A Rapid Micromethod for the Quantitative Analysis of Jojoba Wax and Its Components

M.L. TONNET, R.L. DUNSTONE, Division of Plant Industry, CSIRO, G.P.O. Box 1600, Canberra, A.C.T. 2601, Australia and **A. SHANI,** Department of Chemistry, Ben-Gurion University of the Negev, Be'er-Sheva 84120, Israel

ABSTRACT

A method for the routine determination of jojoba wax ester composition and the ethanolysis products of these esters is described. In the recommended procedure, single or half seeds are crushed onto filter paper disks to provide duplicate 10-20 mg samples of the wax. One paper is extracted with petroleum ether for wax ester analysis and the second sample is transesterified in a sealed bottle using 5% HCl in ethanol at 80 C for 1-2 hr. This preparation is extracted with NaCl and petroleum ether, neutralized with potassium bicarbonate and dried with anhydrous sodium sulfate. The fatty acid ethyl esters and free alcohols are determined by gas chromatography (GC). The method requires only small amounts of seed material, provides duplicate samples of the wax, simplifies the ethanolysis procedures and reduces the time needed for the removal of the acid catalyst.

INTRODUCTION

The jojoba plant (*Simmondsia chinensis* [Link] Schneid.) is unique because its seeds produce an "oil" that is a liquid wax consisting of long-chain wax esters rather than a mixture of triglycerides (Table I). In both its natural or chemically modified states, the wax is the basis for a wide range of products ranging from cosmetics to high-pressure lubricants (1). The wax could also be valuable as a source of long-chain fatty acids and alcohols for the chemical industry (1).

The distribution of chain lengths in the wax esters can be determined readily by gas chromatography (GC). Methods of analyzing the component acids and alcohols are based on acidic transesterification reactions followed by GC of the ester products and alcohols. Ethanolysis is usually chosen as the means of transesterification as the reaction products can be separated using nonpolar columns. Methanolysis, the usual reaction for triglycerides, produces esters and alcohols that are not easily separable by GC and require separation by thin layer chromatography (TLC) or saponification and esterification before GC analysis.

Two methods are currently used for the extraction and ethanolysis of jojoba wax (2,3). In both, crushed seed is initially extracted with a solvent that must be evaporated before dissolving the wax in benzene (2,3) or toluene (4) in preparation for the chosen ethanolysis procedure. Although the methods differ in ethanolysis time and reagent concentrations, they are both time-consuming because of the lengthy procedures required for removing acidic products and washing the organic phase to remove contaminants.

During a study of the effect of temperature on the growth and wax composition of jojoba seed (5), a more rapid method of analysis was seen to be necessary to handle the large number of treatments and replications necessary in such work. A method of expression onto filter paper followed by an ethanolysis procedure based on the transesterification method used by Welch (6) for other oil seeds was investigated. This paper describes the results of that study and its application to the analysis of jojoba wax.

EXPERIMENTAL PROCEDURES

Wax

Jojoba wax was obtained from 2 sources. The first was a commercial sample, from Jojoba Growers and Processors Inc., Apache Junction, AZ, prepared from seed collected from wild plants in the area (Arizona wax). The second was harvested from a trial plantation at Condobolin, N.S.W., Australia (Condobolin seed).

Reagents

The petroleum ether was purchased as petroleum spirit, 40-60 C, analytical grade, from Ajax Chemicals (Australia). Analytical grade ethanol (99.7-100%) and diethyl ether were obtained from BDH Chemicals (Australia). The toluene was an analytical grade 'Pronalys' of M & B Chemicals (Australia). All were used without further purification.

An anhydrous solution of 5% HCl in ethanol was prepared by adding acetyl chloride (BDH) to ethanol (10% v/v). An anhydrous solution of 2% H_2SO_4 in ethanol (v/v) was prepared using 18M H_2SO_4 (BDH).

Sample Preparation for Ethanolysis

Four alternative sample preparations were tested. (a) Arizona wax to be used for ethanolysis by the method of Miwa (2) was dissolved in toluene (a substitute for benzene). (b) Arizona wax (10-20 mg) to be used for ethanolysis by the proposed microprocedure was spread onto a 25 mm filter paper disk (Whatman No. 1). (c) Duplicate samples of wax from a single Condobolin seed were obtained by crushing the seed onto 4, 25 mm filter paper disks under a screw press and removing the 2 innermost papers. (d) Condobolin seed was cracked with a hammer and steel plate into small particles, which were then introduced into the ethanolysis solution.

Ethanolysis by Published Procedure

The method of Miwa (2) was followed, except that toluene was substituted for benzene to solubilize the wax. Ca. 1 mmol (600 mg) of Arizona wax was refluxed with 10 mL of 5% HCl in ethanol for 8 hr. The solvent and HCl were removed by distillation followed by 3 further treatments

TABLE I

Ester Composition (%) of Jojoba Wax Esters Used for Ethanolysis Studies

Carbon number		34	36	38	40	42	44	46	48
Arizona wax	Mean	0.1	1.3	7.1	30.5	49.5	10.0	1.3	0.2
	SD	±0.01	0.02	0.14	0.59	0.20	0.38	0.36	0.21
Condobolin wax	Mean	0.1	1.5	6.6	26.5	52.7	11.2	1.5	0.1
	SD	±0.02	0.20	1.35	2.86	1.87	1.95	0.67	0.04

with ethanol. The products were dissolved in ethyl ether and washed with water. After drying, the residue was dissolved in petroleum ether (40-60 C) for GC.

Ethanolysis by Proposed Procedure

A filter paper disk containing 10-20 mg of Arizona wax was placed in each of 6, 25 mm wide-mouthed glass bottles (75 mm in height). Five mL of 5% HCl in anhydrous ethanol was added to each bottle, which were sealed with a neoprene-lined screw cap and placed in an oven at 80 C for 4 hr. The bottles were then removed from the oven and allowed to cool. To each bottle was added 5 mL of 18% w/v aqueous sodium chloride solution and 6 mL of petroleum ether. The bottle was shaken on a vortex mixer and the contents were allowed to settle. Following phase separation, the organic layer was removed by Pasteur pipette, transferred to a test tube and washed twice with 2% potassium bicarbonate to remove acidic and water-soluble material. Finally, the organic layer was dried over anhydrous sodium sulfate and transferred to an autosampler vial.

Time Course and Extraction Efficiency Studies

Ethanolysis by the proposed procedure was carried out for 1, 2, 4, 8 and 16 hr. To test the efficiency of a single extraction to remove a representative sample from the reaction mixture, 6 samples were extracted a 2nd time and, after being prepared in the described manner, the acid esters and alcohols were analyzed by GC. Recoveries were calculated from the total peak area recorded for each extraction.

Alternative Catalyst

H_2SO_4 in ethanol (2% v/v) was tested as an alternative to the standard catalyst on a batch of 6 replicates.

Gas Chromatography

The wax esters and the samples containing the ethyl esters of the acids and the free alcohols were chromatogrammed on a Varian 3600 Gas Chromatograph equipped with dual flame ionization detectors (FID). The chromatographic system included a Varian 8000 Autosampler and a Varian CDS 111C Data System.

For wax esters, a 45 cm × 3 mm o.d. stainless-steel column packed with 1.5% OV-101 on Gas-Chrom Q was used. Injector temperature was 350 C and detector temperature was 370 C. The column, with a flow rate of 80 mL/min of nitrogen, was programmed from 240 C to 320 C at 5 C/min after an initial hold time of 5 min. Each separation took 25 min.

For the ethanolysis products, a 90 cm × 3 mm o.d. stainless-steel column packed with 10% Apiezon L on Chromosorb W-AW (DMCS) was used. Injector temperature was 320 C, detector temperature 280 C and the column was programmed from 200 C to 240 C at 1 C/min, with an initial hold period of 5 min. With a nitrogen carrier gas flow rate of 60 mL/min, each separation took 60 min.

Standards

Both ethyl and methyl esters of Arizona wax were prepared and separated from the free alcohols by TLC with diethyl ether/petroleum ether (20% v/v) on Merck silica gel F-60 PLC plates. The esters and free alcohols were identified by reference to fatty acid methyl esters obtained from Alltech and to the alcohols obtained from the esters by lithium aluminium hydride reduction. Detector response was found to be the same throughout the range of chain lengths studied, although it differed between alcohols and the esters. The factor of 1.13 that was found for response of ethyl esters to their corresponding alcohols for the column used was in agreement with literature values (3). For this identification procedure, 2 other columns were also used, a 15% EGSS-X on Gas-Chrom Q and 10% Silar 10c on Gas-Chrom Q.

RESULTS AND DISCUSSION

Sample Preparation

All 4 methods of sample preparation provided sufficient wax material and sufficient dispersion to allow ethanolysis, which is a surface reaction, to occur. Similar results for the analyses of the Arizona wax were obtained by Miwa's method and by the proposed method (Table II) but some small differences were observed. Spreading the wax from a crushed seed onto filter paper or dispersing by introducing cracked seed particles into the reaction solution were equally satisfactory in producing a sample suitable for ethanolysis (Table III).

Crushing seed onto filter paper disks has a number of practical advantages over the other 3 methods. Several replicate samples of wax are produced from each seed (or half seed if subsequent germination is required for selection purposes [7]). The necessity of using toluene or benzene

TABLE II

Comparison of the Ethanolysis Products of Jojoba Wax by Two Methods[a]

	Component means	
	Proposed method[b]	Miwa's method[c]
Fatty acid ethyl esters, area (%)	Mean SD	Mean SD
16:0	1.2 ± 0.15	0.8 ± 0.44
18:1	10.8 ± 0.25	11.4 ± 0.35
20:1	67.8 ± 1.20	70.7 ± 1.50
22:1	18.9 ± 1.35	15.6 ± 2.10
24:1	1.3 ± 0.08	1.5 ± 0.59
Alcohols, area (%)		
18:1	0.9 ± 0.02	0.8 ± 0.09
20:1	45.2 ± 1.09	45.5 ± 0.47
22:1	47.4 ± 1.39	45.5 ± 1.11
24:1	6.8 ± 0.94	8.1 ± 0.79

[a]Mean of 6 replicates for Arizona wax.
[b]Ethanolysis carried out for 4 hr.
[c]Ethanolysis products diluted to ca. the same concentration used in the micromethod.

TABLE III

Comparison of 5% Hydrochloric Acid and 2% Sulphuric Acid as Catalysts for the Ethanolysis (16 hr) of Jojoba Wax

Catalyst	Arizona wax		Cracked seed	
	HCl	H_2SO_4	HCl	H_2SO_4
Fatty acid ethyl esters, area (%)				
16:0	2.0	1.1	1.5	1.2
18:1	11.7[a]	11.7	12.1	13.0
20:1	67.0[a]	68.7	66.9[a]	66.5
22:1	18.1	15.2	17.6	16.2
24:1	1.2	3.3	1.9	3.0
Alcohols, area (%)				
18:1	1.1	1.1	1.1	0.9
20:1	43.8	43.8	44.5	39.1
22:1	45.9[a]	44.9	46.1[a]	48.2
24:1	9.4	10.3	8.4	11.8

[a]Includes an unknown component.

TABLE IV

Effect of Time on the Products of the Ethanolysis of Jojoba Wax[a]

	Time (hr)				
	1	2	4	8	16
Fatty acid ethyl esters, area (%)					
16:0	0.5	0.2	0.8	2.0	2.1
18:1	10.4	10.4	10.6	11.1[b]	11.9[b]
20:1	69.5	69.5	69.3	66.7	66.5
22:1	18.7	19.1	18.8	19.8	18.7
24:1	0.9	0.9	0.5	0.4	0.8
Alcohols, area (%)					
18:1	0.6	0.5	0.7	0.8	0.7
20:1	45.8	45.5	45.3	46.3	45.4
22:1	46.9	47.1	47.6	46.6[b]	46.7[b]
24:1	6.7	6.6	6.9	6.1	7.2

[a]Mean of 4 determinations for Arizona wax.
[b]Includes an unknown component.

TABLE V

Effect of Using Only One Extraction on the Accuracy of the Results for the Ethanolysis of Jojoba Wax[a]

	Extraction number		Calculated composition
	1	2	
Fatty acid ethyl esters, area (%)			
16:0	2.6	2.3	2.6
18:1	11.8	11.6	11.8
20:1	65.4	70.3	66.0
22:1	19.2	15.9	18.9
24:1	1.0	0.0	0.9
Alcohols, area (%)			
18:1	0.8	0.2	0.7
20:1	41.6	44.1	41.8
22:1	51.4	52.4	51.4
24:1	6.2	3.3	6.0

[a]Mean of 6 seeds from Condobolin.
[b]Calculated assuming 100% recovery in 2 extractions with an average of 88 ± 8.4% in the 1st extraction.

for solubilization is avoided and the filter paper provides a suitable surface for reaction. In the proposed method, a wax sample was extracted from one of the filter paper disks with petroleum ether for analysis of the wax esters by GC while a duplicate disk was used for ethanolysis. The remaining disks were stored in a refrigerator as spares. Care must be taken not to overload filter paper discs (no more than 35 mg wax) as globules of unreacted wax may remain in the reaction solution after ethanolysis.

Time Course of Analysis

The results for varying times of ethanolysis are given in Table IV. In agreement with previously published results (3), we found that ethanolysis was complete in 1 hr. For reaction times of 4 hr or less, only 1 saturated component, 16:0 acid, was found. Longer ethanolysis time resulted in the production of small amounts (<3%) of unknown compounds, which had retention times corresponding to the ethyl esters of C18, C20 and C22 saturated fatty acids. Such peaks have been observed in previous work (2,3). As positive identification of these compounds was not made in the present work, and because only small amounts were present with long ethanolysis times, only the amounts of the identified compounds are presented in this paper.

Efficiency of One Extraction

Although 3 extractions are usually necessary to remove compounds in a liquid-liquid extraction, the present work requires only that a representative sample be taken. The 1st extract differed in composition from the 2nd extract but was not significantly different from the calculated composition of the mixtures (Table V). A single extraction is therefore sufficient for an accurate estimate of the fatty acid and alcohol composition of jojoba wax.

Phase Separation

No difficulty was experienced in phase separation following mixing on a vortex mixer, and the 2 phases separated completely within 2 min.

Alternative Catalyst

Two percent H_2SO_4 was used by Welch (6) in his published method for methanolysis of triglycerides. This acid did successfully catalyze the ethanolysis of jojoba wax, but a much longer reaction time (8-16 hr) was required than when HCl was the catalyst. The reaction products did not include the unknown acids or alcohols found in HCl catalyzed material (Table III). Where the 2 catalysts were used on duplicate wax extracts from a single seed, small, non-significant differences were found. Differences between crushed seed samples showed variations attributable to the different catalysts and to the differences in composition of the wax in the individual seeds used.

Comparison with Miwa's Method

The results of the analysis of the fatty acid esters and alcohol components of Arizona wax by the method of Miwa (2) and by the proposed method are compared in Table II. The percentage of components assessed by the 2 methods was highly correlated ($r^2 = .99$). The differences that occurred between the components, although statistically significant in some cases, are within the limits of reproducibility quoted for triglycerides (6) and agreement between the 2 methods exists.

Advantages of the Proposed Method

The proposed method simplifies the extraction of duplicate wax samples from single or half seeds and provides a rapid ethanolysis procedure for small samples of 10-20 mg. The only equipment required is suitably stoppered glass bottles,

test tubes, a mixer and an 80 C oven or water bath. The shortened preparation time allows large numbers of samples to be processed in a batch mode. The results generally agree with an accepted method within the limitations of the GC procedure.

The method should prove useful in studying the effect of environmental factors on and plant breeding for wax composition where the available sample may be small and a large number of treatments and replicates must be processed. If automatic injection of the GC is used, large numbers of samples can be processed on a continuous basis by a single operator. This makes the method particularly suitable for industrial use in product development and quality control.

REFERENCES

1. Committee of Jojoba Utilization, Products from Jojoba: A Promising New Crop for Arid Lands, National Academy of Sciences, Washington, DC, 1975.
2. Miwa, T.K., JAOCS 48:259 (1971).
3. Duncan, C.C., D.M. Yermanos, J. Kumanto and C.S. Levesque, JAOCS 51:534 (1974).
4. Miwa, T.K., G.F. Spencer and R.D. Plattner, La Jojoba, Consejo Nacional de Cienca y Tecnologia, Mexico, 1978, pp. 187-197.
5. Dunstone, R.L., M.L. Tonnet, I. Wardlaw and A. Shani, Aust. J. Agric. Res., in press (1984).
6. Welch, R.W., J. Sci. Fd. Agric. 28:635 (1977).
7. Yermanos, D.M., and C.C. Duncan, La Jojoba, Consejo Nacional de Cienca y Technologia, Mexico, 1978, pp. 123-133.

[Received October 24, 1983]

A Flavonoid Antioxidant in Spanish Peanuts *(Arachia hypogoea)*

D.E. PRATT and **E.E. MILLER**, Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907

ABSTRACT

Hot methanol extracts of Spanish peanuts were found to possess antioxidant activity. Thin layer (TLC) and paper chromatography of the methanolic peanut extracts yielded 6 fluorescent bands of which one exhibited potent antioxidant activity. Further separation by TLC showed this band to be a complex mixture of 3 components that were tested for antioxidant activity. One component demonstrated all of the antioxidant activity associated with the parent band. Analysis of this antioxidant by paper chromatography and TLC, chromatographic spray reagents and spectral analysis demonstrated that the compound was dihydroquercetin.

INTRODUCTION

Most varieties of oilseeds contain phenolics that retard oxidation in the native seed as well as in extracted oil. Activities of these compounds are as primary antioxidants, synergists and chelators (1). Among naturally occurring substances of potential antioxidant activity, flavones and flavonones are of particular interest (1). Flavonoids, a major group of plant phenols, are widely distributed in nature and occur as glycosides that, on hydrolysis with acid and heat, produce aglycone and sugar moities (2). Flavonoids are also found in association with proteins and tannins (3). Flavonoids possess both chain-breaking and metal-deactivating functions as antioxidants (1). The flavonoids consist of 2 hydroxylated or methoxylated aromatic nuclei linked by a condensed 3-carbon chain (2).

The present study was initiated to investigate aqueous and methanolic extracts of Spanish peanuts.

PROCEDURES AND METHODS

Extraction of Samples

Fifty g of peanuts *(Arachia hypogoea)* were homogenized in a Waring blender with 250 mL of water containing 2 drops of antifoam A, then boiled for 5 min. The mixture was filtered and washed with 200 mL hot water and the insoluble material was discarded. The filtrate was freeze-dried and the residue was brought to a volume of 50 mL with water. Thus, 1 mL of extract contained the water-soluble components from 1 g of peanuts. Alcohol-soluble components were extracted by a similar technique except absolute methanol was substituted for water and antifoam A was omitted.

Paper Chromatography

Methanolic extracts were repeatedly streaked on Whatman #3MM chromatographic paper (23 x 47 cm) until a total of 3 mL had been applied. The papers were equilibrated for 12 hr and developed in the upper phase of n-butanol/acetic acid/water (4:1:5, v/v/v) (BAW). The chromatograms were dried and examined under short (250 nm) and long (360 nm) ultraviolet (UV) wave lengths before and after exposure to ammonia fumes. Major fluorescent bands were eluted with 80% aqueous methanol (AR grade).

The eluted components were further separated by a 2-dimensional technique using 21 cm square Whatman #3MM paper. Chromatograms were developed ascendingly using tertiary butanol/acetic acid/water (3:1:1, v/v/v) (tBAW) as the solvent, air-dried and redeveloped in the second dimension in 15% acetic acid.

Thin Layer Chromatograph (TLC)

TLC plates precoated with silica gel were also used to separate constituents of the methanolic extract. The plates were activated at 100 C for 15 min and streaked with 0.25 mL extract. The plates were developed using the upper phase of an ethyl acetate/formic acid/water (10:2:3, v/v/v) (EFW) mixture. Bands of interest were scraped from TLC plates, soaked for 30 min in 50 mL spectral grade methanol, filtered and evaporated in vacuo to near dryness on a rotary evaporator at 45 C. The residue was redissolved in 1.0 mL methanol (spectral grade) and filtered through glass wool to remove any residual silica gel. The components were further separated by streaking on silica gel TLC plates (0.5 mL/plate) and developing in BAW.

Tests of Antioxidant Activity

Antioxidants on TLC plates were detected using the carotene spray method of Philip (4). Nine mg β-carotene were dissolved in 30 mL chloroform. Two drops of purified