Article

Quantification of Essential Fatty Acids in the Heads of Nile Tilapia (*Oreochromis niloticus*) Fed with Linseed Oil

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O objetivo do presente trabalho foi avaliar os efeitos do óleo de linhaça sobre as concentrações dos teores de ácidos graxos poli-insaturados n-6 e n-3 (AGPI) em cabeças de tilápia do Nilo (*Oreochromis niloticus*), um resíduo do pescado normalmente descartado durante o processo de filetagem. A quantificação dos ácidos graxos poli-insaturados foi realizada utilizando-se cromatografia gasosa (CG), sendo a concentração dos ácidos graxos expressa em termos de massa absoluta através da utilização do éster metílico (23:0) como padrão interno e do TCFx (fator de correção teórica do FID). As tilápias receberam dietas com níveis crescentes de óleo de linhaça (0,00, 1,25, 2,50, 3,75 e 5,00%) em substituição ao óleo de girassol (controle) durante um período de cinco meses. A ingestão de LNA causa a dessaturação sequencial e alongamento das cadeias carbônicas de ácidos graxos presentes nas cabeças de peixes, levando a um aumento em todos os AGPIs n-3. Este experimento demonstrou claramente que a adição de óleo de linhaça na ração de tilápia do Nilo aumenta significativamente as concentrações (em 100 g^{-1} de cabeça) de LNA (100,8 para 973,6 mg), EPA (4,8 para 82,1 mg) e DHA (24,0 para 125,4 mg). Estas alterações contribuem acentuadamente para melhorar a razão AGPI n-6/n-3 e, desta forma, pode ser utilizada em suplementos alimentícios, transformando as cabeças de tilápia em valiosa alternativa como fonte de alimento para dieta humana.

The objective of this study was to examine the effects of linseed oil on n-6 and n-3 polyunsaturated (PUFA) fatty acid content in the heads of Nile tilapia (*Oreochromis niloticus*), these are normally discarded during the filleting process. The quantification of PUFA was carried out in absolute weights through the use of methyl ester (23:0), as an internal standard, and TCFx (theoretical FID correction factor) by GC gas chromatography. Tilapias were given diets with increasing levels (0.00, 1.25, 2.50, 3.75 and 5.00%) of linseed oil as a replacement for sunflower oil (control) for a period of five months. The ingestion of LNA causes sequential desaturation and elongation in fish heads, leading to an increase in all n-3 PUFAs. This experiment demonstrated clearly that adding linseed oil to the feed of Nile tilapia can markedly increase the amounts (*per* 100 g^{-1} of head) of LNA (100.8 to 973.6 mg), EPA (4.8 to 82.1 mg), and DHA (24.0 to 125.4 mg) in their heads, and thus, may be used to help balance n-6/n-3 PUFA ratios in dietary supplements, and creating a valuable alternative food source in the human diet.

Keywords: tilapia head, linseed oil, fatty acids, quantification, correction factor

Introduction

Globalization, new eating habits, the increasing ingestion of vegetable oils, and industrial expansion are factors that have resulted in a higher human intake of the fatty acids omega-6 series in relation to those of the n-3 series, causing an increase in the n-6/n-3 ratio. This has

lead to the development of numerous research projects related to human diets.

In Brazil, in recent years, there has been an increase in the production and consumption of freshwater farmed fish, mainly the Nile tilapia (*Oreochromis niloticus*). However, studies have shown that these freshwater fish, including the tilapia, have low polyunsaturated fatty acids omega-3 (PUFA n-3) contents including alpha-linolenic acid (LNA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3),

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and docosahexaenoic (DHA, 22:6n-3) fatty acids, when compared to those from the sea or from natural habitats.[1-4]

Alpha-linolenic acid, an omega-3 fatty acid (FA), and linoleic acid (LA, 18:2n-6), an omega-6 FA, are considered strictly essential fatty acids because cannot be synthesized by humans, and araquidonic acid (AA, 20:4n-6) is an abundant FA present in the brain cells as in other cells of the body. The fetus receives AA from the mother to aid in the development of the brain, and during nursing the AA present in the maternal milk simulates the development of the baby's brain.[5] It is well reported, in humans, that an increase in the ingestion of PUFA n-3, especially EPA and DHA, in diet reduces the risk of heart disease[6] and rheumatoid arthritis.[7]

Linseed oil is one of the word's most important vegetable sources of LNA,[8,9] which is a precursor of the long chain n-3 PUFA series, in freshwater fish. In these ones, LNA and LA are metabolized by the same sequential desaturation and elongation enzyme systems, which results in the production of n-3 and n-6, such as EPA, DHA, and AA.[10,11]

Recent studies have indicated that some fish parts, treated as waste, are appropriate for human nutrition.[12,13] Researchers have also shown the existence of significant concentrations of n-3 PUFA in visceras,[14] muscular tissue,[15] head[16] and liver[9] of tilapia.

On the other hand, the accurate analysis of the n-3 PUFA is of growing importance, however the amount of research related to the quantification of FA, absolute weights of FA *per* gram of sample, in Brazilian products is still small. Results are frequently expressed as relative percent data, using only simple normalization methods (area normalization) this is currently used in nutrition works in Brazil. The disadvantages of area normalization are error propagation: the strong interdependence of results. Thus, if one fatty acid is wrongly estimated (or omitted when unknown), the results for the other fatty acids are affected. Furthermore, the results cause difficulties in interpretation and, therefore, in the labeling of nutritional values.[17]

Researchers have quantified the amounts of PUFA in absolute weights *per* gram of sample for informative labeling of nutritional supplements. This conversion requires the use of internal standards, and for accuracy, correction factors (CFx) for the flame ionization detector (FID) response must be applied by Joseph and Ackman,[18] and Milinsk *et al.*[19]

The FID response of FA is generally proportional to the mass of carbon present in the sample. CFx, can be determined either through the analysis of authentic standards or from accurate predictions of loss of response based on measurements made for compounds of related molecular structure. The official methods of the Association of Analytical Chemists and the American Oil Chemists's Society provide clear guidelines for accurate quantification of n-3 PUFA. Both sources stipulate the use of methyl tricosanoate (23:0 FAME) as internal standard and wax-type capillary columns are mandatory.[17]

A gas chromatographic method using a capillary column for analysis of encapsulated fish oil was studied collaboratively in 21 laboratories and the authors recommend the capillary column GC method for quantification n-3 PUFA of fish oils in absolute weights through the use of 23:0 FAME as internal standard by Joseph and Ackman[18] and for accurate determination of PUFA the use of a theoretical correction factor (TCFx) or empirical correction factor (ECFx) has been recommended.[18,20,21] Recently Brazilian researchers have quantified the amounts of LNA, EPA and DHA in freshwater fish, showing the relevance of the correct quantification of FA.[22]

The purpose of the present study was to investigate incremented addition of linseed oil in fish feed and to evaluate its influence on the absolute weights of LNA, EPA, DHA, LA and AA in the heads of Nile tilapia using analytical techniques that are more appropriate for the quantification of fatty acids.

Experimental

Animal management and treatments

The experiments were carried out in the Aquaculture Laboratory of the Biology Department of Universidade Estadual de Maringa, Brazil. Five treatments in five duplications were used with 125 Nile tilapias (*Oreochromis niloticus*) with initial mean individual weight of 88 ± 6 g distributed in 25 ponds (1000 L each). The treatments consisted of the addition of linseed oil (0.00, 1.25, 2.50, 3.75 and 5.00%) as a substitute of sunflower oil (control) in feeds (Table 1). After five months, the Nile tilapia head was removed and kept in polyethylene packing (in N_2 atmosphere) at −18 °C. At the beginning of each analysis, the samples were allowed to equilibrate to room temperature and homogenized.

Analytical determination

The moisture content in heads and feeds was determined as described by AOAC[23] and the total lipids (TL) were determined by the Bligh and Dyer[24] method.

The fatty acid methyl esters (FAME) were prepared by methylation of the total lipids (TL), as described by method of Joseph and Ackman.[18] Methyl esters was separated by gas chromatography using a Varian 3300 (USA) gas

chromatograph fitted with a flame ionization detector (FID) and a fused-silica DB-WAX capillary column (30 m and 0.25 mm i.d.) (J&W Scientific, Folsom, CA). The operation parameters were as follows: detector temperature, 280 ºC; injection port temperature, 250 ºC; column temperature, 170 ºC for 16 min, programmed to increase at 2 ºC min^{-1} up to 210 ºC, with final holding time of 25 min; carrier gas, hydrogen at 0.8 mL min^{-1}, linear velocity of 38 cm s^{-1}, with an oxygen filter coupled to the feed line; nitrogen was used as the makeup gas at 30 mL min^{-1}; split injection at 1:50 ratio. For identification of the fatty acids, the retention times of the fatty acids were compared to those of standard methyl esters (Sigma, St. Louis, MO). Equivalent chain-length values (ECL) were used[17,25] as well as a coupled system of a gas chromatograph-mass spectrometer Shimadzu QP 5000 and fragmentation by electron impact, 70 eV. Retention times and peaks area percentages were automatically computed by a Varian 4290 integrator.

Quantification of the LNA, EPA, DHA, LA, and AA (mg g^{-1} of TL) was made against 23:0 FAME (methyl tricosanoate) from Sigma (USA) as an internal standard (IS), as described by Joseph and Ackman.[18]

$$\text{Fatty acid (mg g}^{-1}\text{ TL)} = \frac{(A_X)\,(W_{IS})\,(TCF_X)}{(A_{IS})\,(W_X)\,(1.04)} \times 1000 \quad (1)$$

where TL = total lipid, A_X is the peak area % (LNA, EPA, DHA, LA, and AA), A_{IS} is the peak area of the 23:0 FAME, W_{IS} is the weight (mg) of IS added to the sample, W_X is the sample weight (mg), TCF_X is the theoretical correction factor,

and 1.04 is conversion factor necessary to express results as mg of fatty acids rather than as FAME. The results were recalculated from mg g^{-1} of TL to mg 100 g^{-1} Nile tilapia head.

In this study saturated FAMEs 14:0, 16:0, 18:0, 20:0, 22:0, 24:0, and polyunsaturated FAMEs LNA, EPA, DHA, LA, and DHA were used to verify the empirical correction factor (ECFx) in relation to the internal standard of 23:0 FAME and compared with the theoretical FID correction factor (TCFx) (Table 2).

Statistical analysis

The values of the means were statistically compared by Tukey's test at 5% with one-way ANOVA. Data were processed using the Statistica software.[26]

Results and Discussion

There were no significant differences ($p > 0.05$) in total lipids and moisture contents of feeds (Table 1) among the treatments II, III, IV and V. The amount of moisture (66.5 to 68.6%) and total lipids (9.6 to 11.4%) found in tilapia heads in this experiment were according to Stevanato *et al.*[13] with values moisture of 67.24% and total lipids 9.56%, *in natura* tilapia heads.

Because unsaturated FAMEs are subject to autoxidation, it is not possible to obtain and maintain high purity standards. It has been demonstrated by Bannon *et al.*[21] that theoretical FID correction factor TCFx may be applied

Table 1. Ingredients and composition of experimental feeds

Ingredients	Treatments[A]				
	I	II	III	IV	V
	(g 100 g^{-1})				
Linseed oil	0.00	1.25	2.50	3.75	5.00
Sunflower oil	5.00	3.75	2.50	1.25	0.00
Corn	16.93	16.93	16.93	16.93	16.93
Soybean meal	51.62	51.62	51.62	51.62	51.62
Wheat meal	20.00	20.00	20.00	20.00	20.00
Sugarcane silage	1.28	1.28	1.28	1.28	1.28
Calcium (carbonate)	1.74	1.74	1.74	1.74	1.74
Dicalcium phosphate	2.41	2.41	2.41	2.41	2.41
Premix[B]	0.50	0.50	0.50	0.50	0.50
Composition					
Total lipids	7.6 ± 0.3a	7.7 ± 0.4a	8.0 ± 0.6a	8.0 ± 0.7a	7.8 ± 0.3a
18:3n-3 LNA	13.6 ± 2.0a	79.4 ± 3.8b	140.2 ± 13.0	202.1 ± 11.6	272.4 ± 8.6e
20:5n-3 EPA	nd	nd	nd	nd	nd
22:6n-3 DHA	nd	nd	nd	nd	nd

[A]Treatments: I (0.00%), II (1.25%), III (2.50%), IV (3.75%), and V (5.00%) of linseed oil completed up to 5.00% with sunflower oil. [B]Premix (mineral and vitamin supplement). Results expressed as averages of the three replicates. Averages followed by different letters in the same line are significantly different ($p < 0.05$) by Tukey's test. nd: not detected; LNA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

for unsaturated FAMEs after verifying that the empirical correction factor (ECFx) for saturated FAMEs are in accordance with TCFx values.

Table 2 presents ECFx values obtained from a FAME standard saturated: 14:0, 16:0, 18:0, 20:0, 22:0, 24:0, and polyunsaturated (double ≥ 2) 18:3n-3, 20:5n-3, 22:6n-3, 18:2n-6, and 20:4n-6, and the TCFx one for methyl tricosanoate. There is a possibility of error when quantifying n-3 PUFA in marine fish oil as their ethyl esters and using 23:0 as the internal standard. Under certain chromatographic conditions, the internal standard peak area may eluate with the 21:5n-3 ethyl ester peak area.[27]

The empirical factors for saturated FAME were closer to the TCFx when compared with the values obtained for polyunsaturated FAME, error factor (E) close to 1.000 (Table 2). This agreement between the ECFx and TCFx values showed that the chemical and instrumental parameters were optimized and therefore the TCFx could be adopted as recommended by Joseph and Ackman[18] for

the quantification of PUFA. The use of a TCFx has been recommended by several researchers.[17,20,21]

Thus, in this study it was used the TCFx in the quantification of polyunsaturated fatty acids are reported in mg g^{-1} of total lipids (LT) by using the equation 1, and the results were recalculated from mg g^{-1} of TL to mg 100 g^{-1} Nile tilapia head (Table 3).

The increase of the concentration of LNA acid in feeds was well established (Table 1), values between 13.6 and 272 mg 100 g^{-1} of feeds, with a significant difference (p < 0.05) among the treatments with additional linseed oil. EPA and DHA were not detected in the feeds. Fatty acid profiles of commercial feeds used in the treatment of species grown in fish farms in Brazil, presented low values of LNA (3.3%) and a high value of LA (38.8%).[28]

The same enzymes serve to elongate both n-3 and n-6 fatty acids. There is therefore a competitive effect in that an excess of LNA could interfere with conversion of LA to AA, a highly desirable fatty acid.[29] In this study, the value

Table 2. Response factor empirical and theoretical in relation to methyl tricosanoate

FAMEs	Response factor		
	Empirical (ECF$_X$)^a	Theoretical (TCF$_X$)^b	E= ECFx / TCFx
14:0	1.065 ± 0.009	1.080	0.986
16:0	1.049 ± 0.009	1.054	0.995
18:0	1.028 ± 0.009	1.034	0.994
20:0	1.027 ± 0.008	1.035	0.992
22:0	0.990 ± 0.009	1.005	0.985
24:0	0.987 ± 0.09	0.995	0.992
18:3n-3 (LNA)	1.050 ± 0.016	1.014	1.036
20:5n-3 (EPA)	1.044 ± 0.023	0.987	1.058
22:6n-3 (DHA)	1.044 ± 0.017	0.971	1.075
18:2n-6 (LA)	1.052 ± 0.018	1.021	1.030
20:4n-6 (AA)	1.048 ± 0.019	0.994	1.054

^{a}Values are means ± SD for six replicates (n = 6). ^{b}Reference 17. FAMEs: fatty acid methyl esters; LNA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; AA: arachidonic acid. ECFx: empirical correction factor; TCFx: theoretical correction factor.

Table 3. Total lipids, moisture and LNA, EPA, DHA, LA and AA contents of the tilapia heads

Composition	Treatments^A				
	I	II	III	IV	V
Total lipids (%)	9.6 ± 0.3a	11.4 ± 0.4b	11.4 ± 0.2b	11.4 ± 0.3b	11.4 ± 0.3b
Moisture (%)	68.6 ± 0.2a	66.2 ± 0.2b	66.7 ± 0.3b	66.6 ± 0.3b	66.5 ± 0.3b
Fatty acids (mg 100 g^{-1} of head)					
18:3n-3 LNA	100.8 ± 16.0a	296.4 ± 15.0b	652.1 ± 28.0c	755.8 ± 25.0d	973.6 ± 32.0e
20:5n-3 EPA	4.8 ± 0.8a	22.8 ± 1.9b	33.1 ± 1.6c	39.9 ± 2.2d	82.1 ± 3.1e
22:6n-3 DHA	24.0 ± 2.1a	44.5 ± 2.5b	61.2 ± 3.8c	95.8 ± 3.1d	125.4 ± 3.4e
18:2n-6 LA	2010.2 ± 150.0a	2209.2 ± 120.2a	1724.8 ± 110.3c	1618.8 ± 98.0d	1417.0 ± 82.0e
20:4n-6 AA	71.0 ± 4.5a	71.8 ± 4.8a	54.7 ± 4.2b	50.2 ± 4.0b	34.2 ± 3.6c

^{A}Treatments: I (0.00%); II (1.25%); III (2.50%); IV (3.75%) e V (5.00%) of linseed oil completed up to 5.00% with sunflower oil. Results expressed as an average of 30 replicates. Averages followed by different letters in the same line are significantly different (p < 0.05) by Tukey's test. LNA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; AA: arachidonic acid.

of the AA ranged from 71.0 mg (treatment I) to 34.2 mg (treatment V) *per* 100 g^{-1} of head.

LNA is a precursor of the n-3 fatty acid series and only LNA was present in the different feeds for this experiment, but with increasing amounts (Table 1). In conversions by elongation and desaturation of the series, EPA and DHA in the heads of the tilapia (Table 3), and some of the LNA, were not converted but stored in the fillets. Increases of the concentrations of LNA, EPA and DHA (in mg 100 g^{-1} of head), were well established in the heads, with a significant difference (p < 0.05) among all the treatments, as the substitution of the sunflower oil by linseed oil was increased.

The LNA, EPA and DHA concentrations in the tilapia head increased significantly in the I to V treatments. While the concentrations of LA and AA still tended to decrease after treatment III with increase of the concentration of LNA in the feeds. Therefore, increasing the amounts of LNA in the feed can markedly increase the amounts of LNA, EPA and DHA in the tilapia heads.

Conclusions

The effects of adding linseed oil to the feed on the fatty acid composition of the heads of Nile tilapia were a very large increase in omega-3 fatty acid concentration (LNA, EPA, and DHA), a decrease in LA and AA (omega-6 fatty acids), and a decrease in n-6/n-3 ratios. The heads of Nile tilapia could be used in diets that require high levels of omega-3 fatty acids, but use of the heads to produce omega-3 fatty-acid-rich oil still requires greater study with respect to its economic viability.

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