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Fatty acids composition of *Caenorhabditis elegans* using accurate mass GCMS-QTOF

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Abstract

The free living nematode *Caenorhabditis elegans* is a proven model organism for lipid metabolism research. Total lipids of *C. elegans* were extracted using chloroform, methanol 2:1(v/v). Fatty acids composition of the extracted total lipids were converted to their corresponding methyl esters (FAMES) and analyzed by gas chromatography/accurate mass quadrupole time of flight mass spectrometry (GCMS-QTOF) using both electron ionization (EI) and chemical ionization (CI) techniques. 28 fatty acids consisting of 12 to 22 carbon atoms were identified, 65% of them were unsaturated. Fatty acids containing 12 to 17 carbons were mostly saturated with stearic acid (18:0) as the major constituent. Several branched-chain fatty acids were identified. Methyl-14-methylhexadecanoate (*iso*-17:0) was the major identified branched fatty acid. This is the first report to detect the intact molecular parent ions of the identified fatty acids using chemical ionization compared to electron ionization which produced fragmentations of the fatty acids methyl esters (FAMES).

Keywords

Nematodes; biomarkers; mass spectrometry; branched fatty acids; chemical ionization; electron ionization; omega fatty acids

INTRODUCTION

The free living nematode *Caenorhabditis elegans* is a major model organism in environmental toxicology, developmental biology, neurobiology, host pathogen interactions, aging research and pharmacology. Experimental advantages of the *C. elegans* model include a fast reproductive cycle, a transparent body, known cell lineages and a fully sequenced genome.^[1] Furthermore, its hermaphroditic nature (self-fertilization) allows raising a large number of homozygous animals in short time, and the presence of males allows mutations to

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be moved between strains. The full developmental cycle of *C. elegans* from eggs to fertile adults takes about 2–3 days at 20°C, and can be cultured easily and inexpensively in the laboratory. More detailed information about its biology can be found in the review by Hulme and Whitesides.^[2]

Lipids play a central role in the biology of nematodes, but also in other model organisms and are often associated with reproduction and lifespan, energy storage, the separation between intracellular and extracellular environments, and may serve as signaling molecules.^[3] *C. elegans* is a proven model organism for lipid metabolism research. Aarnio et al.,^[4] showed that fatty acids profiles in *C. elegans* correlate significantly with the aryl hydrocarbon receptor (AHR) providing more understanding of the molecular actions of AHR that may be extrapolated for higher organisms. Mammalian AHR binds to environmental xenobiotics, including halogenated aromatic hydrocarbons (PCBs, PCDDs and PCDFs) and polyaromatic hydrocarbons (PAHs).^[5–7] General genetics of *C. elegans* are closely related to that of humans, in which 40 to 75% of human genes identified as disease-related; have *C. elegans* analogs.^[8–10] Additionally stem cell populations in *C. elegans* are similar to those of humans.^[11] Horikawa and Sakamoto^[12] found that fatty-acids' metabolism regulates heat, osmotic, and oxidative-stress resistance in *C. elegans* and suggested that stress-resistance mechanisms are regulated by fatty acids metabolism.

There are many examples in scientific literature suggesting that fatty acids profiling may be used as a biomarker for exposure as well as effects of environmental toxicants or environmental stresses.^[13–24] Fatty acids composition of *C. elegans* was examined by several investigators, using gas chromatography and flame ionization detectors (GCFID)^[25–30] or by using low resolution GCMS in electron ionization (EI) mode.^[4, 31, 32] Identification of the fatty acids composition when GCFID is used, is based on comparing retention times with those of analytical standard fatty acids methyl esters (FAMES) which lacks structural conformation or may miss detection of FAMES that has no available analytical standard. When GCMS is used, fatty acid identification is performed by both retention times and mass spectral data, however, in the EI mode molecular ions are rarely detected and with the single mass unit (low resolution), still difficult to conclusively confirm the structure of the target fatty acids. In this work, we analyzed fatty acids profile of the nematode *C. elegans* using high resolution gas chromatography/accurate mass quadrupole time of flight mass spectrometry (GCMS-QTOF) in both electron ionization (EI) and chemical ionization (CI) modes to detect both molecular ions (accurate mass and isotope ratio by CI mode) and the accurate mass fragmentation pattern in the EI mode for further confirmation of the fatty acids structure.

MATERIALS AND METHODS

Chemicals

All solvents and chemical reagents were purchased from VWR (USA). Isooctane and 1% boron trifluoride in methanol were obtained from Sigma-Aldrich (USA). Complete set of certified reference material fatty acids methyl esters, saturated, unsaturated and branched from C4 to C26 were obtained from Sigma-Aldrich (Milwaukee, WI, USA) and were used

for GC retention time comparison. This (CRM) is produced and certified in accordance with ISO Guide 34:2009 and ISO/IEC 17025:2005.

***C. elegans* Culturing and Harvesting**

Wild-type (N2) *Caenorhabditis elegans* was obtained from the Caenorhabditis Genetics Center (CGC) (University of Minneapolis, MN, USA). Worms were maintained on NGM plates, seeded with *E. coli* OP50 as the food source using standard growth conditions,^[33] Wild-type gravid hermaphrodite *C. elegans* were collected from at least 10 NGM plates grown on a lawn of *E. coli* OP50 and maintained at 20°C. In order to obtain a synchronous culture, the nematodes were lysed in a NaOH/NaOCl solution. Eggs were collected in M9 buffer (3g KH₂PO₄, 6g Na₂PO₄, 5g NaCl, 1mL 1M MgSO₄. In one L of water). Eggs were incubated overnight in M9 buffer at 20°C, hatched L1 *C. elegans* were added to NGM plates and allowed to grow until they reached the adult stage (about 46 hours). The adult worms were washed three times with M9 buffer and centrifuged in 30% sucrose solution at 700 rpm for 5 minutes to separate the worms from *E. coli* and any other possible contaminants. The floating worms were washed with water using a 400 mesh sieve, centrifuged at 2000 rpm for 2 minutes to pellet and were stored at -80 °C until further use.

Fatty Acid Methyl Esters (FAMES) Analysis

C. elegans lipids were extracted in three replicates according to the method of Bligh and Dyer^[34] in chloroform, methanol 2:1 (v/v). Hajra solution (1:1 v/v, 0.2 M H₃PO₄:1 M KCl) was added to the samples, vortexed and centrifuged, the lower layer was removed, dried over sodium sulfate anhydrous and evaporated to dryness using evaporating centrifuge Centrifan PE® (Denville Scientific Inc. Holliston, MA, USA) at 25°C. One mL of boron trifluoride-methanol complex solution was added to the dried lipids and the mixture was heated at 70°C for 1 hour using heating blocks. After cooling to room temperature 1 mL of saturated NaCl solution was added and FAMES were extracted in 2 mL of 2,2,4-trimethylpentane, dried over Na₂SO₄, anhydrous, filtered using syringe filter and stored in GC glass amber vials.

Accurate Mass CI/EI GCMS QTOF

FAMES of the *C. elegans* were analyzed separately in the chemical ionization mode and electron ionization mode using an Agilent 7200 accurate mass GCMS QTOF System. GC separation was done using a BPX90 SGEWAX column 15 m × 0.25 mm and 0.25 µm film thickness, run under average velocity of 1 cm/sec with a hold up time of 1 min. Oven temperature programming started at 75°C held for 5 min and heated up to 225°C at a rate of 5°C/min, and was held at 225°C for 5 min. Total run time was 40 minutes, solvent delay 4 minutes, equilibrium time 3 minutes, helium quench gas 4 mL/min and injected volume 1 µL splitless mode. The measurements and post-run analyses were controlled by the software Mass Hunter Qualitative Analysis B.06.01 with Service Pack 3. The same conditions were used for the electron ionization mode except that nitrogen was used as the quenching gas. FAMES were identified based on their retention times, their accurate molecular weights including isotope abundances (using CI mode) and accurate molecular fragmentation in the EI mode. Also their electron ionization fragmentation and mass spectral EI data were also searched using Wiley10 NIST mass spectral data base. GCMS analysis of the fatty acids methyl esters (FAMES) was achieved with base line resolution for all samples. Quantitative

analysis and % composition of fatty acids were calculated based on peak area relative to total peaks area in the total ion chromatograms. Results were calculated based on an average of three FAMES preparation and their standard deviation.

RESULTS AND DISCUSSION

FAMES gas chromatographic separation was achieved to its individual isomers in both CI and EI mode using the BPX90 column as shown in Figure 1. Chemical ionization (CI) mode gave us the ability to detect the molecular ions of each individual fatty acid methyl ester with an accurate mass that were very consistent with the theoretical calculated molecular weights reported in the Lipidomics Gateway /LIPID MAPS (www.lipidmaps.org). CI mode provided accurate molecular weight of the target chemicals along with their natural isotope abundances in the % ratio of M^+ to $[M+1]^+$ to $[M+2]^+$. Figure 2 illustrates an example using the methyl ester of the fatty acid 20:4 having a molecular weight of 319.2616, $[M+1]^+$ and $[M+2]^+$ in a ratio of 24% and 3% respectively relative to the molecular ion as 100%. This accurate mass and isotope abundances confirmed the molecular formula of $C_{21}H_{35}O_2$. Therefore, CI GCMS provides an accurate estimation of the molecular formula of each identified fatty acid in the chromatogram, using this information in addition to comparing retention times of the standard references FAMES can achieve a positive prediction of the identity of the fatty acids in the samples. With the additional information that we can obtain from the EI GCMS which provides accurate mass fragmentation, more conformation about the chemical structure can be deduced, for example branched fatty acids can be identified as an *iso*- or as an *anteiso*- by monitoring the ions corresponding to the loss of an isopropyl radical (loss of 43 mass) or the loss of the isobutyl radical (loss of 57 mass) for *iso*- vs *anteiso*- respectively as shown in Figure 3. EI also provides the ability to search mass spectral data base (WILEY/NIST) for additional conformation of chemical identities. With all of the above resources 28 different fatty acids were identified (Fig. 4), the results of the quantitative analyses of FAMES derived from the lipids of *C. elegans* are shown in Table 1. The identified fatty acids ranged from 12 to 22 carbons in length, all of those containing 12 to 17 carbons were saturated, with the exception of methyl 9Z-hexadecenoate (16:1) and methyl 10Z-heptadecenoate (17:1). The predominant saturated fatty acid was stearic acid (18:0) comprised an average of 6.5% the total fatty acids. Other saturated acids present were methyl dodecanoate (12:0), methyl tridecanoate (13:0), methyl tetradecanoate (14:0), methyl pentadecanoate (15:0), methyl hexadecanoate (16:0), methyl heptadecanoate (17:0), methyl eicosanoate (20:0) and methyl docosanoate (22:0). Several branched-chain fatty acids were identified including methyl 13-methyltetradecanoate (*iso*-15:0), methyl 12-methyltetradecanoate (*anteiso*- 15:0), methyl 14-methylpentadecanoate (*iso*-16:0), methyl 14-methylpentadec-9-enoate (*iso*-16:1), methyl 14-methylhexadecanoate (*iso*-17:0) and methyl 15-methylhexadecanoate (*iso*-17:0). 65% of the identified fatty acids were unsaturated consisting of 18 to 20 carbon atoms, monounsaturated acids accounted for 35.6% of the total fatty acids. Methyl 9Z-hexadecenoate (16:1), methyl 9Z-octadecenoate (18:1) and methyl 9-eicosenoate (20:1) were identified. The 18:1 fatty acid comprised about 62% of the monounsaturated fatty acids. Single forms of methyl 9Z,12Z-octadecadienoate (18:2), methyl 1Z,14Z-eicosadienoate (20:2), methyl 8Z,11Z,14Z-eicosatrienoate (20:3), methyl 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5) were identified as well as two isomers

each of 18:3 (γ -Linolenic acid, methyl ester and methyl 9Z,12Z,15Z-octadecatrienoate) and 20:4 (methyl 5Z,8Z,11Z,14Z-eicosatetraenoate and methyl 8Z,11Z,14Z,17Z-eicosatetraenoate). Several fatty acids components occurred in trace amounts and were identified as 12:0, iso-14:0, 16:1 and 22:0. Fatty acids profile in *C. elegans* was found to be similar to those found in many of the free living and plant-parasitic nematodes with the fatty acid 18:1 being the major component, with only few exceptions. *Panagrellus redivivus* was found to contain 18:2 as its major fatty acid component,^[35] and the bulk of the fatty acid of *Globodera rostochiensis* and *G. solanacearum* consisted of 20:1 and 20:4.^[36,37] Branched fatty acids were also reported to be found in the lipids of the free living nematode *Turbatrix acetii* and plants parasitic *Meloidogyne* spp.^[38], however, in a previous study *iso*- fatty acids were not detected in lipids from *C. elegans*.^[39]

CONCLUSION

Chemical ionization GCMS with accurate mass of the molecular ions provided a powerful tool for identifying the fatty acids profiling of the model organism *Caenorhabditis elegans* particularly the polyunsaturated fatty acids of the omega 3 type (n-3 PUFAs) such as the C20:5 n-3 and C20:4n-3 which comprising 13.4 % and 2.8% of the total fatty acids in *C. elegans* respectively. These fatty acids are highly prone to peroxidation both *in vitro* and *in vivo* and can be used as biomarker for oxidative damage due to exposure to environmental toxicants or stressors.

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Figure 1. Chemical ionization and electron ionization total ion chromatograms of fatty acids methyl esters (FAMES) of *C. elegans* lipids.

Figure 3.
EI fragmentation pattern for the *iso*- and *anteiso*- fatty acid methyl ester 17:0.

Figure 4.
Chemical structure of the identified FAMES of *C. elegans* lipids.

Table 1
Fatty acids composition, retention times (R_t) and their relative percentage as FAMES of *C. elegans* lipids.

#	Chemical Name	R_t (MIN)	FA	CAS#	Exact Mass, Methyl esters	Calculated	Measured	%
1	Methyl Dodecanoate	6.47	12:0	111-82-0	215.2006	215.2003	Trace	
2	Methyl tridecanoate	7.23	13:0	1731-88-0	229.2162	229.2153	0.33 ± 0.01	
3	Methyl 12-methyltridecanoate	8.67	14 <i>iso</i>	5129-58-8	243.2319	243.2308	Trace	
4	Methyl tetradecanoate	9.36	14:0	124-10-7	243.2319	243.2308	1.19 ± 0.31	
5	Methyl 13-methyltetradecanoate	10.12	15 <i>iso</i>		257.2475	257.2467	11.09 ± 1.50	
6	Methyl 12-methyltetradecanoate	10.34	15 <i>ante</i>	5129-66-8	257.2475	257.2467	0.28 ± 0.05	
7	Methyl 14-methylpentadecanoate	11.51	16 <i>iso</i>	5129-60-2	271.2631	271.2623	1.06 ± 0.18	
8	Methyl 14-methylpentadec-9-enoate	11.80	16:1 <i>iso</i>	365897 *	269.2475	269.2464	1.08 ± 0.13	
9	Methyl hexadecanoate	12.15	16:0	112-39-0	271.2631	271.2623	4.17 ± 0.48	
10	Methyl 9Z-hexadecenoate	12.45	16:1	1120-25-8	269.2475	269.2464	Trace	
11	Methyl 14-methylhexadecanoate	12.88	17 <i>iso</i>	2490-49-5	285.2788	285.2782	9.35 ± 1.35	
12	Methyl 15-methylhexadecanoate	13.47	17 <i>ante</i>	6929-04-0	285.2788	285.2782	0.27 ± 0.04	
13	Methyl 10Z-heptadecenoate	13.99	17:1	333621 *	283.2631	283.2621	9.97 ± 1.13	
14	Methyl octadecanoate	14.79	18:0	112-61-8	299.2944	299.2927	6.51 ± 1.33	
15	Methyl 9Z-octadecenoate	15.40	18:1 9Z	112-62-9	297.2788	297.2778	22.20 ± 0.88	
16	Methyl 9Z,12Z-octadecadienoate	16.20	18:2	112-63-0	295.2631	295.2629	6.16 ± 0.75	
17	Methyl 10Z-nonadecenoate	16.43	19:1	333644 *	311.2935	311.2935	2.12 ± 0.16	
18	Methyl γ -Linolenate	16.90	18:3	333650 *	293.2475	293.2467	0.63 ± 0.25	
19	Methyl eicosanoate	17.20	20:0	1120-28-1	327.3257	327.3242	0.23 ± 0.01	
20	Methyl allZ-9,12,15-Octadecatrienoate	17.34	18:3	301-00-8	293.2475	293.2467	0.28 ± 0.06	
21	Methyl 9Z-eicosenoate	17.69	20:1	336505 *	353.3414	353.3415	0.21 ± 0.01	
22	Methyl stearidonate	18.09	18:4	336478 *	291.2319	291.2316	0.25 ± 0.02	
23	Methyl 11Z,14Z-eicosadienoate	18.44	20:2	2463-02-7	323.2944	323.2949	0.66 ± 0.20	
24	Methyl allZ-8,11,14-eicosatrienoate	19.09	20:3	17364-32-8	321.2788	321.2778	4.03 ± 0.03	
25	Methyl docosanoate	19.32	22:0	929-77-1	355.3570	355.3564	Trace	
26	Methyl allZ-5,8,11,14-eicosatetraenoate	19.55	20:4	2566-89-4	319.2631	319.2615	1.71 ± 0.36	

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#	Chemical Name	R _f (MIN)	FA	CAS#	Exact Mass, Methyl esters		%
					Calculated	Measured	
27	Methyl alIZ-8,11,14,17-eicosatetraenoate	20.18	20:4	336470 [*]	319.2631	319.2616	2.83 ± 0.54
28	Methyl alIZ-5,8,11,14,17-eicosapentaenoate	20.71	20:5	2734-47-6	317.2475	317.2462	13.39 ± 1.27

^{*} is the NIST ID#