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Identification of fatty acids in fishes collected from the Ohio River using gas chromatography–mass spectrometry in chemical ionization and electron impact modes

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Abstract

Analyses of fatty acid (FA) composition in freshwater fishes promote understanding of the potential relationship between fish health or human nutrition and specific FAs. Therefore, the chemical identity of FAs in endemic fishes must be established. Paddlefish, sauger, and white bass were collected from the Ohio River. The structural identification of esterified FAs from fish-fillet lipids was conducted using gas chromatography–mass spectrometry (GC–MS). The same 13 FAs, composing more than 90% of the mass of FAs extracted by techniques used in this research, were found in all three species examined. Carbon chain length and degree and position of unsaturation were determined from the characteristic ionization and fragmentation of FA methyl esters (FAMEs) resulting from GC–MS electron impact (EI) and chemical ionization (CI) modes. Assignment of structure to the extracted FAs required complementary interpretation of both EI and CI MS. The EI spectra observed substantiate findings reported in the literature. The novelty of this research is in the thorough interpretation of CI spectra for which less data are available. The observations reported for analyses of fishes will be useful to all researchers studying FAs regardless of sample media.

Keywords: Fatty acids; FAMEs; Gas chromatography–mass spectrometry; Electron impact; Chemical ionization; Fish; Paddlefish; Sauger; White bass; Ohio River

1. Introduction

The fatty acid (FA) composition in fishes results from complex interactions among nutritional, physiological, and environmental effects acting simultaneously on the organism. FAs have three major roles in fishes as fuels, as membrane components, and as precursors of eicosanoids, that are required for cellular regulatory processes [1]. Therefore, identifying the molecular composition of the FA profile is necessary in general for research on the role of FAs in fish health and in human nutrition and vital specifically to examination of the FA composition of freshwater species in the Ohio River mainstem system.

FAs differ in chain length and in the number and position of double bonds. The most common FAs of animal origin are composed of even-numbered chains of 16–22 carbon atoms, with zero to six double bonds in the *cis* configuration [2]. FAs are categorized as saturated FAs (SFAs), having no double bonds; monounsaturated FAs (MUFAs), having one double bond; diunsaturated FAs (DUFAs), having two double bonds; and PUFAs, having more than two double bonds. PUFAs are further classified as n-3 or n-6 FAs in which the first double bond occurs either three or six carbon atoms from the methyl terminus of the FA molecule, respectively.

FAs have considerable potential for use as biomarkers, and the analyses of fish FAs as monitors of habitat conditions could prove to be significant tools for establishing environmental policy. Since fish accumulate pollutants preferentially in fatty tissues, bioaccumulation may be a function of the FA composition.

The most widely used methods to analyze FAs rely on gas chromatography–mass spectrometry (GC–MS) to separate and identify FAs converted to FA methyl esters (FAMEs). GC–MS analyses in the electron impact (EI) mode result in strong fragmentation and rearrangement of FAMEs that often do not yield a molecular ion $[M]^+$ peak representing the molecular weight (MW) of the FAME, particularly as the MW increases [3]. Also, as the carbon chain length increases, the EI fragmentation

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patterns of different FAMEs become more alike. The unambiguous assignment of the positions of double bonds is not solely possible from EI MS of FAMEs because of double-bond migration [4]. Double bond positions in fatty acids can be determined by EI MS on picolinyl or dimethyloxazoline (DMOX) derivatives [5,6], or by derivatization of the double bond [7–9]. Recently, Jalali-Heravi and Vosough [10] used GC tandem mass spectrometry in the EI mode to determine fatty acids in fish oil. Mondello et al. [11] applied 2D space chromatograms derived from GC × GC analysis for identification of specific families of compounds in natural fats and oils. Michaud et al. [12] reported analysis of conjugated linoleic acids as methyl esters using acetonitrile as the reagent gas in chemical ionisation (CI) tandem mass spectrometry.

In this research, GC–MS in the CI mode using methane reagent gas was used in addition to EI to obtain more information about the chemical structures of FAMEs. Combining the information gained from interpretation of CI and EI mass spectra with confirmation using commercially available standards, allowed determination of FA identities.

Previously unknown information regarding the chemical identities of FAs in paddlefish, sauger, and white bass collected from the Ohio River was established using CI and EI MS. The same 13 FAs, composing more than 90% of the mass of FAs extracted by techniques used in this research, were found in all three species examined. Each species differed in the relative amounts of FAs identified. Multivariate comparisons of differences in the FA profile by species, season, and location within the Ohio River mainstem will be published separately. Recently in this laboratory, chemometric analysis [13] was used to discriminate among wild and cultured age-0 largemouth bass, black crappies, and white crappies based on fatty acid composition.

2. Experimental

2.1. Chemicals and reagents

Isooctane, chloroform, methanol, hexane, sodium chloride, and sodium sulfate (ACS reagent grade) were purchased from Fisher Scientific, Atlanta, GA, USA. Boron trifluoride (BF_3) in methanol (12%, w/w) was purchased from Supelco, Bellefonte, PA, USA. Analytical standards of methyl ester (ME) derivatives (99.5% pure) of FAs were purchased from Nu-Chek Prep Inc., Elysian, MN, USA.

2.2. Lipid extraction

Lipids were extracted from fish tissue samples using a chloroform:methanol (2:1, v:v) procedure [13] based on the original methods of Folch et al. [14], as modified by Jahncke et al. [15]. The methods of Jahncke et al. [15] were designed for tissue samples weighing approximately 5 g and required 25 mg of pure lipid in chloroform:methanol, with a split injection ratio of 1:50 for GC analyses. In the present study, modifications were made to accommodate tissue samples weighing approximately 1 g by changing the injector split ratio to 1:10.

Partially frozen tissue samples consisting of the white muscle portion of the fillet were weighed to the nearest 0.0001 g. Tissue samples were transferred to a flat-bottom test tube (100 mL) fitted with a Teflon-lined lid and covered with approximately 30 mL of chloroform:methanol (2:1, v:v). Samples were homogenized 1–2 min with a stainless steel hand-held biohomogenizer (Biospec Products Inc., Bartlesville, OK, USA). An additional 30 mL of solvent was added. Tissue samples were homogenized for another 1–2 min. Any sample remaining on the homogenizer was washed into the test tube with approximately 1 mL of solvent. Test tubes were covered with lids and allowed to stand for 10–15 min. Using a vacuum, each sample was filtered through a Coors No. 60240 funnel (Fisher Scientific) fitted with Whatman No. 1 filter paper (5.5 cm) attached to a 250 mL filter flask. Approximately 10 mL of solvent was used to rinse the test tube and additionally poured through the funnel. The sample filtrate was then transferred to a 100 mL glass-stoppered graduated cylinder. A sodium chloride (NaCl) solution (0.73 g NaCl/100 mL distilled water), corresponding to 20% of the filtrate volume, was added to the cylinder and mixed by inverting the cylinder several times. Stoppered cylinders were refrigerated 12–48 h to promote phase separation. The volume of the lower phase (chloroform) was recorded. When the cylinder reached room temperature, the upper phase (methanol) was removed with a Pasteur pipette. Care was taken not to disturb the lower phase. Approximately 2 g of anhydrous sodium sulfate (Na_2SO_4) was then added to the cylinder and mixed by gently inverting the cylinder. After the mixture was allowed to stand for 20 min, the contents of the cylinder were filtered through Whatman No. 1 filter paper into a 60 mL glass vial fitted with a screw-cap lid.

Aluminum weighing pans (in duplicate) were weighed to the nearest 10th of a milligram. Chloroform extract (5 mL) was transferred to each weighing pan. Pans were placed in a drying oven for 30 min at 105 °C and then transferred to a desiccator for an additional 30 min drying time before weighing. Proper precautions should be followed in a well-ventilated area when evaporating the chloroform extract in weighing pans in a drying oven for 30 min at 105 °C. A vacuum oven or alternative means of evaporating the sample should be utilized. Lipid weight was determined by subtracting empty aluminum pan weights from the weight of aluminum pans containing pure lipids. Duplicate analyses were averaged to yield the total percent lipid of each fillet. The volume of chloroform extract required to achieve a 5 mg sample of pure lipid was used in subsequent derivatization processes.

2.3. Hydrolysis and derivatization

Hydrolysis of lipids to FAs and derivatization to FAMEs were conducted according to modifications by this laboratory and Jahncke et al. [15], based on the original work of Metcalfe et al. [16]. Samples of pure lipid (5 mg) in chloroform extract were transferred to centrifuge tubes with Teflon-lined screw caps. All traces of chloroform were evaporated under a stream of dry nitrogen using an N-EVAP analytical evaporator. Any unused portion of chloroform extract was blanketed with nitrogen and stored at

−80 °C. Sodium hydroxide, NaOH, 0.5 M (1.5 mL) was added to each centrifuge tube; tubes were vortexed for 15–25 s, heated for 5 min in a 100 °C chemical oxygen demand (COD) reactor, and then cooled in a beaker of tap water. A volume of 2 mL of BF_3–methanol was added to each tube. Tubes were vortexed 15–25 s, returned to the 100 °C COD reactor for 20 min, and cooled to 37 °C in a water bath. A volume of 1 mL of isooctane was added to each centrifuge tube. The tubes were vortexed 15–25 s, and 3 mL of saturated NaCl were added. Tubes were repeatedly inverted 180° by hand for 1 min before being centrifuged at approximately 1300 rpm for 2 min to promote phase separation. The upper phase (isooctane) was transferred to a test tube containing approximately a 2 mm layer of anhydrous Na_2SO_4. Tubes were shaken and allowed to stand for 20 min. The isooctane was transferred with a Pasteur pipette to suitable vials for GC analysis. Care was taken to avoid transferring the Na_2SO_4 with the extract.

2.4. GC–MS

EI spectra produced for FAMEs of 14:0, 16:0, 18:0, 18:3n-3, 18:2n-6, 20:4n-6, 16:1n-7, 18:1n-9, and 18:1n-7 were generated in this laboratory. The FA notation follows the naming convention in which the carboxyl carbon is assigned the C-1 position. Other spectra were generated at ADPEN Laboratories Inc., Jacksonville, FL, USA.

In this laboratory, GC–MS (EI) analyses of FAMEs utilized a Hewlett Packard 6890 Series GC System with a 5973 Mass Selective Detector (MSD). An Omegawax 250 fused-silica capillary column (Supelco, 30 m × 0.25 mm I.D., 0.25 μm film thickness) was used to separate FAMEs. Injections of 2 μL were made in a split mode ratio (1:10) using a glass cup liner (Supelco, Bellefonte, PA, USA). Injector temperature was maintained at 250 °C, and the MS source was maintained at 230 °C. Helium carrier gas was used at a flow rate of 1 mL/min. The oven temperature increased at 1 °C/min from 170 °C to 180 °C and at 2 °C/min from 180 °C to 240 °C. The final temperature of 240 °C was maintained for 5 min for a total run time of 45 min. EI spectra were generated using electron-beam energy of 70 eV, and the MS was operated in scan mode only.

At ADPEN Laboratories Inc., EI and CI GC–MS analyses of FAMEs were conducted on an Agilent 5890 Series II Plus GC with a 5972 series MSD. Injections of 2 μL were made in a splitless mode. A Stabilwax column (Restek, 30 m × 0.25 mm I.D., 0.25 μm film thickness) was used to separate FAMEs for MS analyses. Injector temperature was maintained at 250 °C, and detector temperature was maintained at 280 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The initial oven temperature was 110 °C and increased at 2 °C/min until reaching a final temperature of 240 °C, for a total run time of 65 min. EI spectra were generated using electron beam energy of 70 eV, and CI was initiated using methane. The MS was operated in the scan mode only.

3. Results and discussion

GC–MS was used to establish the chemical identities of 13 FAs extracted from paddlefish, sauger, and white bass collected from the Ohio River mainstem system (Fig. 1). Correct assignment of chemical identity is necessary for further research using the data generated. Complementary CI/EI spectra and proposed fragmentation patterns of all FAMEs identified in this research are included as Supplementary Material. Eleven of the 13 EI spectra produced in this research (excepting the FAMEs of 22:5n-6 and 22:5n-3) are available in the Wiley, NIST, and/or Palisade mass spectral databases. However, there are no existing databases of CI mass spectra.

The chemical structures were confirmed by examination of MS fragmentation in CI and EI modes, and by comparison to authentic analytical standards using GC retention time. Spectra derived from fish extracts may include minor matrix interferences when compared to spectra measured for analytical standards.

All FAs identified were saturated, monounsaturated, diunsaturated, or polyunsaturated. All PUFAs had methylene-interrupted double bonds of *cis* geometry and belonged to the

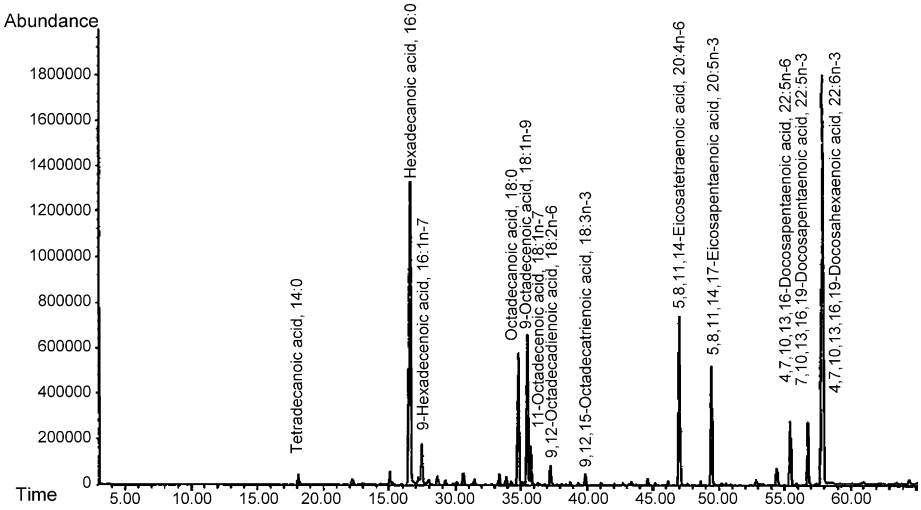

Fig. 1. Total ion gas chromatogram of esterified FAs identified from a sauger fillet.

Table 1
Summary of EI and CI information used to determine MW of FAMEs

FA notation	FA molecular weight (amu)	EI of FAME $[M]^+$	CI of FAME		
			$[M+1]^+$	$[M+29]^+$	$[M+41]^+$
14:0	228	242	243	271	283
16:0	256	270	271	299	311
16:1n-7	254	268	269	297	[b]
18:0	284	298	299	327	[b]
18:1n-9	282	296	297	325	[b]
18:1n-7	282	296	297	325	[b]
18:2n-6	280	294	295	323	[b]
18:3n-3	278	292	293	321	[b]
20:4n-6	304	318	319	347	[b]
20:5n-3	302	ND[a]	317	345	[b]
22:5n-6	330	ND	345	[b]	[b]
22:5n-3	330	ND	345	[b]	[b]
22:6n-3	328	ND	343	371	[b]

[a] ND: not detected.
[b] Scanning scale did not allow for visualization of peak.

n-3 or n-6 family. Therefore, the discussion is restricted to these families of compounds.

3.1. Determination of chain length and number of double bonds

3.1.1. The MW

The single, most important contribution resulting from measurement of the CI spectra was unequivocal establishment of the MW of FAMEs and thereby the MW of the corresponding FAs. The MW of each FAME was identified from the CI protonated molecular ion $[M+1]^+$ (Table 1). The EI molecular ion $[M]^+$ was not detected for highly unsaturated FAMEs (five or six double bonds).

At pressures used for CI, the most abundant ionic species formed from methane was CH_5^+. The CH_5^+ ionic species is a strong Bronsted acid that can readily transfer its proton to another neutral species to form the $[M+1]^+$ ion. Ions at $[M+29]^+$ and $[M+41]^+$ in the CI spectra were formed by adding $C_2H_5^+$ and $C_3H_5^+$, respectively, to the molecule [17] and assisted in confirming the correct assignment of the $[M+1]^+$ ion in CI spectra. Additionally, all CI spectra contained $[M-1]^+$ ions that resulted from a hydride transfer from the original FAME or the loss of H_2 from an $[M+1]^+$ ion [18,19].

Accurate determination of the MW of FAMEs was used to predict the carbon chain length and number of double bonds of FAs. The general molecular formula of a FA is $C_xH_{2x+y}COOH$, where x = the number of non-carboxylic group carbons, and y is equal to 1, −1, −3, −5, −7, −9, or −11, for FAs having 0, 1, 2, 3, 4, 5, or 6 double bonds, respectively, assuming that the FA is unbranched and contains no cyclic or aromatic moieties. Potential molecular formulas of each FA were calculated using the target mass determined by CI MS (Table 1). Assuming that all FAs present have a MW less than that for the saturated FA, 25:0, the elemental composition was calculated for atomic combinations that produced MWs within ±0.5 amu of the target MW, having up to 25 carbon atoms, fifty hydrogen atoms, and a fixed value of two oxygen atoms. The molecular combinations thus calculated were compared to the general molecular formula for an FA as previously defined. For each target MW determined by CI, a single empirical formula emerged that was definitive for the number of carbon atoms and the number of double bonds in the molecule. For example, four possible elemental combinations were calculated for the FA having a target MW of 328: $C_{24}H_8O_2$, $C_{23}H_{20}O_2$, $C_{22}H_{32}O_2$, and $C_{21}H_{44}O_2$. Of the four possibilities, only the elemental composition $C_{22}H_{32}O_2$ is consistent with the general structure of a FA, $C_xH_{2x+y}COOH$ (where $x=21$ and $y=-11$), thereby leading to the conclusion that the FA having MW 328 has 22 carbon atoms and six double bonds.

3.1.2. The most abundant ion (MAI)

When the peak associated with the molecular ion (EI) or protonated molecular ion (CI) was detected, it was not always the MAI (Table 2). In fact, the MAI was the molecular ion only for the $[M+1]^+$ ion in the CI spectra of SFAs and in the 16:1n-7 ME. The EI MAIs of all FAMEs with three or more double bonds, regardless of the position of unsaturation (i.e. n-3 or n-6), were equivalent. Therefore, the MAI was specifically related to the number of double bonds only for FAs having two double bonds or less.

3.1.3. The $[M-31]^+$, $[M-32]^+$, and $[M-33]^+$ ions

Although derived by mechanistically different routes, the $[M-31]^+$ peak was observed in all CI spectra and in 8 of the 13 EI spectra. Only EI spectra for FAs having three or fewer double bonds exhibited the presence of the $[M-31]^+$ peak. Ions at $[M-31]^+$, present in CI spectra (Fig. 2), represented the loss of CH_3OH from the $[M+1]^+$ ion [3,17,19,20] and were reported in CI spectra of saturated FAMEs [18]. Ions at $[M-33]^+$, in CI spectra of unsaturated and monounsaturated FAMEs, illustrated the loss of CH_3OH from the $[M-1]^+$ ion [3].

In EI spectra, ions at $[M-31]^+$ were produced by the loss of CH_3O from $[M]^+$ (Fig. 3). The $[M-31]^+$ ion was produced when a simple homolytic cleavage of the methoxy bond yielded a commonly observed acylium ion [18].

In EI spectra, an $[M-32]^+$ ion in greater abundance than the $[M-31]^+$ ion was observed only in monounsaturated FAMEs, whereas in CI the $[M-31]^+$ ion was in greater abundance than the $[M-32]^+$ ion in monounsaturated FAMEs (Table 2). Therefore, the presence and magnitude of the ratio of $[M-32]^+/[M-31]^+$ were diagnostic for FAMEs having a single double bond.

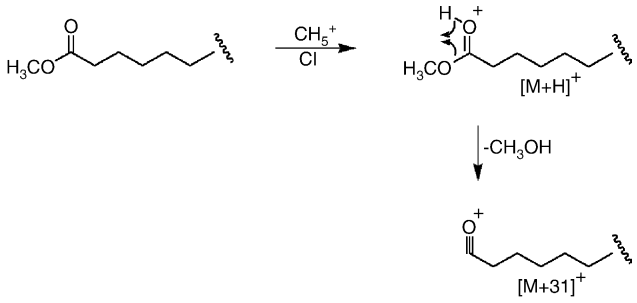

Fig. 2. CI fragmentation scheme resulting in the $[M-31]^+$ ion.

Table 2
Most abundant ions and ratios of abundances for EI and CI spectra

Category	FAME	Chemical ionisation				Electron impact			
		MAI[a] (*m*/*z*)	Ratio			MAI (*m*/*z*)	Ratio		
			$[M+1]^+$/MAI	$[M-31]^+$/$[M+1]^+$	$[M-32]^+$/$[M-31]^+$		$[M]^+$/MAI	$[M-31]^+$/$[M]^+$	$[M-32]^+$/$[M-31]^+$
SFAs	14:0	243	1.00	0.10	0.00[b]	74	0.09	1.17	0.00
	16:0	271	1.00	0.10	0.00	74	0.14	0.70	0.00
	18:0	299	1.00	0.08	0.00	74	0.17	0.45	0.00
MUFAs	16:1n-7	269	1.00	0.58	0.28	55	0.07	3.80	1.11
	18:1n-9	57	0.75	0.42	0.29	55	0.08	4.40	1.30
	18:1n-7	57	0.73	0.45	0.29	55	0.03	6.00	1.47
DUFA	18:2n-6	57	0.19	2.38	0.00	67	0.14	0.95	0.56
n-3 PUFAs	18:3n-3	95	0.46	0.29	0.00	79	0.06	0.63	0.00
	20:5n-3	81	0.23	0.26	0.00	79	0.00	NA[c]	NA
	22:5n-3	95	0.26	0.19	0.00	79	0.00	NA	NA
	22:6n-3	81	0.13	0.44	0.00	79	0.00	NA	NA
n-6 PUFAs	20:4n-6	81	0.73	0.23	0.00	79	0.01	0.00	NA
	22:5n-6	81	0.34	0.32	0.00	79	0.00	NA	NA

[a] Most abundant ion.
[b] Numerator = 0.
[c] Not applicable (denominator = 0).

Principal component analysis (PCA) of the $[M+1]^+$/MAI, $[M-31]^+/[M+1]^+$, and $[M-32]^+/[M-31]^+$ ratios for CI spectra demonstrated clustering of FAMEs into the categories of SFAs, MUFAs, the single DUFA represented, and the PUFAs, but did not distinguish among the PUFAs by degree of saturation, nor was there any indication of the position of unsaturation. Similarly, Mjos [21] demonstrated that PCA applied to data from the 50 to 110 amu mass region in EI MS distinguished between saturated, monoene, diene, and polyene FAMEs having methylene-interrupted double bond systems. Clearly, reactions, occurring as illustrated in Figs. 2 and 3, are affected by the number of double bonds in the molecule, but the effects are indistinguishable when the number of double bonds is equal to or greater than three.

3.1.4. The McLafferty rearrangement

EI ions at 74 and 87 *m*/*z* were attributed to the McLafferty rearrangement [22], a six-membered transition-state process that results in a γ H-transfer and β-cleavage (Fig. 4). The intensity of the McLafferty fragment is controlled by the number of π-bonds in the molecule [3] and is commonly observed in the spectra of esters, ketones, and olefins [18]. A neutral alkene is also

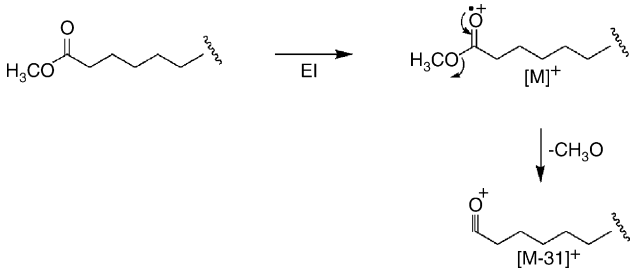

Fig. 3. EI fragmentation scheme resulting in the $[M-31]^+$ ion.

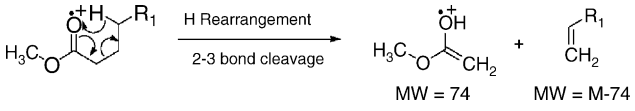

Fig. 4. McLafferty rearrangement resulting in the ion at MW = 74.

formed during the McLafferty rearrangement. Ionized fragments of neutral molecules resulting from the McLafferty rearrangement were observed in the spectra. All CI spectra and selected EI spectra of FAMEs indicated that the ionized fragments of neutral molecules resulting from the McLafferty rearrangement, fragmented further. Double-bond positions likely influenced whether ionized neutral molecules further fragmented.

3.2. Position of unsaturation

While CI spectra were essential for determination of chain length and number of double bonds, EI spectra provided information useful for predicting the position of unsaturation. The prominent EI ion at *m*/*z* = 150 was a diagnostic marker for the methylene-interrupted PUFAs that belonged to the n-6 family. The ion at *m*/*z* = 150 was present in the EI spectra of 18:2n-6, 20:4n-6, and 22:5n-6, while absent in the n-3 family of FAMEs. Double bond position was not identifiable from the limited set of CI spectra examined in this research.

3.3. Conclusion

The scope of the data presented was restricted to the families of compounds represented by13 FAs extracted from freshwater fishes collected from the Ohio River. All FAs identified were saturated, monounsaturated, diunsaturated, or polyunsaturated. All PUFAs had methylene-interrupted double bonds of *cis* geometry

and belonged to the n-3 or n-6 family. CI and EI MS of FAMEs coupled with comparison to the GC retention times and spectra of authentic analytical standards were used to confirm carbon chain length and degree and position of unsaturation. Identification of the MW from CI MS data was key to the determination of chain length and number of double bonds. Examination of the MAI, the $[M-31]^+$, $[M-32]^+$, and $[M-33]^+$ ions, and the McLafferty rearrangement added supporting information to confirmation of the degree of unsaturation. EI MS provided more information related to the position of unsaturation than CI MS. As more research on the CI of FAMEs is conducted, increased understanding of CI fragmentation patterns will result.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2005.08.049.

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