C19:1 cpe was below LOQ; C19:0 cpa, being more stable, was present at approximately the same level as in the crude oil.

Accuracy

In the absence of certified reference materials and analytical standards for C18:1 cpe and C19:1 cpe, accuracy was investigated by spiked recoveries, and comparison to alternative methods and the International Life Sciences Institute (ILSI) Crop Composition Database values [21].

Spiked recoveries were performed in triplicate on three separate days (Table 6). NFA were added as triacylglycerols (TAG), and C19:0 cpa was added as the methyl ester, because the TAG form was not available. The cottonseed sample was spiked after lipid extraction on day 1, and prior to extraction on day 2 and 3. Recoveries of C19:0 cpa ranged from 95.0 to 102.5 %.

Comparison was performed with two alternative methods: HPLC for CPFA and acid-catalyzed esterification GC analysis for NFA. For the HPLC method, crude cottonseed oil (20 replicates over 4 days) and one cottonseed sample (10 replicates over 2 days) were analyzed. For the GC method, two cottonseed samples were analyzed, 20 replicates of each over 4 days.

Compared to the HPLC method, results of the new method are higher and the % RSD is lower (Table 7). Based on the *T*

Table 8 Comparison of nutritional fatty acids by acid and base catalyzed esterification, on a sample basis. Difference in the results can be contributed to the free fatty acid content of the seed

Fatty acid	Cottonseed 1: units g/100 g				Cottonseed 2: units g/100 g			
	A Mean	B Mean	Absolute difference	*P* value*	A Mean	B Mean	Absolute difference	*P* value*
C14:0	0.143	0.140	0.003	1.27E−08	0.189	0.188	0.001	1.23E−01
C16:0	4.32	4.19	0.13	2.43E−11	4.71	4.66	0.05	6.80E−02
C16:1	0.0891	0.0866	0.0025	9.78E−09	0.106	0.105	0.001	2.51E−01
C17:0	0.0154	0.0147	0.0007	1.14E−03	0.0163	0.0157	0.0006	1.55E−01
C17:1	0.0136	0.0133	0.0003	2.62E−01	0.0154	0.0142	0.0012	4.81E−04
C18:0	0.450	0.434	0.016	6.55E−10	0.429	0.421	0.008	5.05E−03
C18:1n9	2.90	2.81	0.09	9.93E−12	3.05	3.00	0.05	1.27E−02
C18:1n7	0.143	0.137	0.006	2.27E−08	0.161	0.158	0.003	4.56E−03
C18:2	9.79	9.56	0.23	3.46E−09	10.1	10.0	0.1	2.07E−01
C18:3n6	0.0270	0.0261	0.0009	9.14E−04	0.0250	0.0239	0.0011	1.60E−03
C20:0	0.0471	0.0452	0.0019	1.52E−09	0.0493	0.0477	0.0016	3.68E−06
C20:1	0.00958	0.00994	0.00036	4.00E−02	0.0118	0.0117	0.0001	7.86E−01
C22:0	0.0215	0.0199	0.0016	3.57E−04	0.0246	0.0229	0.0017	2.61E−04
C24:0	0.0181	0.0159	0.0022	7.30E−03	0.0224	0.0178	0.0046	8.53E−06
Total fatty acids	18.0	17.5	0.4847	2.46E−10	18.9	18.7	0.2239	7.79E−02
FFA (g/100 g seed)			0.30				0.23	
Difference after subtracting free fatty acids			0.18				0.01	

A = acid catalyzed esterification, $n = 20$

B = base catalyzed esterification, $n = 20$

* *P* value >5.00E−02 results equivalent at a 95 % confidence level

Table 9 Comparison to ILSI database, g/100 g on a sample basis

Fatty acid	ILSI database			Reported method	
	Minimum	Maximum	Mean	Cottonseed 1	Cottonseed 2
C14:0	0.101	0.193	0.153	0.140	0.188
C16:0	3.82	5.40	4.54	4.19	4.66
C16:1	0.090	0.133	0.113	0.087	0.105
C18:0	0.41	0.71	0.52	0.434	0.421
C18:1n9	2.1	4.1	3.1	2.95	3.16
C18:2	9.0	12.8	10.6	9.56	10.0
C18:3n3	0.02	0.05	0.03	0.026	0.024
C20:0	0.041	0.065	0.053	0.045	0.048
C22:0	0.021	0.036	0.028	0.020	0.023
C19:0 cpa	0.014	0.048	0.030	0.044	0.046
C18:1 cpe	0.042	0.097	0.071	0.091	0.086
C19:1 cpe	0.037	0.075	0.056	0.047	0.045

test, the results are not statistically equivalent. The differences could be due to better quantitation using the C19:0 cpa standard, and better repeatability of the GC method.

When the data from acid and base-catalyzed esterification was statistically compared (Table 8), all of the fatty acids were statistically different. While statistically different, the numeric differences observed ranged from 0.0001 to 0.23 g/100 and were not considered biologically meaningful. The difference is due primarily to FFA, which are not converted to methyl esters by base transesterification. To confirm this, the FFA content of both seed samples was tested by titration. The new method will not account for FFA, and this will be a fundamental difference from traditional NFA analyses when comparing total fatty acids on a weight/weight basis in cottonseed and crude cottonseed oils. The statistical difference may be difficult to quantify however, as FFA in cottonseed is inherently variable due to moisture content and thermal history from the time of harvest through storage [22]. Both base and acid esterified results were additionally compared to ILSI Crop Composition Database. Both sets of results compare favorably with each other and are well within the published values established for cottonseed oils (Table 9). It is therefore concluded that the impact of non-esterified free fatty acids will be minimal in most cases.

Conclusions

Combining the analysis of NFA and CPFA by GC has been proven valid and practical for compositional studies of

cottonseed and cottonseed oils. This approach efficiently combines two legacy methods into one, and most notably, eliminates the labor intensive HPLC analysis. Conclusive accuracy data for malvalic and sterculic acids, via spike recovery, may be possible in the future as methyl ester and triacylglycerol forms become available.

Acknowledgments The authors would like to thank Holly Baganz, Andy Dyer, David Ellingson, Sharon Habeck, Stephanie Jolitz, Sarah Kanable, Danielle Michalica, Chris Richard, and Matt Yerges.

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