

Comparative Study on Total Lipid Quantification and Oxidative Rancidity Determination in Freshwater Fish Species with Different Fat Levels

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Abstract

The aim of this study was to investigate the efficiency of different extraction methods for total lipid determination and oxidative rancidity evaluation in freshwater fish with different fat levels. Total lipid content was determined by Bligh and Dyer with slight modifications and Soxhlet methods. Malondialdehyde (MDA) quantification was determined by analytical methods with and without heat application. In both freshwater fish species, Bligh and Dyer method extracted greater total lipid content ($P < 0.05$) than Soxhlet method. In addition, the heat method revealed higher MDA values ($P < 0.05$) compared to cold method in both lean fish (*Cichla ocellaris*) and high-fat fish species (*Piaractus brachypomus*). We concluded that, regardless of the fat content in the freshwater fish species, the Bligh and Dyer method and the cold method used for oxidative rancidity were more efficient than Soxhlet method and heat technique, respectively.

Keywords: *Cichla ocellaris*, *Piaractus brachypomus*, Malondialdehyde, Lipid stability, Oxidative rancidity, Total lipid content

1. Introduction

Lipids are important components in foods which directly impact on sensory properties and diet consumer, mainly due to unsaturated fatty acids present on lipid fraction (Chowdhury et al., 2012; Collewet et al., 2013). Therefore, total lipid determination is essential for known food composition. Lipids are composed by nonpolar, neutral and polar components. Muscle foods contain mainly acylglycerides, cholesterol, free fatty acids (nonpolar compounds), and phospholipids or sphingolipids which are more polar lipids (Tocher & Glencross, 2015; Wijaya et al., 2015). Nevertheless, lipid classes are highly variable depending of food matrices (Contarini & Povolo, 2013; Dey et al., 2015). In freshwater fishes, neutral lipids (eg, triglycerides, and fatty acids) represent the lipid class more expressive followed by phospholipids (polar lipids) and glycolipids (Dey et al., 2015) which are amphipathic molecules containing hydrophilic and hydrophobic portions (Ikami et al., 2000).

Several methodologies are used for total lipids determination, however, there is a lack of study comparing lipid extraction methods more often used taking into consideration food matrix evaluated, required analysis time, and factors regarding solvents applied in the analysis such as type and volume, which directly affect the amount of extracted fat (Brum et al., 2009; Kus et al., 2009; Tonial et al., 2009). Soxhlet (1879) and Bligh and Dyer (1959) methods are more commonly used for total lipids determination in fish meat (Palmeira et al., 2014a; Canto et al., 2015; Monteiro et al., 2015). Soxhlet and Bligh and Dyer methodologies are based on fat extraction utilizing organic solvents such as ether and chloroform, respectively, due to great solubility of the fat molecules on these chemical solvents (Brum et al., 2009).

Moreover, studies about lipid deterioration in fish are very important due to high susceptibility of the unsaturated fatty acids to lipid oxidation resulting on formation of intermediate (peroxides and hydroperoxides) and secondary (aldehydes, ketones, carbonyls, alcohols, and acids) compounds which negatively impact flavor and human health (Chaijan, 2008; Bertolin et al., 2011; Duthie et al., 2013; Zaki et al., 2014). Malondialdehyde (MDA) is considered the main secondary compound from lipid decomposition and their quantification is commonly used as indicator of oxidative rancidity in fishes (Monteiro et al., 2012; Palmeira et al., 2014b). There are several methods for MDA determination which basically differ in terms of the extraction method (distillation or acid addition and hot or cold extraction) (Tarladgis et al., 1960; Yin et al., 1993; Monteiro et al., 2012; Karlsdottir et al., 2014). However, the main methods used in fish are those proposed by Tarladgis et al. (1960) and Yin et al. (1993). These methodologies are based on the reaction of one molecule of MDA with two molecules of 2-thiobarbituric acid (TBA) resulting in a stable pink chromophore. These methods are called thiobarbituric acid reactive substance (TBARS) tests and the results are expressed as mg of MDA per 1 kg of sample or TBA-number (Tarladgis et al., 1960; Yin et al., 1993; Monteiro et al., 2012; Karlsdottir et al., 2014).

Despite Soxhlet method (Soxhlet, 1879) is widely used and represents the official methodology for total lipids determination (AOAC, 2012), this methodology presents some disadvantages such as long time required and reflux distillation apparatus leading to

excessive use of water and solvent. Furthermore, petroleum ether is unique solvent used in this method resulting only in apolar lipids extraction. Nonetheless, in Bligh and Dyer method (Bligh & Dyer, 1979) a solvent mixture of methanol-chloroform (2:1, v/v) is used which leads to great efficiency on total lipid extraction (polar, nonpolar and neutral fractions) depending of the food matrix (Bligh & Dyer, 1959; Conte-Junior et al., 2007; Ryckebosch et al., 2012). In addition, Bligh and Dyer method not requires reflux distillation apparatus decreasing the analysis time and solvent amount used during analytical procedure.

Regarding lipid oxidation, MDA measurement proposed by Tarladgis et al. (1960) present some analytical limitations such as distillation step (long time analysis and excessive use of water) and heating process which can catalyze lipid oxidation. On the other hand, MDA methodology described by Yin et al. (1993) is a simple and fast method based on cold extraction without distillation step.

The aforementioned methods are widely used for determination of the total lipid content and lipid oxidation in several food matrices, including freshwater fish species. Nonetheless, to the best of our knowledge, a comparative study between Soxhlet and Bligh and Dyer methods as well as the analytical techniques proposed by Tarladgis et al. (1960) and Yin et al. (1993) was not conducted in freshwater fishes with different fat levels. In this context, the aim of the present study was to investigate the efficiency of analytical techniques for total lipid determination and oxidative rancidity evaluation in freshwater fishes with different fat levels.

2. Materials and Methods

2.1 Samples

Two species of freshwater fishes with different lipid levels were used, *Cichla ocellaris* which can be considered a lean fish (<2%), and *Piaractus brachypomus* which presents high total lipid amount (>8%) (Haard, 1992). Fish samples were collected in a fish farming located in Goiás, Brazil. The mean and the standard deviation of the length (cm) and weight (g) of the samples were calculated. The length and the weight of *Cichla ocellaris* were 36.57 ± 0.51 cm and 861.1 ± 32.59 g, respectively. For *Piaractus brachypomus* the length and weigh were 39.77 ± 1.27 cm and 1205.8 ± 104.59 g, respectively. Samples were transported in insulated container with ice to the laboratory where the analyzes were made. The time from sample obtaining until arrival at the laboratory did not exceed 8 hours.

2.2 Total lipid content

Total lipid content was determined by Soxhlet extraction method using petroleum ether solvent (Soxhlet, 1879) and by Bligh and Dyer method using methanol-chloroform (2:1, v/v) (Bligh & Dyer, 1959).

2.3 Lipid oxidation

Oxidative rancidity was measured according to method described by Tarladgis et al. (1960) and Yin et al. (1993). In the first method, sample (10g) was homogenized with 97.5 mL of distilled water, 2.5 mL of hydrochloric acid (4N) was added, and samples were distilled to obtain 50 mL. An aliquot of 5 mL was transferred to a tube and 5 mL of TBA solution

(20mM) was added. The tube was heated at 60°C for 35 minutes and cooled at 4°C for 10 minutes in running water. After, reading was carried out in UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at 538 nm (Tarladgis et al., 1960). In the second method, sample (5g) was homogenized with 22.5 mL of trichloroacetic acid (11%), filtered with Whatman paper n° 1. An aliquot of 5 mL was transferred to a tube and 5 mL of TBA solution (20mM) was added. The tube was incubated in dark conditions for 20 hours. The reading was performed in UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at 532 nm (Yin et al., 1993).

2.4 Statistical analysis

One-way ANOVA with post test T Student at 5% significance level was used to identify separately differences between the methods applied (Soxhlet versus Bligh and Dyer for total lipid determination, and hot versus cold extraction for MDA measurement) for both species studied. For statistical analysis was used the program XLSTAT version 2012.6.08 (Addinsoft, Paris, France).

3. Results and Discussion

3.1 Total lipid content

Total lipid content in two freshwater fish species evaluated by Soxhlet (1879) and Bligh and Dyer (1959) methods are exhibited in Table 1. The Bligh and Dyer (1959) method extracted greater lipid content ($P < 0.05$) than Soxhlet (1879) method in both *Cichla ocellaris* and *Piaractus brachypomus* species. Previous studies also reported this difference between Soxhlet (1879) and Bligh and Dyer (1959) methods in fish species such as *Clupea harengus*, *Salmo salar*, *Gadus morhua*, *Esox Lucius* (Ewald et al., 1998), *Trachurus trachurus*, *Scomber japonicas*, and *Sardina pilchardus* (Ramalhosa et al., 2012), whereas ricotta cream (Gusso et al., 2012) and meat (Tanamati et al., 2005).

This fact can be explained by solvent mixture used in Bligh and Dyer method resulting in a great lipid extraction with broad range of polarity (Bligh & Dyer, 1959). In this method, total lipid content is extracted by a solvent mixture (chloroform-methanol-water) which is able of recovering non-polar, polar and neutral lipids (Brum et al., 2009). Furthermore, the vigorous homogenization process performed in Bligh and Dyer (1959) method making the lipid extraction more efficient (Brum et al., 2009). Another advantage of this method includes less time required (Martins et al., 2015). On the other hand, the Soxhlet method (1879) is based on use of a single solvent resulting only in non-polar lipid extraction (Lu et al., 2015). Maia et al. (1995) reported that fish genus *Piaractus* contain 94% of neutral lipids. Therefore, the use of a single solvent is not recommended for the lipid extraction of animal tissue demonstrating the major efficiency of the Bligh and Dyer (1959) method in this type of food matrix (Brum et al., 2009). Moreover, the methodology proposed by Soxhlet (1879) presents a long heating step leading to interference in the results due to peroxides formation and acidity increased (Brum et al., 2009). Regarding lipid amount, Bligh and Dyer (1959) method exhibited a great lipid extraction even in low-fat fish species (3.8%) indicating that the lipid content of fish matrix does not influence on lipid extraction capacity of this method (Maia et

al., 1999). Moreover, Ramalhosa et al. (2012) to quantify the content of lipids in the species *Scomber japonicas* showed that the Bligh and Dyer (1959) method also demonstrated superior results in fish with high fat content. Our findings indicate that Bligh and Dyer (1959) method is more efficient for lipid extraction of freshwater fish species up to approximately 12% of fat.

3.2 Lipid oxidation

Malondialdehyde (MDA) values of the two freshwater fish species using methods proposed by Tarladgis et al. (1960) and Yin et al. (1993) are presented in Table 2. The heat method (Tarladgis et al., 1960) exhibited greater MDA values ($P < 0.05$) than cold method (Yin et al., 1993) in both fish species. High temperatures and strong acidic conditions, which are required in Tarladgis et al. (1960) method, can result in possible artefactual peroxidation leading to overestimation of MDA amount (Mendes et al., 2009).

In agreement with our results, Tokur et al. (2006) observed greater MDA values in heat method than cold method in *Sardina pilchardus*, *Mugil cephalus*, *Sarda sarda* and *Pomatomus saltator*. In addition, Ulu (2004) evaluating lipid oxidation methods in chicken meat and meat products, respectively, also observed that heat method exhibit greater MDA values than cold method. Moreover, this author reported that the degree of differentiation between heat and cold methods is proportional to amount of unsaturated fat in the food matrices. The lipid oxidation is directly related to unsaturated fatty acids composition due to chemical instability of the double bonds (Martínez-Yusta et al., 2014) which can explain the great MDA values in the low fat fish species (*Cichla ocellaris*). The fatty acid composition is variable between both freshwater fishes evaluated. According to Inhamuns et al. (2009), *Cichla ocellaris* presents 35.8% of polyunsaturated fatty acids (PUFA), including high amounts of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids whereas *Piaractus brachypomus* contain 32.5% of PUFA (Murthy et al., 2015).

Therefore, our study suggests that cold method is able of generate MDA results more reliable than heat method. Moreover, the method described by Tarladgis et al. (1960) demonstrated a great coefficient of variation than Yin et al. (1993) in both fish species studied, possibly due to high number of analytical steps in this method. According to Fourier et al. (2015) the high variability of results is related to the analytical phase. In addition, the high temperature employed in heat method can be considered an interfering because the heating process may accelerate the occurrence of lipid oxidation and the TBA reaction with other components of the food matrix (Mendes et al., 2009). Similarly to our results, previous studies (Pikul et al., 1989; Wang et al., 1997) demonstrated that heat method presented greater coefficient of variation in the MDA data (15%; 2.9%) than cold method (9.5%; 1.6%) in meat products and fish, respectively.

Lipid oxidation is a chain reaction with the production of free radicals wherein unsaturated fatty acids present in the food matrix react with oxygen to form peroxides, hydroperoxides and secondary compounds such as aldehydes, alkanes and conjugated dienes which are from the hydroperoxides decomposition (Pacheco et al., 2010; Salcedo-Sandoval et al., 2015). Among the compounds from decomposition of polyunsaturated fatty acids, MDA is

considered the main secondary compound, which presents a great importance due to toxic effect on human health (Karlsdottir et al., 2014; Zaki et al., 2014). MDA increased leads to a lower paraoxonase (PON1) level contributing to the greater risk of dyslipidemia, insulin resistance, and high blood pressure (Zaki et al., 2014). Moreover, high MDA levels generate health damages such as inefficiency on nutrient metabolism, detrimental effects on cell functionality and structure, and atherosclerosis (Albert et al., 2013; Palmeira et al., 2014b).

Another consequence of the secondary compounds from lipid oxidation is the negative effects on the sensory aspects of the food such as undesirable odor and flavor which limits the food shelf-life during storage. In addition, the secondary compounds negatively impact on nutritive value (Karlsdottir et al., 2014; Ladeira et al., 2014; Vieira et al., 2015).

In this context, the lipid oxidation measurement is highly relevant for monitoring food quality and human health. Although the importance of this parameter, there are no limits established for MDA values by legislation. Therefore, it is important to define MDA limits in fish as well as establish a standard analytical method for the evaluation of this parameter in order to avoid doubtful values due to different methodologies employed.

4. Conclusion

Bligh and Dyer (1959) technique must be the method choice for lipid extraction in freshwater fish species independently of fat levels. Furthermore, the cold method for MDA quantification proposed by Yin et al. (1993) may be a viable alternative for official monitoring of lipid oxidation in fish, producing accurate and reliable data due to non-interference of the heating step. Moreover, Bligh and Dyer (1959) and Yin et al. (1993) methodologies also require less amount of solvents and analysis time for determination of total lipid content and oxidative rancidity, respectively.

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Table 1. Total lipid content (%) in two freshwater fish species by two different extraction methods

Species	Soxhlet	Bligh and Dyer
<i>Cichla ocellaris</i> ¹	0.88 ^a ± 0.00*(0.11) [#]	1.62 ^b ± 0.00*(0.18) [#]
<i>Piaractusbrachypomus</i> ²	9.20 ^a ± 0.00*(0.01) [#]	11.27 ^b ± 0.01*(0.14) [#]

¹Lean fish; ² Fish with high fat content (Haard, 1992).

^{a,b} Different letters in the same row indicate significant differences ($P < 0.05$); * Mean ± standard deviation; # (Values in parentheses represent coefficient of variation %).

Table 2. Malondialdehyde (MDA) values (absorbance) in two freshwater fish species by two different extraction methods

Species	Tarladgis et al. [¥]	Yin et al. [¥]
<i>Cichla ocellaris</i> ¹	0.186 ^a ±0.079*(42.4) [#]	0.101 ^b ±0.017*(16.8) [#]
<i>Piaractus brachypomus</i> ²	0.120 ^a ±0.078*(65.0) [#]	0.039 ^b ±0.004*(10.2) [#]

¹Lean fish; ² Fish with high fat content (Haard, 1992).

^{a,b} Different letters in the same row indicate significant differences ($P < 0.05$); * Values are means ± standard deviation; [#] (Values in parentheses represent coefficient of variation %).

[¥]Heat and cold methods proposed by Tarladgis et al. (1960) and Yin et al. (1993), respectively. Results are absorbance in 538nm and 532nm, respectively.