Micro Wave-Assisted Fast Extraction of Oil from Thrombolytic Agent Native Earthworm (*Lampito mauritii,* Kinberg) their GC-MS Quantification of the Fatty Acids and Evaluation of Lipids by ATR FTIR Spectroscopy

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Summary: Fatty acids methyl esters (FAMES) were synthesised utilizing the iupac standard, 1979 method 2.301 and their significance on the basis of nutritional research purpose. The earthworms were microwave extracted, then analysed for fatty acids composition according to aoac method. Fourier transform infrared spectroscopy used to identify the distinctive peaks of the fatty acid spectrum, whereas gas-chromatography combined with mass spectroscopy was used to evaluate the fatty acid composition of earthworm (*Lampito mauritii*, Kinberg). The current investigation identified two unsaturated fatty acids (C18:1 and C19:3) and eleven saturated fatty acids (C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C23:0, C24:0, and C25:0). Unsaturated fatty acids (UFA) varied from 26.38 to 29.14%, with oleic acid (C18:1) dominating at 16.88%, 17.75%, and 17.52%, respectively. Saturated fatty acids (SFA) ranged from 70.86 to 73.61%, with stearic acid (C18:0) dominating at 13.24 to 13.99%. Thirteen fatty acids, were identified from *Lampito mauritii,* Kinberg Sindh Pakistan origin, using Agilent 6890 N gas chromatographic device with Agilent autosampler 7683-B injector and Agilent MS-5975 inert XL mass selective detector (Agilent Technologies, Little Fall, NY, USA)reported by us in the literature for the first time. Based on the results the earthworm (*L. mauritii,* Kinberg) contains significant fatty acids that have potential pharmaceutical values.

Key words: Earthworm, *Lampito mauritii* Kinberg*, Sind Pakistan origin,* Microwave extraction, GC-MS, FAMEs, FT-IR, Incubator drying

Introduction

In Pakistan, the indigenous earthworm *Lampito mauritii (L. mauritii)*, Kinberg, is frequently utilized as chicken feed and fish bait. Animals need protein in their diets to develop and stay healthy. Fish meal, which is becoming more and more expensive, is one of the main sources of protein for animal diets.

Many researchers have already reported on *L. mauritii's* potential as a source of protein for animal feed [1-6]. In fact, a variety of earthworm specie has been analysed for composition of fatty acids [7-10]. However, here we first reported composition of fatty acids in *Lampito mauritii,* Kinberg Sindh Pakistan origin, by Gas-chromatography (GC) combined with Mass Spectroscopy (MS) using low temperature technique**,** microwave extraction method.

The components of various earthworm species' tissue were analysed by McInroy [11-15]. Animal fats and oils have the same fatty acids that are present in the tissues of EW (*L. mauritii*) [16]. Earthworm tissues contain a preponderance of long chain fatty acids. Low density lipoprotein, known as bad cholesterol, is reduced by oleic acid, which helps to reduce the risk of cardiovascular disorders [17-25].

Since studies on the fatty ester composition of indigenous earthworm *Lampito mauritii,* Kinberg are limited and quite complex [26-28], the present study in many aspects, investigates, the fatty acid composition in comparision with standards of fatty acids [20]. Animals given to earthworms that has been dried, usually using various drying methods, however, these studies provide microwave extracted fatty acid composition and dried *L. Mauritii* Kinberg

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by using incubator [21, 22]. Additionally, prior research did not use the Agilent 6890 N gas chromatography equipment in conjunction with an Agilent autosampler 7683-B injector and an Agilent MS-5975 inert XL mass selective detector (Agilent Technologies, Little Fall, NY, USA). By taking measures to reduce the likelihood of changes occurring both before and after extraction, the current study aims to give information on the lipid composition of the *L. mauritii*. Therefore the objective of this study was to determine the fast microwave extracted fatty acid profile of *L. mauritii* Kinberg in sindh Pakistan due to its following characteristics.

Bioactive Compounds from Earthworms for Pharmaceutical Industries: Earthworm's clot-dissolving compounds have been tested clinically [29-31]. Thrombotic diseases, diabetes mellitus, chronic lumbago, schizophrenia, vertigo, and digestive disorders have all been found to benefit from oral administration of earthworm powder [32]. Bronchial dilating substance also isolated from earthworms [33]. *L. rubellus* contains 63.06% crude protein. Earthworms contain compounds including lumbrokinase, superoxide dismutase, cholinesterase, glycosidases, calmodulin-binding protein, lysenin, antitumor protein, and fibrinolytic enzymes[34-38].

a). Medicines for Heart Disease: A class of six proteolytic enzymes known as lumbro kinase, useful in the treatment and prevention of ischemic heart disease[39], myocardial infarction, retinal vein thrombosis, peripheral vein embolism, and pulmonary embolism [40]. The cytolytic, agglutinating, hemolytic, mitogenic and tumor static properties of earthworm protein and its coelomic fluid has been discovered [41,42].

b). Cancer Cure by Earthworm: Earthworms leukocytes can recognize human cancer cells. Lumbricin peptide from *Lumbricus terrestris* inhibit mammary tumors in mice. Lumbrokinase enzymes also promises to wage a war on cancer [43].

c). Anti-microbial Products from Earthworms for Production of Antibiotic: The coelomic fluid of earthworms have anti-pathogenic activities for the production of antibiotics [44]. Lauric fatty acid from earthworm known for its anti-microbial properties. It is a precursor to monolaurin which is a more powerful anti-microbial agent that has potential to fight lipid-coated RNA, DNA viruses, several pathogenic Gram-positive bacteria, yeasts and various pathogenic protozoa [42].

2. *Raw Materials for Rubber, Lubricant, Detergent, Soaps and Cosmetic Industries:* Biological compounds from earthworms are also finding industrial application [42].

3. *Nutritive Feed Materials for Poultry, Dairy and Fishery Industries*: Earthworm are rich in high quality protein (65 %) with all essential amino acids, 70-80 % high quality lysine and methionine [45]. According to Sogbesan and Ugwumba [46], EWs are considered a valuable source of animal protein and are typically used as bait for fishing. Their meal is also included in poultry and aquaculture feed as an unconventional diet. Since ancient times, earthworms have also been used ethno medically, particularly in China and Japan, to treat a range of illnesses [47]. Advances in biochemical research have examined the antioxidant, anti-inflammatory and antipyretic properties of EWs extract [48], [21, 22], [49-51]. Furthermore, a wide variety of microorganisms found in soil are an inevitable part of EW's natural diet [52, 53]. A comprehensive review on earthworm has been published [54, 55].

In the light of these studies attempts have been made to extract oil from *Lampito mauritii* using a microwave and the oil composition of different fatty acids was determined.

Experimental

Work plan

Earthworms (*Lampito mauritii*) were collected from the research field of garden of pcsir laboratories complex Karachi, Sindh. *Lampito mauritii* specimen identified and voucher deposited in National Nematological Research Centre, University of Karachi.

Materials and Methods

Reagents, Standards and Samples: All chemicals and reagents used were of analytical grade. Hexane was obtained from Fisher Scientific Ltd. UK. Methanol, potassium hydroxide and anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany).

Collection/ incubator drying of Lampito mauritii: Two kilograms of wild *Lampito mauritii* was gathered at the PCSIR Laboratories Complex's study field in Karachi, Pakistan. Some 400 of earthworm was cleaned under running water and drained. After that, the earthworms were submerged in distilled water for six to eight hours in order to extract the soil from their digestive tract. After being

carefully cleaned with distilled water, the earthworms were gathered in a petridish and stored at 55°C in an incubator. It took around three days to dry. The earthworm pastes were kept at room temperature in the refrigerator.

Sample Preparation: Before being stored in air tight plastic bags until they were needed for extraction, the dried earthworm was crushed using a domestic grinder and passed through a stainless-steel screen with a 1.0 mm mesh size to achieve a consistent particle size.

Microwave Extraction Procedure: A Sharp Carousel Jet Convection microwave (R-8170 (W) Japan) with 650 W of power adjustment was used for the microwave extraction process. A 30 ml screw-capped vial with 12 ml of hexane and roughly 10 g of powdered earthworm were exposed to 650 W of microwave energy for 20 seconds. When the vial reached the boiling point, it was removed from the oven, given a good shake, and then put back in. The same basic procedure was followed, with the exception that the miscella was collected and replaced with fresh solvent after reaching two minutes of cumulative microwave extraction time. This process was repeated until a cumulative ten minutes of microwave exposure had been reached, at which point the miscella was centrifuged, the solvent was removed, and the remaining oil was dried and weighed. To achieve a total of 10 minutes of microwave irradiation, this procedure was carried out four more times [56].

Determination of fatty acid composition: The GC-MS chromatogram obtained was compared with two libraries (NIST & Wily) that offer the best information regarding the identification of fatty acids present in EW samples. FAMEs were prepared using standard IUPAC, 1979 method 2.301 [57] in order to determine the fatty acid composition of the earthworm samples.

GC-MS conditions: The GC-MS analysis for FAMEs was performed on Agilent 6890 N gas chromatography instrument coupled with an Agilent MS-5975 inert XL mass selective detector and an Agilent autosampler 7683-B injector (Agilent Technologies, Little Fall, NY, USA). Fatty acid methyl esters were separated using a capillary column HP-5MS (5% phenyl methyl siloxane) that measured 30 m × 0.25 mm i.d. × 0.25 µm film thickness (Agilent Technologies, Palo Alto, CA, USA).

Fatty acid methyl ester samples were identified by comparing the retention times of FAME peaks from samples with those of standards. The initial temperature of 150ºC was maintained for 2 min raised to 230ºC at the rate of 4ºC/min, and kept at 230ºC for 5 min. The split ratio was 1:50, and helium was used as a carrier gas with the flow rate of 0.8 ml/min. The temperatures of the injector and detector were 240 and 260 degrees Celsius, respectively. In the scan range of 50–550 *m/z*, the mass spectrometer was run in the electron impact (EI) mode at 70 eV.

Calculations and Statistical Analyses: The sample's mean was determined after triplicate analysis. By comparing retention periods, the fatty acids in the examined EW samples were identified at their peak. The GC-MS library results were confirmed using standard methyl esters of palmitic, stearic, oleic, and linoleic acids (Table-**1)**. Liquid state, after the derivative analysis by GC. were done (Liquid by FT-IR).

FTIR Spectral Measurements

A Thermo Nicolet Avatar 320 FTIR spectrometer, controlled by OMNIC software (Thermo Nicolet Analytical Instruments, Madison, WI), was used to acquire all infrared spectra [58]. It was outfitted with a deuterated triglycine sulfate (DTGS) detector, KBr lenses, and a detachable diamond cell smart accessory. All spectra were obtained by co-adding 32 images in the 4000–650 cm^{-1} region at a resolution of 4 cm^{-1} (Table-2). The ATR crystal was meticulously cleaned with propanol to eliminate any remaining contribution of the previous sample, and the remaining solvent was then evaporated using a stream of nitrogen gas before the spectrum of each standard or sample was radioed against a new background spectrum recorded from the bare ATR crystal [59].

Result and Discussion

We have previously reported the isolation and purification of esters [60-66]. Recently, we investigated the fatty acid profile in extracted fat of indigenous *Lampito mauritii,* Kinberg Pakistan origin dried powder by low temperature technique using microwave extraction**,** stored at 55°C in an incubator. The Soxhlet, Goldfisch, and Folch processes were the most widely used techniques for lipid extraction from solid samples in lab settings. These techniques can accurately identify lipids quantitatively, however they all require an extraction duration of eight to twenty-four hours. We've employed a fast, accurate, and cost-effective extraction method to analyze fatty acids in earthworms, previously published in a reputable international journal [56].

Table-1. Analysis of the Fatty Acids composition of extracted oil from *Lampito mauritii,* Kinberg samples.

S.	Systematic Name (Lipid Numbers)	Common Name	EW-1	EW-2	EW-3
1.	Dodecanoic acid (C12:0)	Lauric acid	6.13±0.15	6.53±0.13	6.67±0.15
2.	Tetradecanoic acid (C14:0)	Myristic acid	8.62±0.25	8.65±0.25	8.66±0.22
3.	Pentadecanoic acid (C15:0)	Pentadecylic acid	5.69±0.21	5.89±0.15	5.65±0.16
4.	Hexadecanoic acid (C16:0)	Palmitic acid	12.33±0.35	13.05±0.44	13.46±0.35
5.	Heptadecanoic acid (C17:0)	Margaric acid	4.53±0.13	4.84±0.12	4.94±0.13
6.	Octadecanoic acid (C18:0)	Stearic acid	13.24±0.25	13.99±0.55	13.76±0.64
7.	Octadecenoic acid (C18:1n9)	Oleic acid	16.88±0.64	17.75±0.45	17.52±0.75
8.	Nonadecatrienoic acid (C19:3n1,3,12)	-	12.26±0.32	8.63±0.25	9.27±0.23
9.	Eicosanoic acid (C:20:0)	Arachidic acid	6.43±0.15	6.70±0.13	6.62±0.33
10.	Docosanoic acid (C22:0)	Behenic acid	4.25±0.12	4.18±0.12	3.88±0.11
11.	Tricosanoic acid (C23:0)	Tricosylic acid	3.44±0.08	3.50±0.13	3.27±0.12
12.	Tetracosanoic acid (C24:0)	Lignoceric acid	4.29±0.11	4.32±0.11	4.20±0.15
13.	Pentacosanoic acid (C25:0)	Pentacosylic acid	1.91±0.05	1.96±0.05	2.11±0.05
Total SFA	∑ Saturated Fatty acids (SFA)		70.86	73.61	73.22
Total UFA	∑ Unsaturated Fatty acids (UFA)		29.14	26.38	26.79

EW-1, EW-2, AND EW-3 were the codes assigned to the triplicate samples. Fatty acids were expressed as the proportion of each individual fatty acid to the total of all fatty acids present in the sample. The Fatty acid combinations were calculated: total saturated fatty acids (SFA), total unsaturated fatty acids (UFA). Results are presented as mean ± SD of triplicates.

Table-**2**. FT-IR spectrum results of extracted oil of earthworm *Lampito mauritii,* Kinberg
It illustrates the characteristic bands of the expected functional groups.

Peak no.	Peak Position	Intensity	Assignment
01	3011.1	0.042	=C-H Stretching
02	2922.3	0.20	$-CH_3$ Asymmetrical stretching
03	2855.5	0.130	$-CH_2$ Symmetrical stretching
04	1708.7	0.127	C=O stretching
05	1640.6	0.117	C=C stretching
06	1456.6	0.122	$-CH_3$ Asymmetrical stretching
07	1410.4	0.0972	C-O bending
08	1373.0	0.0958	C-CH_3 bending
09	1204.1	0.143	
10	1054. 5	0.177	C=C-C-O
11	832.9	0.0861	CH=CH
12	715.7	0.128	CH_2 Rocking
13	647.4	0.127	Cis- H structure in olefins
14	523.9	0.138	Difficult to interpret due to unique for each compound

The means for three batches of worms, with five worms per batch, are the results presented in this research. EW-1, EW-2, and EW-3 were the codes assigned to the triplicate samples. The results of the analysis of the fatty acid composition of the extracted EW oil samples displays in Table-**1**. Eleven saturated fatty acids, including C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C23:0, C24:0, and C25:0, and two unsaturated fatty acids, C18:1 and C19:3, are present in all examined samples.

Stearic acid (C18:0) was the most prevalent saturated fatty acid with a range of 13.24 to 13.99%. EW-2 had the highest concentration of stearic acid, while EW-1 had the lowest. Palmitic acid (C16:0) was the second-highest, with a range of 12.33 to 13.46%. EW-3 had the highest concentration of palmitic acid, while EW-1 had the lowest. Myristic acid (C14:0) and lauric acid (C12:0) were at 8.62-8.66% and 6.13-6.673.40%, respectively. Pentadecylic acid (C15:0), margaric acid (C17:0), tricosylic acid (C23:0), and pentacosylic acid (C25:0) are examples of odd-number fatty acids that were found to range between 5.65 and 5.89%, 4.53 and 4.94%, 3.27 and 3.50%, and 1.91 and 2.11%, respectively.

The other components of saturated acid are lignoceric acid (C24:0), which was found to range between 4.20 and 4.32%, and arachidic acid (C20:0) and behenic acid (C22:0), which were likewise found to range between 6.43 and 6.70% and 3.88 and 4.25%, respectively. EW-1, EW-2, and EW-3 have total saturated fatty acids (SFA) of 70.86%, 73.61%, and 73.22%, respectively. Oleic acid (C18:1) is the predominant fatty acid in the unsaturated group; its percentages in EWO-1, EWO-2, and EWO-3 were 16.88%, 17.75%, and 17.52%, respectively. Nonadecatrienoic acid (C19:1) came in second, with percentages of 12.26%, 8.63%, and 9.27%, respectively.

The studied extracted oil sample chromatogram was in a satisfactory peak shape under the optimal chromatographic conditions, according to Fig. 1, which displays example chromatograms of various fatty acids.

Fig. 2 shows a representative ATR-FTIR spectrum of EW-1 sample, it illustrates the predicted functional groups' characteristic bands in Table-**2**. The C-H stretching vibration of a cis double bond is represented by the band at 3011 cm-1, However, the

aliphatic-CH3 and -CH2 fatty acid hydrocarbon chains' symmetric and asymmetric vibrations are represented by the bands at 2922 and 2855 cm-1, respectively [67, 68]. At 1708 cm^{-1}. The carbonyl (C=O) functional groups of the ester and acid, respectively, display distinctive triglyceride stretching bands. The aliphatic group's C=C stretching was detected at 1640 cm^{-1}.

Three extracted oil samples of *L. Mauritii* (Ew-1, Ew-2, And Ew-3) were examined; because the three EW oil samples were chosen from the same location, there was no discernible variation in the fatty acid makeup of any of the samples.

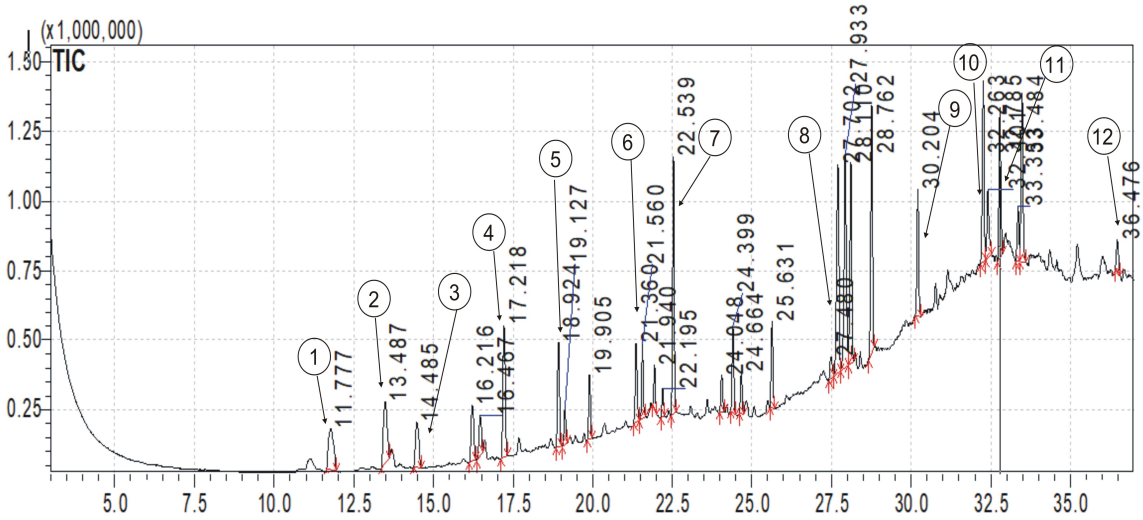

Fig-1: Gas chromatogram (excellent peak shape) of various fatty acids., using Agilent 6890 N gas chromatography instrument coupled with an Agilent MS-5975 inert XL mass selective detector and an Agilent autosampler 7683-B injector (Agilent Technologies, Little Fall, NY, USA) Under ideal chromatographic circumstances

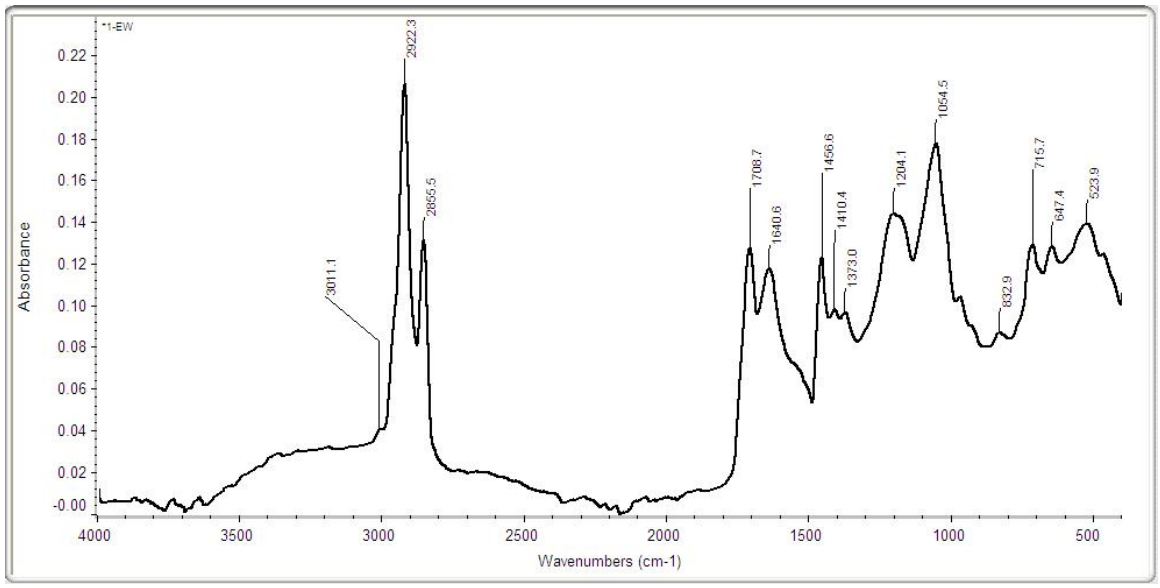

Fig- 2: Representative FTIR spectrum of EW-1 sample, illustrates the characteristic bands of extracted oil of earthworm *lampito mauritii,* kinberg of the expected functional groups in Table-2.

According to the asymmetric stretching of CH_3 aliphatic groups was banded at 1456 cm^{-1} [69]. Bending of the C-O group is the cause of the band 1410 cm^{-1}. The C-CH_3 groups' bending vibrations are the cause of the bands at 1373 and 1204 cm^{-1}. The C=C-C-O group is characterized by the band at 1054 cm^{-1}. In contrast, CH=CH is allocated to the band at 832 cm^{-1}. The CH_2 group's rocking vibration is located at 715 cm^{-1}. Long chain fatty acids are characterized by the out-of-plane vibration of cis-H caused by the overlap of CH2 disubstituted olefins, which is the cause of the band at 715 and 647 cm^{-1} [69].

Since ancient times, EWs have been used in medicine [70]. Extracts made from EWs tissue have long been utilized to cure a variety of illnesses in East Asia and North America [71]. Numerous bioactive compounds that have the potential to be employed as drugs have been found by researchers studying the medicinal effects of EWS and the advancement of biochemical technologies. These compounds were used to treat a range of illnesses since they demonstrated a variety of actions, including fibrinolytic, anticoagulative, anticancer, and antibacterial [43]. Polyunsaturated fatty acids have positive impacts on cardiovascular [18], immunological [72] and lipid regulation aspects of both normal health and chronic illnesses.

Three *L. mauritii* extracted oil samples (EW-1, EW-2, and EW-3) were examined. The total unsaturated fatty acid compositions were 29.14%, 26.38%, and 26.79%. Because the three EW oil samples were chosen from the same location, there was no discernible variation in the fatty acid makeup of any of the samples. Under ideal chromatographic circumstances, the representative chromatogram of several fatty acids has an excellent peak shape (Fig-**1**). The makeup of fatty acids is the same of the three samples in the current investigation.

The findings showed that an important class of fatty acids with possible medicinal uses is present in *Lampito mauritii*, Kinberg Sindh Pakistan. Proteins, peptides, enzymes, and physiologically active compounds can be obtained easily and affordably from agricultural soil or by growing worms through vermiculture. Their enormous therapeutic value can be utilized to investigate novel pharmaceutical industry study.

Conclusion

Many animals are used as food, including locusts, termites, caterpillars, and more. It is necessary to conduct research on these crustaceans' nutritional worth. High concentrations of vital amino acids, such as Essential fatty acids like arachidonic acid and linoleic acids, as well as minerals and amino acids like methionine, leucine, lysine, and valine, show that earthworm meal is a suitable food supplement for fish and poultry. Enhancing local knowledge and understanding of earthworms and other invertebrates as sources of protein, fat, minerals, and vitamins requires work.

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