High-Resolution Triacylglycerol Mixture Analysis using High-Temperature Gas Chromatography/Mass Spectrometry with a Polarizable Stationary Phase, Negative Ion Chemical Ionization, and Mass-Resolved Chromatography

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High-temperature gas chromatography/mass spectrometry (HT-GC/MS) has been employed to study the behavior of mixtures of triacylglycerol molecular species on a polarizable stationary phase (immobilized 65% phenyl methyl silicone). The use of negative ion chemical ionization (NICI) at an ion source block temperature of 300 °C overcomes problems with interpretation of electron ionization (EI) mass spectra produced during the HT-GC/MS. The NICI spectra of triacylglycerols produced under these conditions contain abundant $[RCO_2]^-$, $[RCO_2 - 18]^-$, and $[RCO_2 - 19]^-$ ions, believed to be produced by nucleophilic gas-phase ammonolysis, that are used to identify the individual fatty acid moieties associated with peaks in triacylglycerol total ion chromatograms. The polarizable stationary phase produces significantly enhanced resolution of triacylglycerol molecular species compared to high-temperature stable apolar stationary phases, such as immobilized dimethyl polysiloxanes. The resolution of complex natural mixtures of triacylglycerols can be further improved by use of the Biller–Biemann enhancement technique to produce mass-resolved chromatograms. Investigation of the mass-resolved chromatograms provides important information with regard to the factors that affect elution orders of individual triacylglycerol molecular species. The analysis of mixtures of authentic triacylglycerols by HT-GC/MS via NICI provides data that relate to the analytical limits of the technique for the analysis of triacylglycerols that bear both saturated and polyunsaturated fatty acyl moieties. *(J Am Soc Mass Spectrom 1996, 7, 350–361)*

Natural triacylglycerol mixtures are potentially extremely complex because the number of possible molecular species is equal to the number of fatty acids cubed. Although the complexity is somewhat reduced due to biosynthetic considerations, most triacylglycerol analyses still only provide partial compositional information. The most common approach to the analysis of triacylglycerols is to release, either chemically or enzymatically, the free fatty acids and perform gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS) analysis after appropriate derivatization, for example, methylation [1]. When this latter technique is employed, all information that relates to which of the fatty acids comprise the individual triacylglycerol molecular species is lost. The obvious solution to this problem is to analyze the intact triacylglycerols. Most of the early analyses of intact triacylglycerols by mass spectrometry used the direct insertion probe technique for sample introduction [2, 3]. This approach can provide a certain amount of information with regard to the carbon number distribution and the nature of the fatty acyl moieties present in the triacylglycerol mixtures. However, only limited information is obtained with regard to the distribution of the various fatty acyl moieties amongst the individual triacylglycerol molecular species.

The most significant advances in the analysis of triacylglycerol mixtures have involved the combination of separatory and mass spectrometric techniques, with a view to performance of on-line separations and characterization of triacylglycerol molecular species ([4] and references therein). Notable successes in this area

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include the use of high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). Kuksis and co-workers [5, 6] used a direct liquid inlet to generate solvent-assisted chemical ionization (CI) spectra. High molecular weight adduct ions, for example, $[M + H]^+$, and low molecular weight fragment ions, for example, $[RCO]^+$, were of value to study the composition of individual molecular species. A moving belt interface also has been used for the reversed-phase HPLC/mass spectrometry analysis of triacylglycerols, but problems were encountered in achieving and maintaining chromatographic resolution in on-line analyses [7]. In capillary SFC separation of triacylglycerols is in accordance with carbon number [8]. SFC/mass spectrometry with a dimethyl polysiloxane-coated capillary afforded resolution of triacylglycerols that bear different degrees of unsaturation by means of selected ion monitoring (SIM) of molecular ions obtained by electron ionization (EI). Discrimination between fatty acids at the *sn*-2 position and *sn*-1,3 was established by monitoring $[M - RCO_2CH_2]^+$ ions and by comparisons with reference compounds [9].

In tandem mass spectrometry MS1 effectively replaces the chromatographic step in combined separation–mass spectrometry analyses [10]. This approach has been adopted in tandem mass spectrometry studies of triacylglycerol mixtures via either electrospray ionization [11] or ammonia CI [12]. Henion and coworkers [11] provided one of the first examples of the use of electrospray ionization in conjunction with apolar compounds by using nonpolar carrier solvents. Cationated molecular adduct ions that depended upon the nature of added salts or acid were observed. The cationated species were selected readily by MS1 and yielded informative product ion spectra after low energy collisions. The carbon number and degree of unsaturation of the individual fatty acyl moieties were determined readily, although the location of the double bonds was obscured due to their migration during the collision-induced dissociation (CID) process. A further tandem mass spectrometry approach used direct probe introduction of samples and negative ion chemical ionization (NICI) at a low ion source temperature to generate $[M - H]^-$ ions that were selected by MS1 and subjected to CID. The product ions provided information with regard to the carbon number and degree of unsaturation of the fatty acyl moieties present ([12] and references therein). The investigation revealed 29 deprotonated triacylglycerol molecular species in human milk.

Purely in terms of the numbers of theoretical plates generated by the analytical system, GC offers perhaps considerable potential for the study of triacylglycerol mixtures. Although high-temperature gas chromatography (HT-GC) is used routinely now to profile triacylglycerol mixtures, combined GC/MS has been used surprisingly little to confirm peak identities and to study the factors that influence the elution orders of triacylglycerol molecular species. A notable advance in the area of HT-GC analysis of triacylglycerols was the development of high-temperature stable, medium polarity immobilized stationary phases. One such stationary phase is an immobilized OV-22 (65% phenyl methyl polysiloxane) polymer, which becomes more polar with increasing temperature and is stable up to 360 °C [13]. This stationary phase can separate molecular species that exhibit only small differences in polarity, for example, triacylglycerols that possess different numbers of double bonds. Although the effectiveness of this stationary phase for the HT-GC analysis of triacylglycerols has been demonstrated by several groups [13, 14], little work has been performed by using high-temperature gas chromatography/mass spectrometry (HT-GC/MS). Kuksis and co-workers [15] used this type of phase in the GC and GC/MS analysis of a volatile distillate of butter oil that resembles the lower half of the molecular mass distribution of bovine milk fat. One problem that exists in the analysis of higher molecular weight triacylglycerols derives from the high level of chemical background that results from column bleed into the mass spectrometer during HT-GC/MS analyses that employ EI, which can prevent satisfactory interpretation of spectra [16]. A partial solution to the problem is to carry out GC/MS analyses in the SIM mode [17], in which case the retention times of peaks in appropriate mass chromatograms are used to determine the nature of the various fatty acyl moieties in the individual molecular species separated by the polarizable column. Ions employed in this approach include $[RCO]^+$ and $[M\text{-}OCOR]^+$. It is well recognized [2, 18] that at low ion source temperature (~ 200 °C) triacylglycerols that contain unsaturated fatty acyl moieties yield more intense $[RCO\text{-}1]^+$ and $[RCO\text{-}2]^+$ ions rather than RCO^+. However, the use of these ions for the identification of unsaturated fatty acyl residues in HT-GC/MS analyses that employ EI is problematical [16], presumably due to thermal rearrangements in the hot ion source; a high ion source temperature (~ 300 °C) is essential in HT-GC/MS analyses to ensure chromatographic resolution is maintained and to prevent condensation as relatively involatile high molecular weight triacylglycerols elute from the GC.

An alternative approach that has been developed for the HT-GC/MS analysis of intact acyl lipids [20–26], which include triacylglycerols [19], involves the use of NICI. Ammonia NICI is especially compatible with HT-GC/MS because useful spectra are produced from a wide range of acyl lipids at an ion source block temperature of ~ 300 °C. In the case of triacylglycerols, very simple spectra are produced that are dominated by $[RCO_2]^-$, $[RCO_2 - H_2O]^-$, and $[RCO_2 - H_2O - H]^-$ ions [19]. Although no molecular or pseudomolecular ions are present, the carbon numbers of the triacylglycerols are assigned readily by comparison of retention data for compounds of known carbon number. The aforementioned anions are presumed to

arise via a nucleophilic gas-phase ammonolysis reaction by analogy with the behavior of the steryl fatty acyl esters under analogous source conditions [20, 25].

Presented herein are the results of analyses of triacylglycerols by HT-GC/MS that employs capillary columns coated with an immobilized polarizable stationary phase. Substantial improvements in chromatographic resolution are achieved compared to results obtained when dimethyl polysiloxane-coated capillaries are used. Still further improvements in resolution of triacylglycerol species are achieved by use of mass-resolved chromatography. The advantages of the use of NICI rather than EI are discussed, and the problems associated with the recovery of triacylglycerols that bear saturated and polyunsaturated fatty acyl moieties during HT-GC/MS analyses are assessed critically.

Experimental

Samples

All authentic compounds, which include trilaurin, trimyristin, tripalmitin, tristearin, triolein, trilinolein, trilinolenin, and triarachidin, were supplied by the Sigma Chemical Co. (St. Louis, MO). The authentic compounds were dissolved in hexane for HT-GC/MS analysis to give a concentration of 50–100 ng per component injected on-column. A sample of fresh butter fat triacylglycerol was obtained by dissolution of fresh butter in hexane.

High-Temperature Gas Chromatography / Mass Spectrometry

Two GC/MS systems were employed in this work:

1. A Pye Unicam 204 gas chromatograph linked to a VG 7070H double-focusing magnetic sector mass spectrometer (VG Analytical Ltd., Manchester, UK) via an interface oven that had been modified for high-temperature operation (300–350 °C) [20]. The analytical column was a Quadrex aluminum clad 25-m × 0.25-mm-i.d. capillary coated with 65HT Supercaps stationary phase (65% phenyl methyl polysiloxane, 0.1-μm film thickness; supplied by Alltech, Carnforth, UK). The column was connected directly into the ion source after removal of the terminal 5 cm of the aluminum cladding by dissolution with concentrated aqueous sodium hydroxide [27]. On-column injection was performed by using an SGE (Australia) OCI 3 injector. Previous work [19, 20] had shown that optimum ammonia NICI performance is achieved for acyl lipids that employ an "open" (EI slit) source. There was no direct means to determine the ion source pressure on the VG 7070H mass spectrometer employed for the NICI studies. Hence, the optimum reagent gas pressure was established by raising the GC oven temperature to 350 °C and the source temperature to 300 °C, and then adjusting the gas pressure and source tuning to achieve maximum sensitivity for the column bleed ions (this corresponded to an ion source manifold pressure of $\sim 10^{-4}$ torr). Under these conditions the major reagent gas ion is $[NH_2]^-$, which acts as a strong nucleophile toward acyl lipids such as triacylglycerols [19, 20, 24].
2. A Carlo Erba Mega GC linked to a Finnigan 4500 single-stage quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) via an interface line that had been modified similarly for high-temperature operation. The analytical column used on this instrument was a polyimide-clad 25-m × 0.33-mm-i.d. capillary coated with R330 stationary phase (65% phenyl methyl polysiloxane, 0.1-μm film thickness; supplied by Thames Chromatography, Maidenhead, UK). All analyses on this instrument were performed under EI conditions (70 eV) at an ion source block temperature of 300 °C. The transfer-line temperature was maintained at 360 °C throughout the analyses. Scan control, data acquisition, and processing were performed by a Finnigan INCOS data system.

All analyses were temperature-programmed following on-column injection at 50 °C. The full details of the temperature programming conditions employed are given in the caption to the appropriate figures.

Mass-Resolved Chromatography

The mass-resolved chromatographic enhancements of the HT-GC/MS data were performed by using the proprietary software installed on the Finnigan INCOS data system, which is based on a Biller–Biemann resolution enhancement algorithm [28].

Results and Discussion

Earlier work using HT-GC [29] either alone or in combination with mass spectrometry [19] has shown that essentially only carbon number resolution is obtainable in the analysis of triacylglycerols on apolar stationary phases, for example, dimethyl polysiloxanes. There is little scope to improve GC resolution simply by using longer columns due to the high retention of triaclyglycerols on phases of this type. Although such columns are used widely to profile the carbon number of triacylglycerol mixtures, the poor chromatographic resolution limits their usefulness for direct compositional analysis of individual triacylglycerol molecular species. Whereas we have shown previously [19] that HT-GC/MS with NICI aids determination of the nature of the fatty acyl moieties associated with the envelops of peaks at the different carbon numbers in total ion chromatograms, the problem of co-elution of different triacylglycerol molecular species on apolar phases still precludes detailed analysis. High-temperature stable polarizable stationary phases have been shown to significantly improve the resolution of triacylglycerol

molecular species in stand-alone GC work [13, 14]. In this work we aimed to explore the use of such a phase in combined HT-GC/MS analyses, with particular consideration to the factors that affect elution of the individual triacylglycerols and limitations that might exist that concern the recovery of triacylglycerols of increasing carbon number and degree of unsaturation.

The total ion current (TIC) derived from the analysis of the triacylglycerols of fresh butter obtained by using a polarizable stationary phase in conjunction with HT-GC/MS that employs NICI is shown in Figure 1a. The chromatographic resolution is somewhat reduced compared to that obtained by GC alone [30], presumably due to the different GC conditions (e.g., the use of helium rather than hydrogen carrier gas) and the discontinuous nature of mass spectral data. The latter factor would be less problematical on newer instruments, which are able to achieve faster scan rates

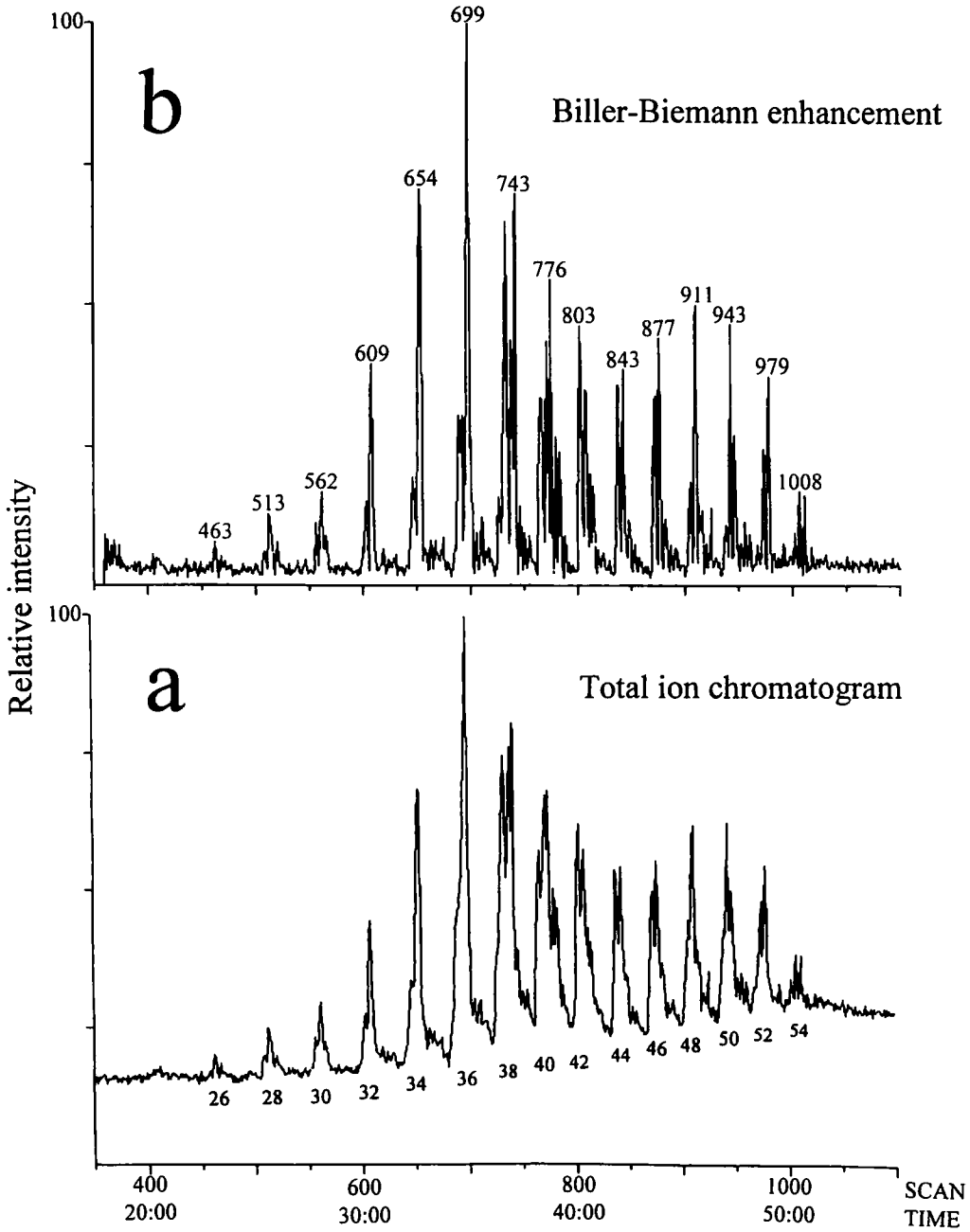

Figure 1. Total ion current (TIC) chromatogram (**a**) and Biller–Biemann mass resolved chromatogram (**b**) obtained for the high-temperature GC/MS analysis of butter triacylglycerols by using a polarizable stationary phase. The numbers under the peaks refer to the total number of acyl carbon atoms in each cluster. The analysis employed on-column injection into a 25-m × 0.25-mm-i.d. aluminum-clad capillary column coated with methyl 65% phenyl polysiloxane (0.1-μm film thickness). The GC oven temperature was held for 2 min at 50 °C before programming to 350 °C at 10 °C min^{-1}.

(seconds per decade) than those attainable on the instrument used in the analysis shown in Figure 1. The region of the TIC from scan 800 to 1050 is shown in Figure 2, together with the m/z 255, 283, and 281 mass chromatograms, to show the distribution of the palmitate ($C_{16:0}$), stearate ($C_{18:0}$), and $C_{18:1}$ moieties, respectively, in the later eluting triacylglycerol components. The clusters of peaks that appear in the mass chromatograms at each carbon number are due to resolution of individual molecular species in accordance with the degree of unsaturation in their component fatty acyl moieties.

The mass spectra for the peaks in the TIC that maximize at scans 975 and 979 are shown in Figure 3. Peaks in this region correspond to triacylglycerols that bear 52 acyl carbon atoms; hence, due to the established fatty acid composition of butter, these components will comprise combinations of one C_{16} fatty acyl moiety and two C_{18} fatty acyl moieties, where the C_{18} fatty acyl moieties are either fully saturated or monounsaturated. With this in mind, interpretation of the nature of the peaks in the TIC and mass chromatograms based on the ammonia NICI spectra is straightforward (see Table 1). The major $[RCO_2]^-$ ions in the spectrum shown in Figure 3a at m/z 255, 281, and 283 derive from $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$ fatty acyl moieties, respectively. The presence of these fatty acyl components is corroborated by the presence of the appropriate $[RCO_2 - H_2O]^-$ and $[RCO_2 - H_2O - H]^-$ ions, that is, m/z 237 and 236 for $C_{16:0}$, m/z 263 and 262 for $C_{18:1}$, and m/z 265 and 264 for $C_{18:0}$. The mass spectrum of the later eluting peak of the C_{52} cluster, which maximizes at scan 979, is shown in Figure 3b. As expected, there is close similarity between this spectrum and that of the earlier eluting peak in this cluster, which confirms that this too derives from a triacylglycerol that bears 52 acyl carbon atoms. A notable difference between the spectra of the two peaks is the high abundance of the $[RCO_2]^-$ ion of m/z 281 and the negligible abundance of m/z 283 in the spectrum of the later eluting peak. Hence, this peak corresponds to a triacylglycerol that bears one $C_{16:0}$ and two $C_{18:1}$ fatty acyl moieties. These observations are entirely consistent with the predicted elution order of these two components on the polarizable stationary phase employed; that is, the triacylglycerol that bears one degree of unsaturation elutes before the triacylglycerol that bears two degrees of unsaturation (see Figure 2). The use of similar arguments enables considerable progress to be made in identification of the other fully and partially resolved peaks in the TIC. The identities of the C_{54} triacylglycerols based on their NICI spectra and mass chromatograms are shown in Figure 2 by way of a further example.

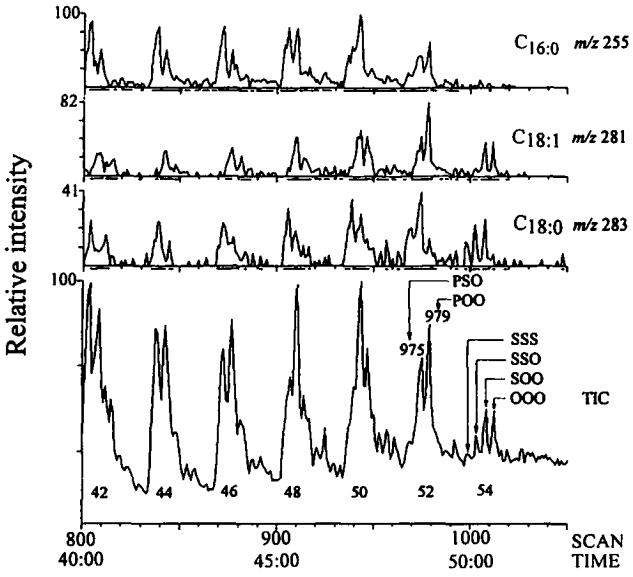

Figure 2. Expansion of the 800–1050 scan number region of the TIC shown in Figure 1. The mass chromatograms correspond to the mass-to-charge ratio values for the $[RCO_2]^-$ ions produced in ammonia NICI for $C_{16:0}$ (palmitate = 16:0; m/z 255), $C_{18:1}$ (octadecenoate = 18:1; m/z 281), and $C_{18:0}$ (stearate = 18:0; m/z 283). All other experimental details are identical to those given in the caption to Figure 1.

Mass-Resolved Chromatography: Biller–Biemann Resolution Enhancement

As previously noted, the chromatographic resolution obtained in HT-GC/MS analyses is somewhat inferior

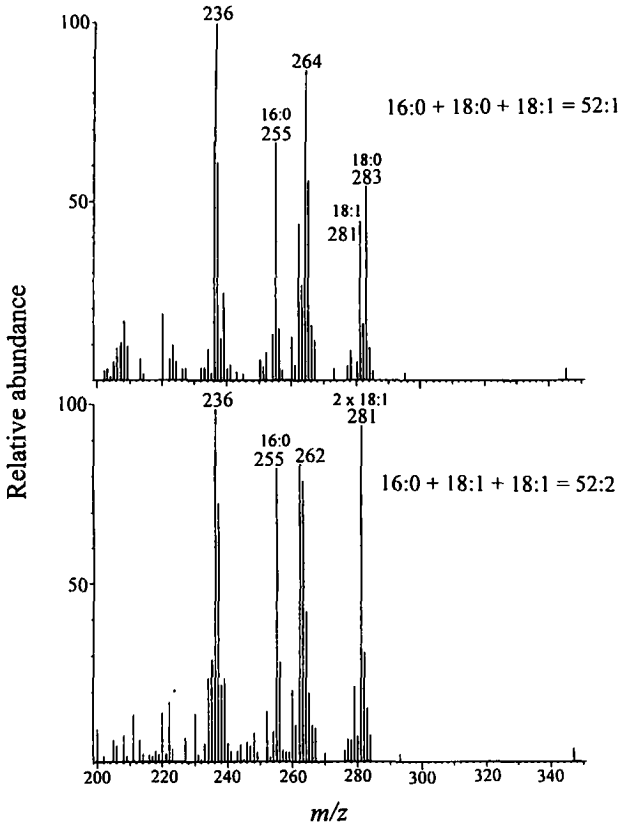

Figure 3. Ammonia NICI mass spectra for the triacylglycerol peaks maximizing at scan numbers 975 (a) and 979 (b) in Figures 1 and 2. See text for further descriptions of the interpretation of these spectra.

Table 1. Diagnostic fragment ions observed in the ammonia NICI mass spectra of triacylglycerol containing acyl moieties of varying carbon numbers and degree of unsaturation

Fatty acyl moiety	Carbon number	*m/z*		
		$[RCO_2]^-$	$[RCO_2\text{-}18]^-$	$[RCO_2\text{-}19]^-$
		Saturated		
Butyric	C_4	87	69	67
Caproic	C_6	115	97	96
Caprylic	C_8	143	125	126
Capric	C_{10}	171	153	152
Lauric	C_{12}	199	181	180
Myristic	C_{14}	227	209	208
Palmitic	C_{16}	255	237	236
Stearic	C_{18}	283	265	264
		Unsaturated		
Oleic	$C_{18:1}$	281	263	262
Linoleic	$C_{18:2}$	279	261	260
Linolenic	$C_{18:3}$	277	259	258

to that obtained when the same analyses are performed by GC with flame ionization detection. The reduced resolution complicates post-run processing of the GC/MS data due to the lack of discrete peaks in some parts of the total ion chromatogram. Thus, considerable effort is required to resolve co-eluting or poorly resolved components by the normal procedures, which involve examination of summed mass spectra obtained following judicious background subtractions and generation of ion chromatograms. A con-

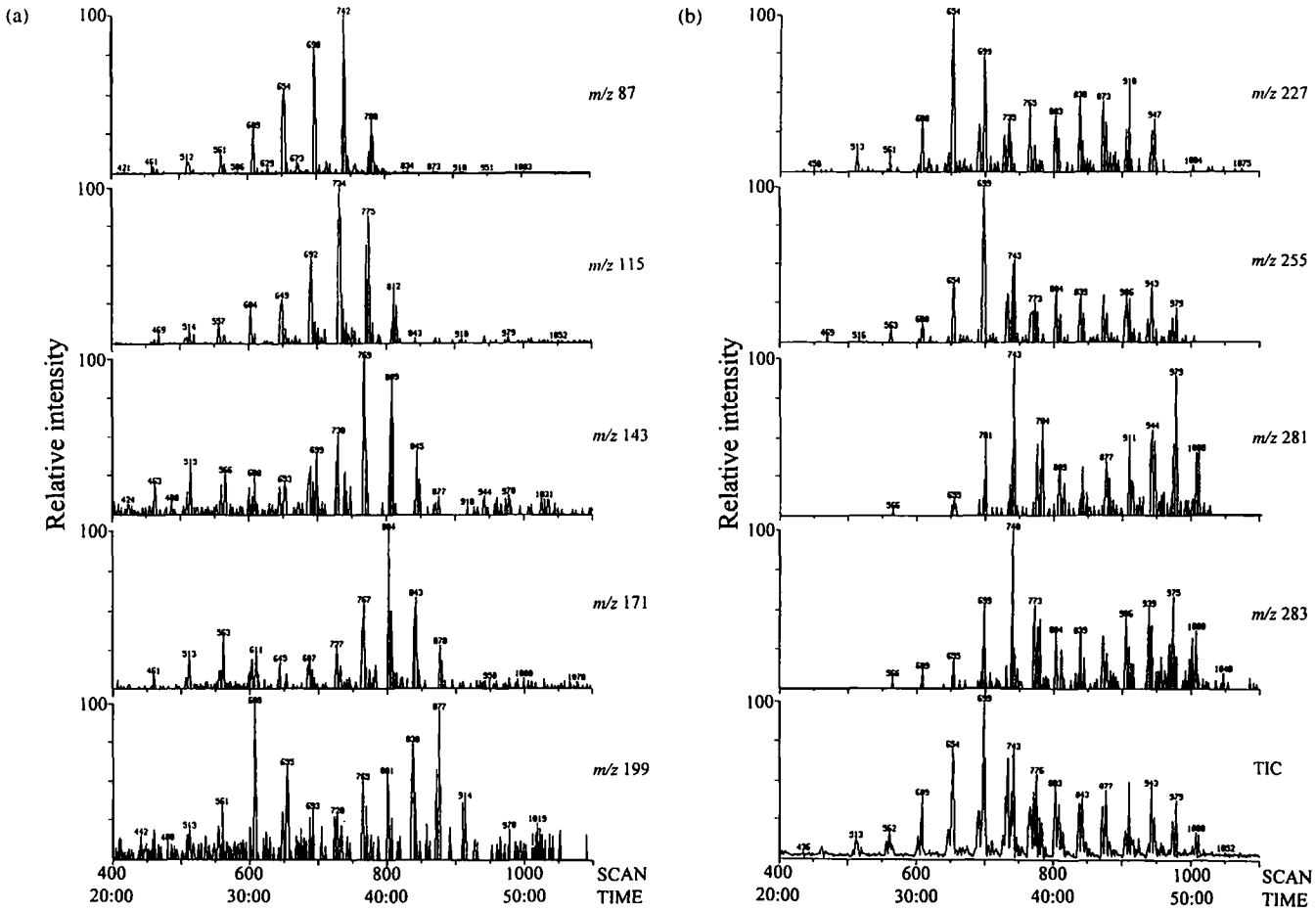

Figure 4. Ion chromatograms for the $[RCO_2]^-$ ions of the major even carbon number fatty acyl moieties present in butter fat.

venient approach adopted here was to employ the Biller–Biemann enhancement technique to produce mass-resolved total ion chromatograms [28]. The mass-resolved TIC obtained from the GC/MS analysis of the triacylglycerols (conventional TIC shown in Figure 1a) is presented in Figure 1b. The Biller–Biemann enhancement algorithm examines each mass that appears in successive scans in the TIC to detect masses that are rising and falling in intensity. Such masses are deemed to be peaks, whereas the remainder, which appear either randomly (noise) or constantly (column bleed), are not included in the final output (e.g., Figure 1b).

Hence, the Biller–Biemann routine serves to enhance the resolution and produce background-subtracted chromatograms automatically. The resulting chromatogram for the butter triacylglycerols is shown in Figure 1b and exhibits a degree of resolution that is comparable to that achievable in GC with FID under optimized chromatographic conditions. This effective enhancement in resolution greatly eases post-run processing. Single or summed mass spectra can be obtained directly from the mass-resolved GC/MS data without need for further background subtraction. Ion chromatograms can be generated for the various $[RCO_2]^-$ or $[RCO_2 - H_2O]^-$ ions to determine the distribution of the various fatty acyl moieties amongst the different triacylglycerol molecular species. Such ion chromatograms serve to emphasize the potential usefulness of combining the analysis of triacylglycerols on the polarizable stationary phase with NICI and resolution enhancement of the GC/MS data. The ion chromatograms shown in Figure 4 represent a significant improvement in compositional analysis compared to that seen when a capillary column coated with an apolar stationary phase is used (cf. ion chromatograms presented in Figure 5 of ref 19). Substantial evidence now can be obtained on the distribution of specific acyl moieties between individual peaks resolved at each carbon number due to differences in the polarity of the resolved components.

As was found previously the spectra recorded for triacylglycerols by using NICI are very easy to interpret on the basis of the masses of $[RCO_2]^-$ ions and corroborating $[RCO_2\text{-}18]^-$ and $[RCO_2\text{-}19]^-$ ions. An example of the interpretation of the mass spectra of the individual peaks resolved for triacylglycerols that bear 42 acyl carbon atoms is shown in Figure 5. The identities of the fatty acyl moieties associated with the various triacylglycerols present in the individual peaks in the mass-resolved TIC are established on the basis of numerical linear combinations of the carbon numbers of all fatty acyl moieties determined through inspection of their individual mass spectra. Similar analyses have been performed on the mass spectra recorded for all the major peaks in the mass-resolved TIC and the data are presented in Table 2. It is clear from the example given in Figure 5 and the data presented in Table 2 that, as anticipated, many of the peaks still

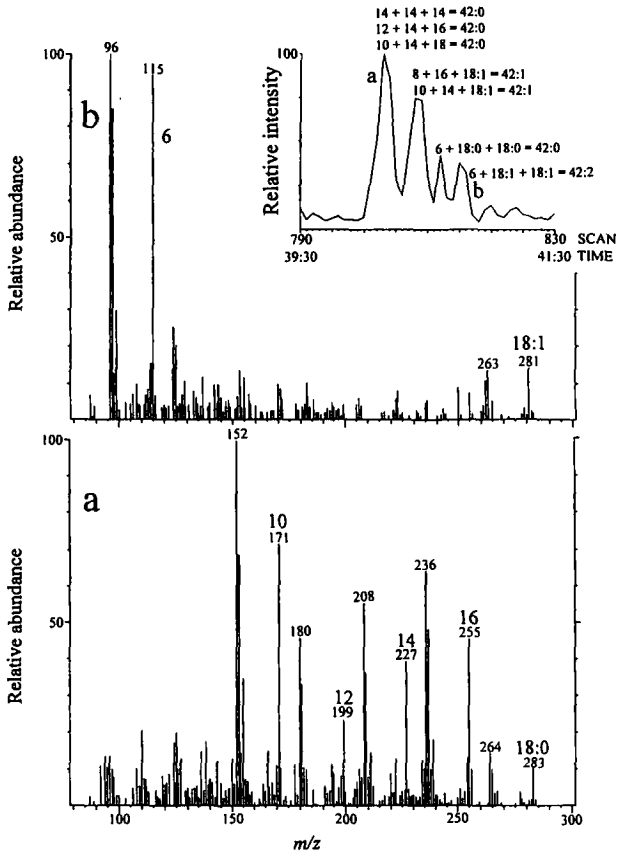

Figure 5. Partial NICI mass spectra and partial mass-resolved ion chromatogram (inset) of the four peaks resolved in the 42 acyl carbon region of butter fat. The letters a and b indicate the peaks in the chromatogram from which the mass spectra were obtained. The numbers on the inset chromatogram and mass spectrum indicate the linear combinations of fatty acyl moieties likely to be present. No positional order of attachment of the fatty acyl moieties to the glycerol backbones is to be inferred.

contain co-eluting components. However, Figure 5 illustrates how considerable progress can be made to identify the combinations of fatty acyl moieties that comprise the co-eluting components, on a purely numerical basis. Comparison of the relative proportions of the individual peaks in the TIC and GC profiles obtained by flame ionization detection suggests slightly enhanced responses for the shorter-chain acyl moieties. This effect has been observed previously in NICI studies of cholesteryl esters that bear short- and long-chain fatty acyl moieties and is presumed to relate to steric factors that influence the nucleophilic attack by $[NH_2]^-$ ions at the ester linkages during the gas-phase ammonolysis reaction [20]. In view of this and additional distortions in the relative proportions, especially of minor constituents during the Biller–Biemann processing, the use of appropriate internal standards is recommended for quantitative analyses.

Closer examination of the identified components serves to highlight those factors that are affect the elution orders due to GC analysis on the polarizable stationary phase. The data presented in Figures 3 and 5

and Table 2 confirm that a combination of the degree of unsaturation and chain length of the fatty acyl moieties is dominant in influencing the order of elution of the individual molecular species at a given carbon number. For the higher carbon number triacylglycerols the combined degree of unsaturation of the three triacylglycerol moieties is the dominant factor. For example, Figure 4 shows that the order of elution for the 52 and 54 carbon number triacylglycerols is based purely on the number of double bonds present

Table 2. Fatty acyl moieties present in the triacylglycerol components resolved in HT-GC/MS analyses using the polarizable stationary phase in conjunction with NICI

Triacylglycerol[a] carbon number	Scan number[b]		Fatty acyl moieties present[c]
	Peak maximum	Range summed	
26	463	462–464	$C_4, C_8, C_6, C_8, C_{10}$
28	509	508–510	C_6, C_8, C_{10}, C_{12}
	514	512–516	$C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}$
	520	519–522	C_4, C_6, C_{10}, C_{14}
30	556	555–558	C_6, C_{10}, C_{12}
	561	560–563	$C_4, C_8, C_{10}, C_{12}, C_{14}, C_{16}$
	566	565–567	$C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{18:1}$
32	603	602–605	$C_6, C_{10}, C_{12}, C_{14}, C_{16}$
	608	607–610	$C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}$
34	647	645–649	$C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}$
	653	651–655	$C_4, C_{12}, C_{14}, C_{16}, C_{18}$
36	690	689–692	$C_6, C_8, C_{10}, C_{14}, C_{16}, C_{18:1}$
	698	696–701	$C_4, C_{14}, C_{16}, C_{18}, C_{18:1}$
38	727	726–729	$C_8, C_{10}, C_{12}, C_{14}$
	732	730–735	C_6, C_8, C_{14}, C_{16}
	738	737–740	C_4, C_6, C_8, C_{18}
	742	741–744	$C_4, C_{16}, C_{18:1}$
40	766	765–768	$C_8, C_{10}, C_{12}, C_{14}, C_{16}$
	771	770–773	C_6, C_8, C_{16}, C_{18}
	775	774–776	$C_6, C_{16}, C_{18:1}$
	780	779–781	$C_4, C_6, C_{18}, C_{18:1}$
	784	783–785	$C_4, C_8, C_{16}, C_{18:1}$
42	803	801–805	$C_{10}, C_{12}, C_{14}, C_{16}, C_{18}$
	808	807–809	$C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18:1}$
	812	812–813	C_6, C_{18}
	815	814–816	$C_6, C_{18:1}$
44	837	835–840	$C_{10}, C_{12}, C_{14}, C_{16}, C_{18}$
	843	842–845	$C_{10}, C_{12}, C_{14}, C_{16}, C_{18:1}$
46	871	870–873	$C_{12}, C_{14}, C_{16}, C_{18}$
	876	875–878	$C_{10}, C_{12}, C_{14}, C_{16}, C_{18:1}$
	882	881–883	$C_{10}, C_{18:1}$
48	905	903–907	C_{14}, C_{16}, C_{18}
	910	909–911	$C_{12}, C_{14}, C_{16}, C_{18}, C_{18:1}$
	915	914–917	$C_{12}, C_{14}, C_{16}, C_{18}, C_{18:1}$
50	938	937–940	C_{16}, C_{18}
	942	941–943	$C_{14}, C_{16}, C_{18:1}$
	947	946–948	$C_{14}, C_{16}, C_{16:1}, C_{18:1}$
52	973	971–975	$C_{16}, C_{18}, C_{18:1}$
	978	977–980	$C_{16}, C_{18:1}$
54	1002	1002–1003	C_{18}
	1006	1005–1007	$C_{18}, C_{18:1}$
	1008	1007–1009	$C_{18}, C_{18:1}$
	1012	1011–1013	$C_{18:1}$

[a] Carbon number assigned by retention time comparison with authentic standards.
[b] Scan numbers refer to Figure 1.
[c] Identification based on NICI generated fragment ions listed in Table 1.

in the resolved triacylglycerols, which in the case of butter reflect the number of oleyl (and other octadecenoic acid positional isomers) moieties associated with the various molecular species at each carbon number. Hence, for triacylglycerol that bears 52 acyl carbon atoms, oleylpalmitylstearyl glycerol (52:1) elutes before dioleylpalmityl glycerol (52:2), whereas for triacylglycerol that bears 54 carbon atoms the order of elution is tristearin (54:0) < oleyldistearyl glycerol (54:1) < dioleylstearyl glycerol (54:2) < trioleyl glycerol (54:3). The greater retention of the compounds that contain increasing unsaturation will be due to enhanced dipole–dipole interactions between the phenyl moieties present in the stationary phase and the double bonds present in the fatty acyl moieties. Although it has yet to be rigorously assessed, it appears that the NICI spectra recorded at a high ion source block temperature do not contain information that relates to the position of substitution, that is, *sn*-1, *sn*-2, or *sn*-3, of the individual fatty acyl moieties onto the glycerol backbone.

At shorter retention times, that is, lower carbon numbers, the presence of shorter-chain fatty acyl moieties, for example, *n*-butyrate, *n*-hexanoate, and *n*-octanoate, appears to become highly influential in establishment of elution orders. Returning to Figure 5 it can be seen that distearylhexyl glycerol (42:0) elutes after either octylpalmityloleyl glycerol and decylpalmityloleyl glycerol (both 42:1), whereas the 42:0 triacylglycerols (Figure 5) with the longer acyl chains eluted prior to the 42:1 triacylglycerols. It is known that the shorter-chain fatty acyl moieties occur predominantly at the *sn*-3 position in the triacylglycerols of butter [31]. Hence, it can be reasoned that a stronger dipole–dipole interaction operates between the relatively unhindered *sn*-3 ester moiety that bears a short-chain fatty acyl group, for example, C_6, and the GC stationary phase than occurs between the stationary phase and the isolated double bond (Δ^9) in an oleyl moiety. It can be seen from Table 2 that in the case of still lower carbon number triacylglycerols, for example, C_{40}, C_{38}, C_{36}, and so forth, those compounds that bear the shorter-chain acyl moieties, specifically at *sn*-3, are relatively more retained than compounds of the same carbon number that contain a combination of rather longer-chain fatty acid moieties. The presence of an *n*-butyrate moiety (also known to be present almost exclusively at *sn*-3 in the triacylglycerols of butter [31]) in particular can be seen to exert strong influence on elution order, due to analogous factors to those set out previously, namely, the degree of steric hindrance at the *sn*-3 ester group influences the magnitude of dipole–dipole interactions with the GC stationary phase; that is, the presence of a short-chain acyl moiety offers relatively less steric hindrance compared to a triacylglycerol that contains the same number of acyl carbon atoms but a longer-chain fatty acyl moiety at *sn*-3. Hence, the triacylglycerol that contains the shorter-chain fatty acyl moiety at *sn*-3 will be more highly retained on GC due to the relatively stronger dipole–dipole interactions that can occur; that is, reduction of the chain length of the alkyl group of the fatty acyl moiety constitutes a reduction in steric hindrance at the ester moiety.

Recovery of Triacylglycerols during HT-GC / MS

The analysis of triacylglycerols by GC/MS is a particularly demanding application of this technique, and the use of HT-GC and HT-GC/MS has been suggested for a number of other compound classes, for example, wax esters, steryl esters, and so forth. It is important to be aware that the high temperatures required for elution of some intact high molecular weight lipids (generally > 300 °C) can cause considerable losses through thermal decomposition or irreversible adsorption. Most thorough assessments of recoveries from HT-GC analyses have been carried out on triacylglycerols. Loss of material was dependent on the molecular weight and degree of unsaturation of a given compound [32]. Hence, although good recoveries are obtained for analyses of many fats and oils, HT-GC and HT-GC/MS currently appear unsuitable for the analysis of triacylglycerols that contain a high proportion of polyunsaturated fatty acyl moieties amongst their acyl lipids.

An indication of the magnitude of the losses of polyunsaturated triacylglycerols in combined HT-GC/MS analyses can be gleaned from Figure 6, which represents the result of the analysis of an equimolar mixture of tristearin (54:0), triolein (54:3), trilinolein (54:6), and trilinolenin (54:9). The inset partial spectrum was obtained by analysis of the same mixture by

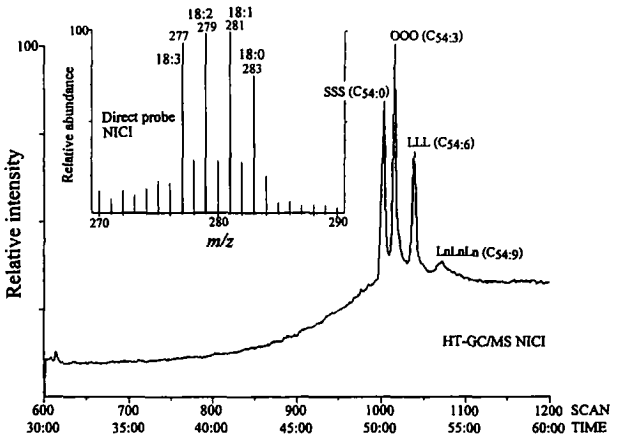

Figure 6. Partial TIC for the GC/MS analysis of a synthetic mixture of triacylglycerols that contain 54 acyl carbon atoms and varying numbers of double bonds (50 ng of each component injected on-column). The inset shows the partial ammonia NICI mass spectrum, $[RCO_2]^-$ ions, of another aliquot of the same mixture introduced via a direct insertion probe (~ 200 ng of each component in the solution added to the probe crucible). Peak identities are SSS ($C_{54:0}$) = tristearin, OOO ($C_{54:3}$) = triolein; LLL ($C_{54:6}$) = trilinolein; LnLnLn ($C_{54:9}$) = trilinolenin.

using a heated direct insertion probe under the same ionization conditions and confirms the approximately equivalent abundance of the four components of the synthetic triacylglycerol mixture. The TIC shown in Figure 6 shows that poor recoveries are seen for the later eluting triacylglycerols, which contain the polyunsaturated fatty acyl moieties. The loss is particularly acute (~ 80%) in the case of trilinolenin (54:9), which is barely detectable above the column bleed. This undesirable behavior would have serious ramifications if this type of analytical approach were to be employed for the study of naturally occurring triacylglycerols of the type that occurs in fish and other marine organisms, owing to the high proportion of polyunsaturated fatty acids in their acyl lipids. These observations are broadly in agreement with those seen in studies that use the same type of HT-GC stationary and a stand-alone GC instrument [32]. Hence, the poor recoveries observed here reflect the undesirable GC behavior of the polyunsaturated species rather than any marked differences in mass spectrometry ionization efficiency.

Losses of saturated triacylglycerols also have been found to occur in HT-GC and are attributed to irreversible saturation (adsorption) of the stationary phase [29]. This is also the case in HT-GC/MS analyses. Analysis of an equimolar mixture of triacylglycerols that contain only saturated fatty acyl moieties showed that losses were most severe in the case of triarachadrin (C_{60}) compared to other saturated triacylglycerols that bear 54, 48, 42, and 36 acyl carbon atoms. Losses of all types of compound are best assessed through the analysis of authentic compounds. Analysis of equimolar mixtures of pure analogs of the compounds to be analyzed allows calibration, or correction, factors to be calculated to account for losses during the GC or GC/MS analyses, if quantitative analyses are demanded. Where a given compound is not available, then only approximate correction factors can be derived from a closely related compound. A useful test for the loss of polyunsaturated components during GC or GC/MS analyses of unknowns is to perform a catalytic hydrogenation and then repeat the analysis. Major losses of polyunsaturated components are revealed by the enhancement of the abundance of existing peaks or by the appearance of new peaks in the chromatogram for the repeat analysis. Alternatively, chemical or enzymatic degradation and subsequent analysis of the simpler lipid moieties released can be used to check for components lost in HT-GC or HT-GC/MS analyses. One approach we have employed recently in the HT-GC/MS analysis of steryl fatty acyl esters that bear polyunsaturated fatty acyl moieties employed a deuterium reduction in the presence of a homogeneous catalyst (Wilkinson's catalyst) to introduce deuterium atoms specifically across double bonds [26]. Selective labeling performed in this way preserves structural information with regard to the carbon number and degree of unsaturation of the fatty acyl moiety. Although originally developed for use in the analysis of diacylglycerols, this technique has yet to be applied to the analysis of intact triacylglycerols [33].

Comparison of EI and NICI Techniques

EI and NICI mass spectra of the individual C_{54} carbon number triacylglycerols were recorded under HT-GC/MS conditions. Figure 7a and b, respectively, shows the partial EI and NICI mass spectra for trilinolein. The EI spectra obtained for tristearin, triolein, and trilinolein are reasonable although the high mass ions (> m/z 200) are present in low abundance. The characteristic $[RCO]^+$ ion that readily can be used to assign the carbon number and degree of unsaturation of the fatty acyl moieties present in individual triacylglycerols is clearly apparent in the spectra of the above-mentioned compounds, at m/z 267, 265, and 263 respectively. These ions are, however, undetectable

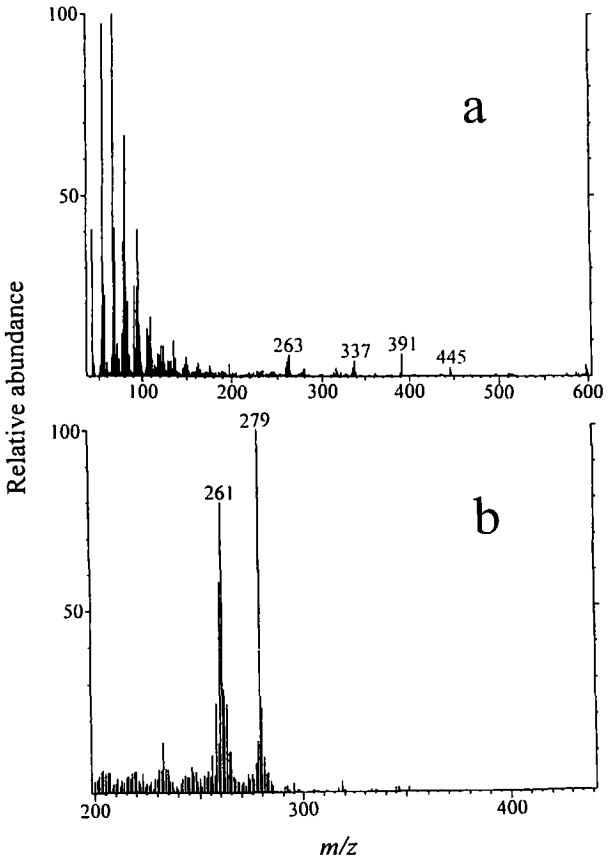

Figure 7. Comparison of partial EI (a) and NICI (b) mass spectra of the trilinolein component from the mixture of triacylglycerols used in the analysis shown in Figure 6. All spectra recorded by HT-GC/MS on the polarizable stationary phase at an ion source temperature of 300 °C.

in the spectrum of the trilinolenin recorded from the same analytical run; this is presumed to be largely the result of the small amount of the latter compound recovered from the HT-GC step due to thermal degradation. It has been reported widely that the $[RCO\text{-}1]^+$ ions rather than $[RCO]^+$ ions are seen in the spectra of triacylglycerols that contain unsaturated fatty acyl moieties [2, 18]. Examination of the spectra obtained here shows that this is not the case in these HT-GC/MS analyses. One explanation would appear to be that there is a thermal contribution to these spectra that results from the unusually high ion source block temperature required for the HT-GC/MS analyses.

Turning to the NICI spectrum from trilinolein shown in Figure 7b, the spectrum is dominated by the familiar $[RCO_2]^-$, $[RCO_2\text{-}18]^-$, and $[RCO_2\text{-}19]^-$ ions listed in Table 1 and used elsewhere in this paper. Clearly these spectra are rather easier to interpret to draw conclusions with regard to the carbon number and degree of unsaturation of the fatty acyl moieties present compared to the EI spectrum of the same compounds (cf. Figure 7a) particularly for triacylglycerols that contain more polyunsaturated fatty acyl moities. Further advantages of the use of NICI become apparent when triacylglycerols that contain short-chain fatty acyl moieties are analyzed. The EI spectrum of triacylglycerol displays complex distributions of fragment ions at low mass that mask the diagnostic $[RCO]^+$ ions, whereas the NICI spectra are relatively "cleaner" in the low mass region, which allows the short-chain fatty acyl moieties to be identified readily (see Figure 5).

Conclusions

HT-GC/MS that employs medium polarity stationary phases in combination with NICI provides a useful approach to the study of complex triacylglycerol mixtures. Although some loss of GC resolution was experienced in combined HT-GC/MS analyses, this was largely recovered by use of a mass-resolved chromatography algorithm [28]. Examination of the mass spectra and mass chromatograms for the individual carbon number peak clusters resolved by using a polarizable stationary phase shows that the elution orders of triacylglycerol molecular species is dependent on both chain length of the triacylglycerol moieties and their degree of unsaturation, that is, short-chain moieties, for example, C_4, C_6, and so forth, at *sn*-3 may be relatively more retained than longer-chain moieties that bear a single degree of unsaturation, for example, $C_{18:1}$. The NICI mass spectra of triacylglycerols that contain unsaturated fatty acyl moieties are easier to interpret in HT-GC/MS analyses than the corresponding EI spectra. However, caution must be exercised in the HT-GC/MS analysis of triacylglycerols that contain polyunsaturated fatty acyl moieties as a result of serious losses, presumably as a result of thermal degradation during the HT-GC analysis. HPLC [34], SFC [9], and tandem mass spectrometry [11, 12] offer the best alternatives for the analysis of thermally unstable triacylglycerol species.

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References

1. Hamilton, R. J.; Hamilton, S. *Lipid Analysis: A Practical Approach;* Oxford University Press: Oxford, 1992.
2. Hites, R. A. *Methods Enzymol.* **1975,** 348.
3. Murata, T. *Anal. Chem.* **1977,** *49,* 2209.
4. Evershed, R. P. In *New Developments in the Analysis of Lipids;* Tyman, J. H. P.; Gordon, M. H., Eds.; Royal Society of Chemistry: London, 1994; pp 123–160.
5. Kuksis, A.; Marai, L.; Myher, J. J.; Pino, D. In *Chromatography of Lipids in Biomedical Research and Clinical Diagnosis;* Kuksis, A., Ed.; Elsevier: Amsterdam, 1987; pp 403–440.
6. Marai, L.; Myher, J. J.; Kuksis, A. *Can. J. Biochem. Cell Biol.* **1983,** *61,* 840.
7. Rawele, N. W.; Willis, R. G.; Baty, J. D. *Analyst* **1990,** *115,* 521–523.
8. Sandra, P.; David, F.; Munari, F.; Mapelli, G.; Trestianu, S. In *Supercritical Fluid Chromatography;* Smith, R. M., Ed.; Royal Society of Chemistry: London, 1988; pp 137–158.
9. Kallio, H.; Laasko, P.; Huopalahti, R.; Linko, R. R.; Oksman, P. *Anal. Chem.* **1989,** *61,* 698–700.
10. Johnson, J. V.; Lee, M. S.; Lee, M. R.; Brotherton, H. O.; Yost, R. A. In *Mass Spectrometry in Biomedical Research;* Gaskell, S. J., Ed.; John Wiley: Chichester, 1986; pp 459–475.
11. Duffin, K. L.; Henion, J. D.; Sheih, J. J. *Anal. Chem.* **1991,** *63,* 1781–1788.
12. Currie, G. J.; Kallio, H. *Lipids* **1993,** *28,* 217.
13. Lipsky, S. R.; Duffy, M. L. *J. High Res. Chromatogr. Chromatogr. Commun.* **1986,** *9,* 725.
14. Kuksis, A.; Myher, J. J.; Sandra, P. *J. Chromatogr.* **1990,** *500,* 427.
15. Myher, J. J.; Kuksis, A.; Marai, L.; Sandra, P. *J. Chromatogr.* **1988,** *452,* 93.
16. Schmid, P. P.; Muller, M. D.; Simon, W. *J. High Res. Chromatogr. Chromatogr, Commun.* **1979,** *2,* 675–676.
17. Ohishima, T.; Yon, H. S.; Koizumi, C. *Lipids* **1989,** *24,* 535.
18. Bhati, A. In *Analysis of Fats and Oils;* Hamilton, R. J., Ed.; Elsevier Applied Science: London, 1986; pp 207–241.
19. Evershed, R. P.; Prescott, M. C.; Goad, L. J. *Rapid Commun. Mass Spectrom.* **1990,** *4,* 345.
20. Evershed, R. P.; Goad, L. J. *Biomed. Environ. Mass Spectrom.* **1987,** *14,* 131.
21. Evershed, R. P.; Prescott, M. C.; Goad, L. J; Rees, H. H. *Biochem. Soc. Trans.* **1987,** *15,* 175.
22. Evershed, R. P.; Male, V. L.; Goad, L. J. *J. Chromatogr.* **1987,** *400,* 187.
23. Evershed, R. P.; Goad, L. J. *Biomed. Environ. Mass Spectrom.* **1988,** *16,* 169.
24. Evershed, R. P.; Prescott, M. C.; Spooner, N.; Goad, L. J. *Steroids* **1989,** *53,* 285.
25. Evershed, R. P.; Goad, L. J. In *Studies in Natural Product Chemistry;* Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, 1991; pp 447–486.

26. Evershed, R. P.; Prescott, M. C.; Goad, L. J. *J. Chromatogr.* **1992,** *590,* 305.
27. Evershed, R. P.; Prescott, M. C. *Biomed. Environ. Mass Spectrom.* **1989,** *18,* 503.
28. Biller, J. E.; Biemann, K. *Analy. Lett.* **1974,** *7,* 515–528.
29. Mares, P. *Prog. Lipid Res.* **1988,** *27,* 107.
30. Termonola, M.; Munari, F.; Sandra, P. *J. High Res. Chromatogr. Chromatogr. Commun.* **1987,** *10,* 263–268.
31. Parodi, P. W. *J. Dairy Res.* **1979,** *46,* 75–81.
32. Gilkison, I. S. *J. High Res. Chromatogr. Chromatogr. Commun.* **1989,** *12,* 481–483.
33. Dickens, B. F.; Ramesha, C. S.; Thompson, G. A. *Anal. Biochem.* **1982,** *27,* 37.
34. Byrdwell, W. C.; Emken, E. A. *Lipids* **1995,** *30,* 173–175.