Routine Analysis of Jojoba Wax Fatty Acids and Alcohols by Single Column Capillary GC

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Through a detailed study of the different steps and reactions of the ethanolysis and acetylation of jojoba wax, two analytical procedures were developed. One method could be a standard method of analysis because it includes a one-pot derivatization of the jojoba wax followed by a single GC analysis with an overall analysis time of three hr. The other method is suitable for the analysis of large numbers of samples for checking, screening, selection and breeding among the different jojoba genera.

The development of the jojoba crop (*Simondsia chinensis* Link Schneider) and the increasing potential industrial utilization of its waxes as chemicals, lubricants, diet food, cosmetics, etc. (1) was discussed in depth at the 6th International Conference on Jojoba, held recently in Israel (2). As a result, a large research project has begun in the field of plant selection and breeding, as recommended elsewhere (3,4), prior to the intensive development of jojoba culture.

Such a screening program requires a large number of chemical analyses of the wax components and constituents of the jojoba genera. However, the few analytical methods published up to now for the determination of fatty acid and alcohol composition are time consuming and/or require two GC injections. This may drastically limit the number of samples analyzed daily.

Initially Miwa (5,6) reported a day-long method that includes ethanolysis, saponification, methylation (three steps), extraction of the alcohols and methyl esters and a single GC injection of a packed column. Duncan and coworkers (7) carried out the ethanolysis of the wax and the extraction of the ethyl esters and free alcohols in a 4- to 5-hr process; the samples were then analyzed as above. Later, Miwa and coworkers (8) published a half-day-long method as follows: alkaline catalyzed methanolysis and successive acid catalyzed methylation; extraction and TLC separation of the methyl esters and fatty alcohols; derivatization of the alcohols as TMS ethers and, finally, separate GC analyses of the two fractions. More recently, Tonnet and coworkers (9) published a 4- to 5-hr long one-pot sample preparation which is suitable for a large number of analyses.

The following method has been developed in our laboratory: ethanolysis of the wax yielding fatty acid ethyl esters (FAEE) and free fatty alcohols (FFAl), then conversion of the alcohols into acetates (FAlA) followed by a single column GC analysis. This procedure takes advantage of the four methods listed above and allows for a large number of daily analyses as well as accurate and detailed determination of the wax composition. Only three hr are needed to carry out a complete analysis owing to the one-pot sample derivatization and the single high resolution GC analysis.

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MATERIALS AND METHODS

Wax sample. The jojoba wax samples were supplied by Commissariat à l'Energie Atomique (CEA). All samples were yellow and sediment free liquids; their acidity was about 0.3%.

Reagents. Anhydrous methanol, ethanol, hexane, benzene and diethylether were analytical grade solvents (Prolabo). An anhydrous solution of 1N HCl in ethanol was prepared by adding acetyl chloride (Merck) to ethanol. Ethanolic HCl, obtained from anhydrous ethanol and 10.5 N aqueous HCl, was also used for a comparative study. The acetylation reagent was a solution of acetic anhydride in pyridine (25:75, v/v). The saponification reagent was obtained by dissolving 6 g of potassium hydroxide in 100 ml ethanol. The water was deionized through an ion exchange column. Silica gel 60 plates without fluorescent indicator (Merck), layer thickness 0.25 mm, were used for TLC analysis.

Hydrogenation of jojoba wax. The hydrogenation of a 100 mg sample dissolved in 70 ml of a toluene/ethanol mixture (1:8, v/v) was carried out under low hydrogen pressure with 50 mg of 10% Pd/C catalyst (10).

GC analysis of the jojoba wax. The analysis was performed on a Carlo Erba HRGC 4160 apparatus equipped with a split-splitless injector and a flame ionization detector (FID) connected to a Delsi Icap 10 integrator. The sample size was 1 μl of a 0.1% w/v solution. The separation was performed on a laboratory-made OV1 coated glass capillary column (11): length 3 m; id 0.28 mm; film thickness 0.1 μm. The experimental conditions were as follows: injector 325 C; detector 350 C; oven, 1 min at 190 C, 190 C to 340 C at 5 C/min, 5 min at 340 C; helium carrier flowrate 3.3 ml/min; septum purge 10 ml/min; splitting ratio 1/60. The injection was made in splitless mode, the splitter being opened 10 sec after.

Procedure for the wax derivatives reference analysis. The reference analysis procedure published earlier by Miwa et al. (5,6) was followed step by step. It includes HCl catalyzed ethanolysis of the wax; saponification, extraction and water washing of the alcohols, then acidification, extraction and water washing of the acids. The alcohols were analyzed without further treatment on an OV1 coated glass capillary column (11) (length 38 m; id 0.3 mm; film thickness 0.1 μm) under the following conditions: Girdel 300 GC apparatus; splitter 250 C; detector 275 C; oven 230 C; helium carrier flow rate 2.7 ml/min; split ratio 1/50; analysis time 40 min. The acids were converted into methyl esters by refluxing in 1 N HCl methanolic solution for one hr; the esters were then extracted with hexane; the

organic layer was water washed, dried over anhydrous sodium sulfate and analyzed on the Carbowax 20 M coated capillary column (12) under the same chromatographic conditions used for the FAEE and FAlA.

Quantitative TLC (13, 14). The silica gel plates were cleaned by developing with diethyl ether to free the silica of any organic matter which would lead to a greyish color after the phosphoric carbonization step. The sample (1% w/v in hexane) was applied as a narrow, 15-mm long band with an automatic Camag Linomat III applicator. After a 10-cm migration from the deposit line, the solvent was evaporated.

Plates were developed by spraying with a mixture of pure phosphoric acid and saturated Cu $(CH_3CO_2)_2$ aqueous solution (50:50, v/v) and then heated at 180 C for six min. The components appear as grey-black bands. Their optical density was measured at 500 nm with a Camag Scanner Photodensitometer coupled with a Delsi Enica 10 integrator. Careful and constant setting of the parameters (shape and weight of the deposit, temperature, freshly prepared solvent and spraying) are required to establish a linear relationship between optical density and weight of each component in the range 0-20 μg. This relationship was determined with known amounts of each jojoba derivative analyzed under the same TLC conditions. Consequently, a suitable deposit of the sample must be done to apply amounts less than 20 μg for components selected for evaluation. Moreover, because the quantitative TLC is accurate only in the Rf range 0.30-0.70, adequate solvents must be used for each class of components. Practically, this was achieved through two TLC plates for each sample, one for low polarity component evaluation and one for high polarity component evaluation. Rf values were as follows:

	Hexane/diethyl ether % v/v	
	94/6	60/40
Wax	0.64	1.0
FAEE	0.51	1.0
FAlA	0.43	0.98
FFAl	0.07	0.47
FFA	0.02	0.18

The composition (w %) was measured through an absolute determination for each component with respect to known amounts of jojoba standards deposited on the same TLC plates. Trace FFA were neglected.

Derivatization procedure. For the ethanolysis step, a 200-mg sample of jojoba wax was refluxed for 90 min with three ml of anhydrous 1 N HCl ethanolic solution and 0.3 ml of benzene in a 25-ml conical bottomed flask. The temperature of the oil bath was set at 95 C. The ground necked flask was equipped with a condenser and a $CaCl_2$ trap. At the end of the run, solvent and HCl were evaporated under reduced pressure to constant weight.

For the acetylation step, the dry crude ethanolysis product (about 220 mg) was heated for 10 min at 95 C in the glassware described above with two ml of the acetic anhydride-pyridine mixture. Then two ml of water were added, and after five min the flask was allowed to cool to room temperature.

The isolation of the jojoba derivatives was carried out as follows. The crude ethanolysis and acetylation mixture was transferred quantitatively into a 100-ml separatory funnel with 10 ml of water and 30 ml of hexane. 10 ml of 1 N aqueous KOH were then added, and the FAEE and FAlA were extracted with two more 20 ml portions of hexane. The combined organic layers were washed twice with 20 ml of water, then acidified with 10 ml of aqueous 4 N HCl solution and washed three times with 20 ml of water. After drying over anhydrous sodium sulfate, the solvent was evaporated under reduced pressure to constant weight. The GC sample was prepared by dissolving the residue in hexane to obtain a 5% solution (w/v).

Preparative TLC of the FAEE and FAlA. The chromatography was performed with three analytical plates (20 × 20 cm; thickness 0.25 mm) to avoid overloading and to obtain better separations. Six mg of the crude ethanolysis-acetylation mixture were deposited on each plate as a 150-mm long band. The plates were developed with a hexane/diethyl ether mixture (94:6, v/v), and the bands were located in a dark room with a visible light source placed on the back side of the plate. For each plate, the two bands were scraped off and the corresponding fractions joined and extracted overnight at room temperature in an ethyl acetate/ethanol mixture (70:30, v/v). The two solutions were filtered carefully and the solvents evaporated. Seven to eight mg of each component were recovered.

GC analysis of the jojoba derivatives. The solutions containing the mixture of the FAEE and the FAlA as well as the solutions of the fractions separated by TLC were analyzed on a Girdel 300 gas chromatograph equipped with a FID connected to a Delsi Enica 10 integrator. The sample size was one μl of a 6% w/v solution for the mixture and one μl of a 3% w/v solution for the separated fractions.

The separation was performed on a laboratory-made Carbowax 20 M coated glass capillary column (12): length 57 m; id 0.3 mm and film thickness 0.1 μm. Conditions were: splitter injector 250 C; detector 275 C; oven 200 C; helium flow rate three ml/min; splitting ratio 1/50.

Simplified procedure for routine analysis. The complete derivatization of each sample is performed in a set of four 30-ml test tubes. Tube number 1 is equipped with an air condenser fitted with a $CaCl_2$ trap. Four drops of jojoba wax from a Pasteur pipette are introduced into this tube along with one ml of 1 N ethanolic HCl and 0.1 ml of benzene; the mixture is then refluxed for 70 min in an oil bath set at 95 C. The solvents and HCl are flushed off in a stream of nitrogen for 10 min at room temperature, and then 0.5 ml of acetylation reagent is added. The test tube is placed in the 95 C bath again, for 10 min.

The simplified extraction procedure includes removal of the test tube; cooling in air; addition of six ml of an aqueous 0.5 N KOH solution; addition of four ml of hexane; stirring for 10 sec on a vortex; transfer of the supernatant via a syringe to test tube number 2 containing four ml of aqueous 0.5 N HCl solution; stirring and transfer of the supernatant to tube number 4 containing dry sodium sulfate and the appropriate amount of hexane to adjust the concentra-

tion before GC analysis. The drying step may be by-passed if no water is transferred.

RESULTS AND DISCUSSION

We first studied the two successive ethanolysis and acetylation steps as a function of time in order to optimize each reaction time; then we simplified the procedure so it would be adaptable for handling a large number of samples.

Ethanolysis. Base catalyzed alcoholysis of jojoba wax was found by Miwa (5) to be a slow reaction. In early experiments we noted that acid catalyzed methanolysis was complete after refluxing for six hr, whereas under the same experimental conditions ethanolysis was complete after 1.5 hr. Moreover, the single column GC resolution of the fatty acid methyl esters and the alcohol derivatives was not satisfactory. Acid catalyzed ethanolysis was chosen for these reasons. We carried out a set of ethanolysis tests for various refluxing times: 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 8.0 and 36 hr. Because of the heterogeneity of the reaction mixture at the beginning, each run was done in an individual flask. TLC analysis of the crude mixture displayed the changes occurring in the composition and allowed for the detection of trace components. However, accurate and reproducible quantitation of the TLC spots requires care and a trained operator for the deposit step as well as for monitoring of the plates. Results reported in Figure 1 show that the ethanolysis of the wax is almost complete after 1.5 hr. Moreover, for longer refluxing times the wax content of the mixture increased to 8% after 8 hr and up to 31.5% after 36 hr. This indicates that, under our experimental conditions, with increasing reaction time, recombination of the ethanolysis products to wax esters occurs. In order to prove this phenomenon, the wax composition was determined (Tables 1 and 2); the C40/C42 ratio was found to be less than 1 in natural jojoba wax (1,5,6,8,9) and is a convenient parameter to follow the recombination reaction. Indeed, GC analysis of the crude ethanolysis mixtures vs time (Table 2) showed that the composition of the reformed wax differed from that of the original sample. The changes of the C40/C42 ratio are shown in Figure 2 (0 hr: 0.78; 8 hr: 1.27).

During the ethanolysis step, we noted the synthesis of small amounts of FAlA due to the nature of the ethanolysis reagent made from acetyl chloride and anhydrous ethanol. As a matter of fact, the resulting ethyl acetate is 10 times more concentrated, on a molar basis, than the total liberated fatty alcohols. This point was checked through preparative TLC of the mixture and a GC analysis of the isolated acetate-like components. We obtained the same chromatogram as that of the acetate derivatives synthesized in the following section.

Obviously, when preparing the reagent from 10.5 N aqueous hydrochloric acid, we did not detect any acetates. Moreover, we found that the recombination

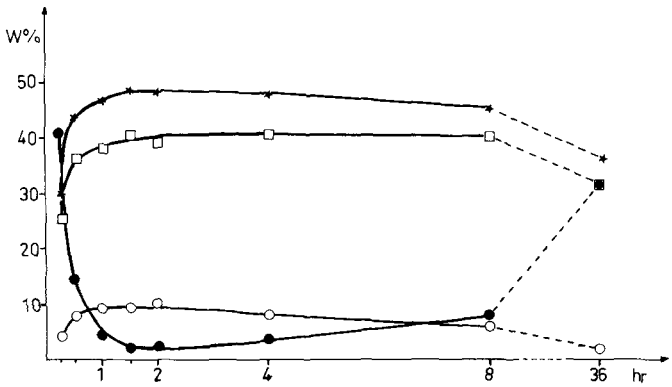

FIG. 1. Composition of crude ethanolysis mixture vs reaction time determined by quantitative TLC. ●, wax; ★, FAEE; □, FFA1; ○, FA1A.

TABLE 1

Natural and Random Composition of Wax Esters[a]

Wax ester chain length	Natural composition	Random[c] composition
32	tr[b]	tr
34	0.2	0.1
35	0.1	tr
36	2.0	1.5
37	0.2	tr
38	11.0	9.8
39	0.3	tr
40	33.6	43.7
41	0.1	tr
42	42.8	34.3
44	8.2	9.2
46	1.3	1.1
48	0.2	0.1

[a]W% determined by GC on OV1 column.

[b]"tr": trace amounts < 0.05%.

[c]Statistical computations from FAEE and FA1A compositions of the original jojoba wax sample.

TABLE 2

Wax Content and Wax Ester Composition vs Ethanolysis Time

Time hr	Wax[a]	Main wax components[b]				
		38	40	42	44	Ratio 40/42
0.0	100	11.0	33.6	42.8	8.2	0.78
0.25	41.0	11.5	34.4	42.3	7.9	0.81
0.5	14.0	10.8	35.0	42.1	8.5	0.83
1.0	3.5(28.5)[c]	11.3	37.2	39.3	7.9	0.95(0.79)
1.5	1.5	11.5	38.8	37.7	8.4	1.03
4.0	4.5(5.5)	12.1	40.1	35.5	8.7	1.13(1.09)
8.0	8.0(2)	10.3	43.1	34.4	9.1	1.25(1.07)
36.0	31.5	9.6	44.1	33.9	8.9	1.30
Random composition		9.8	43.7	34.3	9.2	1.27

[a]W% determined by quantitive TLC.

[b]W% determined by GC on OV1 column.

[c]Values in parentheses: aqueous HCl catalyzed ethanolysis.

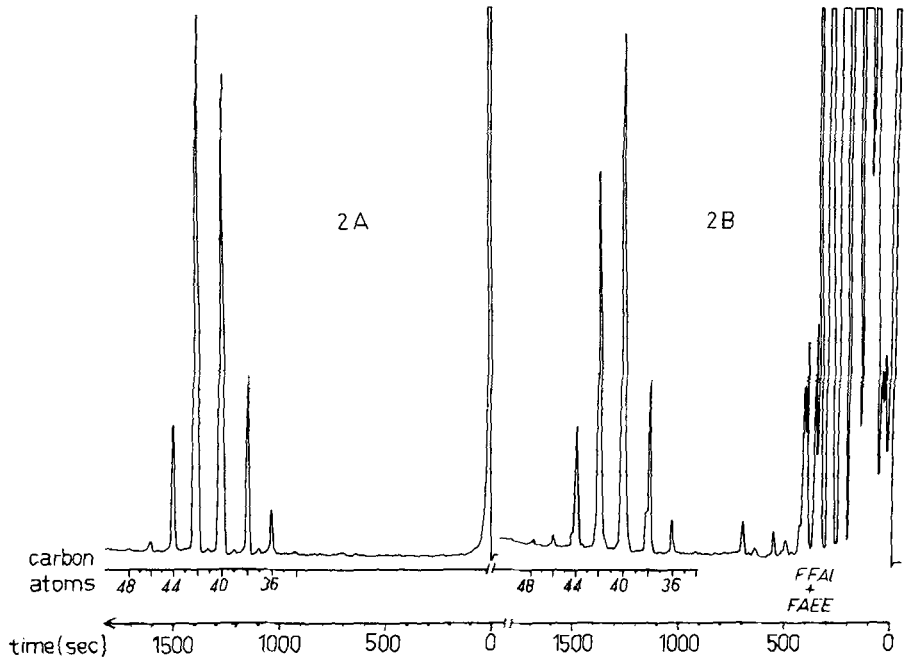

FIG. 2. Gas chromatograms of jojoba wax esters (OV1 column). 2A. Natural wax (C40/C42 = 0.75). 2B. Wax in crude mixture after 8 hr of ethanolysis (C40/C42 = 1.25).

reaction was slower (Table 3). Although this was not the main purpose of the present work, from this result and the evolution of the acetate concentration (Fig. 1 and 2), we might conclude that the FAlA facilitate wax recombination. On the other hand, it is probable that Tonnet and coworkers (9), who used acetyl chloride, also obtained acetates and reformed wax after their 4-hr ethanolysis step. This may explain the presence of unidentified products as well as changes in composition of their mixture with increased reaction times.

In our case, because the second step of the procedure is acetylation of the free alcohols, the side reaction does not interfere with the analysis.

Acetylation. A time course study of the fatty alcohol acetylation performed at 10, 20, 40 and 60 min showed a 100% conversion into acetate derivatives after 10 min. One might expect such a result because some acetates were readily synthesized during the first step. TLC of the crude mixture displayed only three spots corresponding to the FAEE, the FAlA and a small amount (1-2%) of residual wax. This residual wax did not interfere with the GC analysis discussed in the following section.

Single column GC analysis of the jojoba derivatives. Since the mid seventies the use of capillary columns has greatly improved GC separations. In the present work, using a carefully made laboratory Carbowax 20 M coated glass capillary column, we obtained a complete separation of about 40 derivatives of the jojoba wax (Fig. 3). Preparative TLC of the mixture allowed individual GC injections of the two fractions and identification of the FAEE and FAlA (Fig. 3A, 3B); because of the Rf values, each fraction still contained trace amounts of the other. The ethyl ester of a given fatty acid and the acetate of the corresponding primary fatty alcohol have the same molecular weight and very close linear aliphatic structures;

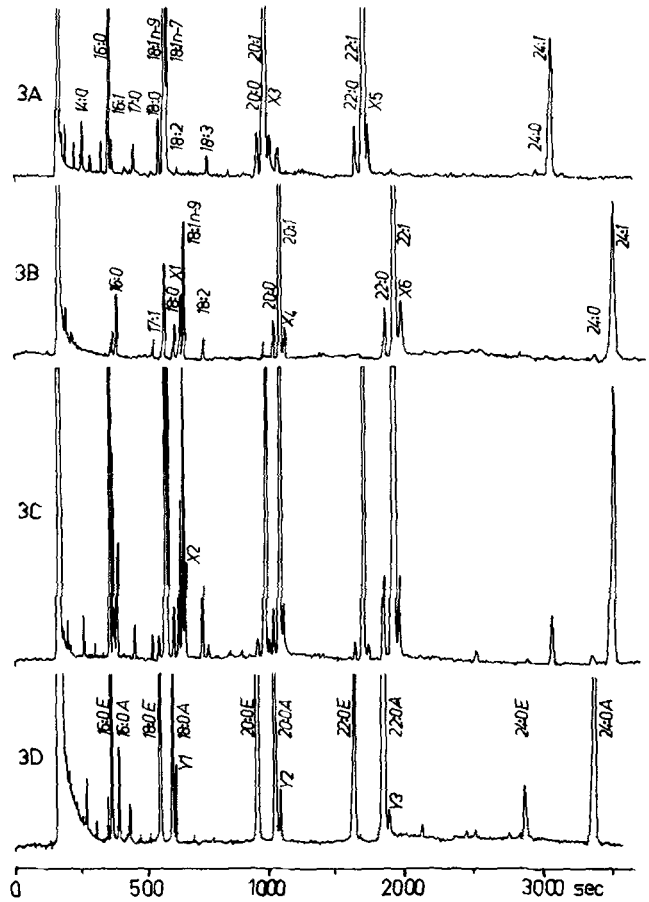

FIG. 3. Gas chromatograms of jojoba wax derivatives (Carbowax 20 M column). 3A, FAEE; 3B, FA1A; 3C, FAEE + FA1A; 3D, hydrogened (FAEE + FA1A). Chart: 5mm/min from 0 + 800 sec and 2.5 mm/min after 800 sec. Unlabelled peaks are contaminants: FAlA in 3A and FAEE in 3B. In 3D compound names are ended by E and A for FAEE and FAlA, respectively.

TABLE 3

Composition of Fatty Acid Methyl Esters and Fatty Alcohol Acetates Derived from Jojoba Wax

	Full[a,b] procedure (anhydrous HCl)	Miwa's[b,c,d] (aqueous HCl)	Routine procedure[a]			
			Ethanolysis 15 min	Ethanolysis 30 min	Ethanolysis 70 min[e]	Standard deviation
Acids						
12:0	tr[f]	tr	tr	tr	tr	
14:0	tr	tr	tr	tr	tr	
15:0	tr	tr	tr	tr	tr	
16:0	1.5	1.7	1.4	1.5	1.6	0.08
16:1	0.4	0.4	0.3	0.3	0.3	0.05
17:0	0.1	0.1	tr	0.1	0.1	0.03
18:0	0.1	0.1	0.1	0.1	0.1	0.02
18:1 (n-9)	13.1	13.0	12.1	11.4	12.4	0.28
18:1 (n-7)	0.8	1.0	0.9	0.8	0.8	0.09
18:2 + X1	0.3	0.4	0.5	0.6	0.5	0.03
18:3	0.2	0.3	0.2	0.3	0.2	0.02
19:0	tr	tr	tr	tr	tr	
20:0	0.1	0.2	0.1	0.2	0.1	0.04
20:1	70.8	69.9	70.9	71.0	70.6	0.35
22:0	0.2	0.2	0.1	0.2	0.2	0.10
22:1	11.2	11.3	12.2	12.1	11.8	0.31
24:0	0.1	tr	tr	tr	tr	
24:1	0.9	0.9	0.9	1.2	1.0	0.15
Unidentified	0.2	0.3	0.2	0.2	0.2	
Alcohols						
14:0	tr	tr	tr	tr	tr	
16:0	0.2	0.1	0.2	0.2	0.2	0.09
17:1	0.1	tr	tr	tr	tr	
18:0	0.2	0.2	0.1	0.1	0.1	0.06
18:1 (n-9)	0.9	0.9	0.9	0.8	0.8	0.10
18:2	tr	tr	tr	tr	tr	
20:0	0.3	0.5	0.3	0.3	0.3	0.04
20:1	51.9	50.8	51.7	51.9	51.6	0.59
22:0	1.0	1.3	1.0	1.1	1.1	0.17
22:1	38.1	38.9	38.7	38.5	38.8	0.30
24:0	0.2	0.3	0.2	0.2	0.2	0.05
24:1	6.1	6.7	6.0	5.9	6.1	0.39
Unidentified	1.0	0.3	0.9	0.9	0.8	

[a]W% of FAEE and FAlA determined by GC, Carbowax 20M.

[b]Mean of 5 replicate runs.

[c]GC of fatty acid methyl esters, Carbowax 20M.

[d]GC of free fatty alcohols, OV1.

[e]Mean of 8 replicate runs.

[f]"tr": trace amounts <0.05%.

nonetheless, we obtained the satisfactory separation shown in Figure 3C.

Hydrogenation of the derived jojoba wax allowed unambiguous recognition of the saturated and unsaturated derivatives (Fig. 3D). The FAEE were identified by co-chromatography with known oils, and the FAlA were identified by analogy with the FAEE series and the equivalent chain lengths. One has to note the presence of small peaks following the major constituents in the C18, C20, C22 FAEE and FAlA series. In the FAlA series since this phenomenon also was observed in hydrogened samples, the corresponding unidentified compounds cannot be positional unsaturated isomers of the main constituents (Fig. 3A, 3B, X1-X6; Fig. 3D, Y1-Y3). We are now working on a more complete identification of the GC peaks.

The average composition of the identified jojoba wax derivatives, determined by our procedure and calculated over five complete runs and two GC analyses for each, is given in Table 3. The analysis also was performed following Miwa's procedure including two GC analytical columns (5,6). The two sets of values are in good agreement; the slight deviations are within the experimental error.

Simplified microquantitative IRHO procedure. The good agreement of our method with Miwa's allowed us to develop a simplified microquantitative procedure suitable for a large number of samples: the derivatization was performed with four drops of sample from a Pasteur pipette in a test tube equipped with an air condenser; the extraction steps were carried out in two other test tubes. This simplified procedure has several advantages over other published methods (5-9); it includes one-pot sample preparation, low reagent consumption, simplified extraction steps and low cost glassware. Following this procedure we did establish that the composition of the

derived wax did not depend on the percentage progress of the ethanolysis as shown in Table 3, where we reported the composition only after 15, 30 and 70 min. As a result, the reaction time could be shortened; however, low residual wax content preserves the capillary column efficiency. Moreover, during the refluxing time, the operator has to prepare the test tubes used for the extraction step. Therefore, we chose 70 min as the ethanolysis time (2% of residual wax). The repeatability of the proposed method was checked over eight replicate runs (two GC injections for each); the standard deviations are within that of the GC (Table 3).

The overall analysis, including sample preparation and GC, can be carried out within three hr. One operator can easily prepare two sets of 12 samples daily, and the corresponding high resolution single column GC analysis (about one hr each) can be carried out overnight using an automatic injector. In conclusion, we succeeded in developing a rapid and accurate microanalytical method that allows for the wide screening of jojoba plants.

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