storage without appreciably changing the over-all fatty acid content of the fat.

REFERENCES

1. American Meat Institute Foundation, "The Science of Meat and Meat Products," W. H. Freeman & Co., San Francisco, 1960, p. 124–131.
2. Lea, C. H., "Lipids and Their Oxidation," The AVI Publishing Co., Inc., Westport, Conn., 1962, p. 3–28.
3. Meyer, L. H., "Food Chemistry," Reinhold Publishing Corp., New York, 1960, p. 32–42.
4. Watts, B. M., Adv. Food Res. *5*, 1–52 (1954).
5. Lundberg, W. O., "Lipids and Their Oxidation," The AVI Publishing Co., Inc., Westport, Conn., 1962, p. 31–50.
6. Watts, B. M., *Ibid.*, 202–214 (1962).
7. Lea, C. H., J. Soc. Chem. Ind. *50*, 215T–220T (1931).
8. Ramsbottom, J. M., Refrig. Eng. *53*(1), 19–23 (1947).
9. Privett, O. S., F. J. Pusch and W. O. Lundberg, Food Tech. *9*, 347–351 (1955).
10. Hiner, R. L., A. M. Gaddis and O. G. Hankins, *Ibid. 5*, 223–229 (1951).
11. Chang, I., and B. M. Watts, JAOCS *29*, 334–338 (1952).
12. Brown, J. B., and D. K. Kolb, Prog. Chem. Fats Other Lipids *3*, 57–94 (1955).
13. Garton, G. A., T. P. Hilditch and M. L. Meara, Biochem. J. *50*, 517–524 (1952).
14. Hilditch, T. P., and R. K. Shrivastava, JAOCS *26*, 1–4 (1949).
15. Luddy, F. E., G. R. Fertsh and R. W. Reimenschneider, *Ibid. 31*, 266–268 (1954).
16. Reiser, R., and H. G. R. Reddy, *Ibid. 36*, 97–99 (1959).
17. Bailey, A. E., "Industrial Oil and Fat Products," 2nd ed., Interscience Publishers, Inc., New York, 1951, p. 142–149.
18. Lange, W., JAOCS *27*, 414–422 (1950).
19. Snedecor, G. W., "Statistical Methods," 2nd ed., Iowa State University Press, Ames, 1956, p. 291–328.

[Received June 1, 1964—Accepted September 29, 1964]

Determination of Free and Bound Gossypol in Swine Tissues[1]

F. H. SMITH, Nutrition Section, Department of Animal Science, North Carolina State of the University of North Carolina at Raleigh, Raleigh, North Carolina

Abstract

Methods have been developed for the determination of free and bound gossypol in the tissues of swine ingesting diets containing free gossypol. Data presented show satisfactory reproducibility and accuracy of the methods.

Introduction

ISOLATION OF GOSSYPOL from the livers of pigs that had consumed toxic cottonseed meal (2) suggested that studies of deposition and accumulation of gossypol in the organs may be helpful in elucidating the metabolism of this compound in animals. An essential phase in the effectiveness of such a study is analytical methods for measuring the free and the bound gossypol. The procedures used for the estimation of these components in cottonseed meal (3,4) were applied to liver tissue of pigs fed various gossypol-containing cottonseed meals (1). The modifications of these methods for application to animal tissues for the improvement of both the analytical mechanics and accuracy are herein described.

Free Gossypol

Reagents. a) Aniline: reagent, freshly distilled, water clear; b) acetic acid (5): glacial reagent; c) ethyl ether: U.S.P. or purified for fat extraction, must be peroxide free; d) solution A: ethanol + 0.2 ml glacial acetic acid/liter; e) solution B: a 60% ethanol-water solution prepared by diluting 715 ml of 95% ethanol to 1 liter with distilled water and subsequently adding 200 ml of ethyl ether and 0.2 ml glacial acetic acid; f) Hyflo Super-Cel: to remove iron, boil 100 g of Hyflo Super-Cel with 600 ml of distilled water and 50 ml of concd HCl for 15 min, filter through a paper in a Buchner funnel and wash with distilled water. Repeat the acid treatment, wash and dry.

Procedure

The tissues are preserved in a frozen state until analyzed. Just before analysis, the tissues are partially thawed, ground in a food chopper, thoroughly mixed and placed in a glass bottle to prevent moisture changes.

Transfer a 10-g sample of the ground tissue to either the jar of a Serval Omni-mixer or a similar comminuting apparatus, add 50 ml of 95% ethanol (solution A) and 20 ml of ether. Homogenize the mixture for 2 min while the blending jar is surrounded with ice water to prevent heating.

In the meantime, prepare a filter by placing a 5.5-cm filter paper in a size 1 Buchner funnel and after applying vacuum pour a suspension of 2–3 g of Hyflo Super-Cel in ca. 15 ml of 95% ethanol on the paper. After washing the jar cap and blades of the homogenizer with solution B delivered from a wash bottle, suspend 2–3 g of Hyflo Super-Cel in the homogenate and filter through the prepared Buchner funnel. Wash the blender jar with solution B and pour the washings over the tissue residue in the Buchner funnel. Thoroughly wash the tissue residue, which will serve as the sample for bound gossypol, with small portions of solution B to remove all of the free gossypol. Exercise care so that the combined filtrate and washings are not less than 120 ml nor more than 130 ml. Using a flask calibrated to contain 130 ml, dilute the filtrate to 130 ml, mix and refilter a portion of the solution through Whatman No. 1 filter paper if turbid. If the volume should exceed 130 ml, the excess is measured and the calculations are made accordingly.

Transfer 10-ml aliquots in triplicate to 25-ml volumetric flasks, one of which is used as the reference solution after diluting to the mark with solution B. To the other 2 flasks add 0.5 ml freshly distilled aniline and heat them on the surface of the steam bath not directly over the steam for 40 min to convert the gossypol to dianilinogossypol. After the flasks have cooled to room temp, add 2 ml of ether to replace that lost during heating and sufficient solution B to bring the volume to 25 ml. After mixing the contents of the flasks, determine the absorbance at a wavelength of 445 mμ, using the aliquot containing no aniline as the reference solution. Determine the gossypol content of the tissue from a standard absorbance-concn curve prepared from pure gossypol.

Prepare the standard curve by dissolving 0.0250 g of pure gossypol in approx 10 ml of ethyl ether, dilute to 100 ml with solution B and mix. Transfer a 10-ml aliquot to a 100-ml volumetric flask, dilute to volume with solution B and mix. In triplicate and at ml intervals, pipet a series of aliquots ranging from 1–8 ml into 25-ml volumetric flasks. Reserve one flask containing an aliquot at each volume level as a reference. Dilute the remaining aliquots to at least 5 ml with solution B, add 0.5 ml freshly distilled aniline and

[1] Published with the approval of the Director of Research of the N.C. Agricultural Exper. Sta. as Paper No. 1836 of the Journal Series.

TABLE I

Free and Bound Gossypol of Liver Tissue from Pigs

Pig No.	% Free gossypol in diets	No. of deter-minations	Free gossypol µg/g		No. of deter-minations	Bound gossypol µg/g	
			Range	Average		Range	Average
1	0.060	2	133–136	135	2	118–118	118
2	0.060	2	133–152	143	2	113–126	120
3	0.060	2	136–139	138	2	128–138	133
4	0.060	2	119–122	121	2	114–125	120
5	0.060	2	109–118	114	2	192–196	194
6	0.000	17	0.0–3.9	1.7	4	2.3–4.3	3.4
7	0.000	14	1.7–3.9	2.6	4	2.2–3.3	2.8

heat on a steam bath gently for 40 min. After cooling, dilute all solutions, including the references to 25 ml with solution B. Carefully avoid contaminating the reference solutions with aniline. Mix well and, using the appropriate reference solution, determine the absorbance at a wavelength of 445 mµ. Prepare the standard curve by ploting absorbance vs. concn in µg of gossypol/25 ml.

The gossypol in µg contained in the aliquot of the test sample diluted to 25 ml after conversion to dianilinogossypol may be determined graphically from the standard curve. The gossypol content of the sample in µg/g of sample may be calculated from the following equation when the extract is diluted to 130 ml and aliquots are diluted to 25 ml.

$$\mu g \text{ gossypol/g} = \text{absorbance/wt sample (g) in a 10-ml aliquot} \times \alpha$$
$$\alpha = \text{absorbance}/\mu g \text{ gossypol in 25-ml standard}$$

The 95% ethanol-ether mixture extracts the free gossypol and reduces the moisture content to a suitable level for the bound gossypol estimation. The ether dissolves the tissue fat, preventing solution turbidity, and the ether lost during vacuum filtration must be replaced.

Bound Gossypol

Reagents. a) Aniline: freshly distilled, water clear; b) solution B: same as for free gossypol; c) hexane: redistilled.

Procedure

Transfer the tissue residue remaining from the free gossypol extraction from the Buchner funnel to a sheet of white paper. Remove the layer of Hyflo Super-Cel from the tissue residue with a spatula without disturbing the tissue residue. Chop the tissue residue from the free gossypol determination by means of scissors, pulverize and transfer to a 250-ml ground-glass stoppered Erlenmeyer flask. Moisten the residue with 4 ml of solution B, add 2 ml of the freshly distilled aniline to the flask, and mix until all of the residue is moistened with the mixture. Place the flask on the metal surface of a steam bath not in direct contact with the steam and heat gently for 45 min with occasional shaking to prevent overheating the tissue on the bottom of the flask. Remove the flasks from the steam bath, and immediately add ca. 20 ml of glass beads (diam 6 mm) and add 50 ml of redistilled hexane from a pipet while swirling the flask in a pan of hot water (60–70C) to expel the air. Insert the glass stopper tightly with a twisting motion to prevent leakage. Shake vigorously for 1 hr, then filter the extract through paper into a 50-ml volumetric flask. Read the color intensity as absorbance at a wavelength of 440 mµ using hexane as the reference solution. If necessary because of color density, dilute suitable aliquots to 25 ml with hexane. Determine the gossypol content of the tissues from a standard absorbance-concentration curve prepared from pure gossypol.

Prepare a standard absorbance-concn curve for bound gossypol by dissolving 0.0250 g of pure gossypol in ca. 10 ml of ethyl ether and making the solution to 100 ml with hexane. Transfer an aliquot of 10 ml to a 100-ml volumetric flask, dilute to 100 ml with hexane and mix. In triplicate and at ml intervals, transfer a series of aliquots ranging from 1–8 ml into 25-ml volumetric flasks. Reserve one flask of each group as a reference. To the remaining flasks, after diluting the 1- to 4-ml aliquots to 5 ml with hexane, add 0.5 ml of freshly distilled aniline and heat gently on the steam bath for 45 min. Allow to cool and make references and samples to 25 ml with hexane exercising care to avoid contaminating the references with aniline. After mixing, read the absorbance using the appropriate reference solution at a wavelength of 440 mµ. Plot absorbance vs. concn in µg gossypol/25 ml and calculate the extinction coefficient based on a volume of 25 ml.

The bound gossypol content of the test sample may be determined graphically from the standard curve; however, the values scaled from the curve must be doubled if no further dilutions are necessary since the gossypol was extracted in a volume of 50 ml and the standards were prepared in a volume of 25 ml. The µg gossypol/g of sample may be calculated, using the extinction coefficient α according to the equation following when a 10-g sample is used:

$$\text{micrograms gossypol/g of sample} = \frac{2 \times \text{absorbance}}{10 \times \alpha}$$
$$\alpha = \text{absorbance}/\mu g \text{ gossypol in 25 ml of standard}$$

Results and Discussion

These methods have been used for the determination of free and bound gossypol in the various organ tissues of swine fed diets containing 0.06% free gossypol for 15–17 days. Representative data for duplicate samples of liver tissue from 5 pigs, No. 1–5, are presented in Table I. Values obtained by the described methods for free and bound gossypol in liver tissues of two non-gossypol consuming pigs, No. 6 and 7, also are presented in Table I.

A comparison of the free and bound gossypol values for the liver tissues from the gossypol-consuming pigs with the corresponding values from the non-gossypol-consuming pigs shows a marked difference. The low values for free and bound gossypol in the livers of the control animals are insignificant when compared with those obtained from the pigs fed diets containing gossypol.

An experiment was carried out to determine the recovery of gossypol added to liver tissue of pigs No. 6 and 7. Ten ml of solution B containing 0.25 mg of gossypol was mixed with 10-g samples of ground liver tissue of pigs No. 6 and 7, respectively, and then was heated for 15 min on the steam bath with occasional stirring. The amt of gossypol recovered, using hexane as the reference solution for bound, were: pig No. 6, free 0.0715 mg, bound 0.1775; pig No. 7, free 0.0658

mg, bound 0.1888 mg. The total percentage recoveries were 98.6 and 101.8%. When the residues from 10 g of liver of pigs No. 6 and 7 previously extracted for free gossypol were extracted with hexane without the addition of aniline and the extracts were used as the respective reference solutions for bound gossypol, the total recoveries were 88.4 and 91.2%.

The modification of the original method for total gossypol (4) by substituting hexane for chloroform resulted in bright yellow liver tissue-extracts for bound gossypol from gossypol-consuming pigs. These extracts gave spectral curves characteristic of curves prepared from the conversion of pure gossypol to dianilinogossypol under the conditions of this method. In contrast, chloroform-extracts for bound gossypol at times have a pinkish cast which affects the absorbance values. Some extracts of spleen tissue have a slight pinkish-brown tint even when the extraction is made with hexane. This extraneous material affects the spectral curve slightly and shows a peak at approx 380 mμ.

By these methods, free and bound gossypol have been found and measured in liver, kidney, spleen, heart, lung, pancreas, lymph nodes and diaphragm muscle tissues of swine which have consumed diets containing free gossypol. The methods also have been applied to tissue of rats and goats with positive results.

ACKNOWLEDGMENTS

The study in which the proposed methods were adapted to animal tissue analysis was partially supported by the National Cottonseed Products Association, Inc., and research grant AM 07039 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Department of Health, Education and Welfare.

REFERENCES

1. Clawson, A. J., F. H. Smith and E. R. Barrick, J. An. Sci. *21*, 911–915 (1962).
2. Smith, F. H., JAOCS *40*, 60–61 (1963).
3. Smith, F. H., Ind. Eng. Chem. Anal. Ed. *18*, 43–45 (1946).
4. Smith, F. H., JAOCS *35*, 261–265 (1958).
5. Smith, F. H., Ind. Eng. Chem. Anal. Ed. 18, 658, (1946).

[Received July 17, 1964—Accepted October 2, 1964]

Vernonia anthelmintica (L.) Willd. The Effect of Storage on the Epoxy Content of the Seed Oil and Trivernolin[1]

W. E. SCOTT and C. F. KREWSON, Eastern Regional Research Laboratory,[2] Philadelphia, Pennsylvania

Abstract

Accessions of *Vernonia anthelmintica* (L.) Willd. seed from India and Pakistan varied somewhat in the amt of oil that they contained, but the oils did not vary significantly in their epoxy content. Storage of the whole seed for periods up to three years did not affect the quality of the oil, but the activity of the seed enzyme system seemed to increase with time. The data also show a rapid development of free fatty acid once the seed is ground. The epoxy content of low FFA *Vernonia* oil and trivernolin changed only slightly when these products were stored at room temp for six months. However, the viscosity of the samples that were exposed to light increased greatly indicating changes in the physical nature of the products. Similar behavior was exhibited by both products when they were stored under nitrogen at 100C and by trivernolin at 4C.

Introduction

THE PRODUCTS OBTAINED from controlled utilization of the enzyme activity of *Vernonia anthelmintica* (L.) Willd. seed and the methods of deactivating and of activating the enzyme system have been investigated and discussed previously (1,2). In these investigations it was found that the enzyme system was very highly active in crushed or ground seed and produced gross changes in the composition of the oil obtained from such seed. However, it was not known if the enzymes were active during prolonged storage of mature whole seed; if so, lipolytic activity in the whole, uncrushed seed would affect adversely the composition of the oil. Another unknown was the effect that storage had on the epoxy content of *Vernonia* oil and its chief component, trivernolin. This knowledge is essential since these natural products are potential stabilizers and plasticizers of poly(vinyl chloride) (3).

The purpose of this paper is to present the results of the following studies on *V. anthelmintica* seed oil: 1) a comparison of the composition of the oil obtained from several seed accessions; 2) the effect of storage of the whole seed on the composition of the oil; and 3) the effect of storage on the epoxy content of low free fatty acid (FFA) *Vernonia* oil and trivernolin. Also, some observations on the effect of storage of the whole seed on the activity of the seed enzymes were made.

Experimental Procedures

Materials and Methods. Seed used in these studies was collected in India and Pakistan. Some of the seed was supplied by Quentin Jones of the Crops Research Division, ARS, USDA; some was obtained through a commercial seed broker. Consequently, the complete history of the seed is not known.

AOCS procedures were used to obtain analytical data. A 40-hour Soxhlet extraction using a previously described technique (4) was used to determine the amt of oil in the seed. Since this extraction technique degrades the oil, composition analyses were done on oil obtained by the rapid extraction technique (4). Meth-

TABLE I
Comparison of Aanalyses of *V. anthelmintica* Seed Accessions

Origin and date rec'd.	Moisture when rec'd.	Oil in seed (mfb)[a]	Analysis of oil[b]		
			FFA[c]	Oxirane oxygen	I.V. (Wijs)
	%	%	%	%	
India					
Feb. 1960	6.21	23.9	2.0	3.90	105.8
March 1961	7.80	25.5	2.0	3.90	106.3
Aug. 1961	9.20	23.3	2.1	3.95	104.4
July 1962	7.70	22.0	1.6	3.92	104.3
Pakistan					
Sept. 1962	8.04	27.2	2.0	3.95	103.4
June 1963	8.00	26.4	1.9	3.77	106.1

[a] Yield obtained by exhaustive extraction of ground seed. mfb—moisture free basis.
[b] Oil for analyses obtained by rapid extraction technique.
[c] Calc. as epoxyoleic acid.

[1] Presented at the AOCS Meeting, Minneapolis, 1963.
[2] A laboratory of E. Utiliz. Res. & Dev. Div., ARS, USDA.