Quantitative Determination of Cyclopropenoid Fatty Acids in Cottonseed Meal[1]

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Abstract

A rapid analytical procedure for determining the residual cyclopropenoid fatty acids (CPA) in cottonseed meal has been developed. The procedure involves room-temperature extraction of crude CPA-containing lipids with a hexane-water-acetone azeotrope solvent, followed by a benzene-methanol wash. The crude lipids are then converted to methyl esters by methanolysis with sodium methoxide. Extraction with petroleum ether, followed by washing with aqueous acetone, results in a substance which is free from interfering materials. The purified methyl esters are then analyzed for CPA by a spectrophotometric modification of the Halphen reaction.

Introduction

MOST COTTONSEED MEALS contain residual levels of cyclopropenoid fatty acids (CPA), which have been implicated as a cause of certain unusual biological effects on poultry and on the storage quality of eggs (2). During laboratory studies on the development of practical methods for removing or inactivating these components in commercial cottonseed meal, a suitable method for monitoring the progress of the work was required. Since none of the available analytical procedures for the analysis of fats and oils for CPA were directly applicable to cottonseed meal (4), a sensitive analytical procedure was developed for this purpose. The method can be used for quality control although the method as such does not account for the total amount of residual "fixed" lipids in the meal. However it is a reliable method for obtaining relative data on low levels of CPA.

The method consists of a solvent extraction of residual oil by a hexane-acetone-water azeotrope, interesterification of the extracted material to its methyl esters, removal of interfering materials by an aqueous acetone wash, and analysis of the CPA content by a spectrophotometric adaptation of the Halphen reaction (1).

The meals used for this study are representative of the four commercially available cottonseed meals. The method as described has also been applied successfully to the analysis of the CPA content of chemically treated meals.

Reagents and Apparatus

Reagents

Hexane-Water-Acetone (HWA) Azeotrope. Mix 56.5, 1.5, and 42.0% (by volume) of technical-grade hexane, water, and acetone respectively, and distill the mixture at 48.0C.

Benzene-Methanol Azeotrope. Mix 57.8 and 42.2% (by volume) of technical-grade benzene and methanol respectively, and distill the mixture at 57.5C.

Sodium Methoxide Solution (1%). Dissolves 10.5 g of clean metallic sodium in one liter of absolute methanol. This reagent should be made fresh at least once a month and stored in a tightly stoppered bottle.

Petroleum Ether. Use 30 to 60C petroleum ether.

Aqueous Acetone (50%). Mix 500 ml of reagent-grade acetone with 500 ml of distilled water.

n-Butanol. Use certified reagent-grade n-butanol throughout the procedure.

Sulfur in Carbon Disulfide (1%). Mix 5 g of technical-grade roll sulfur with 500 ml of reagent-grade carbon disulfide.

Morpholine Solution (4%). Dissolve 4 ml of reagent-grade morpholine in 100 ml of n-butanol.

Apparatus

General Laboratory Ware. Use commercially available laboratory ware as specified in Experimental Procedures.

Halphen Reaction Flasks. Fabricate the Halphen reaction flasks from low-actinic glass flasks by extending the necks of 50-ml Erlenmeyer flasks with 25-mm tubing and terminating them with a 24/40 standard-tapered joint at the top. The over-all length is approximately 8 in., and the extension tubing used does not have to be low-actinic glass. Vented caps for the flasks are constructed by sealing a 1-in. length of 1-mm capillary tubing to the hollow glass stopper (1).

Experimental Procedures

Extraction of Meal

A 20- to 30-g sample of cottonseed meal is placed in a 150-ml medium-porosity, fritted-glass Buchner funnel so that the meal does not fill more than half the total volume of the funnel. The funnel is attached to a suction flask of at least 250-ml capacity. The following filtration technique is used to extract the meal.

Sufficient solvent, about 80 ml, is placed on the meal to 1 cm above the top. The meal is stirred with a spatula to ensure removal of any trapped air and to prevent channeling. A small amount of suction can then be applied to maintain a dripping rate of not more than 10 ml/min. Four separate volumes of the HWA azeotrope solvent are used, and the meal need not be allowed to dry completely before subsequent portions of solvent are added. A final extraction rinse is performed by using about 80 ml of the benzene-methanol azeotrope.

The extracts are periodically pooled in a 300-ml ground-glass, round-bottom flask, and the solvents are removed completely by stripping under reduced pressure on a rotary evaporator. A water bath is maintained at 50 to 60C to aid removal of the solvents.

Preparation of the Sample for Analysis

Sufficient petroleum ether just to redissolve the crude extract is added to the flask. A 10-ml portion of a 1% sodium methoxide solution is added, and this mixture is refluxed for 30 to 40 min with boiling chips to prevent bumping. The reaction mixture is allowed to come to room temperature before it is

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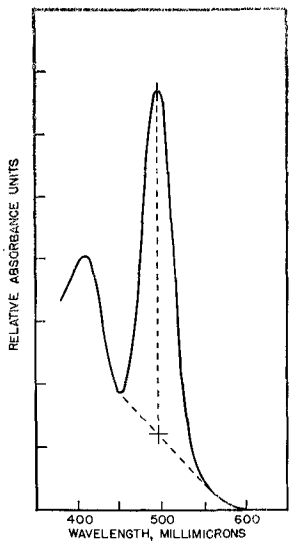

FIG. 1. Typical Halphen reaction spectrum.

transferred to a 250-ml separatory funnel. The reaction flask is rinsed with several portions of petroleum ether and finally with a 2- to 3-ml portion of methanol. The rinsings are then transferred to the separatory funnel. The total volume of solvents used is about 50 ml.

The mixture is shaken thoroughly and then allowed to separate. The separated lower phase is drawn off into a second 250-ml separatory funnel and re-extracted with a fresh portion of petroleum ether. The lower phase is then discarded. The two petroleum ether fractions are washed with several 50-ml portions of aqueous acetone solution. Washing is continued until no further color can be removed and the petroleum ether phase is clear. The aqueous acetone washings are discarded.

The petroleum ether fractions are then combined and shaken with about 40 to 50 g of anhydrous sodium sulfate. The solution is further dried by filtering it through an additional 25 g of sodium sulfate. The Na_2SO_4 remaining in the separatory funnel as well as that on the filter paper is rinsed with several small portions of petroleum ether. The solvent is finally evaporated on a rotary evaporator to yield a residue free from materials that interfere with the Halphen reaction.

Spectrophotometric Analysis of Sample

The CPA in the residue is determined by the spectrophotometric modification of the Halphen reaction, as described by Bailey et al. (1). The residue obtained in the preparative step is transferred directly to the Halphen reaction flask by rinsing it with portions of n-butanol until a volume of 25 ml has been obtained. A 0.5-ml aliquot of the 4% morpholine reagent and then a 5-ml aliquot of the 1% sulfur in carbon disulfide reagent are added. After the mixture is allowed to react for 2.5 hr at 110C, it is transferred to a low-actinic, glass volumetric flask and diluted to 50 ml with n-butanol. This solution appears to be stable for 3 to 4 hr. Further dilution can be done during this time if the CPA concentration is too large to be measured.

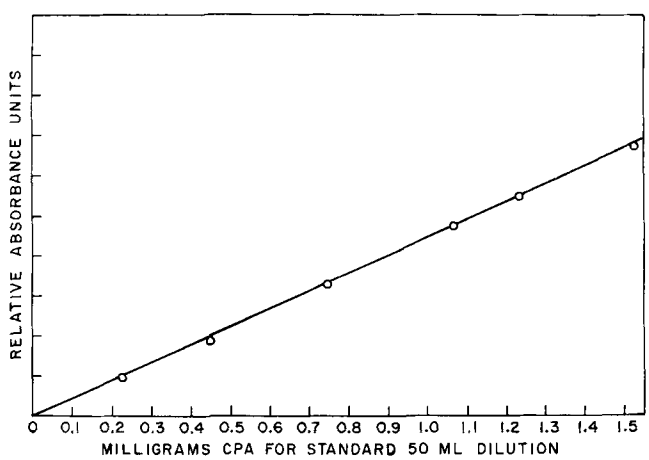

FIG. 2. Calibration curve for Halphen reaction.

The absorption spectrum of the solution is determined by using a recording spectrophotometer with a 1-cm cell and water as a reference. If the CPA content of the sample is low, longer cell paths are used. A scan rate of 25 A/sec over a range of 600 to 400 mμ gives a good spectrum for CPA analysis. The net absorption attributable to CPA is measured for the peak maximum at 495 mμ by a background technique. As shown in Fig. 1, the net absorption is calculated by subtracting the background absorption from the total peak height.

The amount of CPA (as malvalic acid) is then read directly in milligrams from a calibration curve (Fig. 2), which is based on the analysis of reference oils of known CPA content. This curve is constructed by analyzing various aliquots of these reference oils and by using the above spectrophotometric modification of the Halphen reaction. The net absorption observed for each sample is then plotted against milligrams of CPA present.

CPA is then reported as ppm in the meal sample by using the equation:

$$\text{ppm} = \frac{\text{mg of CPA} \times \text{dilution factor}}{\text{sample size in grams}} \times 1000$$

The milligrams of CPA are read directly from the calibration curve by using the above procedure when the dilution factor is one. If dilution is necessary because of a high amount of CPA in the meal, a dilution factor must be applied in the equation.

Duplicate analysis per meal is normally sufficient to give reproducible results.

Discussion

In dealing with complex natural products, such as cottonseed meal, no analytical method can be more reproducible than the accuracy or replicability of the technique by which the individual analytical samples are obtained. When small samples are taken from large batches of meal, it is essential that the technique give representative samples. Sampling techniques with probes or similar devices are described in various testing manuals and need not be discussed.

For relatively small batches of meal, the standard quartering technique is applicable and adequate. For example, when samples are obtained from a 10-lb batch by taking random amounts of meal, the analytical results can vary by as much as 25%. When samples are obtained by the quartering technique, the variation between samples of the same meal is always less than 5%, as shown in Table I.

The sample size of the meal to be analyzed should be based on the expected amount of CPA; it is best found by experimentation. This procedure is directly applicable to a convenient working sample of meal containing a total of 0.1 to 1.0 mg of CPA. If the

sample contains higher concentrations of CPA, it will have to be diluted during spectrophotometric analysis.

After reliable and representative meal samples have been obtained, the extraction, preparation, and analysis procedures described above are simple and straightforward. Caution is required, however, in the application of vacuum in the solvent-extraction and stripping operations. Extract solutions of certain meals tend to foam greatly when suction is first applied, and care must be taken to avoid losses.

In these solvent-extraction studies of cottonseed meal the benzene-methanol azeotrope was found to be a slightly better solvent than HWA. However the former solvent extracted too much extraneous material, the preparation procedure was considerably more cumbersome, and duplication of analyses was poor. A final benzene-methanol rinse is included in the analytical extraction procedure only to optimize the removal of residual CPA.

If the completeness of CPA removal is in doubt, this final benzene-methanol extract or one additional such extract can be collected and analyzed separately. The efficiency of the extraction technique was checked by adding a crude cottonseed meal extract of known CPA content to several meal samples and then determining the CPA content. The recoveries were found to be 98.0, 100,2, and 101.0%, based on the amount of CPA added. Prolonged exhaustive extraction also gave comparable results.

The procedure for preparing the extracts for analysis was checked by adding refined cottonseed oil of known CPA content to crude extract samples. The recovery was also within the range of 99.5 to 100.5% of the total CPA added. It should be pointed out that the procedure, as set up, will not include the free CPA in the meal since they will not be removed in the ethereal washing of the alkaline interesterification product. However it is assumed that the ratio of free CPA to total free fatty acids is in the same relative proportion as their corresponding glyceride and phosphatide distribution; therefore any error due to this factor should be minimal.

It was found necessary at this stage in the procedure to extract the alkaline phase of the interesterification product rather than acidify and then extract since, on acidification, the reaction product became highly colored and this material was also extracted. The inclusion of this material into the ether fraction prevented any reliable analytical data via the Halphen reaction since interfering absorption bands completely mask the 495-mμ band. Another problem encountered in extracting the acidified reaction products was the tendency for these to form emulsions which were extremely difficult to break. Inadvertent losses due to these emulsions frequently led to erratic recoveries of the esters.

The minimum reflux time for the sodium methoxide reaction depends upon the amount of CPA present. Thirty minutes are ample time although extremely large samples of extract or samples containing very high amounts of CPA, *e.g.*, more than 50 mg, require slightly longer reaction times. When the reaction time is extended beyond 1 hr however, CPA values decreased. With a reflux time of 1 hr, average CPA values are about 5% lower whereas a 1½-hr period lowers the analyses values by about 10%.

A study of the stability of the CPA showed that, when the crude extract is stored in solution at room temperature, the CPA values decrease rapidly, and significant changes occur within 24 hr. However the solvent-free extract can be stored in the refrigerator for several days without loss of the CPA. The cleaned samples obtained by the preparative procedure are considerably more stable, and no change is noted after two weeks of storage at 0C.

TABLE I
Replicability of CPA Analysis of Various Commercial Cottonseed Meals

Meal sample[a]		CPA (as malvalic acid), ppm	Deviation ppm	Deviation %[b]
Direct solvent-extracted				
1A		76.0	+0.4	0.5
B		77.3	+1.7	2.2
2A		76.4	+0.8	1.0
B		72.8	−2.8	3.7
	Aver.	75.6	±1.4	1.9
Prepressed solvent-extracted				
1A		20.6	−0.4	1.9
B		22.8	+1.8	8.6
C		20.6	−0.4	1.9
D		19.8	−1.2	5.7
	Aver.	21.0	±1.0	4.8
Screw-pressed				
1A		151	−2	1.3
B		149	−4	2.6
2A		159	+6	3.9
B		154	+1	0.6
3[c]		161	+8	5.2
4[c]		143	−10	6.5
	Aver.	153	±5	3.3

[a] Replicate samples (A, B, C, D) were taken during the same quartering operation (1 or 2).
[b] Percentage of deviation from the mean.
[c] Obtained by probe technique. Average CPA content for the two samples is 152 ppm. Although deviation is large, the average CPA content is comparable. Average deviation of CPA in this meal without these samples is ±3 ppm or 2.0%.

For this analytical procedure the limit of detectable CPA is 0.02 mg. Smaller quantities of CPA can be determined if longer cell paths are used with the spectrophotometer. One advantage afforded by this procedure is that relatively small meal samples are taken. The resulting lipid fractions obtained are adequate for reproducible analyses.

In carrying out this procedure, some concern was exhibited as to the levels of CPA in the cottonseed meal after the extraction for analyses. In essence, this material represents the CPA associated with the so-called "fixed oil." In order to gain some information regarding this point, several cottonseed meals were subjected to the saponification method reported by Szutowicz (5). For this study, meals that were previously extracted for CPA analysis as well as the original meals were subjected to saponification. The products were analyzed either as the free acids or as methyl esters by using both sodium methoxide and diazomethane for esterification.

The data obtained from this experiment are presented in Table II. Although in all cases higher amounts of extractable lipids were recovered from the meals by the saponification technique, the resulting products were unsuitable for CPA analysis. In all saponified samples only traces of CPA could be detected by the Halphen reaction. Investigation showed that the saponification procedure itself was not the limiting factor since, when a cottonseed oil was subjected to the saponification technique, approximately 76% of the original CPA content was recovered. Therefore it would seem that, although HWA extraction of the available lipids does not account for all of the "fixed" lipids of the meal, it is the most feasible method for characterizing the residual CPA content of cottonseed meal.

When applied to the analysis of CPA in cottonseed salad oils and to a crude oil extracted from raw cottonseed meats, the modified Halphen spectrophotometric procedure yielded values comparable with

TABLE II

Comparison of Methods: Cottonseed Meals

Meal No.	Type of meal	IITRI Analytical Extraction			Saponification			
		Crude extract, %	Recovered esters, %	CPA found, ppm	Crude extract, %	As triglyceride,[a] %	Ester yield,[b] %	CPA found, ppm
A	Commercial screw-press, untreated	3.7	2.3	69.02	5.1	5.4	104	T[j]
		3.9	2.5	67.68	5.1	5.4	98.5	T
					5.2	5.4	19[c]	T
							54[d]	T
B	Meal residue from A after anal. ext.	....	....	....	3.0	3.2	84.0	T
		....	....	....	2.2	2.2	102	..
		....	....	....	2.5	2.6	82.5	T
		....	....	....	2.2	2.3	[g]	T
C	Commercial screw-press HWA-treated	0.5	0.4	0.74	2.3	2.4	99.2	T
					2.7	2.8	90.1	T
					2.4	2.5	91.3	..
D	Meal residue from C after anal. ext.	....	....	....	1.9	2.0	104	0
		....	....	....	1.9	2.0	104	0
E	Commercial hexane ext.-HWA-treated	0.01	0.01	0.95	1.8	1.8	108	..
		1.0	0.23[c]	0.46	2.1	2.2	101	0
			0.09[d]	Trace	2.4	2.5	96.8	0
F	Meal residue from E after anal. ext.	....	....	....	2.0	2.0	90.4	..
		....	....	....	2.1	2.2	94.5	T
		....	....	....	2.1	2.2	90.0	T
G	Commercial hexane ext.-HWA-treated	1.0	0.2	1.06	1.6	1.6	106	T
		0.8	0.2	1.17	1.5	1.6	105	T
H	Meal residue from G after anal. ext.	....	....	....	0.90	0.95	148	..
		....	....	....	0.96	1.0	112	T
		....	....	....	0.84[h]	0.87	104	T

[a] Calculated as triglyceride by multiplying the crude extract weight obtained from saponification by 1.045.
[b] Methyl ester yield based on calculated triglyceride value.
[c,d] Sodium methoxide esterification: [c] = sample obtained from basic medium; [d] = aqueous phase of the same sample acidified with sulfuric acid and re-extracted with petroleum ether.
[e] Each of the analytically extracted samples was quartered to obtain duplicate aliquots for saponification.
[f] The two analytically extracted samples were pooled and then quartered to obtain triplicate aliquots for saponification.
[g] Sample analyzed as the free acids.
[h] Acidified with acetic acid instead of HCl.
[j] Trace = less than 0.5 ppm.

those obtained by a precise hydrogen bromide titration method (3), as shown in Table III. Although somewhat less precise than the titration procedure, the Halphen spectrophotometric reaction was used for the analysis of CPA in meal extracts since its inherent sensitivity allowed the detection of extremely low levels of residual CPA.

If a reaction bath that accommodates 15 Halphen reaction flasks is used, 45 ester fractions can be analyzed in one day. Since four days are needed to extract and esterify these samples, a total of 45 samples of meal can be analyzed in one week by using this procedure.

TABLE III

Comparison of HBr Titration Technique with Halphen Spectrophotometric Method

Oil	CPA (as malvalic acid), %	
	HBr titration	Halphen
Commercial salad oil		
A	0.03	0.02
	0.03	0.03
B	0.14	0.11
	0.14	0.14
Crude cottonseed oil		
C	0.49	0.46
	0.49	0.48

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