Rapid Ethanolysis Procedure for Jojoba Wax Analysis by Gas Liquid Chromatography

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ABSTRACT

A rapid ethanolysis procedure for preparing jojoba wax for gas liquid chromatographic analysis is described. The wax esters are hydrolyzed by refluxing 4 drops of jojoba wax in 5% HCl in anhydrous ethanol in a test tube. The resulting fatty acid ethyl esters and fatty alcohols are separated and quantitated by a single gas liquid chromatographic run. Analysis of duplicate samples by this procedure gives essentially the same results as a procedure which requires 10 times more sample and reagents and considerably more time.

INTRODUCTION

The development of methods for rapid gas liquid chromatographic (GLC) analyses of triglycerides has made it possible to survey large numbers of oil samples from various oilseeds and to obtain information on the available variability in terms of fatty acids in cultivated and wild species. In addition, these analytical techniques made it possible to analyze large numbers of samples from segregating populations and to elucidate the mode of inheritance of fatty acid composition in a number of oilseeds. The lack of rapid analytical methods for waxes has inhibited the development of comparable information for wax producing plants. The need for such information has been felt recently, in particular, with the desert species jojoba (*Simmondsia chinensis* [Link] Schneider) which is the source of a unique liquid wax with an impressive list of potential applications (1). Miwa (2) reported a procedure for analysis of jojoba wax and reported data on seed samples collected in five locations. His procedure for HCl catalyzed ethanolysis, however, is time consuming and requires considerable sample size (600 mg). The need for a fast analytical procedure requiring smaller amounts of sample led us to the modification of Miwa's procedure described in this report by which 50 mg samples of wax can be prepared for analysis without affecting the accuracy of the data.

MATERIALS AND METHODS

The steps of the rapid ethanolysis procedure are as follows: 4 drops of liquid jojoba wax (ca. 50 mg) are refluxed for 4 hr with 1 ml 5% HCl in anhydrous ethanol plus 0.1 ml anhydrous benzene and a boiling chip in a marble capped test tube on a heating block at 95 C. The solvents and HCl are evaporated by removing the marble and gently blowing dry nitrogen gas into the tube on the heating block without changing the temperature setting. When practically all of the solvent has evaporated, 1 ml absolute ethanol is added to the tube and evaporated. This step is repeated two more times. The tube is cooled to room temperature, and 2 ml ethyl ether and 2 ml distilled H_2O are added. The tube then is shaken and centrifuged, and the ether phase is collected for injection into the gas chromatograph. The equipment needed consists of only a heating block, an air distribution system, and a centrifuge. The entire procedure can be carried on by one person, and the number of samples he can prepare is limited mainly by the number of places for tubes available in the heating block.

Miwa's procedure (2) differs from the above in that (A) ca. 10 times more sample and reagents are required, (B) solvent and HCl are removed after ethanolysis by distillation, (C) samples are refluxed for 8 hr, and (D) more equipment and glassware is required.

To obtain comparative data between the rapid ethanolysis technique proposed in this report and the one described by Miwa, duplicate subsamples drawn from 31 wax samples were analyzed by GLC for fatty acids and alcohols after ethanolysis of one of the subsamples by Miwa's procedure and the other by ours. Each of the 31 wax samples was obtained from the seed of a different individual jojoba

TABLE I

Analysis of 31 Jojoba Wax Samples after Ethanolysis by 2 Procedures

	Component means		Standard deviations		Correlation coefficients
	Our method	Miwa's method	Our method	Miwa's method	Between methods
Fatty acid ethyl esters:					
16:1	.2	.2	.1	.1	.909[a]
16:0	1.2	1.2	.5	.5	.974[a]
18:1	12.3	12.4	5.2	5.1	.999[a]
20:1	72.7	72.5	2.0	2.3	.856[a]
22:1	12.2	12.2	3.9	3.7	.976[a]
22:0	.4	.4	.5	.4	.226
24:1[b]	1.0	1.2	1.1	1.2	.919[a]
Alcohols:					
18:1	1.2	1.3	.6	.6	.991[a]
20:1	46.6	46.7	13.0	12.8	.999[a]
20:0	2.4	2.5	1.2	1.3	.938[a]
22:1	39.0	38.9	8.9	8.7	.995[a]
22:0	3.3	3.3	1.0	1.0	.740[a]
24:1	6.9	7.1	4.7	4.6	.992[a]
24:0[c]	.9	.8	.5	.7	.455

[a]Significant at .1% level.

[b]Only 27 plant samples contained measurable amounts of acid 24:1.

[c]Only 15 plant samples contained measurable amounts of alcohol 24:0.

plant from native populations in Southern California. Wax was extracted by pressing each seed sample in a Carver Laboratory press at 700 kg/cm^2. Separation of the fatty acid ethyl esters and the alcohols was achieved on the same type of column Miwa used, a 100 cm 1/8 in. outside diameter stainless steel column containing 20% Apiezon L on 100/120 mesh Chromosorb W (AW DMCS). Initial column temperature was 240 C for 16 min, followed by a 16 min linear temperature rise to 260 C which was maintained until the run was complete. Total time of each run was ca. 45 min. The helium carrier gas flow was 20 cc/min. The gas chromatograph was a Beckman GC-4 equipped with dual hydrogen flame ionization detectors. The peaks were integrated with a Datex DIR-1 digital integrator.

Additional comparative data were obtained by analyzing 14 subsamples of wax all drawn from a single large bulk sample. Seven of the subsamples underwent ethanolysis by our proposed technique and the others by Miwa's technique. Fatty acid and alcohol analysis by GLC was performed as above.

To test if the degree of ethanolysis was the same for each method and to determine the best ethanolysis time for our procedure, weighed subsamples of the bulk sample were refluxed for 8 hr by Miwa's method and for various times between 1 min and 4 hr by our procedure. Before evaporation or distillation of the HCl- ethanol, an internal standard was added for GLC quantitation. One ml 1% 1-nonadecanol (10 mg) in alcohol was added to subsamples after ethanolysis by Miwa's procedure, and 0.1 ml (1 mg) was added to the subsamples after ethanolysis by our method. These samples then were analyzed by GLC as usual. It previously had been determined that the ratio of detector response for our GLC for equal wt of a fatty acid ethyl ester and a fatty alcohol was 1.13. This was used as a response factor for the fatty acid ethyl esters relative to the 19 carbon internal standard. Results for fatty acid ethyl esters were not corrected to free acids.

RESULTS AND DISCUSSION

Results of the analysis of the 31 individual plant wax samples by both ethanolysis procedures are essentially the same (Table I). The correlation coefficients between the 2 methods for each component are highly significant except for acid 22:0 and alcohol 24:0, which occurred in quantities of less than 0.5% of the sample. Inaccurate integration of these peaks by our integrator was the source of variation which produced these decreased correlation coefficients. Our automatic integrator was set to integrate the major components accurately at the expense of the minor components late in the run. The two ethanolysis procedures also gave comparable results in analyzing the bulk jojoba wax subsamples (Table II).

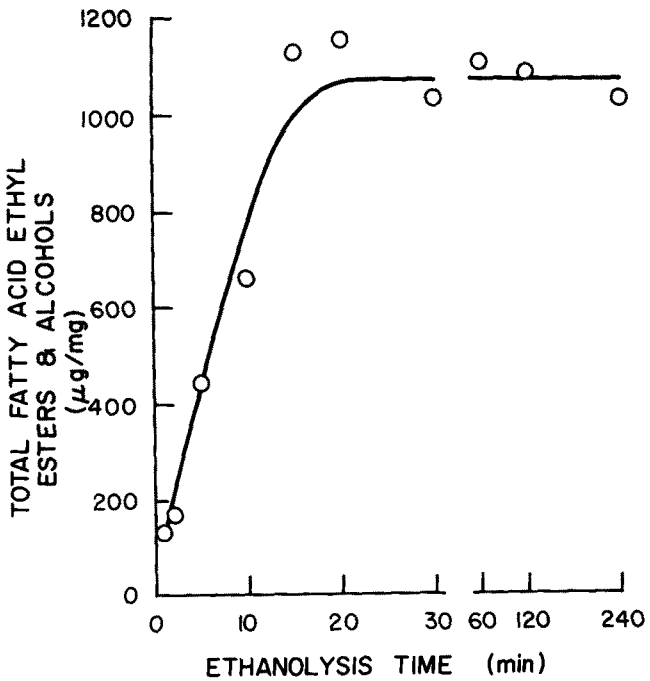

FIG. 1. Total fatty acid ethyl esters and alcohols measured in jojoba wax as a function of ethanolysis time.

The difference between total components recovered by each of the two ethanolysis procedures was not statistically significant. The mean of the total components measured from seven duplicate subsamples after ethanolysis by our procedure was 1041 μg/mg of wax (standard deviation was 86.1). The mean of the total components for 3 duplicate subsamples of the same bulk jojoba wax sample prepared by Miwa's procedure, was 1012 μg/mg wax (standard deviation, 20.1). Both procedures, therefore, hydrolyze the wax esters completely. The increased wt of the fatty acids upon conversion from wax esters to ethyl esters accounts for total wt being greater than 1000 μg/mg wax. (The increased variation in the totals from our procedure

TABLE II

Analysis of Bulk Jojoba Wax Subsamples after Ethanolysis by 2 Procedures[a]

	Component means		Standard deviations	
	Our method	Miwa's method	Our method	Miwa's method
Fatty acid ethyl esters:				
16:1	.3	.4	.01	.01
16:0	1.8	1.9	.03	.04
18:1	12.8	13.1	.15	.23
20:1	71.6	71.5	.33	.52
22:1	12.4	12.2	.18	.39
22:0	.6	.6	.35	.37
24:1	.4	.5	.15	.18
Alcohols:				
18:1	1.4	1.5	.08	.07
20:1	47.6	48.0	.85	.84
20:0	3.3	3.1	.69	.94
22:1	37.8	38.0	.85	.90
22:0	4.0	3.4	.83	.78
24:1	5.7	5.6	.31	.49
24:0	.4	.3	.17	.33

[a]Seven duplicate subsamples for each ethanolysis procedure.

probably reflects the difficulty in accurately measuring 0.1 ml internal standard).

Using the internal standard for quantitation, we found that samples could be refluxed for as little as 15 min by our procedure without decreasing significantly the total wax components measured (Fig. 1). However, the size of the saturated alcohol peaks and the 22:0 acid peak increased with longer ethanolysis. For example, behenyl alcohol (22:0) increased from ca. 1% of all alcohols after 15 min ethanolysis to ca. 2% after 4 hr and to ca. 4.5% after 24 hr in one of our samples. This increase occurs with both ethanolysis procedures. The source of these additional saturated compounds is unknown but it is doubtful that they come from wax esters which are more resistant to ethanolysis. Since these saturated compounds comprise such a small proportion of the total ethanolysis product (ca. 2%), the imprecision of their determination has little effect on the overall analysis. Higher levels of analytical accuracy would be necessary for detailed biochemical analysis of jojoba wax. At this point, however, when large numbers of samples need to be analyzed for an overall survey of the gross available variability among plants or populations and for the identification of possible mutant strains, the level of imprecision associated with this technique can be tolerated. Obviously, comparisons of the wax composition of various samples requires a uniform ethanolysis time so we chose a 4 hr ethanolysis time because it is convenient and gave the same results as Miwa's 8 hr ethanolysis.

A major advantage of our procedure is the decreased sample requirement. For many of our seed samples, four drops of wax can be recovered from a single seed; however, even smaller amounts of wax can be analyzed. We have prepared wax samples as small as 10 mg by this procedure without affecting the results of the analysis.

As the results of jojoba wax analysis obtained by these two ethanolysis procedures are essentially the same, the speed and decreased sample requirement of the proposed method make it a preferable method for routine analysis of large numbers of samples of jojoba wax.

REFERENCES

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