Analysis of Triacylglycerols in Leaves of Citrus by HPLC[1]

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ABSTRACT

Lipids were extracted from leaves of cold-hardened and unhardened citrus plants and the crude triacylglycerol (TG) fraction of each sample was separated from other lipid components by silica gel column and thin layer chromatography (TLC). To determine TG levels and molecular species, the crude TG was subjected to High presure liquid chromatography (HPLC), 12 fractions collected and these fractions quantified by gas liquid chromatography (GLC). Levels of TG/g fresh leaf were 139 μg in unhardened sour orange, 2460 μg in cold-hardened sour orange and 672 μg in cold-hardened Valencia orange. Twenty-one molecular species were determined in the 36-46 equivalent carbon number range (ECN). Five of the TG species that contained linoleate accounted for over 60% of total TG in hardened sour orange and Valencia leaves. Hardened sour orange leaves contained 2-5 times more of these major TG species than hardened Valencia leaves. The increase in these TG species may relate to cold tolerance since sour orange seedlings are more cold hardy than Valencia budded on sour orange.

INTRODUCTION

Tolerance to cold weather is induced in plants by their exposure to near freezing temperatures. Cold hardening increases the structural membrane lipids, e.g., phospholipids, and also increases the unsaturation of their fatty acid moieties (1). Linoleic acid levels in 9 leaf lipids of young potted citrus plants subjected to a cold-hardening regime were greater than the levels in respective lipids of control plants (2). These linoleic increases with cold-hardening, however, were found to the greatest degree in the triacylglycerol (TG) fraction and not in the expected phospholipids. Also, these increases were greater in cold hardy than in less hardy citrus varieties (2,3). To determine the possible relationship that increased TG might have with the cold hardening of membrane lipids, a more definitive analysis of TG molecular species in citrus leaves was needed.

Very efficient qualitative analysis of TG molecular species in seed oils (4-11) and human plasma (12) are obtainable by high performance liquid chromatography (HPLC) using reverse-phase columns. HPLC has not been used perviously for analysis of TG in vegetative tissues because, with few exceptions (13-15), only trace levels of TG are present in these tissues. The refractive index detector used in the majority of the oilseed studies (4-10) does not selectively respond to TG but may respond to any contaminates in sample. Linear responses for fatty acids of various degrees of unsaturation also are not obtainable with this detector, thus, for quantitation, auxiliary GLC analysis of the fatty acid component are needed (4). Detection of the carbonyl moieties of the TG with an HPLC infrared (IR) detector offers a linear relationship between TG concentration and peak response on the recorder (7,11,16). In vegetative tissue, however, pigmented acyl lipids are not readily separated from TG (17,18). Therefore, supplementing HPLC-IR quantitation with GLC analyses of the eluents fatty acid components would seem adviseable.

Three objectives were accomplished in this present study. A HPLC-GLC method was devised for analyzing trace levels of TG molecular species in vegetative tissue. Profiles of TG species in leaves from unhardened and cold-hardened citrus plants were compared and levels of the major TG species in leaves from 2 cold-hardened citrus selections, which differ in their cold tolerance, were compared.

METHODS

Citrus plants used were 8-month-old seedling sour orange (*Citrus aurantium* L.) and Valencia (*C. sinensis* L. Osbeck) budded to sour orange rootstock. Plants were grown under greenhouse conditions and cold-hardened in environmental chambers as previously reported (2,3,19). Lipids extracted from unhardened and hardened leaves were separated into neutral, glycolipids and polar lipids by silica gel columns (2,3). Citrus leaf neutral lipids remaining from the study (3) were streaked (30-60 mg in $CHCl_3$) on 500 μ Silica Gel G plates (Analtech, Inc., Newark, DE) and developed in $CHCl_3$. Bands were detected with Rhodamine 6G under short ultraviolet (UV) light, and the area corresponding in Rf value to flax TG was eluted from the absorbant with $CHCl_3$. In samples of unhardened leaves, the level of TG was often too low to be detectable as a discrete fluorescent band under UV light. From 4 to 6 platings of the neutral control leaf lipid were needed to obtain sufficient material (0.1 to 0.2 mg) for an HPLC analysis, whereas one plate was sufficient for experimental, hardened leaf lipid samples. The TG samples were dissolved in $CHCl_3$ for HPLC fractionation.

A Varian 5000 (Varian Instrument Group, Palo Alto, California) liquid chromatograph containing a 10 μL Valco loop injector, a 5 μ C-18 MPLC guard column (Brownlee Labs, Inc., Santa Clara, CA) and a Varian Micropak 15 cm × 4 mm MCH 5 μ-N-CAP C-18 column was used in conjunction with an IR detector set at 5.75 nm containing a 1 mm NaCl cell (duPont, Instruments Division,Wilmington, DE). Chromatograms were recorded and integrated on a Hewlett-Packard 3390A electronic integrator (Hewlett-Packard, Palo Alto, CA). Solvents, HPLC grade in glass, were acetonitrile (J.T. Baker, Phillipsburg, NJ), chloroform (Mallinckrodt, Paris, KY) and methylene chloride and methyl t-butyl ether (Burdick and Jackson, Muskegon, MI). Flax seed and coconut oils were extracted from fresh produce from local markets. Synthetic triacylglycerol standards were obtained from Applied Science, State College, PA, or Nu Chek Prep, Elysian, MN. HPLC parameters were optimized for analyses of highly unsaturated TG present in citrus leaves. Combinations of acetonitrile with chloroform or methylene chloride were tested to determine the best separation of flax-seed oil and of citrus-leaf TG species. Flax-seed oil served both as a qualitative and as a quantitative HPLC-TG standard having very few impurities and TG molecular species at levels that were similar to TG of hardened citrus leaves. Optimal solvent ratios were: System A—acetonitrile/chloroform, 85:15; System B—acetonitrile/methylene chloride, 80:20. These 2 isocratic systems eluted the majority (97%) of flax and leaf TG species within 30 min; however, to remove the remaining 3% consisting of more saturated TG species, a gradient system was devised. A near optimal ternary gradient system of acetonitrile/methylene chloride/methyl t-butyl ether for total elution of flax or citrus leaf TG was: (elapsed time-ratio of solvents) 0 min—80:20:0, 13 min—80:20:0, 20 min—60:28:12,

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28 min–60:28:12. For confirmation of TG species in the HPLC bands, 12 fractions were collected under isocratic conditions with a flow rate of 1.1 mL/min. Methyl heptadecanoate was added to each fraction as an internal standard and the fractions were transesterified with NaOH-BCl_3-MeOH (2,3) to fatty acid methyl esters (FAME). The FAME were analyzed by GLC (2).

RESULTS AND DISCUSSION

Flax and citrus leaf TG contain 5 major acids: palmitic (P), stearic (S), oleic (O), linoleic (L) and linolenic (Ln). There are 35 possible combinations of the 5 major acids on the glycerol molecule, when no isomers are distinguished. The retention times of coconut oil TG served as the basis for log retention-carbon number plots (4-6). Injections of flax oil, TG standards and leaf TG were interspersed with the coconut injections. Observed theoretical carbon numbers were determined for each major HPLC peak. With the component 3 acids in each TG known, the mean correction fractors (U_i) for linolenic, linoleic and oleic acids were determined (8). Theoretical carbon numbers were calculated for each possible combination by subtracting the sum of the mean correction factors for individual acids present in the molecule from the equivalent carbon number, e.g., $TCN=ECN-(\Sigma_1^3 U_i)$ (8). The 2 solvent systems complement each other in resolving critical TG pairs (TG with same ECN), e.g., system A resolved LnLL from LnLnO whereas system B resolved LnLnO from LnLnP. Under isocratic conditions, the localization of each major leaf TG species on the HPLC chromatogram was determined. Peaks on the chromatograms that could not be assigned to a TG species were considered contaminants. The 2 isocratic systems eluted the majority of flax and leaf TG species within 30 min. Leaf TG consisted of 21 structures with ECN 36-46 (Table I). They were eluted from the HPLC in 12 fractions (Fig. 1) and quantified (Table II).

From calculated TCNs (Table I) and HPLC profiles, We concluded that a maximum of 3 TG species were present in any one of these 12 ECN-36 to ECN-46 TG fractions (Table II). Quantities of each TG were calculated from their ratios to the internal standard, methyl heptadecanoate, along with HPLC quantitation and the original TG analyses (3). Values reported in Tables II and III were obtained from triplicate HPLC analyses and subsequent GLC analyses on each of the 12 eluted fractions.

In each of the 12 fractions, lipid material was present that could not be accounted for by the 1-3 TG species present. This extra lipid material consisted of 2 types. Type 1 material was acyl lipid, e.g., carotenoid esters, detected by their carbonyl absorption on the HPLC-IR detector and as FAME by the GLC-FID detector. The level of Type 1 lipids was the difference between the level of FAME detected by GLC and the level of fatty acids that could be assigned to the TG species theoretically present.This type material was greatest in the t_0 to LnLnL area of the chromatograms (Fig. 1) and accounted for the greatest amount of impurities in control sour orange leaf. Type 2 material was nonacyl lipid that integrated as carbonyl material by the IR detector but was not detected as FAME by GLC. This type

TABLE I

HPLC Analyses of Triacylglycerols in Flax-Seed Oil and in Citrus Leaves

ECN[a]	C:m[b]	Triacylglycerol	Major TG		Solvent system A[c]		Solvent system B[c]	
			Citrus leaf	Flax oil	k'	TCN[d]	k'	TCN
36:0	54:9	LnLnLn	X	X	3.2	34.7	3.6	34.4
38:0	54:8	LnLnL	X	X	4.1	36.4	4.6	36.2
40:0	54:7	LnLL	X		5.2	38.1	5.9	38.0
	54:7	LnLnO		X	5.6	38.6	6.2	38.2
	52:6	LnLnP	X	X	6.0	39.2	6.8	39.0
42:0	54:6	LLL	X		6.8	39.8	7.7	39.8
	54:6	LnLO	X	X	7.3	40.3	8.1	40.0
	52:5	LnLP	X		7.5	40.8	8.6	40.7
	54:6	LnLnS		X	7.9	41.2	8.9	41.0
44:0	54:5	LLO	X		9.0	42.0	10.5	41.8
	54:5	LnOO		X	9.7	42.4	10.4	41.9
	52:4	LLP	X		9.6	42.5	11.2	42.5
	54:5	LnLS				42.8		42.7
	52:4	LnOP	X	X	10.4	43.0	11.1	42.7
	50:3	LnPP			10.7	43.6	12.5	43.5
46:0	54:4	LOO			12.3	44.1	13.5	43.7
	54:4	LLS				44.5		44.5
	52:3	LOP	X	X	13.0	44.7	14.7	44.5
	54:4	LnOS		X		45.0		44.7
	50:2	LPP	X		13.7	45.3	16.8	45.3
	52:3	LnPS				45.6		45.5
48:0	54:3	OOO		X	15.9	46.3	17.6	45.7
	54:3	LOS				46.7		46.5
	52.2	OOP		X	17.0	46.9	19.4	46.5
	52:2	LPS				47.3		47.3
	50:1	OPP				47.4		47.2
	54:3	LnSS				47.6		47.5
	48:0	PPP				48.0		48.0

[a]Equivalent carbon number.
[b]Number of carbons/number double bonds.
[c]See text.
[d]Theoretical carbon number.

material was greatest in the LLL-LnLP peak area of sour orange (Fig. 1).

To determine purities of the 3 leaf TG fractions isolated by TLC (3), fatty acid balance sheets were prepared (Table III). Values for fatty acids of nonpurified TG included fatty acids for all acyl materials within the TLC-isolated TG fraction as reported previously (3). Values for the fatty acids of purified TGs are the summations of each fatty acid's contribution to the 21 HPLC-GLC determined TG species as reported in Table II. Nearly two-thirds of the fatty acids previously assigned to sour orange TG in control samples (3) can now be assigned to non-TG sources. Because this extra lipid material was minor (2-18%) in the hardened samples, structures of leaf non-TG components were not determined.

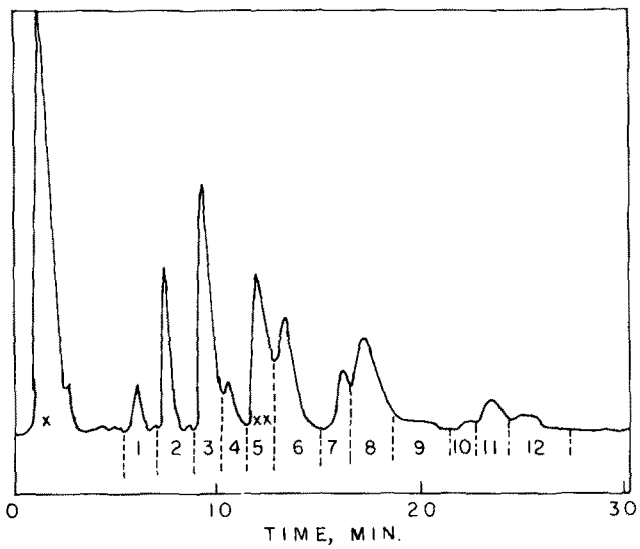

FIG. 1. HPLC of triacylglycerols from hardened sour orange citrus leaves on a 150 X 4 mm, C_{18} 5 μ-MCH column eluted with acetonitrile/chloroform (85:15, v/v). Peaks were detected with an IR detector. The 12 numbered fractions, quantified by GLC analyses, contained type 1 (x) and 2 (xx) extra lipid components.

The 16.7-fold increase in TG on hardening partially confounded sequential trends of TG species between unhardened and hardened sour orange, and between sour orange and hardened Valencia. One trend involved 8 TG species: LLP, LLL, LLO, LPP, LnLnP, LnLnLn, LnLnO and LnPP. Relative percentage values for the first 4 TG species were greater in hardened Valencia and sour orange than unhardened sour orange whereas for the last 4 TG species the reverse was true (Table II). Because all TG species increased during hardening, these percentages show that the 4 linolenate TG species changed much less than the respective linoleate TG species. With only one exception, LOO, the levels for the 21 individual Valencia TG species were between the respective values of unhardened and hardened sour orange (Table II). Five TG species (LnLnL, LnLL, LLL, LnLP and LLP) accounted for 62% and 70% of the total TG in leaves of hardened sour orange and Valencia, respectively. Individually, the levels of these species in sour orange were 2-5 times the levels in Valencia leaves. This is evidence that hardening increases levels of specific TG species and that the magnitude of these increases directly relates to the cold tolerance of the citrus selection.

With unhardened plants, HPLC analyses of leaf TG may have limited use because the lower levels of TG that can be detected are marginal. Under our optimized parameters for sour orange seedling, 0.1-0.2 mg TG corresponding to 354 μg impure, 139 μg pure TG/g fresh leaf was barely acceptable as an integratable HPLC chromatogram. Impure TG extracts (159 μg/g [3]) of Valencia on sour orange, a less cold-tolerant citrus selection than sour orange, failed to produce an integratable chromatogram. To date, an acceptable TLC system has not been found to remove these acyl-lipid contaminates from unhardened citrus leaf TG before HPLC analysis.

Conclusions about the role linoleic-rich triacylglycerols play in the cold protective mechanism of citrus cannot be drawn from the data presented in this study. That specific

TABLE II

Triacylglycerols of Unhardened and Hardened Citrus Leaves

Triacylglycerol	HPLC fraction number	μg TG/g fresh leaf (rel. %)		
		Unhardened sour orange	Hardened sour orange	Hardened Valencia
LnLnLn	1	12 ± 2 (8.6)	46 ± 21 (1.9)	26 ± 1 (3.9)
LnLnL	2	16 ± 5 (11.5)	206 ± 3 (8.4)	93 ± 7 (13.8)
LnLL	3	18 ± 3 (13.0)	387 ± 87 (15.6)	112 ± 8 (16.7)
LnLnO	4	11 ± 3 (7.9)	70 ± 1 (2.8)	22 ± 1 (3.3)
LnLnP	4	14 ± 4 (10.1)	75 ± 1 (3.0)	36 ± 2 (5.4)
LLL	5	4 ± 1 (2.9)	319 ± 43 (13.0)	65 ± 1 (9.7)
LnLO	5	7 ± 2 (5.0)	137 ± 18 (5.6)	24 ± 1 (3.6)
LnLP	6	20 ± 3 (14.5)	272 ± 1 (11.1)	124 ± 1 (18.4)
LnLnS	6	2 ± 1 (1.4)	24 ± 1 (1.0)	7 ± 1 (1.0)
LLO	7	2 ± 1 (1.4)	119 ± 1 (4.8)	10 ± 5 (1.5)
LnOO	7	1 ± 1 (0.7)	15 ± 1 (0.6)	4 ± 2 (0.6)
LLP	8	9 ± 1 (6.5)	341 ± 1 (13.8)	75 ± 1 (11.2)
LnLS	9	2 ± 1 (1.4)	41 ± 9 (1.7)	12 ± 1 (1.8)
LnOP	9	6 ± 3 (4.3)	84 ± 18 (3.4)	9 ± 1 (1.3)
LnPP	9	5 ± 2 (3.6)	36 ± 8 (1.5)	16 ± 1 (2.4)
LOO	10	3 ± 1 (2.2)	24 ± 6 (1.0)	tr
LLS	10	tr	24 ± 6 (1.0)	tr
LOP	11	5 ± 3 (3.6)	123 ± 2 (5.0)	11 ± 1 (1.6)
LnOS	12	tr	17 ± 5 (0.7)	5 ± 1 (0.7)
LPP	12	2 ± 1 (1.4)	78 ± 21 (3.2)	10 ± 1 (1.5)
LnPS	12	tr	22 ± 6 (0.9)	11 ± 1 (1.6)

TABLE III

Comparison of Fatty Acids in Purified and Nonpurified[a] Triacylglycerols in Citrus Leaves

Triacylglycerol	Purified/ nonpurified[a]	Fatty acid 16:0	18:0	18:1	18:2	18:3	Total	TG purity[b]
		μg/g Fresh leaf						
Unhardened sour orange	P	23	1	13	42	60	139	
Unhardened sour orange	NP	90	9	41	89	125	354	39.3
Hardened sour orange	P	382	43	209	1193	633	2460	
Hardened sour orange	NP	333	45	260	1235	648	2521	97.6
Hardened Valencia	P	106	11	30	238	287	672	
Hardened Valencia	NP	150	12	42	357	257	818	82.2

[a]Nonpurified values from Table 1 of reference 3.
[b]Purity based on total fatty acids in column 8.

TG molecular species increase under a cold hardening regime does, however, lay the groundwork for future studies in which changes in lipids of citrus leaves subjected to freeze regimes will be examined.

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A Comparison of the Stability of Oils from Brazil Nut, Para Rubber and Passion Fruit Seeds

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ABSTRACT

The oxidation at 46 C of oils from Brazil nut, *Bertholletia excelsa* H.B.K.—Lecythidaceae (BNO), and from seeds of Para rubber, *Hevea brasiliensis*—Euphorbiaceae (PRO), and passion fruit, *Passiflora edulis*—f. flavicarpa—Passifloraceae (PFO), was followed over 115 days through the measurement of peroxide, acidity values, refractive indices, combustion energies and infrared (IR) spectra. The addition of 3 ppm Cu^{2+} to PFO oil shortened the induction period by 12%. The oxidation of BNO and PRO exhibited first-order kinetics in the production of hydroperoxide (RO_2H), up to the maximum values of the concentration of RO_2H. On the other hand, the oxidation of PFO and PFO + Cu^{2+} displayed first-order kinetics at higher concentrations of RO_2H and possibly half-order kinetics at low hydroperoxide concentrations in the first 15 days. Therefore, the 3 oils studied and PFO + Cu^{2+} did not show the same stability pattern over the 115 days of the experiment. The application of kinetic data, a side from the other parameters, allows the definition of 2 different stability patterns. From 0-15 days the oxidation rates led to the following order of stability: PFO + Cu^{2+} < PFO < BNO < PRO. From the 15th day to the end of the period corresponding to the maximum concentration of RO_2H, the rate constants led to the pattern: PFO + Cu^{2+} < BNO < PFO < PRO. Considering the whole period of the experiment, the changes in viscosity and the values of the induction periods point toward the first-mentioned stability pattern, demonstrating that without kinetic data these 2 parameters are insufficient to determine such patterns.

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INTRODUCTION

Bertholletia excelsa H.B.K.—Lecythidaceae and *Hevea brasilliensis*—Euphorbiaceae are native species of the Brazilian Amazon area, although they are cultivated in other topical countries. The fruit of the *Bertholletia* furnishes a kernel known as Brazil nut that produces a unstable clear yellow oil (1). The oil yield is ca. 70% (1,2). The fruit of *H. brasiliensis* (Para rubber) furnishes seeds that produce a dark red oil, with a high acidity and an oil yield of ca. 50% (3). *Passiflora edulis* f. flavicarpa—Passifloraceae produces a fruit (passion fruit), whose seeds are considered as a by-product of this fruit, is being processed in many countries in order to obtain juice. The seeds furnish a pale yellow oil with an average yield of 20% (4).

Nutritive products containing fats and vegetable oils, when stored, may yield undesirable organoleptic properties as a result of oxidation reactions that produce hydroperoxides (RO_2H) as principal products (5,6). The presence of natural or synthetic antioxidants in the above materials