4. Aust, S.D., and B.A. Svingen, Ibid., Vol. 5, 1982, pp. 1-28.
5. Kuehl, F.A., J.L. Humes, R.W. Egan, E.A. Ham, J.C. Beveridge and C.G. Van Arman, Nature 265:170 (1977).
6. Roubin, R., J.M. Mencia-Huerta and J. Benveniste, Eur. J. Immunol. 12:141 (1982).
7. Hamberg, M., J. Svensson and B. Samuelsson, Proc. Natl. Acad. Sci. USA 72:2994 (1975).
8. Moncada, S., and J.R. Vane, in Biological Aspects of Prostaglandins and Thromboxanes, Kharash, N., and J. Fried, eds., Academic Press, NY, 1977, pp. 155-177.
9. Samuelsson, B., and S. Hammarstrom, Prostaglandins 19:645 (1980).
10. Sirois, P., and P. Borgeat, Int. J. Immunopharmac. 2:281 (1980).
11. Holroyde, M.C., R.E.C. Altounyan, M. Cole, M. Dixon and E.V. Elliot, Agents and Actions, 11:573 (1981).
12. Porter, N.A., R.A. Wolf, E.M. Yarbro and H. Weenen, Biochem. and Biophys. Res. Commun. 89:1058 (1979).
13. Weenen, H. and N.A. Porter, J. Am. Chem. Soc. 104:5216 (1982).
14. Marcuse, R., Rev. Fr. Corps Gras 7:391 (1973).
15. Ikeda, N., and K. Fukuzumi, JAOCS 54:360 (1977).
16. Cillard, J., P. Cillard and M. Cormier, Ibid. 57:225 (1980).
17. Witting, L.A., Arch. Biochem. Biophys. 129:142 (1969).
18. Cillard, J. and P. Cillard, JAOCS 57:39 (1980).
19. Gruger, E.H., Jr., and A.L. Tappel, Lipids 5:326 (1969).
20. Riisom, T., R.J. Sims and J.A. Fioriti, JAOCS 57:354 (1980).
21. Cillard, J., Etude de l'effet prooxygène de l'α-tocophérol en milieux aqueux, thèse de Docteur Es-Sciences Pharmaceutiques, UER Médicales et Pharmaceutiques, Université de Rennes (1978).
22. Farag, R.S., S.A. Osman, S.A.S. Hallabo and A.A. Nasr, JAOCS 55:703 (1978).
23. Gardner, H.W. and P.A. Jursinic, Biochim. Biophys. Acta 665: 100 (1981).

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Thin Layer Chromatography/Flame Ionization Analysis of Transesterified Vegetable Oils[1]

B. FREEDMAN, E.H. PRYDE and **W.F. KWOLEK**[2], Northern Regional Research Center, Agricultural Research Service, USDA, Peoria, IL 61604

ABSTRACT

A quantitative method was developed for analyzing mixtures containing fatty esters and tri- (TG), di- (DG) and monoglycerides (MG) obtained by the transesterification of vegetable oils. Analyses were performed by thin layer chromatography (TLC)/flame ionization detection (FID) with an Iatroscan TH-10 instrument. Stearyl alcohol served as an internal standard. From plots of area and weight ratios of methyl linoleate and tri-, di- and monolinolein, linear equations were developed from which response factors were calculated. Hydrogen flow rate and developing solvent strongly influenced resolution and baseline stability. Variations in scan speed affected completeness of burning and response factors, but not measured composition. Sample weight also affected response factors but not measured composition. A computerized procedure for data analysis was developed so that 30 samples can be completed in 2-3 hr. Relative standard deviations were 1-2% for major components in the 90-100% range and 6-83% for minor components in the 1-2% range.

INTRODUCTION

Analysis of lipids by thin layer chromatography (TLC) with flame ionization detection (FID) (Iatroscan analyzer) is claimed to be sensitive, linear and reproducible (1), comparable to conventional chemical analyses, superior to gravimetric recovery and less time-consuming than comparable methods (2,3). Others report that: response factors varied with the nature and amount of compound applied and with scan speed (4,5,6); pyrolysis was incomplete at some scan speeds (4); slight deviations in rod geometry with respect to the detector also influenced quantitation (4,5); results varied from rod to rod, and one lot of rods to another (7); and, finally, FID design might affect quantitation (8).

The Iatroscan analyzer appeared to offer a rapid, simple and convenient method to quantitate reaction mixtures from the transesterification of vegetable oils (9,10). Our objectives were to determine the suitability of this technique for quantifying transesterifications and to shed light on the variability of response factors with type of compound, scan speed, completeness of pyrolysis and rod-to-rod variation.

[1]Presented at the AOCS meeting, Chicago, May 1983.
[2]Biometrician, North Central Region, Agricultural Research Service, U.S. Department of Agriculture, stationed at the Northern Regional Research Center, Peoria, Illinois 61604.

EXPERIMENTAL

Reagents

Reference materials used in standard solutions in this study were: methyl linoleate; tri-, di- and monolinolein; 1,3- and 1,2-dilinolein; stearyl alcohol; palmitic acid. Reference materials and 2 TLC reference standards (solutions 49 and 50) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN), and were chromatographically pure (>99%). Chloroform was MCB Omnisolv (spectrograde). Developing solvents were A.C.S. reagent grade.

Instrumentation and Operating Conditions

Analyses were performed with an Iatroscan TH-10 Analyzer MK III (Iatron Laboratories, Tokyo, Japan: worldwide distributor, Newman-Howells Assoc., Winchester, Hants, U.K.). The flame ionization detector used hydrogen and air flow rates of 160 mL and 2,000 mL/min. Scan speeds were 2,3 and 4 corresponding to 40 sec/rod, 35 sec/rod and 30 sec/rod. A Linear Instrument Corp. (Irvine, CA) recorder, Model 561, was used at 1 mV full-scale deflection at chart speeds of 10 cm/min, 15 cm/min or 20 cm/min.

Chromarods

Both type S and SII Chromarods were used, but the former predominated; the silica gel particle size is a maximum of 10 in type S and 5 μm in type SII (11). The manufacturer's directions were followed in the storage, use and reactivation of these rods (12). They were stored in a glass chamber in which the atmosphere was saturated with water vapor. Before being spotted, rods were scanned as blanks on the

instrument to obtain the proper degree of hydration. After ca. 10-15 analyses, rods were reactivated by soaking overnight in chromic-sulfuric acid cleaning solution, followed by thorough washing with tap and distilled water. The rods were placed in an oven at 110 C for 1 hr, then scanned as blanks.

Analytical Procedure

Standard solutions were prepared to have a concentration of 3-20 mg/ml in chloroform. A Hamilton 10 μL syringe was used to spot 1-2 μL of solution on rods in small increments although a frame of 10 rods was often processed singly, processing 3 frames at one time was more efficient. The rods, after spotting, were placed in a glass tank containing a conventional TLC plate to equilibrate solvent vapors. Developing solvents were petroleum ether (b.p. 35-60 C) (PE)/diethyl ether (DE) acetic acid (AA) (90:10:1 or 90:10:0.25). The rods were developed for 20 min, air-dried for 5 min, oven-dried at 110-130 C for 5 min and then analyzed on the Iatroscan. An approximate percentage composition based on the integrated signal and a precise percentage composition based on the internal standard were computed. Scans were repeated when the determination of completeness of combustion was desirable.

Developing Solvents

Changes in the solvent system PE/DE/AA (90:10:1) included: reduction or elimination of AA; substituting formic acid for AA and hexane for PE; decreasing the ratio of PE/DE; using chlorinated solvents and benzene; combinations of single and double development. Type S rods were used for these evaluations.

Vacuum-Drying Process

Procedures for gaining optimal reproducibility with the Iatroscan have recently been described by Iatron Laboratories (13,14). First, a uniform amount of moisture was produced in a vacuum desiccator on the rods after they were spotted; then the rods were exposed to the vapors of developing solvent before development. This vacuum-drying process was evaluated with type SII rods.

Standard Solutions and Quantitation

The compositions of the 5 standard solutions used to obtain parameters for quantitation are shown in Table I. Each solution also contained a known amount of stearyl alcohol. Solution concentration was ca. 6 mg/mL, of which 1-2 μL were applied to the type S rods. Analyses were based on 5-10 replicates per compound within each standard solution. These standard solutions were used to determine linear regression equations and response factors that, in turn, were employed to quantitate composition of unknown solutions as discussed later.

TABLE I

Composition for Standard Solutions (wt % ± Standard Deviation)[a]

Component	Solution number				
Methyl linoleate	13	14	16	19	20
Actual	97	94	1	30	40
Found	97.4 ± 2.2	95.2 ± 1.4	0.6 ± .5	33.7 ± 1.1	41.5 ± 1.7
Trilinolein					
Actual	1	2	97	10	30
Found	0.5 ± .4	1.7 ± .9	94.7 ± 2.3	9.2 ± 1.0	31.5 ± 1.1
Dilinolein					
Actual	1	2	1	40	20
Found	0.5 ± .3	2.0 ± 1.3	1.0 ± .6	40.6 ± 4.0	19.8 ± 4.1
Monolinolein					
Actual	1	2	1	20	10
Found	1.2 ± .8	1.7 ± .1	2.2 ± .7	20.0 ± 3.5	9.7 ± .4

[a]Analyses were based on 5 replicates per compound within each standard solution.

RESULTS AND DISCUSSION

Effect of Hydrogen Flow Rate on Resolution and Baseline Stability

Comparison of the recommended flow rate of 160 mL/min with a rate of 285 mL/min showed that the lower rate was clearly superior in resolution and baseline stability (Fig. 1). Although hydrogen flow rate, scan speed and chart speed differed between the upper and lower chromatograms, subsequent analyses showed that the higher hydrogen flow rate (285 mL/min) and not scan speed or chart speed had caused the inferior results. Most investigators have used a hydrogen flow rate of 160 mL/min. Bradley et al. (1) reported that of 3 hydrogen flow rates—160, 180 and 188 mL/min—the lowest coefficient of variation for response factors was obtained at 180 mL/min. We used a rate of 160 mL/min in subsequent analyses.

Effect of Solvent on Separation

Of the systems tested, the solvent combination PE/DE/AA (90:10:1) worked best for the transesterification product analysis (Fig. 2-1). Although affording a better separation of diglyceride (DG) and monoglyceride (MG), more polar systems decreased separation of methyl ester (ME) and triglyceride (TG). Less polar systems increased the resolution of ME and TG, but at the expense of DG and MG separation. Lowering the AA content from 1% to 0.25% often improved separation of ME and TG without adversely affecting the separation of DG and MG. Sensitivity of such solvent systems to small changes in organic acid content has also been noted by others (15).

In the presence of TG, free fatty acids (FFA) present a more difficult separation. Yet such mixtures are encountered routinely by those working with plasma or neutral

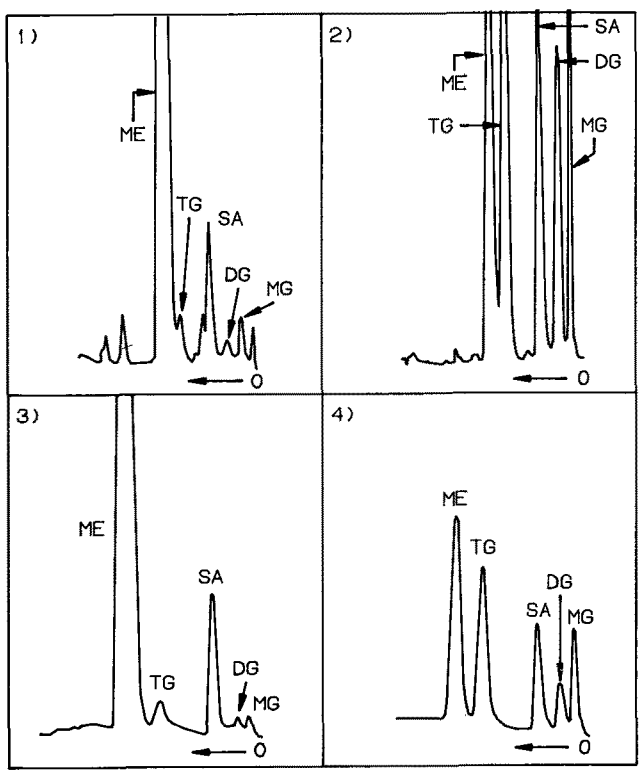

FIG. 1. Effect of hydrogen flow rate on resolution and baseline stability—ME, methyl ester; TG, triglyceride; SA, stearyl alcohol; DG, diglyceride; MG, monoglyceride; 0, origin. 1-1 and 1-2: flow rate, 285 mL/min; scan speed 2 (40 sec/rod); chart speed, 10 cm/min. 1-3 and 1-4: flow rate, 160 mL/min; scan speed 4 (30 sec/rod); chart speed, 20 cm/min.

lipids. Although FFA occurred only in trace amounts in our reactions, we examined separation of FFA from other components of our mixture. The solvent system used in Figure 2-1 was unable to resolve TG and FFA (Fig. 2-2). The failure of this or similar systems to separate TG and FFA has been reported (1,5,15). Double development with this solvent (Fig. 2-3) partially resolved 1,3- and 1,2-DG, gave a much better separation of DG and MG, but also failed to resolve TG and FFA. By using a solvent system that separated TG and FFA in neutral lipids (15), we resolved all the components of our mixture (Fig. 2-4). However, we continued to use PE/DE/AA (90:10:1) because of better results in the critical separation of ME and TG for our application.

Comparison of Chromarods S and SII

Comparison of type SII with S rods (both previously unused) showed that the SII rods gave distinctly better resolution. Whereas ME and TG were almost completely resolved, and DG and MG were partially resolved on type S rods, both pairs were completely resolved on type SII rods. These observations confirmed the superiority of the latter rods for our application. Except for their slightly higher cost, type SII rods appear to have no disadvantages when compared with type S rods.

Quantitation

Linear regression equations were developed from plots of area and weight ratios of standard solutions containing internal standard. In equation 1:

$$A_C/A_S = a + b \cdot (W_C/W_S) \qquad [1]$$

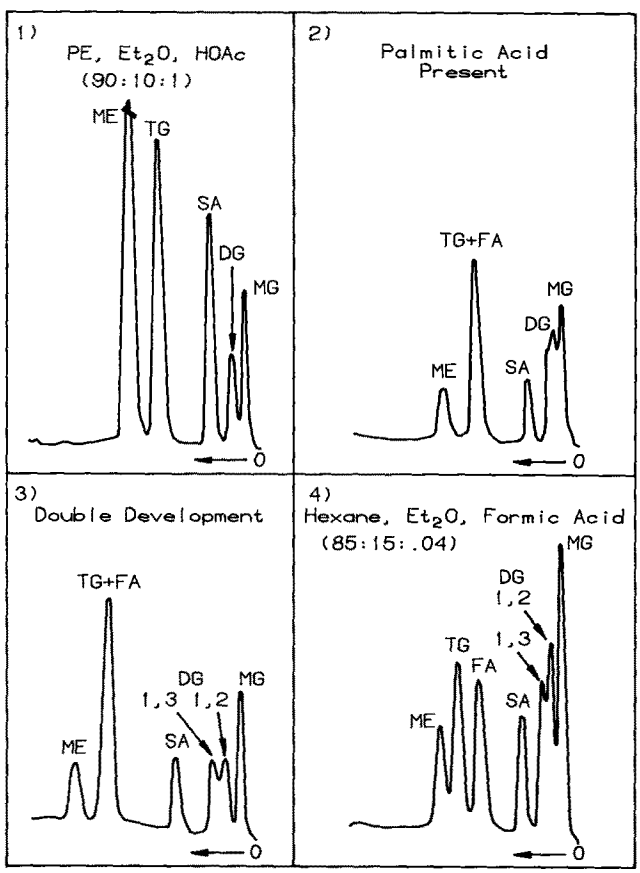

FIG. 2. Effect of developing solvent on separation. For abbreviations, see Figure 1; FA, fatty acid.

A_C is the area of the compound, A_S is the area of the internal standard, a the intercept, b the slope, W_C the weight of the compound and W_S the weight of the internal standard. By plotting A_C/A_S against W_C/W_S, we determined a and b for each compound in the standard solutions. This information was used in equation 2 to calculate the weight of compound in an analytical solution where the weight of the compound was unknown:

$$W_C = [(A_C/A_S - a)/b] \cdot W_S \qquad [2]$$

The weight percentage of the compound, and then percentage composition of the mixture, were calculated from equation 3:

$$\text{Compound Weight \%} = (W_C/\Sigma W_C) \cdot 100 \qquad [3]$$

Percentage composition was also determined using response factors (RF) assuming a=0, and defining RF as 1/b. Thus, RF was calculated for each compound from the reciprocal of the slope, and W_C by equation 4:

$$W_C = RF \cdot (A_C/A_S) \cdot W_S \qquad [4]$$

Percentage composition of the mixture was then determined using equation 3.

The compositions of the 5 standard solutions used to determine linear regression equations are shown in Table I. Solution 13 corresponds to a transesterification reaction mixture in which most of the TG has been converted to ME. In these mixtures, small amounts of TG, DG and MG were invariably present (10). In solution 13, 1% by weight of each of these 3 components corresponds to 0.05-0.1 μg applied to the Chromarod-S. This weight range is barely detectable by the instrument and agrees with the detectable limit of 0.1 μg reported by other workers (2,3). We employed solution 14 to ensure that these minor components would be detected. Solution 16 corresponds to a vegetable oil starting material that, of course, would not contain ME. Solutions 19 and 20 contain intermediate quantities of these 4 components. We used stearyl alcohol as an internal standard; it has been used previously in lipid analysis (3), and performed very satisfactorily in our application.

Plots of A_C/A_S vs W_C/W_S for these standard solutions are shown in Figure 3. Each compound showed acceptable linearity over the range of interest. The intercepts and slopes derived from these plots were used to calculate percentage composition of the standard solutions. Agreement between experimental and theoretical values was excellent; differences were 1-2% or less.

Results from another large-scale trial in which standard deviations were determined are shown in Table I. Agreement between experimental and theoretical values was reasonable in most cases, at least as far as our intended application was concerned. Relative standard deviations (RSD) ranged from 1.5% to 9.8% for the major component of each solution. However, for the minor components of solutions 13, 14 and 16 RSD ranged from 5.9% to 83.3%. As these minor components correspond to loadings of 0.05-0.2 μg, the high RSD associated with these components suggest that good reproducibility cannot be obtained in this low μg range. Even at 0.4 μg, a RSD range of 5.2-25.8% has been reported for neutral lipids (16). Thus, several workers have suggested that 1-10 μg is a reasonable range for accurate determinations (2,16). The high RSD for samples below this range does represent a limitation of the method when minor components must be analyzed. Although 10 μg is an upper limit suggested by some, others have used or recommended 25 μg (3) or 30 μg (11,15) as upper limits.

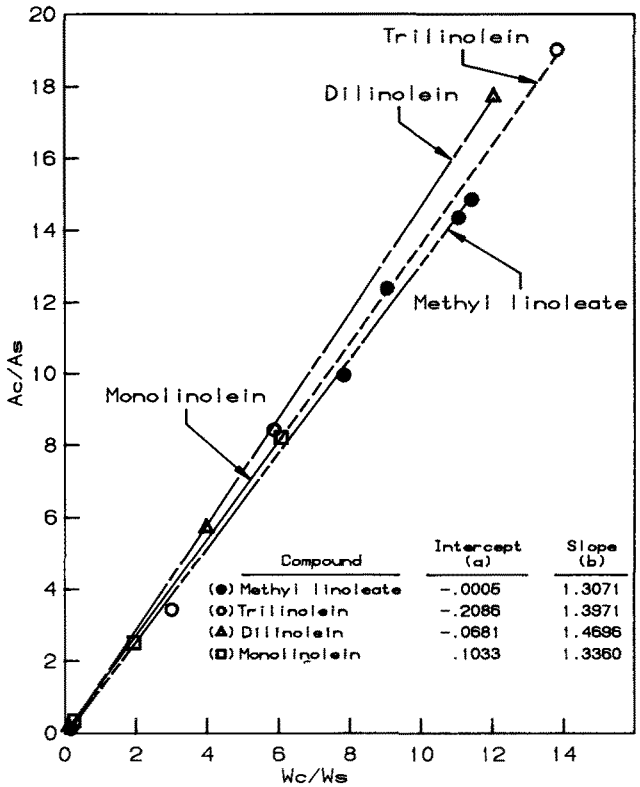

FIG. 3. Plots of area and weight ratios for standard solutions 13-20. A_C/A_S, area of compound/area of internal standard; W_C/W_S, weight of compound/weight of internal standard.

Vacuum Drying Process

We compared the regular analysis and the vacuum-drying process by evaluating the 5 standard solutions 13-20. The results obtained with 2 of these solutions are shown in Table II. The RSD calculated by the vacuum-drying process for A_C/A_S ratios were generally somewhat higher than those obtained by regular analysis; therefore, this modified procedure did not improve reproducibility. Comparable results were observed with the other 3 standard solutions. Although conditioning the rods at 65% relative humidity has been found to improve reproducibility and also to permit improved FFA-TG separations (17), we carried out no further work in this area.

Effect of Scan Speed on Completeness of Burning

Larger sample size may improve the reproducibility of minor components, if adequate resolution can be maintained. To successfully apply larger samples (>10 μg), these samples must be completely burned in the FID. The effect of scan speed on completeness of burning (Table III) showed that combustion was complete at scan speed 2 except for a small amount of noncombusted material with 18 μg, as determined by a second scan. Combustion was incomplete with both 12 μg (solution 16, 20) and 18 μg (solution 49) at scan speeds 3 and 4; thus, these speeds were unsatisfactory with 12-18 μg of our mixtures. Below ca. 10 μg, no unburned material was observed at scan speed 4. Improved resolution was also observed at scan speed 2 when compared with 3 and 4. Thus, we considered scan speed 2 suitable for our purpose. Crane et al. (4) have noted that, at scan speed 2, 5-10 μg of phosphatidylcholine was completely burned, whereas at scan speed 5 (25 sec/rod), 10% of the sample remained unburned (4). Scan speed 2 has also been employed in quantitative lipid analyses by others (16).

TABLE II

Comparison of Results Obtained by the Regular and Vacuum Drying Processes[a]

Solution #		Methyl linoleate		Trilinolein		Dilinolein		Monolinolein	
		Reg.	Vac.	Reg.	Vac.	Reg.	Vac.	Reg.	Vac.
14	Ac/As[b]	25.7	25.2	0.18	0.18	0.15	0.16	0.22	0.28
	RSD, %[c]	13.9	18.5	54.1	69.0	32.4	37.4	24.6	32.9
20	Ac/As	13.8	14.2	9.8	10.3	4.9	4.7	2.3	2.9
	RSD, %	19.6	15.2	14.8	13.1	11.7	12.3	14.0	17.9

[a]Each ratio is the mean of 12 replicates with Chromarods-SII.

[b]Area of compound/area of internal standard.

[c]Relative standard deviation.

TABLE III

Effect of Scan Speed on Completeness of Combustion

Scan Speed		Total non-combusted area (arbitrary units)[a]			
Gear number	Seconds/rod	Solution 13 (12.3 μg/rod)	Solution 16 (12.2 μg/rod)	Solution 20 (12.3 μg/rod)	Solution 49[b] (18.0 μg/rod)
2	40	0	0	0	1,066
3	35	0	388	2,693	8,363
4	30	0	14,198	9,186	47,536

[a]Each solution was replicated 6 times per scan speed with Chromarods-SII.

[b]Composition of Solution 49: 40% ME, 25% TG, 20% DG and 15% MG.

TABLE IV

Effect of Scan Speed on Response Factors and Measured Composition[a,b]

Scan speed		Response factors ± SD (Solution 49)			
Gear number	Seconds/rod	Methyl linoleate	Trilinolein	Dilinolein	Monolinolein
2	40	0.99 ± .09	0.83 ± .07	0.83 ± .08	0.51 ± .10
3	35	0.98 ± .13	0.92 ± .11	0.88 ± .12	0.62 ± .23
4	30	0.91 ± .11	0.99 ± .10	1.14 ± .20	0.84 ± .22
		Composition (wt % ± SD) (Solution 49)			
		Methyl linoleate	Trilinolein	Dilinolein	Monolinolein
		40	25	20	15
2	40	39.8 ± 2.1	24.9 ± 1.3	19.5 ± 2.5	15.9 ± 3.0
3	35	39.8 ± 2.3	25.0 ± 1.3	20.1 ± 1.6	15.0 ± 2.1
4	30	40.5 ± 2.8	25.5 ± 1.4	19.5 ± 3.6	14.5 ± 2.3

[a]Chromarod-SII spotted with 1 μl (18 μg) of Solution 49. Developing solvent: PE-DE-AA (90:10:0.25).

[b]16 Replicates were divided between trial 1 (6 replicates) and trial 2 (10 replicates) for each value shown.

Effect of Scan Speed on Response Factors and Measured Composition

Because accurate RF are critical to quantitation, and because others have shown that RF varies with scan speed (1,4), we studied the effect of scan speed on RF for our mixtures. At the same time, we studied the effect of scan speed on the measured composition of a standard solution.

The RF obtained (Table IV) were examined by analyses of variance to determine whether scan speed produced significant variation. For TG, DG and MG, RF increased significantly ($P<0.01$) with scan speed, but variation for methyl linoleate was not significant. At scan speeds 2 and 3, a decrease occurred in RF between methyl linoleate and monolinolein because of different chemical structures. Finally, SD (and RSD) was lower at scan speed 2 than at 3 or 4.

The data for measured composition as influenced by scan speed (Table IV) showed little variation for a given compound. Indeed, analyses of variance showed no significant effects of scan speed on measured composition for any of the 4 compounds.

Effect of Sample Weight on Response Factors and Measured Composition

RF have been found to increase, often non-linearly, with

TABLE V

Effect of Sample Weight on Response Factors and Measured Composition[a,b]

Quantity applied (μg)	Solution #	Response factors ± SD			
	49	Methyl linoleate (40%)	Trilinolein (25%)	Dilinolein (20%)	Monolinolein (15%)
20		0.79 ± .12	0.66 ± .08	0.71 ± .07	0.49 ± .06
15		0.83 ± .10	0.69 ± .08	0.72 ± .08	0.50 ± .09
5		1.37 ± .21	1.04 ± .18	1.12 ± .18	0.75 ± .12
	50	Methyl linoleate (15%)	Trilinolein (20%)	Dilinolein (25%)	Monolinolein (40%)
20		1.14 ± .16	0.75 ± .10	0.69 ± .06	0.41 ± .03
15		1.61 ± .21	1.09 ± .10	1.15 ± .16	0.61 ± .08
5		1.51 ± .21	1.14 ± .16	1.23 ± .33	0.60 ± .13
		Composition (wt % ± SD)			
	49	Methyl linoleate (40%)	Trilinolein (25%)	Dilinolein (20%)	Monolinolein (15%)
20		39.5 ± 1.6	25.2 ± 1.4	20.2 ± 1.2	15.1 ± 1.0
15		40.0 ± 2.3	25.0 ± 1.2	20.1 ± 1.8	14.9 ± 1.1
5		40.1 ± 5.1	24.9 ± 2.5	20.0 ± 2.9	15.0 ± 1.8
	50	Methyl linoleate (15%)	Trilinolein (20%)	Dilinolein (25%)	Monolinolein (40%)
20		15.0 ± 1.2	20.0 ± 1.7	25.0 ± 2.5	40.0 ± 1.2
15		15.0 ± 0.6	20.1 ± 1.1	25.1 ± 2.0	39.8 ± 1.8
5		14.5 ± 1.3	19.2 ± 1.0	24.1 ± 4.9	42.2 ± 4.4

[a]Scan Speed 2 used with Chromarod-SII. Developing solvent: PE-DE-AA (90:10:0.25).

[b]For Solutions 49 and 50, means were based on 7 and 6 replicates, respectively.

increasing sample weight for various lipids in the 1-10 μg range (4,7). We studied the effect of sample weight in the 5-20 μg range on RF and measured composition and found the RF increased as sample size decreased (Table V). This observation differed from earlier reports in which RF increased as sample size increased (4,7), but might be accounted for by differences in sample weight, lipid type, scan speed, developing solvent and so forth. A general decrease in RF in progressing from methyl linoleate to monolinolein as noted in Table IV is also observed in Table V. SD were somewhat lower for 20 μg than 5 μg. Sample weight did not affect measured composition as shown by the data in Table V, and confirmed by analyses of variance. The experimental compositions determined for Solution 49 and their SD are about the same as those shown in Table IV, and are indicative of the accuracy and precision we were able to obtain with this method.

Characteristics of the Method

Disadvantages. The major disadvantage of this method is the lower accuracy and precision compared with such techniques as gas chromatography (GC) and high performance liquid chromatography (HPLC). This is particularly true where minor components in the 1-2% range must be analyzed. As noted earlier, the RSD of our minor components ranged from 6% to 83 %. In contrast, the RSD of these minor components are less than 5%, and accuracy is also significantly improved when GC is employed (work in progress).

Change in RF with sample weight reported by us and others (4,7) requires care to maintain satisfactory quantitation. Rod-to-rod and lot-to-lot differences in Chromarods must be expected. Variation in reproducibility of results because of variation in ambient humidity around the rods suggests that humidity must be carefully controlled. Our attempts to improve reproducibility by maintaining constant humidity around the rods were not successful. Other researchers have been successful in maintaining a constant humidity, which has resulted in a lower RSD (13,14, 17). Finally, RF should be updated frequently, perhaps daily. Although this is time consuming, it will help improve accuracy and precision.

Advantages. Despite these drawbacks, many successful quantitative analyses of lipids have been made using the Iatroscan methodology. Several of these, involving neutral or plasma lipids, have been cited (1,2,3,7). Other lipid classes including TG, DG and MG, FFA, wax ester, fatty alcohol and hydrocarbon, have also been quantitated by this method (16). Esterification and glycerolysis reactions have been studied successfully (18). Ackman has shown that the method is applicable to a wide variety of organic classes including drugs, vitamins, alkaloids, amino acids and pesticides (11). Finally, in our studies on transesterification of vegetable oils (9,10), we found the method to be satisfactory for monitoring reaction mixtures, which has enabled us to pinpoint those reaction variables that lead to high yields of fatty esters.

The TLC/FID method is also simple to learn and use. By processing 3 frames of 10 rods each at one time, and using a computerized procedure for data analysis, we have been able to analyze 30 samples in 2-3 hr, a rate that cannot be matched by GC or HPLC. Only μg samples are required for analyses. Any separation achieved with TLC can probably be duplicated by TLC/FID by using suitable developing solvents. The quantitation resulting from the latter method strongly enhances the usefulness of TLC.

Finally, the TLC/FID method is constantly being improved and refined by numerous researchers throughout the world. The first full-day symposium on this subject, held at the 1984 American Oil Chemists' annual meeting, attests to the interest in this subject. Significant improvements in the method will probably be made.

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REFERENCES

1. Bradley, D.M., C.R. Richards and N.S.T. Thomas, Clin. Chim. Acta 92:293 (1979).
2. Sipos, J.C., and R.G. Ackman, J. Chromatogr. Sci. 16:443 (1978).
3. Van Tornout, P., R. Vercaemst, H. Caster, M.J. Lievens, W. de Keersgieter, F. Soetewey and M. Rosseneu, J. Chromatogr. 164:222 (1979).
4. Crane, R.T., S.C. Goheen, E.C. Larkin and G.A. Rao, Lipids 18:74 (1983).
5. Christie, W.W., and M.L. Hunter, J. Chromatogr. 171:517 (1979).
6. Christie, W.W. in Fats and Oils: Chemistry and Technology, edited by R.J. Hamilton and A. Bhati, Applied Science Publishers Ltd., 1980 p. 14.
7. Farnworth, E.R., B.K. Thompson and J.K.G. Kramer, J.

Chromatogr. 240:463 (1982).
8. Hammond, E.W., Chem. Ind. (London) 20:710 (1981).
9. Freedman, B., and E.H. Pryde, in Vegetable Oil Fuels–Proceedings of the International Conference on Plant and Vegetable Oils as Fuels, American Society of Agricultural Engineers, St. Joseph, MI, 1982 p. 117.
10. Freedman, B., E.H. Pryde and T.L. Mounts, JAOCS 60:737 (1983).
11. Ackman, R.G., in Methods in Enzymology, Volume 72, edited by J.M. Lowenstein, Academic Press, New York, 1981 pp. 205-52.
12. Iatroscan TH-10 Mark III Instruction Manual, 1980, Appendix II, pp. 17-18.
13. Iatron Laboratories, Inc., Optimizing Reproducibility with the Iatroscan TLC/FID Analyzer, January 1982.
14. Iatron Laboratories, Inc., Adjustment of Chromarod Activity by Vacuum Drying Process, OpM/137, November 1982.
15. Kramer, J.K.G., R.C. Fouchard and E.R. Farnworth, J. Chromatogr. 198:279 (1980).
16. Kaitaranta, J.K., and N. Nicolaides, Ibid. 205:339 (1981).
17. Parrish, C.C., and R.G. Ackman, Ibid. 262:103 (1983).
18. Pore, J., J.P. Houis and I. Rasori, Rev. Fr. Corps Gras 28:111 (1981).

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♣Short-Chain Phorbol Ester Constituents of Croton Oil[1]

GARY T. MARSHALL and **A. DOUGLAS KINGHORN,*** Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Health Sciences Center, University of Illinois at Chicago, Chicago, IL 60612

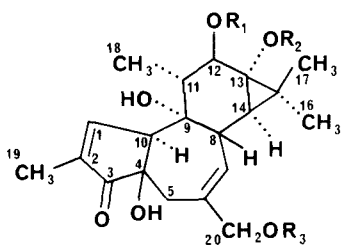
ABSTRACT

Five phorbol diesters, three 4-deoxy-4α-phorbol diesters, two phorbol monoesters, and one 4-deoxy-4α-phorbol monoester were isolated from a commercial sample of croton oil and characterized spectroscopically. Their purification was achieved using combinations of droplet countercurrent chromatography, low-pressure column chromatography over phase-bonded silica gel, and preparative thin layer chromatography. All of these isolates were shown to possess short-chain ester functionalities, with 12-*O*-tiglylphorbol-13-isobutyrate, 12-*O*-(2-methyl)butyrylphorbol-13-isobutyrate, 12-*O*-(2-methyl)butyrylphorbol-13-acetate, 12-*O*-tiglyl-4-deoxy-4α-phorbol-13-isobutyrate, 12-*O*-tiglyl-4-deoxy-4α-phorbol-13-acetate, 12-*O*-(2-methyl)butyryl-4-deoxy-4α-phorbol-13-acetate, phorbol-12-tiglate and 4-deoxy-4α-phorbol-13-acetate being new compounds.

INTRODUCTION

The isolation of pure biologically active constituents from the potent skin-irritating and tumor-promoting seed oil of *Croton tiglium* L. (croton oil) was achieved for the first time by 2 independent groups in the mid-1960s (1,2). Hecker and coworkers eventually isolated 11 diesters of the parent diterpene alcohol, phorbol, that were extractable from croton oil into hydrophilic organic solvents, with a further 3 compounds being obtained on the hydrolysis of a mixture of phorbol triesters present in a lipophilic solvent extract of croton oil (3). These compounds were termed A factors and B factors, respectively, depending on if the longer of 2 acyl moieties present in each molecule was affixed to the C12 or C13 position of phorbol (3). More recently, high pressure liquid chromatography (HPLC) has shown that additional diesters of the phorbol type are present in croton oil, and 3 such diterpenes with short-chain ester functionalities at both C12 and C13 were detected (4). The most abundant croton oil phorbol diester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, phorbol myristate acetate), is now widely used in biochemical experiments as a standard tumor-promoting agent (5).

In the present communication, we wish to report the isolation and characterization of 7 short-chain esters of phorbol and 4 short-chain esters of 4-deoxy-4α-phorbol from croton oil. These new croton oil constituents were separated from a number of less polar, long-chain ester phorbol analogs, of known structure, primarily as a result of applying a refinement of a droplet countercurrent chromatographic (DCCC) solvent system that was developed in this laboratory for the isolation of phorbol and 4α-phorbol from hydrolyzed croton oil (6). Compounds generated in the present study were characterized spectroscopically, and the relative positions of ester substitution were assigned after hydrolysis and partial synthesis experiments were carried out.



Compound	R_1	R_2	R_3
I	Tiglate	Isobutyrate	H
II	2-Methylbutyrate	Isobutyrate	H
IV	Tiglate	H	H
V	Acetate	Acetate	H
VI	Tiglate	Acetate	H
VII	2-Methylbutyrate	Acetate	H
X	H	Acetate	H
XII	Acetate	Acetate	Acetate
XIV	2-Methylbutyrate	H	H
XVII	Acetate	H	H
XVIII	H	H	H

Fig. 1.

EXPERIMENTAL

Apparatus

Instrumentation used to measure optical rotations, ultraviolet (UV), infrared (IR) and 360 MHz ^1H nuclear magnetic resonance (NMR) spectra, and low-resolution mass spectra (MS) has been described previously (7). DCCC was

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*To whom correspondence should be addressed.