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ARTICLE *in* LIPIDS · FEBRUARY 2002

Impact Factor: 1.85 · DOI: 10.1007/s11745-002-0870-x · Source: PubMed

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Dibutyrate Derivatization of Monoacylglycerols for the Resolution of Regioisomers of Oleic, Petroselinic, and *cis*-Vaccenic Acids

Frédéric Destaillats[a], Joseph Arul[a], James E. Simon[b], Robert L. Wolff[c], and Paul Angers[a,*]

[a]Department of Food Science and Nutrition, and Dairy Research Center (STELA), Université Laval, Sainte-Foy, Québec, Canada G1K 7P4, [b]Department of Plant Science, Rutgers University, New Brunswick, New Jersey 08901, and [c]INRA-UNL, 21065 Dijon Cedex, France

ABSTRACT: Dibutyrate derivatives of monoacylglycerols of oleic, petroselinic, and *cis*-vaccenic acids were prepared by diesterification of monoacylglycerols with *n*-butyryl chloride. The resulting triacylglycerols were analyzed by gas chromatography (GC) with a 65% phenyl methyl silicone capillary column and separated on the basis of both fatty acid composition and regiospecific position. The petroselinic acid derivatives eluted first, followed sequentially by the oleic and *cis*-vaccenic acid derivatives, with the *sn*-2 positional isomer eluting before the *sn*-1(3) isomer in each case. Separation of the peaks was almost baseline between petroselinic and oleic acids as well as between oleic and *cis*-vaccenic acids. To assess the accuracy of the method, mixtures of triolein, tripetroselinin, and tri-*cis*-vaccenin in various known proportions were partially deacylated with the use of ethyl magnesium bromide and derivatized and analyzed as above. The results showed that this method compares favorably to the existing methods for analysis of oleic, petroselinic, and *cis*-vaccenic fatty acids by GC with respect to peak separation and accuracy, and it also provides information on the regiospecific distribution of the fatty acids.The method was applied to basil (*Ocimum basilicum*) and coriander (*Coriandrum sativum*) seed oils. *cis*-Vaccenic, oleic, and linoleic acids were mainly distributed at the *sn*-2 position in basil seed oil, and higher proportions of linolenic, palmitic, and stearic acids were distributed at the *sn*-1(3) position than at the *sn*-2 position. In coriander seed oil, petroselinic acid was mainly distributed at the *sn*-1(3) position, and both oleic and linoleic acids were mostly located at the *sn*-2 position, whereas palmitic, stearic, and *cis*-vaccenic acids were located only at the *sn*-1(3) position.

Paper no. L8786 in *Lipids 37*, 111–116 (January 2002).

Petroselinic (*cis*-6 18:1) and *cis*-vaccenic (*cis*-11 18:1) acids are two fatty acid isomers of oleic acid (*cis*-9 18:1), differing in the position of the double bond. These isomers are major fatty acid constituents in the lipids of plants of the *Araliaceae* (1) and *Apiaceae* (formerly *Umbelliferae*) (2–9) families and are also minor constituents of seed lipids of basil (unpublished results) and of other plants (10–15). Regio- and stereospecific distribution of fatty acids in oils containing petroselinic and *cis*-vaccenic acids is not well documented (16,17), attributable to the difficulties involved in the analysis of mixtures of these isomers, which tend to coelute in gas chromatography (GC) (18–21). Until recently, their analysis has been laborious and required isolation of the 18:1 isomeric fatty acids, followed by oxidative cleavage of the double bond to produce, for example, azelaic and nonanoic acids from oleic acid and adipic and lauric acids from petroselinic acid. Analytical methods allowing direct and accurate quantitative analysis by GC of mixtures of derivatized oleic, petroselinic, and *cis*-vaccenic acids were developed by Wolff and Vandamme (20,21) with isopropyl derivatives, by Thies (22) with *n*-butyl derivatives, and by Liu and Hammond (23) with 2-phenylethyl derivatives. The latter can also be analyzed by high-performance liquid chromatography (HPLC). These methods use larger ester alcohol moieties than methanol, and this results in reduced relative polarity of the esterified acid, with the double bond in the hydrocarbon moiety making the primary contribution to the polarity of the esters. Thus, the position of the polar center, i.e., the double bond, plays a significant role in the adsorption of the ester to the hydrophobic polymer coating on the chromatographic column, where the ester with the double bond at position 6 is least strongly adsorbed and should elute faster than the esters with the double bond at position 9 or 11, given that there is no difference in their vapor pressure or boiling point. This reasoning led us to hypothesize that the use of dibutyrate derivatives of monoacylglycerols (24) would permit not only the separation of the fatty acid isomers oleic, petroselinic, and vaccenic acids, but also the determination of their regiospecific distribution.

The objectives of the present work were first to evaluate the separation of dibutyrate derivatives of monoacylglycerols of oleic, petroselinic, and *cis*-vaccenic acids by GC on the basis of both fatty acid composition and regiospecific distribution; second, to determine the accuracy of this method by analyzing dibutyrate derivatives of monoacylglycerols obtained by partial deacylation of mixtures of triolein, tripetroselinin, and tri-*cis*-vaccenin in known proportions; and third, to validate the method by analyzing natural samples of basil and coriander seed oils.

*To whom correspondence should be addressed at Department of Food Science and Nutrition, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4.
E-mail: paul.angers@aln.ulaval.ca

Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; TAG, triacylglycerol; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. Monooleoylglycerol, monopetroselinoylglycerol, monostearoylglycerol, mono-*cis*-vaccenoylglycerol, triolein, tripetroselinin, and tri-*cis*-vaccenin were purchased from Nu-Chek-Prep (Elysian, MN). Ethylmagnesium bromide (3.0 M in diethyl ether), *n*-butyryl chloride, and triethylamine were obtained from Aldrich (Milwaukee, WI).

Oil extraction. Mature dry seeds (10 g) of basil (*Ocimum basilicum*) (25) and of coriander (*Coriandrum sativum*), purchased from a local seed retailer, were washed and finely ground in a mortar. Oil was extracted with hexane in a Soxhlet apparatus and recovered by filtering and drying the extract over anhydrous sodium sulfate and evaporating the solvent under vacuum at 30°C on a rotary evaporator (21% yield for basil oil and 23% for coriander oil). The oils were stored under nitrogen at –35°C until further use.

Derivatization of monoacylglycerols. Standard monoacylglycerols (10 mg) of oleic, petroselinic, *cis*-vaccenic, and stearic acids were solubilized in dry chloroform (0.5 mL). Triethylamine (100 L) and *n*-butyryl chloride (50 L) were sequentially added, and the reaction mixture was held at 60°C for 20 min in a closed vial with constant stirring. After cooling to room temperature, the reaction mixture was diluted with *n*-octane (1 mL), washed sequentially with 1-mL volumes each of cold diluted hydrochloric acid (0.1 N), water, and brine (saturated aqueous solution of sodium chloride), and dried over anhydrous sodium sulfate. Aliquots (50 L) were diluted with *n*-octane (200 L) and analyzed by GC.

Partial deacylation of triacylglycerols (TAG). Mixtures of standard TAG triolein, tripetroselinin, and tri-*cis*-vaccenin in different proportions (Table 1) and basil and coriander seed oils were partially deacylated with ethylmagnesium bromide accordig to a literature procedure (24). After workup, the residue was solubilized in dry chloroform, derivatized with *n*-butyryl chloride as described above for standard monoacylglycerols, and analyzed by GC.

GC of triacylglycerols. Analysis was performed using a gas chromatograph (Hewlett-Packard Model 6890, Series II; Palo Alto, CA), equipped with a flame-ionization detector and connected to a ChemStation (Hewlett-Packard). Sample volumes (1.0 L) in *n*-octane were injected onto a 65% phenyl methyl silicone capillary column (Restek, Bellefonte, PA; 30 m × 0.25 mm i.d., 0.10 m film thickness) with a split ratio of 1:50. Injector and detector temperatures were set at 400°C, while the oven temperature was programmed from 190 to 300°C at 2.5°C min^{-1} and to 360°C at 15°C min^{-1} and held for 5 min at this temperature, for a total duration of 70 min. The linear velocity of the carrier gas (hydrogen) was 47 cm s^{-1} at 190°C. Other chromatographic conditions used were isothermal oven temperatures from 190 to 260°C, in 5°C increments, other parameters being left unchanged. Peak areas and corrected response factors based on standards were used for quantitation. Resolution values were determined using peak base width.

Experimental considerations. The oil samples were crude and possibly contained components other than TAG, such as phospholipids. These, however, do not interfere with the regiospecific analysis of TAG by the present method, which can be performed directly on the crude lipid extracts without further purification of TAG by thin-layer chromatography (TLC) or other methods (26).

RESULTS AND DISCUSSION

GC of TAG. Dibutyrate derivatives of monoacylglycerols of oleic, petroselinic, and *cis*-vaccenic acids were separated by GC by both fatty acid composition and regiospecific position. The order of elution was similar to that reported for other GC methods employed in the analysis of these fatty acid isomers (20–22), with petroselinic acid derivatives eluting first, oleic acid derivatives second, and *cis*-vaccenic acid derivatives third. For each fatty acid derivative, the *sn*-2 positional isomer eluted before the *sn*-1(3) isomer (Fig. 1). The separation was nearly baseline (R_S = 1.28) between the petroselinic and oleic acid de-

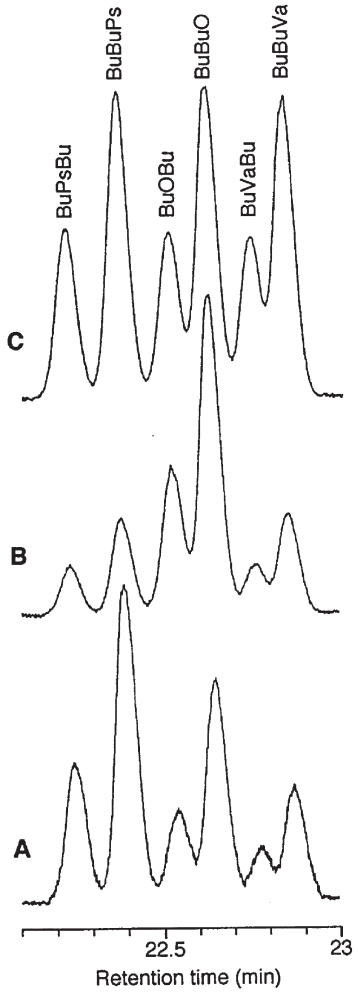

FIG. 1. Gas chromatograms of dibutyrate derivatives of monoacylglycerols obtained after partial deacylation of mixtures of tripetroselinin, triolein, and tri-*cis*-vaccenin by ethyl magnesium bromide, followed by derivatization with *n*-butyryl chloride. Bu, 4:0; Ps, *cis*-6 18:1; O, *cis*-9 18:1; Va, *cis*-11 18:1. Proportions of tripetroselinin/triolein/tri-*cis*-vaccenin in the initial mixtures were (A) 3:2:1, (B) 1:3:1, and (C) 1:1:1.

rivatives, and also close to baseline between the oleic and *cis*-vaccenic acid derivatives (R_S = 1.10). The resolution of positional isomers was highest for petroselinic acid derivatives and lowest for *cis*-vaccenic acid derivatives, with R_S values of 1.37, 0.95, and 0.88 between the *sn*-2 and the *sn*-1(3) positional isomers of the petroselinic, oleic, and *cis*-vaccenic acid derivatives, respectively. However, separation between *sn*-2 and *sn*-1(3) isomers of the derivatives of the 18:1 isomers (as well as other fatty acids) depends on the quality of the column; columns with the same stationary phase can exhibit some variability in their resolution owing to variations in the polymer coating.

Analysis of dibutyrate derivatives of monoacylglycerols by this method does not require separation of glyceride species prior to derivatization because well-defined chromatographic resolution, free of overlap, of all species present in the samples is obtained with the use of proper chromatographic conditions (Fig. 2). The species included dibutyrate derivatives of monoacylglycerols, monobutyrate derivatives of diacylglycerols, TAG, and tertiary alcohols, some of which may have been partly derivatized or dehydrated during the derivatization procedure. However, we have encountered some overlapping of the elution regions for derivatives of mono- and diacylglycerols in samples containing C_8 to C_{20} fatty acids, but not for individual species (data not shown).

The primary chromatographic conditions reported in the Materials and Methods section indeed allowed separation of dibutyrate derivatives of petroselinic, oleic, and *cis*-vaccenic acids. However, these conditions resulted in overlapping of petroselinic and stearic acid derivatives (Fig. 3, A1). Isothermal elution at 205°C resulted in separation of petroselinic and stearic acid derivatives, but the latter overlapped with oleic acid derivatives (Fig. 3, A4). Although we did not succeed in obtaining acceptable separation of stearic, petroselinic, and oleic acid derivatives under a single set of chromatographic conditions, the combined chromatograms obtained under two sets of chromatographic conditions (Fig. 3, A2 and A3) made possible the quantitation of acid derivatives in a given sample. A first set of conditions resulting in overlap of petroselinic and stearic acid derivatives but resolving oleic acid derivatives, and a second set of conditions resulting in overlap of oleic and stearic acid derivatives while resolving petroselinic acid derivatives permitted accurate determination of the values of these acid derivatives in coriander seed oil (Fig. 3, B).

Fatty acid composition of TAG mixtures. The accuracy of the present method for the analysis of petroselinic, oleic, and *cis*-vaccenic acids in samples, and for the determination of the regiospecific distribution of these acids, was evaluated by analyzing dibutyrate derivatives of monoacylglycerols from partial deacylation of three mixtures of triolein, tripetroselinin, and tri-*cis*-vaccenin in defined proportions. The experiments were carried out in triplicate, and the results are presented in Table 1. The expected and experimental values are in good agreement for all three mixtures, although minor deviations were noted. The values for the *sn*-1(3) isomer were slightly underestimated by roughly 1% for petroselinic acid, whereas they were slightly overestimated for oleic acid. Experimental values for the *sn*-2 isomers of petroselinic and oleic acids and the *sn*-1(3) isomer of *cis*-vaccenic acid were on target. Total fatty acid compositions of the mixtures calculated from the values for the *sn*-1(3) and *sn*-2 positions were also in agreement with the expected values.

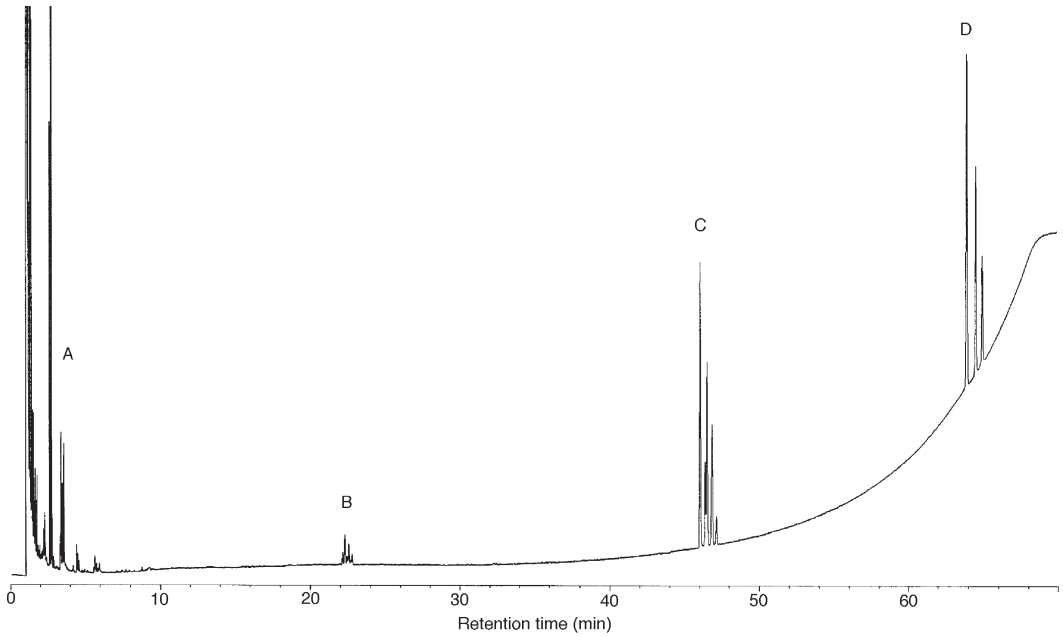

FIG. 2. Gas chromatogram of products resulting from partial deacylation with ethyl magnesium bromide of a 3:2:1 mixture of tripetroselinin, triolein and tri-*cis*-vaccenin, followed by derivatization with *n*-butyryl chloride. (A) Tertiary alcohols and similar compounds; (B) dibutyrate derivatives of monoacylglycerols; (C) monobutyrate derivatives of diacylglycerols; (D) triacylglycerols.

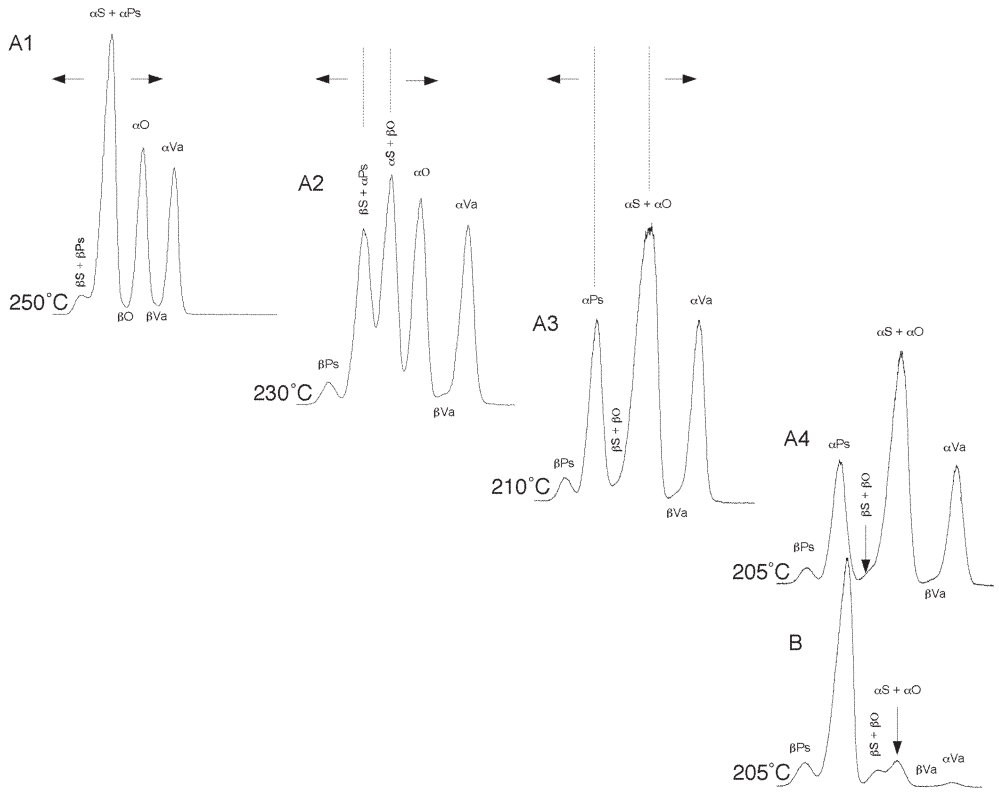

FIG. 3. Partial gas chromatogram of a mixture of dibutyrate derivatives of monoacylglycerols of stearic, petroselinic, oleic and *cis*-vaccenic acids under selected isothermal conditions at 250°C (A1), 230°C (A2), 210°C (A3) and 205°C (A4); and of dibutyrate derivatives of monoacylglycerols obtained after partial deacylation of coriander (*Coriandrum sativum*) seed oil under isothermal conditions at 205°C (B). α and β refer to *sn*-1(3) and *sn*-2 positional isomers, respectively. (Derivatives of other fatty acids not shown.)

Regiospecific analysis of basil and coriander seed oils. The analytical method was further validated by the analysis of basil (*Ocimum basilicum*) and coriander (*Coriandrum sativum*) seed oils. The results (Table 2) showed that in basil seed oil, linolenic acid was the most abundant fatty acid in both the *sn*-1(3) and *sn*-2 positions, amounting to 55.8 and 51.3%, respectively. The results also showed that $C_{18:1}$ isomers were mostly distributed at the *sn*-2 position, whereas the saturated fatty acids, palmitic and stearic acids, were predominantly located at the *sn*-1(3) position. In coriander seed oil, petroselinic acid was the major acid both at the *sn*-1(3) position (80.1%) and at the *sn*-2 position (40.9%). Oleic and linoleic acids were mainly distributed at the *sn*-2 position, whereas palmitic, stearic, and *cis*-vaccenic acids were distributed only at the *sn*-1(3) position. The calculated fatty acid compositions of both seed oil TAG obtained from the results of the regiospecific analysis were in good agreement with the experimental values obtained by GC analysis of fatty acid methyl ester derivatives (25) of basil seed oil and of fatty acid isopropyl ester derivatives (20) of coriander seed oil.

We have developed a simple, fast, and accurate method for the regiospecific analysis of petroselinic, oleic, and *cis*-vaccenic acids in triacylglycerols and validated it with natural oils of basil and coriander seeds. The simplicity of the protocol more than compensates for the possibility of small errors, indicated by the analysis of known mixtures of TAG

TABLE 1
Experimental Values for Fatty Acid Distribution Between *sn*-1(3) and *sn*-2 Positions of Known Mixtures of Triolein, Tripetroselinin, and Tri-*cis*-vaccenin

Tripetroselinin/ triolein/ tri-*cis*-vaccenin	Fatty acid composition (mol%)[a]								
	Petroselinic			Oleic			*cis*-Vaccenic		
	sn-1(3)	*sn*-2	TAG[b]	*sn*-1(3)	*sn*-2	TAG	*sn*-1(3)	*sn*-2	TAG
33.3:33.3:33.3	32.5 ± 0.2	34.1 ± 0.5	33.0 ± 0.3	34.8 ± 0.3	34.3 ± 1.0	34.6 ± 0.5	32.7 ± 0.3	31.6 ± 0.6	32.3 ± 0.4
20.0:60.0:20.0	18.7 ± 0.5	20.5 ± 0.9	19.3 ± 0.7	61.4 ± 0.8	59.5 ± 0.7	60.7 ± 0.7	19.9 ± 0.3	20.0 ± 0.7	19.9 ± 0.1
50.0:33.3:16.7	48.4 ± 0.4	49.9 ± 0.4	48.9 ± 0.2	33.9 ± 0.1	32.8 ± 0.2	33.5 ± 0.1	17.7 ± 0.2	17.3 ± 0.5	17.6 ± 0.3

[a]Values are means of three replicate analyses ± standard deviations.
[b]Triacylglycerol, calculated from values for *sn*-1(3) and *sn*-2: [2 × *sn*-1(3) + *sn*-2]/3.

TABLE 2
Experimental Values (mol%) for Fatty Acid Distribution Between *sn*-1(3) and *sn*-2 Positions and Fatty Acid Composition in Basil (*Ocimum basilicum*) and in Coriander (*Coriandrum sativum*) Seed Oils

	Basil				Coriander			
Fatty acid	*sn*-1(3)[a]	*sn*-2[a]	Calculated[b]	Experimental[c]	*sn*-1(3)[a]	*sn*-2[a]	Calculated[b]	Experimental[c]
Palmitic	10.5 ± 0.2	2.6 ± 0.1	7.9	8.1	5.4 ± 0.1	—[d]	3.6	4.3
Stearic	5.7 ± 0.1	—	3.8	2.8	2.5 ± 0.2	—	1.7	1.0
Petroselinic	—	—	—	—	80.1 ± 0.4	40.9 ± 0.2	67.0	69.7
Oleic	8.6 ± 0.3	13.2 ± 0.4	10.1	13.0	5.5 ± 0.3	28.3 ± 0.4	13.1	11.1
cis-Vaccenic	1.0 ± 0.2	2.4 ± 0.2	1.5	0.8	1.6 ± 0.1	—	1.0	0.7
Linoleic	18.4 ± 0.3	30.6 ± 0.3	22.5	22.8	4.9 ± 0.1	30.8 ± 0.4	13.5	13.2
Linolenic	55.8 ± 0.6	51.3 ± 0.5	54.3	52.7	—	—	—	—

[a]Values are means of three replicate analyses ± standard deviations.
[b]Calculated from values for *sn*-1(3) and *sn*-2: [2 × *sn*-1(3) + *sn*-2]/3.
[c]Experimental values obtained by gas chromatographic analysis of fatty acid methyl (basil) and isopropyl (coriander) esters (means of duplicate analyses).
[d]Concentrations lower than 0.1% are not reported.

of petroselinic, oleic, and *cis*-vaccenic acids. The existing methods for regiospecific analysis of triglycerides also have limitations with respect to accuracy, owing to additional steps in the protocols (TLC, back-calculations, etc.) (27–32). Thus, this method constitutes an improvement over the existing methods for the analysis of these acids in fats and oils: it provides a better separation of the isomers by GC and also provides information on triacylglycerol structure.

ACKNOWLEDGMENTS

We acknowledge financial support of the Natural Sciences and Engineering Research Council of Canada and of Conseil de Recherche en Pêche et en Agroalimentaire du Québec (CORPAQ).

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[Received April 9, 2001, and in revised form November 26, 2001; revision accepted November 30, 2001]