TLC as a Tool for Quantitative Isolation of Conjugated Trienoic FA

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ABSTRACT: Conjugated trienoic FA—α-eleostearic, β-eleostearic, and punicic acids—were preferentially isolated in pure form from the total methyl esters of the respective oils by simple silica gel TLC using hexane/diethyl ether as the mobile phase (94:6). The subsequent saponification of the separated methyl ester fractions upon cooling at –20°C yielded individual acids in crystalline form. Melting points, conventional UV spectroscopy, and GLC confirmed the identity of the conjugated trienoic FA. The method finds potential application in bench-scale reaction optimization using pure FA and in the preparation of standard reference materials of these acids, as these oils are widely used in the coatings industry.

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KEY WORDS: Conjugated fatty acids, geometrical isomers, isomerization, saponification, transesterification.

Conjugated trienoic FA such as α-eleostearic, punicic and licanic acids are an industrially important group of compounds found in vegetable oils. They are not very common in yeasts, bacteria, fish oil, or animal fats, but conjugated dienoic, trienoic, and tetraenoic FA and their oxygenated derivatives are encountered in various seed oils in abundance (1–3). The oils containing these FA are very important raw materials in the manufacture of organic coatings and polymers (4–7), as the conjugated unsaturation facilitates good polymerization and imparts adhesive properties when properly treated. As these acids are heat and light sensitive, they cannot be stored for long times and thus standard reference materials are difficult to procure. The isolation and purification of conjugated acids were effected by the crystallization technique devised by earlier workers (8). Silver-ion TLC was used to achieve separation of conjugated esters from other esters (9). Also, the separation of positional and geometrical isomers was facilitated by HPLC by using the silver-ion mode again (10). Further, the characterization of hydroxy eicosatetraenoic acids and conjugated dienes was carried out by a UV spectroscopic method (11). The analysis of carotenoids using non-aqueous reversed-phase HPLC with photodiode array (PDA) detection was accomplished by Tan *et al.* (12). However, isolation by direct silica gel TLC has not been reported before for the enrichment of α-eleostearate.

Four varieties of bittergourd [*Momordica charantia*: varieties, Long Green Monsoon, Summer Crop (S.C), Coimbatore Long (C.L), and Local] and one variety of snake gourd (*Trichosanthus anguina*) were studied in our laboratory for the content of conjugated FA in the respective seed oils, and different analytical methods were used for identification and quantification. Interestingly, simple TLC yielded very useful results because the methyl esters of the above oils, when separated by TLC using normal silica gel plates, presented well-resolved bands accounting for approximately 96% of conjugated trienoic FA of any geometrical configuration. We first attempted to isolate α-eleostearate in clean form by using hexane/ether (94:6) as the mobile phase system to monitor the biosynthetic pathway of conjugated FA (13). We extended this work using a very simple silica gel TLC method and optimized the conditions with respect to mobile phase, saponification, isolation of pure FA, and identification using different analytical methods including high-performance TLC (HPTLC) for quantification. Use of direct silica gel TLC has not been reported earlier for the enrichment of α-eleostearate. We therefore describe a simple procedure to quantitatively isolate and identify trienoic conjugated FA.

MATERIALS AND METHODS

Materials. The seeds of *M. charantia* and *T. anguina* were procured from the Seed Corporation (Hyderabad, India). All solvents were of chromatographic grade (Merck, Darmstadt, Germany).

Extraction procedure. The kernels of *M. charantia* and *T. anguina* seeds were each ground with anhydrous Na_2SO_4. The oil was extracted by cold percolation with light petroleum and concentrated in a rotary evaporator at 40°C. The extracted samples were stored under nitrogen at –15°C until use.

Esterification. The total methyl esters of the oils were prepared by transesterification. For this, the oil sample (100 mg) was dissolved in hexane (3 mL) in a round-bottomed flask. Sodium methoxide in anhydrous methanol (5 mL) was added, the flask was flushed with nitrogen, and the solution was shaken on a Griffin flask shaker for 30 min. Water (10 mL) was added, and the contents were transferred into a separatory funnel and extracted with hexane (10 mL). The hexane layer was filtered through anhydrous Na_2SO_4. The methyl esters were purified by TLC on silica gel G plates using hexane/diethyl ether (90:10 vol/vol) as developing solvent and stored under nitrogen at –15°C until further use.

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Isomerization of the total methyl esters of extracted oils. Iodine solution (2 mL), prepared by dissolving iodine in pure *n*-hexane (0.65 g/100 mL hexane), was added to the methyl esters (50 mg) dissolved in 3 mL of hexane. The reaction mixture was shaken well and left overnight at ambient temperature (14). The isomerized product was extracted with *n*-hexane and purified by eluting through a small column of silica gel. The isomerized methyl esters were stored under nitrogen at −15°C until further use.

Isolation of conjugated fractions by TLC. Methyl α-eleostearate, β-eleostearate, and methyl punicate were isolated from total methyl esters of *M. charantia*, isomerized *M. charantia,* and *T. anguina*. The total methyl esters of these oils as obtained were spotted on preparative silica gel TLC plates (Merck F254) and developed twice with hexane/diethyl ether (94:6). TLC yielded two close but well-resolved bands (15) at an R_f of 0.44 and 0.50 (Fig. 1) as obtained upon densitometric scanning of the plate at 254 nm under absorption/reflection mode using HPTLC (Model 4.05; CAMAG, Muttenz, Switzerland). A little fluorescent broad band corresponding to conjugated esters formed the lower part of the main band (R_f 0.44) was separately scraped off and extracted with light petroleum ether using a small elution column of silica gel (75-μm size). The contents were concentrated under vacuum and stored under nitrogen at −15°C.

Saponification of conjugated esters. The total extracted fraction of conjugated fatty esters as obtained by TLC was subjected to mild saponification. For this, 0.5 N KOH (2 mL) was added to each sample; the securely stoppered flasks were shaken mechanically for 5 min and left overnight under a nitrogen atmosphere. The mixture was subsequently hydrolyzed with mild acid (0.6 N HCl). The crude sample of conjugated FA thus formed was extracted with petroleum ether. Excess solvent was removed and the concentrated FA mixture cooled at −20°C overnight; the conjugated FA precipitated out as white crystalline needles, which were subsequently filtered through a small sintered funnel-G4. The pure FA thus obtained were analyzed by conventional UV spectroscopy (Fig. 2) and their respective m.p. were determined. The results are presented in Table 1.

GLC of the total methyl esters. In a separate study, the total methyl esters of four varieties of *M. charantia* and *T. anguina* oils were analyzed on a Hewlett-Packard gas chromatograph (HP 6890) equipped with an FID and fitted with 10% SE-30 coated on Chromosorb W AW (80–100 mesh) and Silar 10C on Chromosorb W AW (80–100 mesh) (250 cm × 4 cm i.d.) columns. The injector and detector temperatures were each 300°C, and column temperature was maintained at 190°C. Nitrogen was used as carrier gas with a flow rate of 40 mL/min. Methyl esters containing conjugated trienoates were analyzed by GLC on a nonpolar as well as a polar column (i) to establish the identification, (ii) to standardize the method, and (iii) to estimate the content of conjugated trienoates.

UV analysis. UV analysis was performed on a UV double-beam spectrophotometer (Shimadzu, Kyoto, Japan). FA isolated by TLC, total oil methyl esters, and oils of *M. charantia* and *T. anguina* were analyzed in cyclohexane. Conjugated triene contents were determined by dissolving weighed lipid samples (around 5 μg/mL) in cyclohexane and reading the sample absorbance at 270 nm. The $E^{1\%}_{1cm}$ was calculated from the absorbance measurements and the sample concentration. The relative triene content was obtained from the original $E^{1\%}_{1cm}$ value of the conjugated trienoic FA.

RESULTS AND DISCUSSION

Table 1 summarizes the physical properties and composition of the seed oils of four varieties of *M. charantia* and one variety

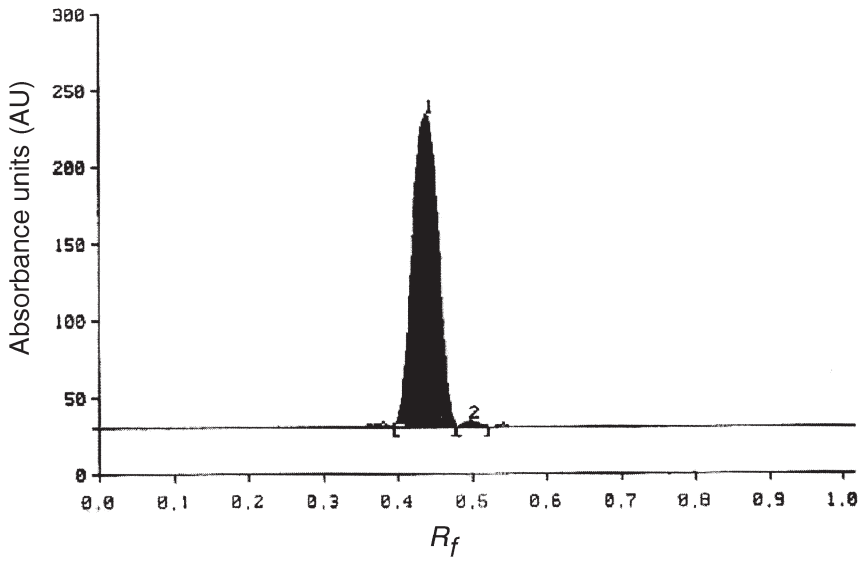


FIG. 1. Typical densitogram to illustrate the separation pattern as obtained from the total methyl esters of *Momordica charantia* oil. Peak 1 at R_f 0.44 represents the lower band containing conjugated trienoic acid, and peak 2 at R_f 0.50 represents the nonconjugated FA.

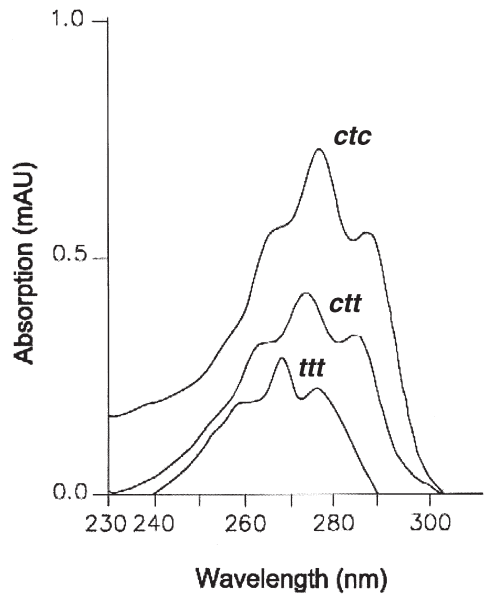


FIG. 2. UV spectra of all three geometrical isomers: punicic acid (*ctc*), α-eleostearic acid (*ctt*), and β-eleostearic acid (*ttt*). For abbreviation see Figure 1.

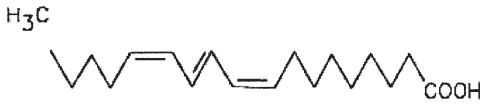
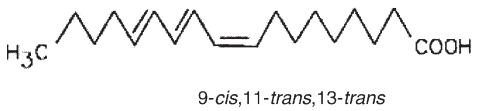
of *T. anguina*. Interestingly, the mobile phase that was used yielded results that were unexpected but that were novel and advantageous. Further, the isolated FA, when subjected to UV analysis, yielded the very characteristic spectrum of trienoic conjugated FA, as depicted in Figure 2 [UV was the only method to reliably identify the conjugated FA irrespective of their geometry, position, and chain length (Table 1)].

UV analysis was performed on known amounts of extracted oils, methyl esters, and FFA in cyclohexane. The amount of conjugated FA present in these samples was calculated from the $E^{1\%}_{1cm}$ coefficients, and the values were compared with GLC data. The results, as recorded in Table 2, along with the spectral characteristics of the methyl esters and purified acids, are as expected and compared well with respect to the composition of the trienoic FA. Thus, the compositions obtained by both analyses agreed well. The λ_{max} were observed at 262, 273, and 284 nm for the ester and the acid of α-eleostearate; 259, 268, and 279 nm for β-eleostearate, and 265, 275, and 287 nm for punicate. In the IR spectrum peaks at 925 (weak), 957 (medium), and 985 cm^{-1} (strong), which are very characteristic of *cis-trans-trans* conjugation; 940, 970, and 990 cm^{-1} for all-*trans;* and 923, 958, and 975 cm^{-1} for *cis-trans-cis* were observed. The purified acids, as prepared, had m.p. at 44.8, 44.0, 45.6, and 46.7°C for α-eleostearic acid obtained from four varieties of *M. charantia* and 41.7°C for punicic acid (reported 48°C for pure α-eleostearic acid and 44.0°C for punic acid). However, with respect to β-eleostearic acid, the studies were limited to UV, IR, and GLC analyses, but from UV data it was assumed that β-eleostearic acid was formed. Conjugated trienoates emerged after C_{18} esters on the SE-30 column, and separation was not facilitated on a polar column as they normally are isomerized and polymerized on this column (13). Hence, by adding varying amounts of α-eleostearate to a methyl ester sample containing 16:0, 18:0, 18:1, 18:2, 18:3, and 20:0, it was observed that conjugated 18:3 was not eluted with any of these components. Since the trienoate undergoes alteration on the polar column (13), the compositions of the various components were calculated by ignoring the peak area of trienoate. However, the trienoate content was obtained from the analysis of the SE-30 column and, using this value, the

TABLE 1
Properties and Composition of Four Varieties of *Momordica charantia* Seed Oils and One Variety of *Trichosanthus anguina*

	Variety					
	Long Green Monsoon	Summer Crop	Coimbatore Long	Local	β-Eleostearate	*T. anguina*
Methyl esters						
λ_{max} (nm)	262	263	262	263	259	265
	273	273	273	272	268	275
	284	284	284	286	279	287
υ_{max} (cm^{-1})	923(W)	923(W)	928(W)	925(W)	932	925
	960(M)	958(M)	963(M)	955(M)	965	958
	987(VS)	985(VS)	990(VS)	981(VS)	990	975
Conjugated triene (%)						
by UV	61.8	57.0	57.5	51.0	—	46.3
by GLC on SE-30	60.0	59.0	56.0	50.1	—	47.7
FA						
λ_{max} (nm)	262	263	262	263	259	265
	273	273	273	272	268	275
	283	284	284	286	279	287
υ_{max} (cm^{-1})	925(W)	925(W)	920(W)	925	940	923
	957(M)	957(M)	957(M)	958	970	958
	985(VS)	985(VS)	985(VS)	987	990	975
m.p. (°C)	44.8	44.0	45.6	46.7	69.6	41.7

TABLE 2
Three Geometrical Isomers with Their Absorption Maxima

Conjugated trienoic acids[a]	UV (nm)
 9-*cis*,11-*trans*,13-*cis* octadecatrienoic acid	265 275 287
 9-*cis*,11-*trans*,13-*trans* octadecatrienoic acid	262 273 284
 9-*trans*,11-*trans*,13-*trans* octadecatrienoic acid	259 268 279

[a]*ctc,* punicic acid; *ctt,* α-eleostearic acid; *ttt,* β-eleostearic acid.

values from the polar column were corrected. This was accomplished with *M. charantia* esters containing known amounts of methyl stearate. The composition thus obtained was comparable to the actual composition.

In the present study, as is visible from the spectra, α-eleostearic acid present in *M. charantia* absorbed at λ_{max} 273 nm whereas punicic acid, with a slight bathochromic shift, absorbed at 275 nm. The all-*trans* isomer β-eleostearic acid, with λ_{max} at 268 nm, was distinct from its two other geometrical isomers, indicating that this method is suitable to separate even the all-*trans* isomer. From Figure 2 it is evident that the differences are subtle but distinguishable from one another for identification.

Thus, silica gel TLC was found to yield two close but well-resolved bands, the upper narrow but discrete one (R_f 0.50) containing mostly nonconjugated esters and the lower broad (R_f 0.44) band containing 95–96% pure conjugated esters as shown by UV analysis. The method is essentially reproducible. We have routinely used this chromatographic separation method since our first observation (13) although details such as saponification and physical parameters were not studied in our previous experiments. The method has potential industrial application for oils such as tung oil, which contains α-eleostearic acid and is used widely in the surface coatings business in general and in India in particular. The method is useful in identifying the main FA, particularly with respect to percent composition. Further, with this method, isolation of pure conjugated FA up to 150–200 mg for bench-scale experiments was often accomplished with great ease in a very short time. However, conjugated dienoates from dehydrated castor oil could not be resolved from the normal esters by this method. In summary, TLC facilitated a very simple and rapid analytical methodology for the quantitative isolation of conjugated FA. This was achieved without the need for highly sophisticated technology, and simple analytical instrumentation such as UV and GLC enabled us to accomplish the quantitative results. The method is reliable and rapid, and FA are relatively stable for the duration of the experiment. This technique is applicable for the preparation of standard reference materials and for reaction optimization in bench-scale reactions in industrial applications.

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REFERENCES

1. Solodovnik, V.D., Chemistry of Natural Aliphatic Acids Containing a Conjugated System of Double Bonds, *Russ. Chem. Rev. 36*:272–283 (1967).
2. Hopkins, C.Y., Fatty Acids with Conjugated Unsaturation, in *Topics in Lipid Chemistry*, edited by F.D. Gunstone, Elek Science, London, 1972, Vol. 3, pp. 37–87.
3. Hopkins, C.Y., and M.J. Chisholm, Conjugated Unsaturation in Vegetable Seed Oils, *J. Am. Oil Chem. Soc. 45*:176–182 (1968).
4. Calderwood, R.L., and F.D. Gunstone, Vegetable Oils III—*Mallotus philippinensis* Muell. Arg. Seed Oil, *J. Sci. Food Agr. 5*:382–387 (1954).
5. Lakshminarayana, G., R. Subba Rao, Y. Sita Rama Sastry, T. Chandrasekhar Rao, V. Kale, and P. Vijaya Lakshmi, Composition of Fatty Acids Obtained by Decomposition of Castor Oil Fatty Acid Estolides, *J. Am. Oil Chem. Soc. 59*:238–240 (1982).
6. Gold Blatt, L.A., L.L. Hoppe, Jr., and E.T. Paynet, A Preparation of Speciality Coatings Like Wrinkle Finishes from Conjugated Trienoic Acids, *Paint J. 43(20)*:81 (1959).
7. Husain, S., K. Sita Devi, G.S.R. Sastry, and V. Madhusudan, Phenolic Lacquers from Lesser Known Conjugated Oils, *Paint India 46(2)*:15–17 (1996).
8. Hopkins, C.Y., and M.J. Chisholm, The Conjugated Triene Acid of *Catalpa ovata* Seed Oil, *J. Chem. Soc.*: 573–575 (1962).
9. Gunstone, F.D., and R. Subba Rao, New Tropical Seed Oils Part I—Conjugated Trienoic and Tetraenoic Acids and Their Oxo Derivatives in the Seed Oils of *Chrysobalanus icaco* and *Parinarium laurinum*, *Chem. Phys. Lipids 1*:349–359 (1967).
10. Christie, W.W., and G.H. Breckenridge, Separation of *cis* and *trans* Isomers of Unsaturated Fatty Acids by HPLC Using Silver Ion Mode, *J. Chromatogr. 469*:261–269 (1989).
11. Ingram, C.D., and A.R. Brash., Characterization of HETEs and Related Conjugated Dienes by UV Spectroscopy, *Lipids. 23*: 340–344 (1988).
12. Tan, B., Analysis of Carotenoids Using Non-aqueous Reversed-Phase HPLC and Photo Diode Array Detection, *J. Am. Oil Chem. Soc. 66*:770–776 (1980).
13. Lakshminarayana, G., T.N.B. Kaimal, V.V.S. Mani, K. Sita Devi, and T. Chandrasekhar Rao, Fatty Acid Changes During Maturation of *Momordica charantia* and *Trichosanthes anguina* seeds, *Phytochemistry 21*:303–305 (1982).
14. Sastry, G.S.R., B.G.K. Murthy, and J.S. Aggrawal, Isomerised Safflower Oil, *Paint Manuf. 40*:32–34 (1970).
15. Husain, S., and K. Sita Devi, Separation and Identification of Isomeric Conjugated Fatty Acids by HPLC with Photo Diode Array (PDA) Detection, *Lipids 28*:1037–1040 (1993).

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