Food Research International 62 (2014) 1087–1094

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Insect lipid profile: aqueous versus organic solvent-based extraction methods

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ARTICLE INFO

Article history:
Received 17 February 2014
Accepted 30 May 2014
Available online 6 June 2014

Keywords:
Insect lipid
Edible insects
Triacylglycerol profile
Tenebrio molitor
Alphitobius diaperinus
Acheta domesticus
Blaptica dubia
ω-3/ω-6 ratio

ABSTRACT

In view of future expected industrial bio-fractionation of insects, we investigated the influence of extraction methods on chemical characteristics of insect lipids. Lipids from *Tenebrio molitor*, *Alphitobius diaperinus*, *Acheta domesticus* and *Blaptica dubia*, reared in the Netherlands, were extracted by two industrial extraction processes (aqueous and Soxhlet) and one laboratory method (Folch extraction). Chemical characterization in terms of fatty acid composition (GC-FID), triacylglycerol profile (GC) and lipid classes (TLC) was performed on all the extracted lipids. The major findings on lipid chemical characterization were the following: (1) *T. molitor* had the highest lipid content around 13%; (2) the highest yield was obtained using Folch extraction, and the lowest yield using the aqueous method (from 19 to 60% related to the lipid recovery of Folch extraction); (3) ω-3 fatty acids, which are related to health benefits, were most abundant in lipids from aqueous extraction, while ω-6 fatty acids were most abundant in Folch extractions, except for *B. dubia*; (4) lipids from Folch and Soxhlet extractions contained free fatty acids and partial glycerides, which were absent in aqueous extractions; (5) triacylglycerol distribution is similar among insect species, with high levels of ECN 50–54 and low amounts of ECN 36–38. In conclusion, aqueous extraction gave the lowest lipid yield, but provided a lipid extract low in ω-6/ω-3 ratio and with less polar lipids than Soxhlet and Folch extractions. These characteristics are desirable in edible lipids. This is the first time that the triacylglycerol profile of insect lipids is reported. It is also the first time that C18:1 and C18:2 are reported as separated isomers and that trans isomers of C16:1 and C18:1 are reported in insect lipids.

1. Introduction

Most of the attention on insects as a food source focuses on protein content. However, lipids are also a main component of insects and are produced during protein isolation (Yi et al., 2013). Lipids are a source of energy and of essential fatty acids (FA), therefore they could be used to combat malnutrition in developing countries (Smit, Muskiet, & Boersma, 2004). In populations with inadequate total energy intake, such as seen in many developing regions, dietary fats are important macronutrients that contribute to increase energy intake to more appropriate levels (FAO, 2010). Insect lipids can contribute to human nutrition by supplying energy and essential fatty acids (Ramos-Elorduy, 2008). Generally, the lipid content of insects ranges from less than 10% to more than 30% on a fresh weight basis and are relatively high in the unsaturated C18 FA, including oleic acid (18:1 cis9), linoleic acid (18:2 cis9,12) and linolenic acid (18:3cis9,12,15) (DeFoliart, 1991).

Approximately 1900 insect species are consumed globally as human food in the world *e.g.* in Africa, Asia and Latin America (van Huis, 2013). As a food source, insects are potentially nutritious, rich in protein, minerals and vitamins. Insect production is a potential agricultural business because insects have a high nutritional value and their rearing has a low environmental impact (Oonincx et al., 2010; Ramos-Elorduy, 2008; van Huis, 2013). Despite this fact, in most developed countries people dislike eating insects due to their “dirty” and “scary” image (Chen, Feng, & Chen, 2009). Extracting insect proteins and using these as a food ingredient may increase consumer acceptance. Recently, a study has been performed on extraction and characterization of proteins from five different insect species using an aqueous extraction method (Yi et al., 2013). Next to several protein-rich fractions, a lipid fraction was obtained as a by-product from the extraction. We studied the lipid composition of the extracts of four insect species obtained by this aqueous method and compared it with two other extraction methods: Soxhlet, a method with industrial application and with Folch an analytical method usually applied at a laboratory scale. Both Soxhlet and aqueous lipid extractions are of industrial relevance.

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http://dx.doi.org/10.1016/j.foodres.2014.05.052
0963-9969/

Lipid content and types of lipids in insects vary according to their species and life stage. Total lipid content for caterpillars (Lepidoptera) ranges from 8.6 to 15.2 g/100 g fresh In contrast, grasshoppers and related species (Orthoptera) have a relatively low lipid content, which range from 3.8 g to 5.3 g/100 g fresh insects (Bukkens, 1997). Insect crude lipids obtained by organic solvent-based extractions are constituted of several types of lipids *e.g.* triacylglycerols, phospholipids, sterols, glycolipids. Although several types of lipids are present in the extracts, about 80% of the lipid content is present in the form of triacylglycerols (Gilby, 1965), which serve as energy deposit for periods of high energy demand for example for prolonged flights (Beenakkers, Vanderhorst, & Vanmarrewijk, 1985). The second most important lipid class in crude insect lipid consists of phospholipids, which have an important role in membrane cell structure. The content of phospholipids in crude fat is usually below 20% but its content varies between life stage and insect species (Ekpo, Onigbinde, & Asia, 2009; Gilby, 1965). Cholesterol is the most important type of sterol present in insects. Cholesterol is also part of the structure of cell membranes. It serves as a precursor for vitamin D, bile salts and steroid hormones. Ekpo et al. (2009) studied the cholesterol content in lipids of termites (*Macrotermes bellicosus*), a caterpillar (*Imbrasia belina*), and beetle larvae of *Oryctes rhinoceros* and *Rhynchophorus pheonicis*, which are four types of insects consumed in Nigeria. They found that the average cholesterol content in insect lipid fraction was 3.6%. The presence of several types of lipids is justified by the biological role of each one of them. On the other hand, the absolute lipid content of insects depends mainly on their life stage and on their physiological requirements (Beenakkers et al., 1985).

Lipids from vegetable and animal sources are industrially extracted with non-polar solvent or with aqueous extractions (Dijkstra & Segers, 2007). Non-polar solvent extractions are based on the capacity of the non-polar solvents to dissolve lipids while aqueous extractions are based on the insolubility of lipids in water (Ricochon & Muniglia, 2010). The use of different lipid extraction methods results in different yields (Perez-Palacios, Ruiz, Martin, Muriel, & Antequera, 2008) and in extraction of different lipid classes (Christie, 1993). Similar lipid extraction yields have been reported for meat and meat products by using Soxhlet and Folch extractions. However, Folch is an analytical method applied mainly in the laboratory, while Soxhlet is an extraction method widely used in industry. The solvents used include carbon disulfide, petroleum naphtha, benzene, trichloroethylene, alcohol, pentane, supercritical carbon dioxide, and especially commercial hexane (Kemper, 2013). Lower yields have been consistently reported with aqueous based lipid extraction when compared with non-polar solvent extractions (Ricochon & Muniglia, 2010). Because of safety, quality, and environmental issues, aqueous extractions are industrially applied to extract animal fat and vegetable oils.

Several studies have been published on FA composition of insects. While most of these studies focused on wild-caught edible insects (Bukkens, 1997; Rumpold & Schluter, 2013), some included reared insects to be used as animal feed (Finke, 2002) using mainly organic solvent-based extractions. It is known that the method of extraction affects the types of lipids extracted; hence, the FA composition of the lipids is expected to be affected. The degree in which the FA profile is affected will depend on the proportion of lipid classes originally present in the sample and this varies from species to species. So far no assessment on the effect of extraction method of insect lipid composition has been done. Such knowledge is of relevance in view of future expected industrial bio-fractionation of insects. For these reasons, it was decided to perform a complete chemical lipid analysis on reared insects commercially available in the Netherlands and to assess the effect of the lipid extraction method used. The four insect species under study are reared as food for human consumption and for insectivorous animals.

2. Materials and methods

Four insect species were selected based on their commercial availability in the Netherlands. The insect species studies were as follows: Yellow mealworm (*Tenebrio molitor*) and Lesser mealworm (*Alphitobius diaperinus*) (Coleoptera); house cricket (*Acheta domesticus*) (Orthoptera); and the Dubia cockroach (*Blaptica dubia*) (Blattodea). The first three insect species are considered for human consumption and the fourth as animal feed. All insect species were obtained from the commercial supplier Kreca V.O.F. (Ermelo, the Netherlands). The feed for *T. molitor* and *A. diaperinus* consisted mainly of wheat, wheat bran, oats, soy, rye, corn, carrot and beer yeast, and the feed for *A. domesticus and B. dubia* consisted mainly of carrot and chicken mash.

2.1. Extraction methods

All live insects were fastened for about 24 hours. After this period, the insects were stored for half an hour in the freezer at −50 °C. Next, the frozen insects were put in liquid nitrogen and subsequently grinded using a blender (Braun Multiquick 5, 600 W, Kronberg, Germany). The frozen, grinded insects were freeze-dried until arriving at stable weight and its moisture content was determined (GRI Vriesdroger, GR Instruments B.V., Wijk bij Duurstede, the Netherlands). Insects were stored at −20 °C for subsequent operations. All experiments were performed in triplicate.

2.1.1. Insect lipids extracted with Soxhlet extraction

Total lipid content was determined using representative samples of 10 g freeze-dried insect powder. The lipids were extracted using a Soxhlet apparatus for 6 hours (Biosolve, CAS nr. 110-54-3) using petroleum ether (CAS nr. 0101316465) as a solvent. Afterwards, petroleum ether was removed using a rotary evaporator (R420, Buchi, Switzerland) at 350 mbar in a water bath at 40 °C for about 30 min, and further increase up to 60 °C until no solvent was seen. The lipid extracts were stored under nitrogen atmosphere at −20 °C for further analysis.

2.1.2. Insect lipids extracted with Folch extraction

Total lipid content was determined using representative samples of 5 g freeze-dried insect powder. Grinded insects were mixed with 200 mL dichloromethane/methanol (2:1) solution. The mixture was shaken for 20 seconds. Next, the mixture was sonicated (Sonicator Elma transsonic T700, Germany) for 10 min and then shaken for 2 hours on a rotary shaker (Edmund Bühler Gmbh SM-30, Hechingen, Germany). After adding 25 mL of demineralised water, the mixture was centrifuged at 1006 *g* for 20 min at room temperature. The upper layer containing non-lipid compounds was removed from the bottle using a glass pipette. Subsequently, the lower layer containing the lipids solubilized in organic solvents was filtered using a paper filter. To avoid losses, the bottles and the filters were flushed with dichloromethane two times. Next, the organic solvents were evaporated for 1.5 hours using a rotary evaporator at 800 mbar flushed with nitrogen and with a water bath at 40 °C. The amount of Folch lipid extracts (FLE) was calculated. The lipid extracts were stored under nitrogen atmosphere at −20 °C for further analysis.

2.1.3. Insect lipids extracted with an aqueous extraction

The extraction method was based on an aqueous method described by (Yi et al., 2013) with some modifications. First, 200 g of frozen insects was mixed with 600 mL of demineralized water and blended for 1 min, followed by 15 min of sonication. The obtained insect suspension was sieved through a stainless steel filter sieve with a pore size of 350 μm. After that, the insect suspension was centrifuged at 15,000 *g* for 30 min at 4 °C. Three fractions were obtained from the filtrate. From top to bottom: the lipid fraction, the supernatant, and the pellet. Subsequently, the lipid fraction was centrifuged at 15,000 *g* for 30 min at

40 °C. After the second centrifugation, a transparent anhydrous lipid extract was obtained in the upper layer of the centrifuged tube. Two other fractions were obtained, these were a thin cream layer and a lower layer with water and water soluble compounds. The amount of aqueous lipid extracts (ALE) was calculated. The lipid extracts were stored under nitrogen atmosphere at −20 °C for further analysis.

2.1.4. Feed lipid extracted with Soxhlet extraction

Insect feed was freeze-dried until arriving at stable weight (GRI Vriesdroger, GR Instruments B.V., Wijk bij Duurstede, the Netherlands). Afterwards, lipids were extracted using a Soxhlet apparatus for 6 hours (Biosolve, CAS nr. 110-54-3) using petroleum ether (CAS nr. 0101316465) as a solvent. Afterwards, petroleum ether was removed using a rotary evaporator (R420, Buchi, Switzerland) at 350 mbar in a water bath at 40 °C, and further increased up to 60 °C until no solvent was seen. The lipid extracts were stored under nitrogen atmosphere at −20 °C for further analysis. All experiments were performed in duplicate.

2.2. Chemical characterization

2.2.1. Determination of fatty acid composition

The FA composition of the lipid extract from four insect species and insect feeds were analysed as fatty acid methyl esters (FAME) prepared by transesterification in accordance with international standard ISO5509:2000(E). The determination of FA composition was performed by means of gas chromatography with flame ionization detector (GC-FID) (Thermo Scientific Trace GC Ultra) using a WCOT fused silica column (100 m × 0.25 mm i.d., Coating Select Fame, Varian, the Netherlands) in accordance with international standard ISO15885:2002(E)IDF184:2002(E).

2.2.2. Qualitative determination of lipid classes

A qualitative determination of lipid classes of insect lipid extracts was achieved by silica gel thin layer chromatography (TLC). The extracted lipids and reference compounds were dissolved in dichloromethane (40 μg/μL). In short, 5 μL of lipid extracts and reference compounds was applied in spots to a silica gel plate of 20 × 20 cm, with a thickness of 500 μm (Analtech Inc., Newark, DE). The plates were developed in glass chambers using hexane/diethyl ether/acetic acid (70:30:1, v/v) as mobile phase (Kaluzny, Duncan, Merritt, & Epps, 1985). The following references for lipid classes were used: L-α-phosphatidylcholine (CAS nr. 8002-43-5) for phospholipids, monopalmitin (CAS nr. 32899-41-5) for monoacylglycerols, dipalmitin (CAS nr. 26657-95-4) for diacylglycerols, free cholesterol (CAS nr. 57-88-5) for free sterols, palmitic acid (CAS nr. 57-10-3) for free fatty acids (FFA), tripalmitin (CAS nr. 555-44-2) for triacylglycerols, and cholesteryl butyrate (CAS nr. 521-13-1) for sterols. Iodine vapour (CAS nr. 7553-56-2) was used to reveal the lipid classes.

2.2.3. Determination of triacylglycerol (TAG) profile by equivalent carbon number (ECN)

Equivalent carbon number (ECN) was determined in insect lipid extracts. ECN is a quantitative determination of the size distribution of the TAG in lipids. This information complements that of the FA composition. The determination of TAG profile was performed by means of gas chromatography with flame ionization detector (GC-FID) (CP-380, Varian) using a WCOT SimDist fused silica column (5 m × 0.53 mm i.d., DF = 0,17 μm, Varian, The Netherlands) in accordance with the reference method of ComissionRegulation(EC)No.273/2008 Annex XX. A certified reference of TAG containing a wide range of TAG (from 24 to 54 carbons) was used as calibration standard (IRMM/BCR519, Fluka, the Netherlands). The ECN of a triacylglycerol is an indirect way to determine the molecular weight of TAG because it is related to the number of carbons and to the number of double bonds in the TAG. ECN is determined by the formula ECN = $N - 2n$ in which N is the number of carbon atoms in the three FA that make up the TAG and 'n' is the total number of double bonds present in the TAG (Christie & Han, 2010).

2.3. Statistical analysis

To test for significant differences between the extraction methods, two way ANOVA was performed, followed by pos-hoc LSD test. IBM SPSS statistics software (version 21; IBM Corp., Armonk, NY) was used. A significance level of $p < 0.05$ was used throughout the study.

3. Results and discussion

3.1. Total lipid content and extraction yield from different extraction methods

The proximate lipid content of four insect species was determined on live weight basis by using Folch, Soxhlet and an aqueous based method (Table 1). Extracted yield was calculated based on total lipid content of SLE/FLE as well as that of ALE/FLE (Table 1). The proximate total lipid content of four insect species ranged from 1.6 to 7.8% using aqueous extraction, from 6.0 to 12.7% using Soxhlet extraction and from 7.5 to 12.9% for Folch extraction. As expected, the highest quantities of lipids were obtained using Folch extraction as well as Soxhlet extraction, and the least amounts were extracted using the aqueous method. No significant difference on lipid content was seen between Soxhlet and Folch extractions, except for *A. domesticus* ($p < 0.001$). The lipid content obtained by aqueous method was significantly different from Soxhlet and Folch (Table 1). Aqueous extraction led to 40.9, 58.3 and 60.3% of lipid yield (ALE/FLE) for *B. dubia*, *A. diaperinus* *T. molitor*, respectively. In contrast, the lipid yield of *A. dometicus* was only 19%. A similar trend was seen for Soxhlet extraction, with Soxhlet extraction the lipid yield obtained was very close to the yield obtained by Folch extraction, reaching almost 100% for *T. molitor*, *A. diaperinus* and *A. domesticus*; however for *B. dubia* the lipid yield was only 74.8%. The crude fat content of *T. molitor* was about 12.7 g/100 g fresh insects, which is comparable to previous findings (Finke, 2002; Ghaly & Alkoaik, 2009; Jones, Cooper, & Harding, 1972). The crude fat content for *A. domesticus* (adult) was comparable to the ranges described in literature, which is approximately 6–7 g/100 g insects (Barker, Fitzpatrick, & Dierenfeld, 1998; Finke, 2002). For *A. diaperinus* and *B. dubia*, no crude fat data are available in the literature.

3.2. Chemical characterization: influence of extraction method on lipid composition of insect lipid extracts

3.2.1. Fatty acid composition

The FA profiles found in the four insect species as analysed by GC-FID (Table 2) are in accordance with the profiles found in previous studies on lipids where high amounts of unsaturated FA (USFA) were found relative to saturated FA (SFA) (Finke, 2002; Paoletti, 2005; Rumpold & Schluter, 2013; Thompson, 1973). The most abundant USFA in each of the four insect lipid extracts was C18:1 cis9 and C18:2 cis9,12; the most abundant SFA was C16:0. These three FA accounted from 84.7 to 89.8 g/100 g of the FA in the lipid extracts (Table 2). In the four insect species studied, the average amount of USFA ranged from 64 g/100 g in *A. diaperinus* and *A. domesticus* to 75 g/100 g in *T. molitor* and *B. dubia*. Other minor FA present in insect lipid extracts included trans, ω -3 and CLA FA.

In the current study, C18:1 cis9 and C18:1 trans12, and C18:1 cis11 and C18:1 trans15 co-eluted in the GC column. Since the cis isomers are the most abundant in nature we expect that the detected peak in the chromatogram corresponds mainly to the cis isomers. Lately the chromatographic techniques in lipid analysis had become more powerful. Better isomer separations have been achieved and the detection limit has dramatically decreased. Therefore, it is now possible to

Table 1
Total lipid content of four insect species after aqueous, Soxhlet and Folch extraction expressed on live weight basis. Extraction yields of aqueous and Soxhlet extractions relative to Folch extraction (mean ± S.D., $N = 3$).

Insect species	Extracted lipid (g/100 g fresh insects)			Yield (%) (ALE/FLE)	Yield (%) (SLE/FLE)
	Aqueous	Soxhlet	Folch		
T. molitor	7.8 ± 0.4^{A}	12.7 ± 2.4^{B}	12.9 ± 0.2^{B}	60.3 ± 0.4	98.4 ± 2.4
A. diaperinus	5.5 ± 1.0^{A}	10.7 ± 0.5^{B}	9.4 ± 1.0^{B}	58.3 ± 1.4	113.5 ± 1.1
A. domesticus	1.6 ± 0.1^{A}	6.0 ± 0.3^{B}	8.0 ± 1.1^{C}	19.2 ± 1.1	74.8 ± 1.1
B. dubia	3.1 ± 0.3^{A}	7.6 ± 0.2^{B}	7.5 ± 0.3^{B}	40.9 ± 0.4	100.5 ± 0.4

Statistical analysis: one-way ANOVA-LSD. Figures with different letters between rows are significantly different ($p < 0.05$).
ALE, aqueous lipid extracts; SLE—Soxhlet lipid extracts; FLE—Folch lipid extracts.

detect very low amounts (from 0.01 g/100 g of lipid approximately) of a FA present in the lipid fraction. These advances in the techniques allow us to challenge previous knowledge regarding trans FA in unprocessed lipids. In the present study, we confirm the presence of C16:1 trans9 and C18:1 trans11 in the lipid extracts of the insects under study. C16:1 trans9 has been previously reported in ruminant milk fats (Destaillats, Jean-Denis, Arul, Wolff, & Angers, 2002) but not in insect lipids. C16:1 trans9 and C18:1 trans11 could be products of some bacteria biohydrogenation because trans isomers cannot be synthesized *de novo* by the insect. It is well known that insect gut possesses a wide microbial biota with potentially complex interactions with the diet, insect developmental age or genotype (Douglas, 2013). Therefore, it is plausible that these microbes produce trans FA. Moreover, neither C16:1 trans9 nor C18:1 trans11 was present in the insect diet (Table 2). C18:1 trans11 was found only in *A. domesticus* lipid extracts which makes this FA specific for this insect species. The proportion of C18:1 cis9 in the lipid extracts was higher as compared with the proportion of this FA in the diet. Moreover, C18:2 cis9,12 and C18:3 cis9,12,15 in the lipid extracts strongly decreased as compared with the proportion of this FA in the diet. A possible synthesis pathway for C18:1 cis9 in insect lipids is that C18:2 cis9,12 and C18:3 cis9,12,15 are completely biohydrogenated to C18:0 by insect gut microbiota. Afterwards, C18:0 is desaturated by Δ9 desaturases producing C18:1 cis9. Previously, Thompson (1973) suggested that C18:1 cis9 is the product of Δ9 desaturase which adds a double bond to C18:0. However, little is known about the biohydrogenation of unsaturatred C18 to C18:0 in insect gut. It is known that ruminal microbial biohydrogenation of unsaturated C18:0 produces C18 trans isomers. In addition, this trans isomers are found only in *A. domesticus* (C18:1 trans11) and in *B. dubia* (C18:2 cis9trans11). To confirm our suggestion, studies on biohydrogenation of FA in insect gut are required.

Table 2
Fatty acid composition (g/100 g) of lipids extracted from four insect species (mean ± S.D.). Lipids were extracted with an aqueous and two organic solvent lipid extraction methods ($N = 2$).

	Feed		*Tenebrio molitor*			*Alphitobius diaperinus*			*Acheta domesticus*			*Blaptica dubia*		
Fatty acid	A[a]	B[b]	Soxhlet[c]	Folch	Aqueous	Soxhlet[c]	Folch	Aqueous	Soxhlet[c]	Folch	Aqueous	Soxhlet[c]	Folch	Aqueous
C12:0	0.89	0	0.23	0.14	0.28	0	0	0	0.30	0.16	0.27	0.16	0.00	0.20
C14:0	0.96	0	3.11	3.18	3.60	0.65	0.63	0.73	1.80	1.55	1.65	1.05	1.12	1.22
C16:0	17.21	12.60	18.52	17.31	17.96	25.18	23.33	24.68	25.99	23.69	24.81	18.05	20.18	19.30
C16:1 trans 9	0	0	0.70	0.69	0.65	0.74	0.70	0.83	0.68	0.60	0.75	0.23	0.09	0.26
C16:1 cis9	0.59	0	2.09	2.05	2.25	0.22	0.11	0.26	2.09	1.78	2.04	5.17	4.95	5.51
C17:0 anteiso	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05
C17:0	0	0	0	0	0	0.40	0.39	0.39	0.20	0.11	0	0.21	0.12	0.22
C17:1 cis9	0	0	0	0	0.10	0	0	0.06	0	0	0	0.18	0.09	0.18
C18:0	1.40	2.80	2.43	2.82	2.53	8.55	8.65	7.78	6.09	6.76	4.61	3.71	4.27	3.67
C18:1 trans 11	0	0	0	0	0	0	0	0	0.21	0.12	0.23	0	0	0
C18:1 cis9 / C-18:1 trans 12	15.43	25.17	49.50	49.15	49.15	38.49	37.35	39.20	29.14	26.63	30.23	51.38	51.51	49.48
C18:1 trans 15 / C-18:1 cis11	1.07	1.04	0.22	0.12	0.23	0.34	0.17	0.37	0.82	0.81	0.77	0.61	0.58	0.61
C18:2 cis9,12 (ω-6)	54.99	53.76	21.82	23.35	21.67	23.28	26.79	23.61	29.11	34.35	31.80	17.36	15.65	17.62
C18:2 cis9, trans 11	0	0	0	0	0	0	0	0	0	0	0	0.15	0.08	0.18
C18:3 cis6,9,12 (ω-6)	0	0	0	0	0	0	0	0	0	0	0	0	0.10	0
C18:3 cis9,12,15 (ω-3)	5.61	4.50	0.84	0.85	0.89	1.14	1.14	1.24	1.56	1.59	1.74	1.24	1.05	1.28
C20:0	0	0.17	0	0	0	0.38	0.36	0.37	0	0.09	0	0.14	0.08	0.14
C20:1 cis11	0.61	0	0	0	0.05	0	0	0	0	0.06	0	0	0	0.12
C20:4 cis5,8,11,14 (ω-6)	0	0	0	0	0	0	0	0	0	0.09	0	0	0	0
C20:3 cis11,14,17 (ω-3)	0	0	0	0	0	0	0.08	0	0	0	0	0	0	0
C20:5 cis5,8,11,14,17 (ω-3, EPA)	0.77	0	0	0	0	0	0	0	0.64	0.46	0.75	0	0	0
C22:6 cis4,7,10,13,16,19 (ω-3, DHA)	0.49	0	0	0	0	0	0	0	0	0	0	0	0	0
Total SFA	20.45	15.56	24.28	23.52	24.43	35.14	33.35	34.05	34.37	32.35	31.33	23.30	25.77	24.78
Total USFA	79.55	84.45	75.17	76.21	74.99	64.19	66.33	65.55	64.23	66.48	68.28	76.31	74.08	75.22
Total CLA FA	0	0	0	0	0	0	0	0	0	0	0	0.15	0.08	0.18
Total ω-3 FA	6.87	4.50	0.84	0.86	0.89	1.14	1.22	1.24	2.20	2.04	2.48	1.24	1.05	1.28
Total ω-6 FA	54.99	53.76	21.82	23.35	21.67	23.28	26.79	23.61	29.11	34.44	31.80	17.36	15.74	17.62
Total MUFA	17.69	26.20	52.51	52.01	52.43	39.77	38.31	40.70	32.92	29.99	34.01	57.56	57.21	56.14
Total PUFA	61.86	58.25	22.66	24.20	22.56	24.42	28.01	24.85	31.31	36.48	34.28	18.75	16.88	19.08
ratio ω-6/ ω-3	8.00	11.96	25.98	27.15	24.35	20.42	21.96	19.04	13.26	16.85	12.82	14.06	14.95	13.76
Total unknown	0	0	0.56	0.27	0.58	0.67	0.32	0.41	1.41	1.17	0.39	0.39	0.15	0

Values are means of duplicate analysis.
[a] Feed A—*Tenebrio molitor* and *Alphitobius diaperinus*.
[b] Feed B—*Acheta domesticus* and *Blaptica dubia*.
[c] Petroleum ether was used as solvent for extraction of lipids.

The most abundant ω-3 FA found in insect lipids was C18:3 cis9,12,15 (α-linolenic acid), which was present (0.84–1.74 g/100 g) in all four insect species. Previous work on terrestrial and aquatic insects have reported only traces (0.09–0.28 g/100 g) of EPA (C20:5 cis5,8,11,14,17) (Fontaneto et al., 2011) and DHA (C22:6 cis4,7,10,13,16,19) in insect lipids or even reported their absence. In this study we found EPA in lipids extracted from *A. domesticus* (mean = 0.62 g/100 g). EPA was not found in the other three insect species studied. The presence of EPA and DHA in insect lipids has been related to their diet and to the need of these FA for specific physiological purposes (Beenakkers et al., 1985; Fontaneto et al., 2011; St-Hilaire et al., 2007). EPA was not present in the feed from *A. domesticus*, indicating that this insect was able to biosynthesize EPA probably from C18:3 cis9,12,15 (Christie, 2013). Previous studies showed that *Gryllus assimillis*, an insect species related to *A. domesticus*, is able to increase its EPA content when C18:3 cis9,12,15 is increased in the diet (Komprda, Zornikova, Rozikova, Borkovcova, & Przywarova, 2013). In our study, C18:3 cis9,12,15 was also present in *A. domesticus* diet and it is likely that this FA is a precursor of EPA.

The highest ω-6/ω-3 ratio in insect lipids was found in *T. molitor*, where the ratio was about 27:1. *A. domesticus* was the insect with the lowest ω-6/ω-3 ratio (17:1). The ω-6/ω-3 ratio of the four insect species is higher than the ratio recommended by FAO, which is 10:1 (FAO, 2010). The high amounts of C18:2 cis9,12, which is the main ω-6 FA present in our lipid extracts, could be responsible for the high ω-6/ω-3 ratio. In our study, C18:2 cis9,12 was the most abundant FA in both diets accounting for more than 53% of the FA present in the feed (Table 2). Since the FA composition of the insect lipids is influenced mainly by insect feed (Bukkens, 1997), an insect diet with low amounts of C18:2 cis9,12 might reduce the presence of this FA in insect lipids with a consequent reduction in ω-6/ω-3 ratio.

Higher amounts of PUFA and ω-6 FA were extracted by Folch compared to Soxhlet or aqueous extraction, except for *B. dubia*. Omega-3 FA content was higher in lipids obtained by aqueous extraction compared to those from Folch or Soxhlet method. These differences in FA have a direct effect on the ω-6/ω-3 ratio. The ω-6/ω-3 ratios of the lipids from the aqueous extraction were lower than the ones from Folch extraction. *A. domesticus* showed the greatest difference between extraction methods.

3.2.2. Lipid classes

Lipid classes are separated in silica gel TLC plates according to their degree of polarity: polar lipids such as phospholipids will stay close to the origin while non-polar lipids such as triacylglycerols will run to the upper part of the plate. As expected, triacylglycerols were the largest and strongest spot at the upper part of all the TLC plates indicating that this is the most important lipid in the extractions (Fig. 1). The method of extraction affected the lipid composition of the lipid extracts. The methods that involve organic solvents are able to extract a wide range of lipid classes while the aqueous method extracted mainly non-polar lipids, namely triacylglycerols, carotenoids and cholesterol esters (Fig. 1). In our samples, phospholipids were present in organic solvent extracts but not in the aqueous extraction. It is likely that during the aqueous extraction the phospholipids, which are the most polar lipids, remain in the aqueous layer because in the presence of water, phospholipids hydrate and become water soluble (Dijkstra, 2011). Hydration does not occur when organic solvents are used, therefore, all phospholipids will stay in the solvent layer.

Monoacylglycerols, diacylglycerols and FFA were extracted with Soxhlet and Folch methods. These three types of glycerides are typical products of lipolysis, which can be formed during sample preparation (Kramer & Hulan, 1978). Kramer and Hulan (1978) showed that the temperature at which the sample is homogenized, prior to enzymatic inactivation by the extracting solvents, affects the lipolitic activity and therefore the amounts of lipolized products. During grinding and drying of our samples for Folch and Soxhlet extractions, our samples were kept frozen at temperatures below −20 °C to reduce degradation of the compounds. However, by TLC we show that FFA, monoacylglycerols and diacylglycerols were present in our lipid extracts. One plausible explanation for the presence of partial glycerides is that diacylglycerols are present in the insects itself. It is known that diacylglycerols are the main form in which lipids are transported in insects (Horne, Haritos, & Oakeshott, 2009), therefore, they will be present in the lipid extracts. However, the presence of FFA cannot be explained in this way and high amounts are not expected in tissues because they perturb the membrane structure (Christie, 2012). Another plausible explanation for the presence of partial glycerides is that these insects contain cold active lipases. These cold active lipases have a low optimum

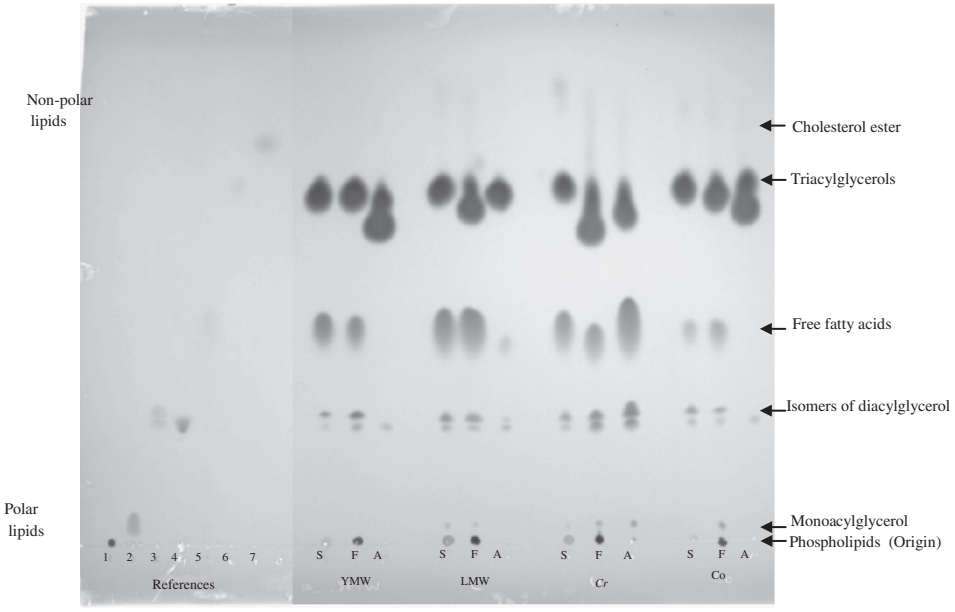

Fig. 1. Lipid class separation of extracted lipids from 4 insect species in silica gel thin layer chromatography. Mobile phase used—hexane:diethyl ether:acetic acid (70:30:1). References: 1. L-α-phosphatidylcholine (phospholipids), 2. monopalmitin (monoacylglycerol), 3. dipalmitin (diacylglycerol), 4. free cholesterol, 5. palmitic acid (free fatty acid), 6. tripalmitin (triacylglycerol), 7. cholesterylbutyrate. Samples: S—Soxhlet extraction with petroleum ether, F—Folch extraction, A—aqueous extraction, YMW—*Tenebrio molitor*, LMW—*Alphitobius diaperinus*, Cr—*Acheta domesticus*, Co—*Blaptica dubia*.

temperature, have high activity at very low temperatures and are active under low water conditions (Joseph, Ramteke, & Thomas, 2008). The conditions during freeze-drying of our samples could be appropriate for the activation of cold active lipases that will degrade the lipids in our insect samples. If Soxhlet extraction is used for industrial extraction of insect lipids, a refining process will be necessary to eliminate phospholipids, FFA and partial glycerides such as mono- and diacylglycerols.

The lipids extracted from aqueous extraction lacked mono- and diacylglycerols and contained less FFA than Soxhlet and Folch extractions. This could be explained by the fact that in the presence of sodium or calcium salts, FFA form soaps and increase their solubility in water (Rustan & Christian, 2005). Calcium and sodium are micronutrients commonly found in insects (Bukkens, 1997). These salts could have been solubilized in the supernatant of our aqueous extractions, which could have bind FFA forming soaps, which in turn will have increased the solubility of these lipids. Mon- and diacylglycerols are insoluble in water but can form stable emulsions (Dolezalkova, Janis, Bunkova, Slobodian, & Vicha, 2013; Miklos, Xu, & Lametsch, 2011). If mono- and diacylglycerols were produced during sample preparation, it is likely that they remain in the cream layer seen after the second centrifugation during the aqueous extraction.

In lipid from *A. domesticus* extracted from aqueous extraction polar and non-polar lipids were found; we suggest that *A. domesticus* contains phospholipids with a low hydration capacity that will allow them stay in the lipid phase. The reason for the presence of partial glycerides and FFA in these insect lipids is unclear. If *A. domesticus* lipids extracts free of phospholipids are desirable, a change in the pH during extraction can be applied (Dijkstra, 2011).

3.2.3. Determination of equivalent carbon number (ECN)

The distribution of FA within the glyceride molecule is genetically controlled; therefore, each oil and fat has a unique and typical pattern (Bonvehi & Coll, 2009). The information on ECN helps to identify the presence of foreign lipids and it is typically used in the industry to detect adulterations. The extracts from the four insect species in this study shared similar patterns (Fig. 2). All four lipids had large concentrations of glycerides with an ECN 50–54 and low concentrations of glycerides with ECN of 36–38. *A. domesticus* lipid extracts had the highest amount of glycerides with ECN of 36–38. The ECN pattern of our extracted lipids from insects differs from most vegetable oils and animal fats (Bergqvist & Kaufmann, 1993; Bonvehi & Coll, 2009). The most abundant glycerides in vegetable oils have an ECN from 44 to 48 (Bonvehi & Coll, 2009), while the most abundant glycerides in tallow have an ECN of 48–50 (Bergqvist & Kaufmann, 1993). Vegetable oils and animal fats had no glycerides below ECN 38, except for bovine milk fat (Haddad, Mozzon, Strabbioli, & Frega, 2012; Kuksis, Marai, & Myher, 1973), which has a large amount of short and medium chain FA. Regarding differences among extraction methods, we observed that the ECN pattern changed with the type of extraction. Glycerides with ECN of 36–38 were extracted with Folch and Soxhlet extractions but not in aqueous extraction. It is likely that phospholipids and diacylglycerols are the responsible glycerides for the peaks at ECN 36–38 because no short chain FA, which are typically in this range of ECN, were found in any of these lipids. Moreover, with lipid class determination, we show that phospholipids and diacylglycerols were present in Folch and Soxhlet but not in aqueous extractions (Fig. 1). Phospholipids and diacylglycerols are glycerides with two FA which will give a lower carbon number than triacylglycerols which have three FA.

Further, the ECN analysis allows quantification of free cholesterol, which cannot be synthesize de novo and is synthesize from plant sterols. Cholesterol was present in amounts lower than 3.6% (Table 3). The highest proportion of free cholesterol in all four insect species was seen for the Folch extraction and the lowest proportions were found for aqueous extraction. Our results are in agreement with the data found by Ekpo et al. (2009) who studied the cholesterol content on four types of oil insects consumed in Nigeria.

4. Conclusions

We extracted lipid fractions from four insect species by using two industrially relevant methods (Soxhlet and aqueous extractions) and an analytical method (Folch extraction). *Tenebrio* contained the highest amounts of lipids among four insect species. With respect to the extraction yield, the highest yield was obtained using Folch extraction. The

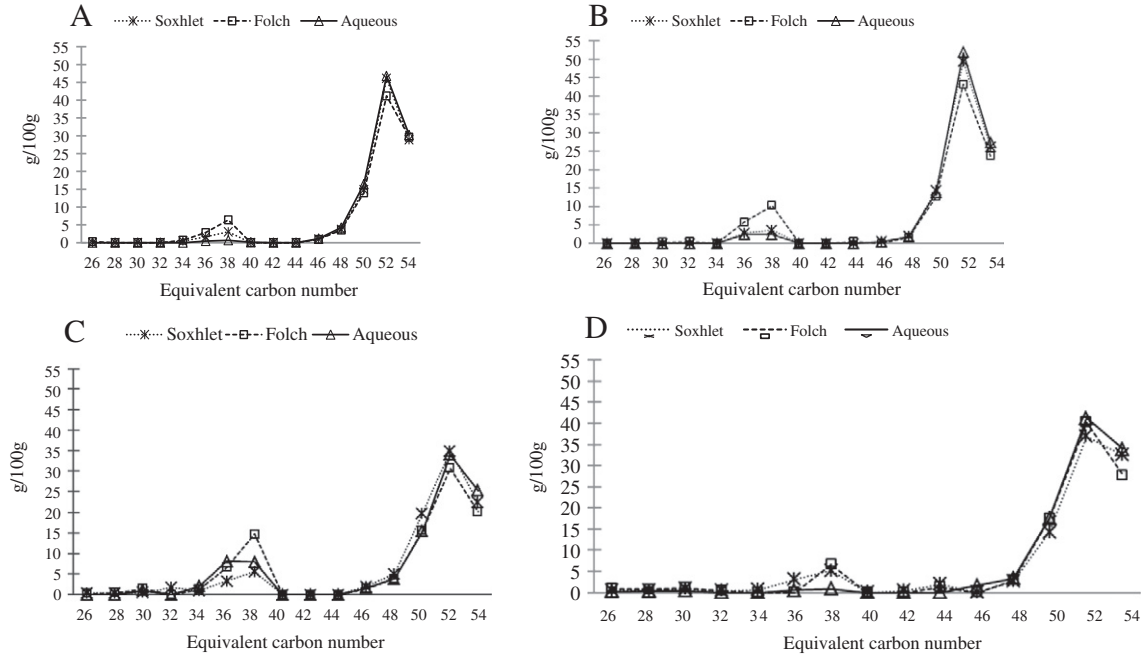

Fig. 2. Triacylglycerol composition of insect fat expressed as equivalent carbon number (g/100 g). Lipid extracted by Folch, Soxhlet and by aqueous method. A. *Tenebrio molitor*; B. *Alphitobius diaperinus*; C. *Acheta domesticus*; D. *Blaptica dubia* ($N = 2$).

Table 3
Free cholesterol (g/100 g) in lipids extracts from four insect species (mean ± S.D, $N = 2$).

Insect species	Aqueous method		Soxhlet method		Folch method	
T. molitor	N.D.		0.41	± 0.02	0.59	± 0.01
A. diaperinus	0.37	± 0.01	1.51	± 0.01	1.76	± 0.05
A. domesticus	0.67	± 0.01	3.32	± 0.03	3.58	± 0.03
B. dubia	0.24	± 0.001	0.49	± 0.01	0.91	± 0.004

N.D.—not detected.

lowest yields for lipid extractions were obtained using the aqueous method. Relative to the lipid recovery of Folch extraction, aqueous extraction could recover 40–60% for *T. molitor*, *A. diaperinus* and *B. dubia*. The lowest lipid yield, around 19%, was obtained for *A. domesticus*. The lipid yield of Soxhlet extraction was close to 100% for *T. molitor*, *A. diaperinus* and *A. domesticus*, and about 75% for *B. dubia*.

The method of extraction affected the quantity and types of lipids and the FA profile of the insect lipid extracts. Higher ω-6/ω-3 FA ratios were extracted by Folch method while aqueous extraction had the lowest ratio, hence having the highest ω-3 FA content. Essential FA EPA was present in the extracted lipids from *A. domesticus*. Lipid extracts obtained by Folch and Soxhlet contained polar and non-polar lipids while lipid extracts obtained by aqueous method contained mainly non-polar lipids. Therefore, if Soxhlet extraction is the chosen method for insect lipid extraction, a refining process should follow the extraction since FFA and monoacylglycerols are undesirable in lipid extracts. This process will not be necessary with an aqueous extraction because the lipids from these extractions lack phospholipids and partial glycerides. The ECN pattern in insect lipid extracts is similar between all four insect species. They are rich in CN 50–54 and have low amounts of CN 36–38. This pattern is unique and does not resemble any other vegetable or animal lipid source. It is the first time that a detailed isomer separation of C18:1 and C18:2 is reported and trans isomers of C16:1 and C18:1 are reported in insect lipids.

Acknowledgments

We thank the Dutch Ministry of Economic Affairs and the Consejo Nacional de Ciencia y Tecnología (CONACYT, Distrito Federal, Mexico) for financial support. The authors thank Hanneke van de Rond from Food Quality and Design from Wageningen University for carrying out the lipid extractions. We kindly thank Herman van den Brink, from Qlip laboratories (Zutphen, The Netherlands) for carrying out the GC analysis. Many thanks to Arnold van Huis from the Entomology Laboratories from Wageningen University for his critical review to this work.

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