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METHOD

Optimised preparation of fish tissue samples for the determination of fatty acids by gas chromatography-mass spectrometry

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Fatty acids play an important role in the nutrition of fish and in assessing the quality of fish products for human consumption. The usual quantification of fatty acids using gas chromatography-mass spectrometry (GC-MS) requires the extraction and derivatization of fatty acids to fatty acid methyl esters (FAMEs). We compared the yields of fatty acids from liver samples of *Oreochromis niloticus* and the recovery of the internal standard C23:0 with five different methods for the production of FAMEs: (1) a method based on standard procedures according to EN ISO 12966-2:2017, (2) a modification of the first method with replacement of H_2SO_4 by HCl for acid-catalyzed methylation, (3) a modification of the first method with use of HCl for acidic methylation and replacement of the solvent isooctane by MTBE, (4) chloroform extraction and subsequent acidic methylation, as well as (5) the rapid method EN ISO 12966-3:2016. The modified standard method according to EN ISO 12966-2:2017 with HCl for acid-catalyzed methylation and the extractant MTBE proved to be the most successful method with the highest fatty acid yields and recovery of the internal standard.

Introduction

Fatty acids play an important role in the nutrition of fish. On the one hand, they are crucial for basic life functions such as growth and reproduction. On the other hand, in particular some marine fish are a valuable source of human health promoting long-chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (Tocher et al. 2019). As the PUFA profile of fish can be influenced by their diet (Babalola et al. 2011), the supply of PUFAs in aquaculture feed is of crucial importance, not only for the balanced nutrition of the fish, but also in terms of the nutritional value of the final product for the consumer. The determination of fatty acids in biological samples such as fish tissues by gas chromatography-mass spectrometry (GC-MS) requires sample preparation

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that includes extraction with organic solvents and transesterification to fatty acid methyl esters (FAMEs), whereby first extraction and then methylation or the reverse is possible (Ostermann et al. 2014).

The extraction with a solvent consisting of chloroform and methanol (MeOH) as introduced by Folch, Lees, and Stanley (1957) and modified by Bligh and Dyer (1959) is an efficient standard for lipid extraction from animal tissues. However, due to health and environmental concerns, alternative extraction procedures have been sought in recent years (Chiu and Kuo, 2020; Saini et al. 2021). Ostermann et al. (2014) figured out, that lipid extraction with methyl *tert*-butyl ether (MTBE)/MeOH is as efficient as the Bligh-Dyer method, which can replace the toxic and environmentally harmful chloroform. In the standard procedure EN ISO 12966-2:2017, however, isooctane is used as a solvent. Various reagents are described for acid-catalysed methylation, including hydrochloric acid (HCl), acetyl chloride (CH_3COCl), sulfuric acid (H_2SO_4) and boron trifluoride (BF_3) (Chiu and Ching-Hua Kuo 2020). Some of these can cause safety issues, such as exothermic reactions, and BF_3 is toxic, so these substances are not easy to handle. Furthermore, Christie (2019) states that H_2SO_4 is a strong oxidising agent and therefore cannot be recommended for the acidic methylation of PUFAs. For alkaline methylation, sodium methoxide ($NaOCH_3$), potassium hydroxide (KOH) or sodium hydroxide (NaOH) in MeOH is used (Chiu and Kuo, 2020; EN ISO 12966-2:2017). The base-catalysed transesterification with trimethylsulfonium hydroxide (TMSH) is described as a rapid method in EN ISO 12966-3:2016, where methylation during injection into the gas chromatograph saves sample preparation time. The standard EN ISO 12966-2:2017 combines alkaline methylation of fatty acids with sodium methylate (NaOH in MeOH) and acidic methylation with methanolic H_2SO_4 to implement as many fatty acids as possible to FAMEs.

With the intention of developing a simple, sensitive method for the quantification of fatty acids from fish tissues with the lowest possible amounts of solvents hazardous to health and the environment, we have modified the standard method EN ISO 12966-2:2017. With this method paper we compare the fatty acid yields and recovery of the internal standard obtained with the standard procedure according to EN ISO 12966-2:2017 (Method 1) with two modifications, firstly by replacing H_2SO_4 with HCl for the acid-catalysed methylation (Method 2) and secondly by using HCl for acidic methylation and replacing the solvent isooctane with MTBE (Method 3). In addition, these methods were compared with a chloroform extraction and subsequent acid methylation (Method 4) as well as with the rapid method EN ISO 12966-3:2016 (Method 5).

Methods

Samples

All fatty acid determinations described below were performed with the same liver sample of *Oreochromis niloticus* from our institute's stock, which was previously freeze-dried, homogenized under liquid nitrogen in a mortar and re-dried to remove excess moisture. Aliquots (approximately 20 mg) were stored at -80 °C until further processing. All aliquots were prepared and measured with four replicates.

Method 1 (M1): Sodium methylate and sulfuric acid derivatisation and isooctane extraction (EN ISO 12966-2:2017)

Alkalic- and subsequent acid-catalysed transesterification and extraction were performed based on DIN EN ISO 12966-2:2017. First, 2 mL of sodium methylate in MeOH (0.2 mol/L) were mixed with liver samples, 200 µL of internal standard (C23:0, 1000 µg/mL in acetone; Neochema, Bodenheim, Germany) was added and boiled for 20 min. Using a few drops of phenolphthalein as a pH indicator, 1 M H_2SO_4 in methanol was added until neutralization, and an excess of 0.2 mL 1 M methanolic H_2SO_4 was added and boiled for 5 min. The samples were shaken for 15 min with 4 mL of sodium chloride (0.4 g/mL). Isooctane (1 mL) was added and mixed well. After centrifugation for 5 min at 500 x g, the organic phase was transferred to an amber GC vial and stored at -20 °C until determination by GC-MS.

Method 2 (M2): Sodium methylate and hydrochloric acid derivatisation, isooctane extraction

Derivatisation and extraction were performed as described in Method 1, but 1 M H_2SO_4 was replaced by 1 M HCl in MeOH (using concentrated HCl, not anhydrous HCl).

Method 3 (M3): Sodium methylate and hydrochloric acid derivatisation, MTBE extraction

Derivatisation and extraction were performed as described in Method 1, but 1 M H_2SO_4 was replaced by 1 M HCl in MeOH and isooctane extraction was replaced by MTBE extraction.

Method 4 (M4): Chloroform-methanol extraction, sulfuric acid derivatization

The method was performed according to Dhellemmes et al. (2020) with minor modifications. Liver samples (20 mg) were extracted with 12 mL chloroform/methanol (2:1, v/v) containing butylated hydroxytoluene (0.2 mg/mL) as antioxidant, 170 µL of internal standard (C23:0) was added. The samples were shaken for 3 h and centrifuged for 4 min at 4500 x g.

The supernatants were evaporated using a rotary evaporator in a nitrogen atmosphere at 40 °C. For derivatisation, the dried extract was redissolved in 2.5 mL of MeOH, 2.5 mL of 5 % H_2SO_4 in MeOH was added and heated to 70 °C for 3 h. Liquid extraction of FAME sample was performed with 4.5 mL hexane for 15 min and centrifuged for phase separation. The supernatant was collected and the extraction was repeated twice with 2 mL of hexane. Hexane extracts were pooled and neutralized with potassium hydrogencarbonate (2.8 g/L), centrifuged for 1 min at 4500 x g. Samples were washed with 1 mL ultra-pure water. Samples were stored in an amber GC vial at -20 °C until determination by GC-MS.

Method 5 (M5): MTBE extraction, TMSH derivatization (EN ISO 12966-3:2016)

A rapid method for the base-catalyzed methylisation with trimethylsulfonium hydroxide (TMSH) was performed according to DIN EN ISO 12966-3:2016 with some modifications regarding the sample quantity and volumes. Liver samples were extracted by shaking vigorously for 10 min in 1 mL MTBE. After centrifugation, 120 µl of the samples were placed in an amber GC vial, mixed with 10 µl of internal standard (C23:0), 370 µl of MTBE and 250 µl of TMSH. The samples were stored at -20 °C and fatty acids identified by using GC-MS analysis.

GC-MS analysis and data evaluation

Gas chromatographic analysis was performed on a 7890 B gas chromatograph equipped with a 7000C Triple Quadrupole GC/MS system (Agilent Technologies, Santa Clara, CA, USA) and a CP Sil 88 capillary column (100 m × 0.25 mm, 0.2 µm, Agilent). Helium was used as carrier gas at a flow rate of 1 mL/min. The temperature gradient started at 80 °C maintained for 1 min, increased within 35 min (4 °C / min) to 220 °C, maintained for 5 min, increased within 5 min (4 °C / min) to 240 °C, maintained for 15 min and ended at a total runtime of 61 min. The injection volume was 1 µL and the injection port was set to 250 °C. Peaks were automatically detected, assigned to the individual fatty acids and their concentration calculated using the peak areas (MassHunter software, Agilent). Supelco 37-component FAME-Mix (Merck, Darmstadt, Germany) was used as standard for calibration. As the yields of fatty acids and recovery of internal standard (ratio of the measured to the added concentration) are to be evaluated in the comparison of methods, the results are given as they were measured and are not corrected with the internal standard for possible variations. Kruskal-Wallis tests and Dunn post-hoc tests were performed to analyse significant differences between the methods using IBM SPSS Statistics 22.0.

Results and Discussion

The most abundant fatty acids detected are consistent with previously published data on the fatty acid composition of liver of tilapia (Molnár et al. 2012). For the most prevalent fatty acids (Figure 1 A-E), such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2n6) and DHA (C22:6n3), a uniform picture of the measured yields emerged. Lowest yields were obtained using the rapid method with MTBE extraction and TMSH derivatisation, as described in EN ISO 12966-3:2016 (M5). The yields were significantly lower than with the most successful method using MTBE extraction and alkaline and acidic derivatisation with sodium methylate and methanolic HCl (M3) ($p = 0.001$), and DHA was even below the limit of quantification, while it was quantifiable with all other methods. The failure of this method to quantify DHA in the present study is consistent with the finding of a generally inadequate transesterification efficiency of TMSH for PUFA (Ostermann et al. 2014). Due to this shortcoming, the rapid TMSH method (M5) is omitted from the following evaluation.

The replacement of H_2SO_4 by HCl as the acid catalyst for transesterification in the standard EN ISO 12966-2:2017 (M2) had hardly any increasing influence on the yields. The additional replacement of the solvent isooctane by MTBE (M3), on the other hand, led to a significant improvement in yield for all fatty acids ($p < 0.007$), emphasising that the extraction of lipids from biological samples is a decisive step in sample preparation (Ostermann et al. 2014). The classic chloroform/MeOH extraction with subsequent acidic methylation with methanolic H_2SO_4 (M4) consistently produced yields that were between those of the standard method (M1) and the modified method with MTBE extraction and alkaline and acidic methylation (M3).

The addition of the internal standard C23:0 at the beginning of the sample preparation allows an estimation of the losses during the entire process. The recovery of C23:0 follows the same pattern as the yields of fatty acids from the liver samples (Fig. 1 F). Thus, differences in the sensitivity of the methods can be compensated by correction with the recovery rate of the internal standard, although a high sensitivity is desirable for accurate measurements. The modified standard method EN ISO 12966-2:2017 with the replacement of the extractant isooctane by MTBE and HCl as acid catalyst (M3) resulted in the highest recovery of 79 %, which was significantly higher than with the original method (M1) ($p < 0.005$).

The standard method EN ISO 12966-2:2017 (M1) replaces the extractant chloroform of the classic methods according to Folch, Lees, and Stanley (1957) or Bligh and Dyer (1959) with the less toxic isooctane, which is favourable in terms of health and environmental concerns. Similar to the standard method (M1) the modified standard method (M2 and M3) requires little equipment and is time-effective, allowing approximately 20 samples to

be prepared for GC-MS analysis within 6 hours. While replacing the acidic catalyst H_2SO_4 with potentially unfavourable oxidative properties by HCl (M2) had little enhancing effect on the efficiency of transesterification, the use of MTBE instead of isooctane as extractant (M3) led to a significant increase in fatty acid yield and made the method the most effective in our comparison. Furthermore, unlike isooctane, MTBE is not classified as harmful to health or the environment. Due to the highest fatty acid yields, good reproducibility, the reasonable time effort and reduced health risk, we consider the modified standard method (M3) to be well suited for the routine processing of fish tissue samples for fatty acid quantification by GC-MS.

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Author contributions

V.S. and K.K. designed the study, V.S. and J.R.I. conducted the measurements, V.S., K.K and C.S. drafted the manuscript. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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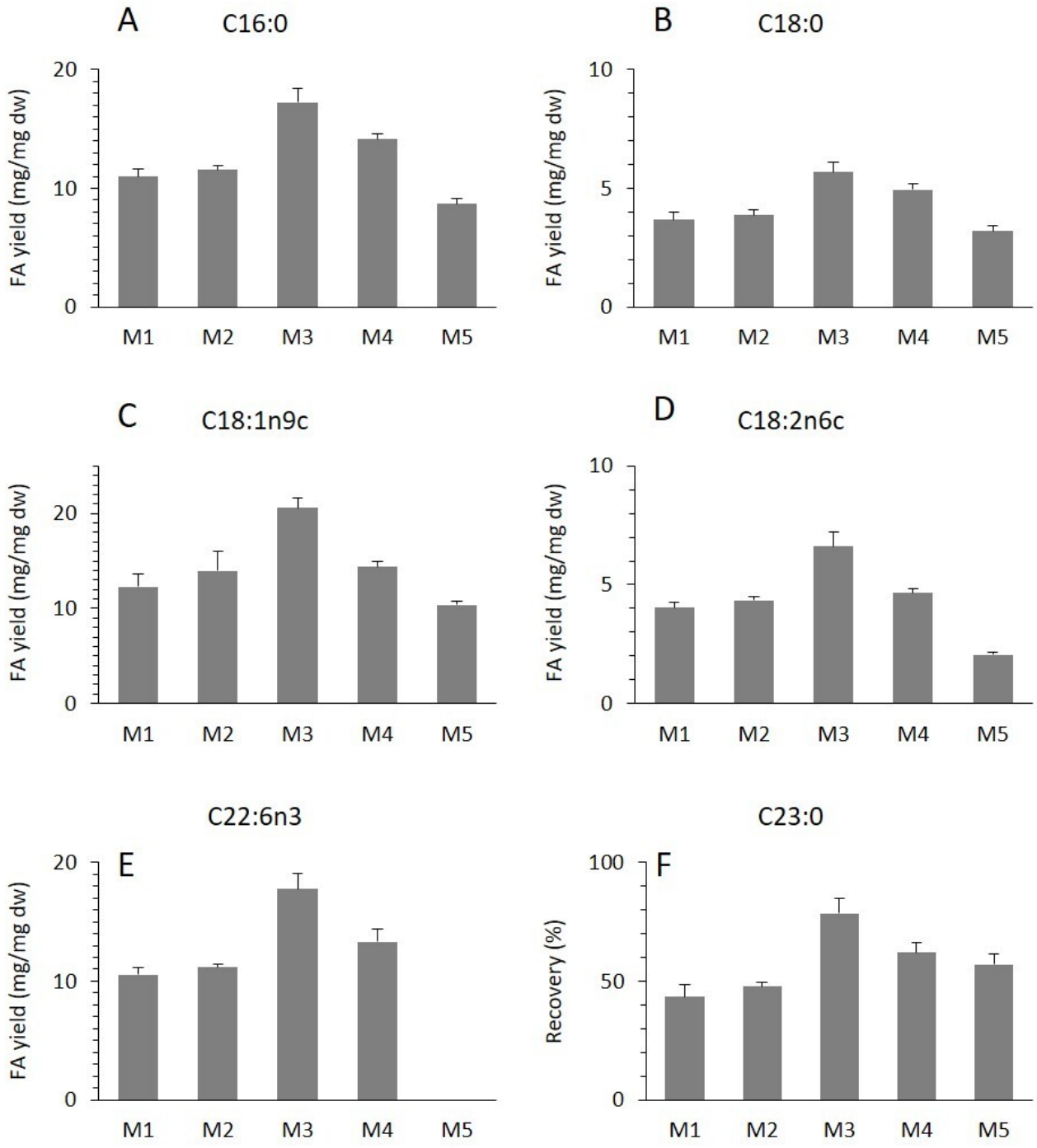

Figure 1. Fatty acid yields (mg/mg dw) from the liver of *Oreochromis niloticus* and recovery of internal standard (C23:0) obtained by different methods for the production of FAMEs (mean ± SD): (M1) Sodium methylate and sulfuric acid derivatisation, isooctane extraction (EN ISO 12966-2:2017); (M2) Sodium methylate and hydrochloric acid derivatisation, isooctane extraction; (M3) Sodium methylate and hydrochloric acid derivatisation, MTBE extraction; (M4) Chloroform-methanol extraction, sulfuric acid derivatization; (M5) MTBE extraction, TMSH derivatization (EN ISO 12966-3:2016); (A) palmitic acid (C16:0), (B) stearic acid (C18:0), (C) oleic acid (C18:1n9), (D) linoleic acid (C18:2n6), (E) docosahexaenoic acid (DHA, C22:6n3), (F) internal standard (C23:0).

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