学術情報リポジトリ

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メタデータ	言語: eng 出版者: 公開日: 2009-08-25 キーワード (Ja): キーワード (En): 作成者: SHIBAHARA, Akira, YAMAMOTO, Kohei, KINOSHITA, Akemi, MIYATANI, Shuichi メールアドレス: 所属:
URL	https://doi.org/10.24729/00005803

J Rehabil Health Sci 2003; 1: 23-28

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Fatty Acids of the Total Lipids from Earthworms*

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Recieved November 13, 2003; accepted December 19, 2003

The total lipids extracted from earthworms were converted to fatty acid methyl esters, and the esters were analyzed to examine their composition by capillary gas chromatography. In the gas chromatogram over 100 peaks were observed, indicating the presence of at least 100 kinds of fatty acids. On the basis of comparison with cochromatographed standards and of argentation thin-layer chromatographic behaviors, a series of saturated fatty acids (12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0) and di- and polyunsaturated fatty acids (linoleic acid, α-linolenic acid, arachidonic acid, EPA, DHA, etc.) were determined. An aliquot of the fatty acid methyl ester mixture was then derivatized to dimethyl disulfide adducts, and the adducts were analyzed to locate the original double-bonds of the methyl monoenoates by gas chromatography/mass spectrometry. Based on the mass spectral evidence and mass chromatographic tracing, 41 kinds of double-bond positional isomers of the monounsaturated fatty acids were identified. Among them, the predominant component was *cis*-7-icosenoic acid which occupied 5.5% of the total fatty acids in the earthworm lipids. To our knowledge, *cis*-7-icosenoic acid is a novel fatty acid found in nature. Thus the earthworm lipids could be characterized due to the complexity of the fatty acid profile.

Key words: earthworm; double-bond positional isomers of monoenoic fatty acids; *cis*-7-icosenoic acid; capillary gas chromatography; gas chromatography/mass spectrometry

Introduction

Chemical constituents of common human diets have been actively studied using modern instrumental analysis. However, unsolved problems remain concerning the make-up of food in uncommon diets. Our research group focused its attention on the lipids, especially fatty acids, of edible creatures which are or have been used as food for humans. For this first part of our project, we analyzed the fatty acids of earthworm lipids. Earthworms are used even now as a food source in some countries and also in Chinese medicine (as an antipyretic).

Relatively little is known about the fatty acids[1-6] of earthworm lipids. Even though high-resolution capillary gas chromatography (GC) has been used to analyze the fatty acid compositions, double-bond positional isomers of monoenoic fatty acids were not fully determined.[7] For this study, we used capillary GC together with another system of gas chromatography/mass spectrometry (GC/MS) and were able to get more information about the fatty acids of the lipids from earthworms.

* Studies on the Lipids of Edible Creatures. Part 1.

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Materials and Methods

Live earthworms (ca. 1 g), donated from Nippon Paper Industries Co., were washed with distilled water two times and then were immersed in boiling water for 10 min to inactivate lipolytic enzymes.[2] After being placed on a filter paper to remove moisture, the earthworms were homogenized with chloroform/methanol (2:1, v/v) according to the method of Folch *et al.*[8] The procedures for extracting the total lipids and for removing the nonlipid contaminants were the same as those described in our previous paper.[9]

The total lipids obtained were converted to fatty acid methyl esters (FAMEs) by methanolysis (0.5 M KOH/methanol) and successive methylation (14% BF_3/methanol).[9] The resulting FAMEs were purified by passing them through a silica gel mini column and were analyzed on a fused silica ULBON HR-SS-10 capillary column (50 m × 0.25 mm i.d., 0.25 μm film, chemically bonded type, Shinwakako, Kyoto, Japan) in a Shimadzu GC-17A gas chromatograph with a splitless injector and a flame ionization detector. The column

Abbreviations: AgTLC, argentation thin-layer chromatography; DMDS, dimethyl disulfide; FAME, fatty acid methyl ester; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry.

temperature was programmed at 100℃ for 2 min isothermally, then to 210℃ at a rate of 6℃/min and held at 210℃ for 25 min. The carrier gas was helium (linear gas velocity: 45.3 cm/sec).

After analyzing the FAMEs by capillary GC, an aliquot of the remaining FAMEs was subjected to the I_2-catalyzed reaction with dimethyl disulfide (DMDS).[10,11] The reaction mixture was passed through a mini column packed with silica gel to remove the by-products originating from methyl dienoates or polyenoates.[11] The purified DMDS adducts of methyl monoenoates were analyzed on a fused silica DB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 μm film, chemically bonded type, Agilent Technologies, Baloalt, CA) linked to a Shimadzu QP-5050A mass spectrometer with a computer on-line system. The column temperature was programmed at 120℃ for 2 min isothermally, then to 300℃ at a rate of 5℃/min and held at 300℃ for 5 min. Electron impact mass spectra were measured at an ionizing energy at 70 eV by scanning from 50 to 450 *m/z* (0.5 sec/cycle).

Argentation thin-layer chromatography (AgTLC) was carried out in the same manner described earlier.[9] All chemicals were of the highest reagent grade available, and all solvents were distilled before use.

Results and Discussion

Fig. 1 shows the gas chromatogram of the FAMEs prepared from the total lipids of the earthworms. Over 100 peaks were observed. The cochromatographed standards composed of authentic saturated FAMEs with even chains, ranging from 12:0 to 24:0, eluted at the same positions as peaks 1, 3, 5, 8, 12, 15 and 17. As for authentic saturated FAMEs with odd chains (13:0, 15:0 and 17:0), they eluted at the same positions as peaks 2, 4 and 7. The retention times of peaks 10, 11, 14, 16, 18 and 19 coincided to those of cochromatographed authentic methyl 18:2(9,12) (linoleate), 18:3(9,12,15) (α-linolenate), 20:4(5,8,11,14) (arachidonate), 20:5(5,8,11,14,17) (EPA), 22:5(7,10,13,16,19) and 22:6(4,7,10,13,16,19) (DHA), respectively. An aliquot of the FAME mixture (Fig. 1) was then fractionated according to the number of double bonds and to the *cis-trans* configuration by AgTLC. No band corresponding to the authentic FAME standards having *trans* double bonds was observed on the AgTLC plates, indicating that we could ignore the effects of *trans* fatty acids in the analysis of the FAME mixture described below. Capillary GC analysis of the recovered FAMEs from each band separated on the AgTLC plates revealed that the identification of these dienoic and polyenoic acids described above was correct. Identification of the esters eluting at the major peaks not numbered in Fig. 1 (probably a series of the esters of isoprenoid fatty acids[3,4]) was omitted in the present study.

An aliquot of the FAME mixture shown in Fig. 1 was then derivatized to DMDS adducts, and the adducts were analyzed by GC/MS to determine the original double-bond positions of the methyl monoenoates. Fig. 2 shows the mass chromatogram traced with the total ions and the calculated *m/z* values of molecular ions corresponding to those of the DMDS adducts originating from even-chained methyl monoenoates. Mass chromatographic responses at *m/z* 306, 334, 362, 390, 418 and 446 indicate the presence of DMDS adducts originating from methyl 12:1, 14:1, 16:1, 18:1, 20:1 and 22:1, respectively. From another mass chromatogram traced with the calculated *m/z* values of molecular ions corresponding to those of DMDS adducts originating from odd-chained methyl monoenoates (data not shown), the presence of methyl

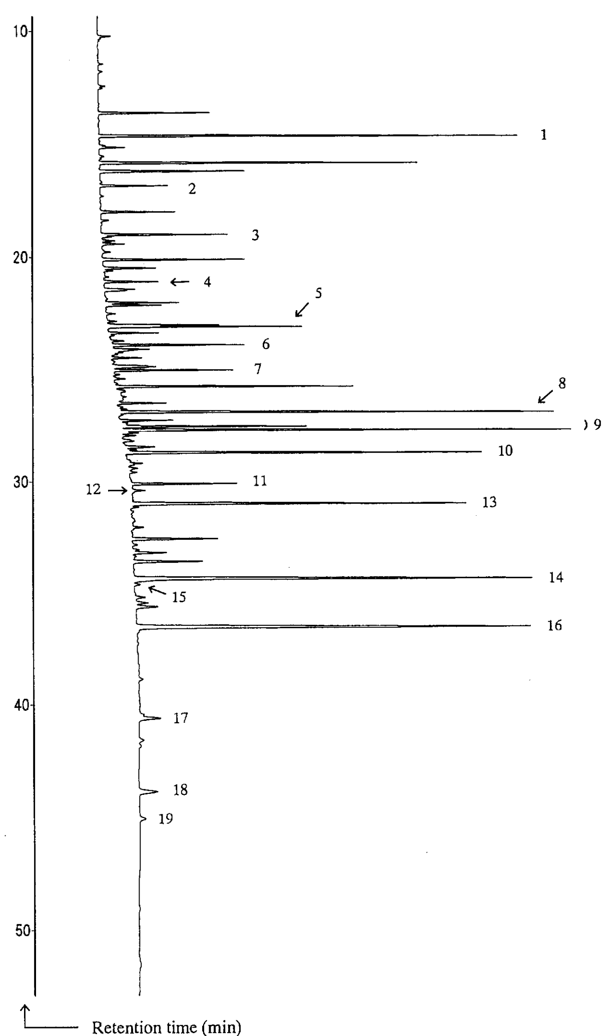


Fig. 1 Capillary gas chromatogram of fatty acid methyl esters prepared from earthworm lipids.
Peak identifications are given in the text. 1=12:0, 2=13:0, 3=14:0, 4=15:0, 5=16:0, 6=16:1, 7=17:0, 8=18:0, 9=18:1, 10=18:2(9,12), 11=18:3(9,12,15), 12=20:0, 13=20:1, 14=20:4(5,8,11,14), 15=22:0, 16=20:5(5,8,11,14,17), 17=24:0, 18=22:5(7,10,13,16,19), 19=22:6(4,7,10,13,16,19). Peaks not numbered are those of unidentified fatty acids and those of 12:1, 13:1, 14:1, 15:1, 17:1, 19:1, 21:1, 22:1, 20:2 and 20:3.

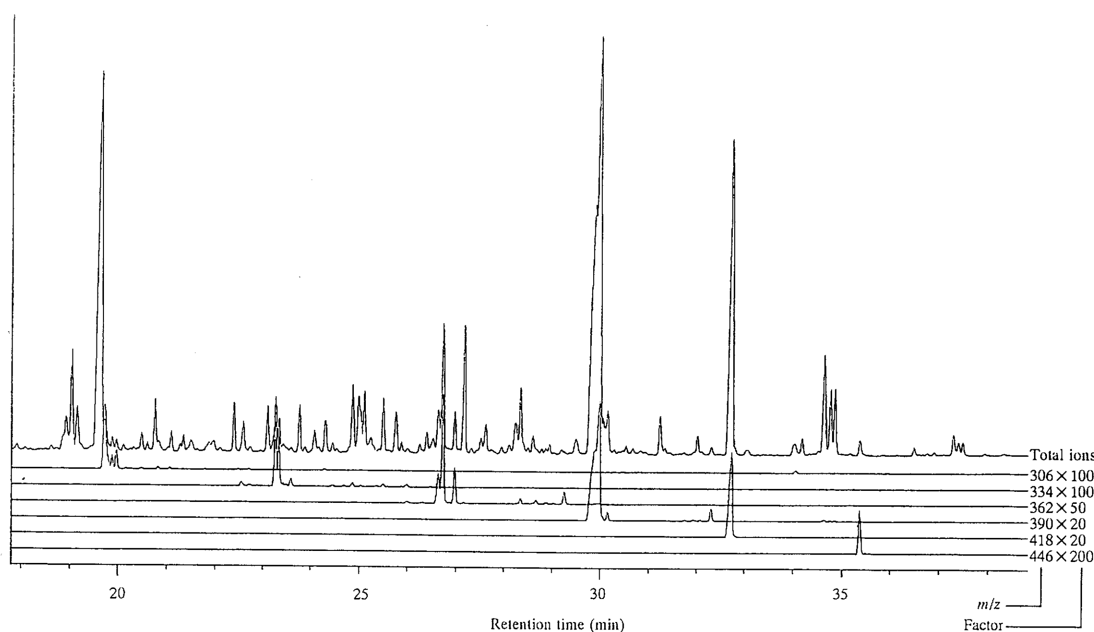
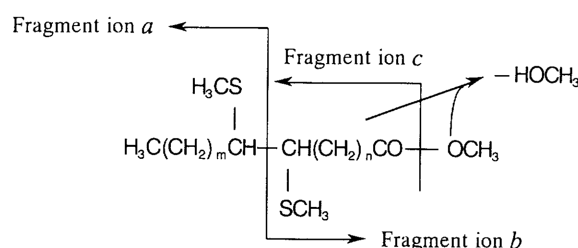


Fig. 2 Mass chromatogram of dimethyl disulfide adducts of methyl monoenoates.
The ions at *m/z* 306, 334, 362, 390, 418 and 446 are the molecular ions of the adducts of methyl 12:1, 14:1, 16:1, 18:1, 20:1 and 22:1 fractions, respectively. For easy readability, we magnified the mass chromatographic responses by using different numerical factors of magnification.

13:1, 15:1, 17:1, 19:1 and 21:1 was determined.

Electron impact ionization of the DMDS adducts of the methyl monoenoate gave a recognizable molecular ion and a set of characteristic ions *a*, *b* and *c* derived from the fragmentation, as illustrated in Scheme 1. The cleavage between the methylthio-substituted carbons produced the characteristic ions *a* and *b*; the characteristic ion *c* was yielded due to the loss of methanol from the ion *b*.[10] By using the molecular ion together with this set of characteristic ions *a*, *b* and *c*, the structure of the adducts was confirmed. From this elucidation we could locate the original double-bonds of the methyl monoenoates, even though methyl monoenoates are composed of several double-bond positional isomers.

Mass spectra shown in Fig. 3 are from the peaks of the DMDS adducts of the methyl 16:1 fraction in the mass chromatogram (Fig. 2). A set of the fragment ions at *m/z* 215, 147 and 115 (Fig. 3A) corresponds to the characteristic ions *a*, *b* and *c* (Scheme 1), confirming that the structure of the DMDS adducts was methyl 4,5-bis(methylthio)hexadecanoate. We could then conclude that the original acid is 16:1(4) and the Δ-4 isomer exists. By using other sets of the fragment ions at *m/z* 201, 161 and 129, at *m/z* 187, 175 and 143, at *m/z* 173, 189 and 157 (Fig. 3A), at *m/z* 145, 217 and 185 (Fig. 3B), and at *m/z* 117, 245 and 213 (Fig. 3C), a series of Δ-5, Δ-6, Δ-7, Δ-9 and Δ-11 isomers of 16:1 was determined. Mass chromatographic analysis using the characteristic ions *a* or *b*[12] shows that the main isomer is the Δ-9 isomer.



Scheme 1 Fragmentation pattern of dimethyl disulfide adducts derived from methyl monoenoate.
Characteristic ions *a*, *b* and *c* are useful for determining the original double-bond positions of the methyl monoenoates.

Fig. 4 shows the mass spectrum obtained from the top of the mass chromatographic peak corresponding to the DMDS adducts of the methyl 20:1 fraction. Together with the molecular ion at *m/z* 418, a set of the fragment ions at *m/z* 229, 189 and 157 (corresponding to the characteristic ions *a*, *b* and *c*) was observed. This indicates that the original acid is 20:1(7). From another mass spectrum obtained from the peak tailing corresponding to the DMDS adducts of the methyl 20:1 fraction (data not shown), the presence of Δ-11 and Δ-13 isomers was also confirmed. The ratio of these Δ-7, Δ-11 and Δ-13 isomers determined by mass chromatography using the characteristic ions *a* and *b* was 100:5:1. These data indicate that 20:1 of the earthworm lipids is composed of 20:1(7), 20:1(11) and 20:1(13), and the predominant component is

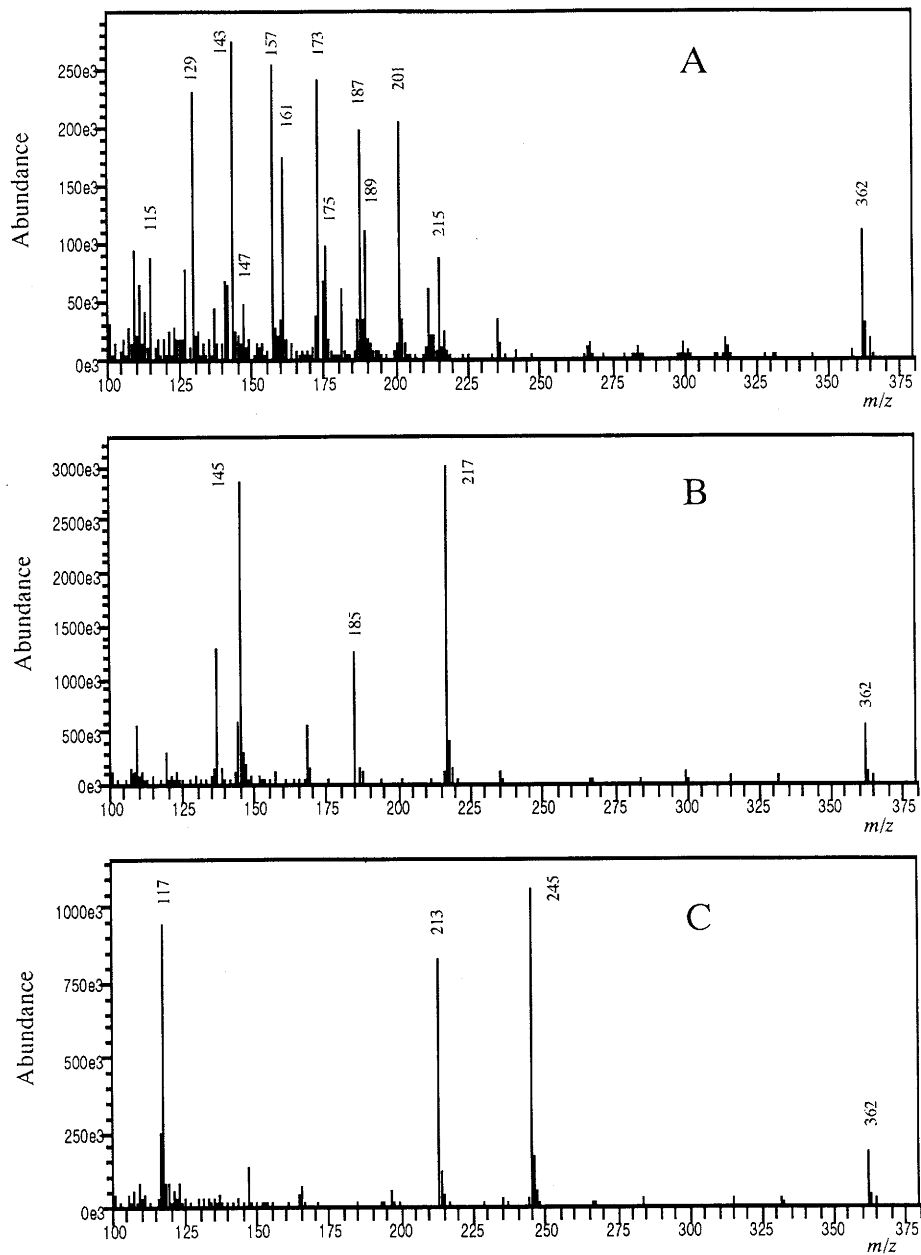


Fig. 3 Mass spectra of dimethyl disulfide adducts of the methyl 16:1 fraction. Mass spectral assignments are given in the text.

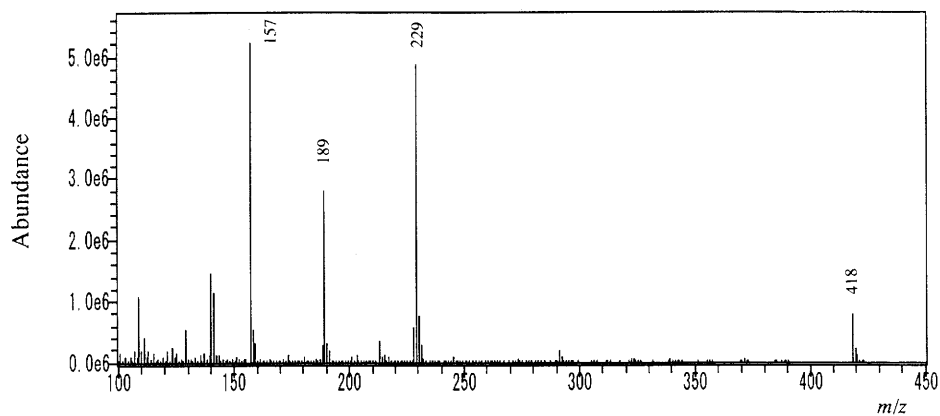


Fig. 4 Mass spectrum of dimethyl disulfide adducts of the methyl 20:1 fraction. Mass spectral assignments are given in the text.

20:1(7).

In previous reports[5,6] on earthworm lipids, the double-bond position of 20:1 was assigned at the Δ-11 based on data from only using GC or only using capillary GC. But now we know that only using GC or only using high-resolution capillary GC often fails to determine the positions of double bonds in monoenoic acids. In that previous research[5,6] the retention time of methyl 20:1(11) might have been slightly different from that of methyl 20:1(7) on the gas chromatogram; however, commercially prepared 20:1(7) was not available at that time (nor at present).

Our GC/MS method clearly reveals the exact structure of monoenoic acids. The amount of 20:1(7) in the total fatty acids of the earthworm lipids we tested was 5.5% of the total fatty acids (Table 1). In our preliminary experiments on this fatty acid in several kinds of earthworms collected in Japan, we always detected this fatty acid as the main component of the earthworm monoenoic acids, though its amount varied (4-8%). To our knowledge, 20:1(7), "*cis*-7-icosenoic acid," is a novel fatty acid found in nature. Monoenoic acid having a double bond at the Δ-7 position is rare in naturally occurring fatty acids.

Table 1 Major fatty acid composition of the total lipids from earthworms

Fatty acid	wt% of total fatty acids
12:0	5.3
12:1[a]	Tr
13:0	0.9
13:1[b]	Tr
14:0	1.6
14:1[c]	Tr
15:0	0.7
15:1[d]	0.7
16:0	2.4
16:1[e]	2.9
17:0	1.6
17:1[f]	Tr
18:0	6.2
18:1[g]	8.6
18:2 (9,12)	4.9
18:3 (9,12,15)	1.8
20:0	Tr
20:1[h]	5.5
20:4 (5,8,11,14)	8.3
20:5 (5,8,11,14,17)	9.1
22:0	Tr
22:1[i]	0.5
22:5 (7,10,13,16,19)	0.6
22:6 (4,7,10,13,16,19)	Tr
24:0	0.6
Others[j]	37.8

Each value of monoenoic acids is calculated from the data from capillary GC of the FAMEs and from GC/MS of the DMDS adducts.
Tr, trace (below 0.4 wt%).
[a] Mixture of Δ-5 (main) and Δ-7 isomers.
[b] Mixture of Δ-5 (main) and Δ-7 isomers.
[c] Mixture of Δ-5 (main), Δ-7 and Δ-9 isomers.
[d] Mixture of Δ-4, Δ-5 (main), Δ-7, Δ-9 and Δ-10 isomers.
[e] Mixture of Δ-4, Δ-5, Δ-6, Δ-7, Δ-9 (main) and Δ-11 isomers.
[f] Mixture of Δ-4, Δ-5, Δ-7, Δ-9 (main) and Δ-11 isomers.
[g] Mixture of Δ-5, Δ-7, Δ-9, Δ-11 (main) and Δ-13 isomers.
[h] Mixture of Δ-7 (main), Δ-11 and Δ-13 isomers.
[i] Mixture of Δ-7, Δ-8 and Δ-9 (main) isomers.
[j] Including 19:1(5), 19:1(7), 19:1(9), 19:1(11), 21:1(7), 21:1(8), 21:1(9), 20:2, 20:3, minor acid (below 0.4 wt%) and unidentified acids.

In the same manner described above, the double-bond positional isomers of the monoenoic acids in the earthworm lipids were elucidated. These results are summarized in the legend of Table 1. The fatty acid composition of the earthworm lipids is very complex, as other researchers reported previously.[1,4,5] In the present work, the total lipids were extracted from the whole body of the earthworms, and the fatty acid profile was examined. The profile might have been affected by the presence of fatty acids originating from body tissues, organs, gut microorganisms and also ingested soil containing plant and animal materials. But Albro *et al.*[5] analyzed the endogenous fatty acids of earthworms fed under a controlled diet and under other controlled environmental factors, and they found that the complexity of the fatty acid profile was unchanged.

It is true that the earthworm synthesizes a variety of fatty acids, especially a series of double-bond positional isomers of monoenoic acids, by itself. To accomplish this, the earthworm must have many enzyme systems involving *de novo* synthesis for saturated fatty acids, chain-elongation reaction for long chain fatty acid formation, and desaturation for unsaturated fatty acid formation. Even in lower animals, such as the earthworm, why do so many enzyme systems exist? We are trying to answer this question.

As a food source, the earthworm can be ranked as one of the top class foods, having a variety of unsaturated fatty acids with *cis* double bonds. There are some foods having complex fatty acid profiles, but all these foods have *trans* fatty acids produced from catalytic hydrogenation of commercial oils.

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