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EDITED BY
Youji Wang,
Shanghai Ocean University, China

REVIEWED BY
Hüseyin Sevgili,
Isparta University of Applied Sciences, Türkiye
Carlos Alfonso Alvarez-González,
Universidad Juárez Autónoma de Tabasco, Mexico

*CORRESPONDENCE
Héctor Nolasco-Soria
hnolasco04@cibnor.mx

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Fish digestive lipase quantification methods used in aquaculture studies

Héctor Nolasco-Soria*

Aquaculture Program, Lab. de Fisiología Comparada y Genómica Funcional, Centro de Investigaciones Biológicas del Noroeste, S.C., La Paz, Baja California Sur, Mexico

The proportion of fats or oils in natural or artificial feed is generally 6%–30%, since this is essential to cover fish's energetic and structural requirements. Therefore, studies of the ontogeny or response of lipase activity to food treatments are widespread. A systematic review of articles published over 5 years (2016–2020) on lipase activity in fish in aquaculture was carried out; however, this was taken only as a representative example. Any 5-year period between 201-2022 would have shown similar results in terms of the actual lipase method used. As a result of this review, it was found that the methods used by the authors are very varied and have significant differences in terms of the type of substrate, substrate concentration, bile salt type and concentration, pH, temperature, incubation time, measurement of hydrolysis products, and definition of lipase units. The above does not mean that comparison of these studies is of no value, but that it is significantly limited. The most used methods (with *p*-nitrophenyl derivates, β-naphthyl derivates, and emulsified natural oils as substrate) can be reviewed to determine the most appropriate standard curves or the corresponding molar extinction coefficient for defining the lipase units. Standardizing current lipase analytical procedures should improve the reliability of comparative studies of aquaculture fish species.

KEYWORDS

aquacultural fish, pyloric caeca-intestine, lipase, enzyme unit, standardizing

1 The importance of digestive lipase activity

In fish, fertilized eggs contain oil droplets. Lipases hydrolyze the lipid content to obtain an energy reserve for embryo development. Even after hatching, the fish larva may still contain traces of the lipid globule, which is later absorbed during its development (Bik et al., 2020). Feeds used in fish aquaculture generally contain up to 20% lipids. Lipids are also present in natural fish food, including phospholipids, sterols, triglycerides, waxes, and free fatty acids, all of which are characterized by high levels of polyunsaturated fatty acids (Bakke et al., 2011). However, the main functional aspects of fish digestive lipases have yet to be deeply studied (Nolasco et al., 2011).

There are two types of lipases in mammals and fish: pancreatic lipase (*pl* gene) and lipase activated by bile salts (*bsal* gene). However, pancreatic lipase is the most important for mammals, and lipase activated by bile salts is the most important for fish (Tang et al., 2022). The fish pancreas synthesizes and secretes lipases into the intestine, which generally function at an alkaline pH, and hydrolyzes lipid food substrates to release the fatty acids absorbed by intestinal enterocytes (Bakke et al., 2011; Rønnestad et al., 2013). The pH in the fish intestine lumen ranges from 6 to 9 (Chakrabarti et al., 1995; Solovyev and Izvekova, 2016). In general, the optimum pH reported for fish lipases is alkaline (i.e., 7 to 9) (Mukundan et al., 1985; Gjellesvik et al., 1992; Iijima et al., 1997; Iijima et al., 1998; Nayak et al., 2004; Kurtovic et al., 2010; Nolasco et al., 2011; Xiong et al., 2011; Görgün and Akpınar, 2012; González-Félix et al., 2018). There are no conclusive data regarding the lipase requirements of Na^{+} or Ca^{+2} due to the small number of studies on this topic. Mullet (*Mugil cephalus*) lipase was not activated by Ca^{+2} (Aryee et al., 2007); *Sparus aurata* lipase has an optimum salinity at 50 mM NaCl and was not shown to be activated by Ca^{+2} or other divalent ions (chloride salts of Mg^{+2}, Mn^{+2}, Fe^{+2}, Co^{+2}, Cu^{+2}, Hg^{+2}, Pb^{+2}, and Zn^{+2}) that were inhibitors (Nolasco et al., 2011). In contrast, a phospholipase A2 from the pyloric ceca of red sea bream, *Pagrus major*, was activated by 8 mM of Ca^{+2} (Iijima et al., 1997); salmon (*Oncorhynchus tshawytscha*) lipase and hoki (*Macruronus novaezelandiae*) lipase were activated by ions (Kurtovic et al., 2010); and stingray (*Dasyatis pastinaca*) lipase increased its activity at 0.5 mM Ca^{+2} (Bouchaâla et al., 2015). The adjustment in the method should also consider the physiological ion concentration of the fish intestine (Nolasco-Soria, 2020).

Micelle formation enormously increases the fraction of lipid molecules accessible to the action of water-soluble lipases in the intestine. Lipase hydrolysis is valid for lipid substrates forming micelles with bile salts, i.e., phospholipids, galactolipids, monoglycerides, and lipolytic enzymes showing a preference for this type of lipid aggregates. Regarding the position of the lipase relative to the emulsified substrate, it is thought that the active site of the lipase is oriented toward the water–oil interface (Brockerhoff, 1973). The gallbladder of vertebrates secretes bile acids or their conjugated forms (bile salts). They collaborate in the action of lipases acting as emulsifiers of their substrates. Taurocholate and taurochenodeoxycholate are the principal bile salts of teleost fish (Une et al., 1991; Alam et al., 2001; Nolasco et al., 2011). In *in vitro* studies, chenodeoxycholyl-L-cysteinolic acid and taurochenodeoxycholate (12 mM concentration) were better activators for red sea bream pyloric ceca phospholipase (Iijima et al., 1997). Salmon lipase was better activated by sodium taurocholate (10 mM), and hoki lipase was equally activated by sodium cholate and sodium taurocholate (5, 10 mM) (Kurtovic et al., 2010). Red sea bream lipase was better activated by sodium taurocholate and sodium cholate, but was not activated by sodium deoxycholate (Iijima et al., 1998), similar to mullet (*Mugil cephalus*) lipase (Aryee et al., 2007). Stingray lipase increases its activity at 1 mM sodium taurodeoxycholate, but a concentration higher than 4 mM inhibits lipase activity (Bouchaâla et al., 2015).

Reviews on digestive physiology in larval fish report that lipase activity has been found during all stages of ontogenic development (Rønnestad et al., 2013; Yúfera et al., 2018). For example, in green catfish (*Mystus nemurus*) larvae, lipase activity showed a basal activity level from day 1 to day 25, and subsequently an increase in lipase activity between 30–40 dph (Srichanun et al., 2012). In leopard groupers (*Mycteroperca rosacea*) larvae, lipase activity was detected before mouth opening and during feeding, with a significant peak at 30 dph (Martínez-Lagos et al., 2014). In Chinese perch (*Siniperca chuatsi*) larvae, a low lipase activity was detected before the mouth opened. In contrast to other carnivorous fish, lipase activity did not increase suddenly after the first feeding, but gradually increased until 15 dph, then suddenly decreased at 25 dph, and continued declining until 30 dph (Tang et al., 2021).

Lipids are common nutrient substrates for farmed fish, and lipases are secreted in the intestine for their digestion. Subsequently, fish lipases can be used as enzymatic reagents to determine the *in vitro* digestibility of food oils (according to the methodology of Espinosa-Chaurand and Nolasco-Soria, 2019) to select the most digestible ones and allow the possible formulation of alternative diets for aquaculture.

According to Yúfera et al. (2018), the most commonly used substrates to measure lipase activity are *p*-nitrophenyl myristate (*p*-NPM), β-naphthyl caprylate (β-NC) (colorimetric methods, based on the color generated by releasing *p*-nitrophenol (*p*-NP) or β-naphthol (β-N), respectively), and 4-methylumbelliferyl (MU) substrates (MU-butyrate, MU-heptanoate or MU-oleate) (fluorometric methods, based on the fluorescence generated by releasing of 4-methylumbelliferone, MU). Emulsions of water-insoluble short-chain triacylglycerols (e.g., tributyrin) or long-chain triacylglycerols (e.g., triolein) are used as lipase substrates by titration of the released protons with NaOH, or by calculating the moles of NaOH required per unit of time to maintain a constant pH during the lipase hydrolysis of tryglyceride ester bonds.

The methods used by the authors differ in the type of substrate used. Therefore, the hydrolysis products generated (what is measured) are different, making it necessary to use the standard curves or the specific molar extinction coefficients (MEC) to measure the hydrolyzed ester bonds per unit of time. If the measurements are made at different pHs, temperatures, ion concentrations, or types or concentrations of bile salt, this will directly affect the lipase activity. If the definitions of the lipase unit differ, the comparison of results becomes more complex (Yúfera et al., 2018). Considering the above, the objective of this study was to review the authors' methods to identify their differences and their effects on the quantification of lipase activity, in addition to carrying out experimental analyses to evaluate some of the most critical methodological points for quantifying lipase activity, to propose more *ad hoc* strategies closer to fish physiology, and lipase unit calculation tools for studies in fish in aquaculture.

2 Lipase determination

2.1 Fish lipase activity

Lipase methods were explored with a Web of Science Core Collection™ (www.webofknowledge.com; Thomson Reuters™) bibliographic search using the keywords "lipase AND fish",

resulting in a total of 936 published articles from 2016 to 2020 (5 years) (Supplementary Material A contains a complete list of reviewed and excluded articles) being found. After removing the studies that did not focus on the lipase of aquaculture fish, a final database of 339 articles was used for their review (Supplementary Material 1), and additional references used by authors were reviewed.

2.1.1 Fish species

Lipase activity has been studied in at least 95 fish genera (127 species) and 10 fish hybrids (Table S1, Supplementary Material 2), with the most studied genera being *Oreochromis*, *Cyprinus*, *Oncorhynchus*, *Ctenopharyngodon*, and *Carassius* (29.2%, 102 studies).

2.1.2 Digestive organs

The results indicated (Table S2, Supplementary Material 2) that the studied tissues belonged to either intestine [whole, anterior, medium, posterior (69.0%, 234 studies)], emptied intestine [mechanically or by washing with distilled water (2.7%, 9 studies)], intestinal content [digesta (2.9%, 10 studies)], whole digestive tract (7.4%, 25 studies), whole larvae (8.6%, 279 studies), or dissected larvae (2.7%, 9 studies), for the preparation of lipase extracts. It is essential to know the fish's anatomy to be studied for the identification of the digestive segments. This system generally comprises the mouth–esophagus, stomach (or pseudostomach), and intestine. The pancreas or hepatopancreas is connected to the first portion of the intestine. Likewise, the pyloric cecum in fish that have it is identified as a cluster of lobes. The number of larvae was from 1 to 600 per sample, and the weights of tissue for lipase crude extract preparation were from 40 mg to 30 g. Unfortunately, 88.2% of the studies did not indicate the amount of tissue used. In general, the authors have used 1 to 66 volumes of extraction solvent: 1–2 (1.8%), 2–4 (9.4%), 5–6 (7.1%), 8–10 (31.0%), 14–25 (2.1%), 30–40 (4.4%), 66 (0.3%), 20,000 (0.3%), and not indicated (41.8%). In the specific case of larvae, the authors used 5 to 66 volumes of extraction solution.

2.1.3 Extraction solution

Regarding the extraction solution, 16.2% of the authors used physiological saline solution (0.65%–0.90%), 12.7% used water (distilled, deionized, Milli-Q, ultrapure), 7.4% used Tris (10 mM–100 mM, pH 7.2–7.5), 6.5% used phosphate-buffered saline (PBS) (pH 7.4–7.5), 6.2% phosphate (20 mM–100 mM, pH 7.0–8.0), and the rest used other solutions or did not specify the solution used for tissue homogenization (Table S3, Supplementary Material 2).

2.1.4 Extract clarification

Enzymatic extracts clarification has been performed at relative centrifugal force (RCF) values that ranged from 125 to 33,000 × *g* and centrifugation times that varied from 3 min to 60 min (at 4°C). In this regard, the *g**min values varied from 1,875 to 900,000. Some authors reported the RCF applied but not the centrifugation time; others reported the rpm applied but did not give the data of the rotor used or their radius (r), which makes it impossible to calculate the RCF applied. Only 29.8% of the studies used a *g**min value higher than 200,000 (Table S4, Supplementary Material 2).

For enzyme extract preparation, the method proposed by Nolasco-Soria (2020) is recommended, also considering the time of sacrifice to avoid the effect of the circadian cycle (Yúfera et al., 2018). In the case of larvae, the size (i.e., the weight and length) and thermal units of development should also be considered (Gisbert et al., 2018; Yúfera et al., 2018). In addition, samples should be kept cold while they are being handled, and long freezing times before their enzymatic analysis should be avoided (Solovyev and Gisbert, 2016).

According to differences in the fish intestinal pH (Solovyev and Izvekova, 2016) and the working physiological pH of fish lipases (pH that gives stability to the enzymes), as previously reported, the recommendation is to perform lipase extraction with a buffer at pH 7.0–9.0, or, better, in distilled water if the intestinal pH is unknown or if the optimum pH of fish lipases is expected to be determined. The extract is then doubly clarified by centrifugation of at least 15,000 × *g*, 15 min (225,000 *g**min) at 4°C to reduce turbidity and keep it at −80°C (Nolasco-Soria, 2020).

2.2 Determination of lipase activity

Table 1 shows the methods used by the authors. The reader who knows the rationale of the methods may recognize the difficulty of comparing lipase values reported in fish studies.

One of the oldest published procedures for quantifying lipase activity used by some authors (3.2%) was from Cherry and Crandall (1932). The titrimetric protocol described by these authors is as follows:

"In a flask, 1 ml of serum is added to 2 ml of substrate olive oil emulsion (50% olive oil containing 5% of acacia gum as emulsifier and 0.2% of sodium benzoate as preservative), 3 ml of distilled water, and 0.5 ml of phosphate buffer (0.33 M, pH 7.0). After 24 hours of incubation at 40°C, the reaction is stopped by the addition of 3 ml of alcohol (95%). Then, three drops of phenolphthalein (1%), as an indicator, were added, and mixture titrated with NAOH (50 mM), recording the volume in ml required bringing the solution to the faintest permanent pink, as lipase activity."

These authors also suggested that the term lipase should be reserved for the enzyme capable of hydrolyzing ester bonds on true lipids (fats and oils) and that esterase be used for the enzyme acting upon other esters (Cherry and Crandall, 1932). However, this method, indisputably valuable in determining the presence of true lipases, is tedious because samples need to be individually measured in glass flasks. In addition, this procedure does not permit the miniaturization of volumes to be carried out at a plastic microplate level. Similar methods cited by the authors include Bier (1955) [including Furné et al., 2005, which cited Bier (1955) (6.8%)], King (1965) (0.6%), Tietz and Fiereck (1966) (0.3%), Gotthilf (1974) (0.3%), Ogunbiyi and Okon (1976) (0.3%), Linfield et al. (1984) (0.3%), Borlongan (1990) (5.3%), Zamani et al. (2009) (0.6%), and Ismat et al. (2013) (0.6%). A more straightforward, turbidimetric method using an olive oil emulsion was developed by Shihabi and Bishop (1971). A modified method using olive oil as a substrate and

TABLE 1 Reference protocols (type of method) and their frequency of use for assessing the activity of lipase in larvae and digestive tract of fish.

Reference studies	Studies	Number of studies	%
NANJING kit (A054–2-1) (including Verduin et al., 1973; Wang et al., 2017; Chen et al., 2018; Cai et al., 2020) **(SS, K, C)**	2, 5, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19, 20, 23, 43, 44, 46, 50, 53, 63, 97, 99, 101, 103, 105, 106, 111, 112, 114, 124, 134, 135, 137, 140, 144, 150, 153, 159, 176, 177, 181, 184, 187, 190, 191, 193, 208, 210, 215, 223, 230, 231, 233, 234, 235, 240, 244, 246, 248, 249, 251, 262, 268, 275, 283, 284, 285, 288,289, 292, 302, 307, 318, 332, 334, 267	76	22.4
Iijima et al., 1998 (including Babaei et al., 2011) **(SS, EP, C)**	6, 26, 27, 29, 31, 56, 67, 71, 72, 80, 84, 86, 91, 94, 109, 119, 125, 139, 147, 152, 156, 157, 179, 226, 258, 266, 279, 282, 296, 297, 299, 300, 301, 305, 310, 311, 322	37	10.9
Bier, 1955 (including Furné et al., 2005 that cited Bier, 1955) **(NS, EP, T)**	1, 17, 47, 69, 83, 142, 166, 172, 173, 197, 225, 232, 236, 237, 238, 290, 291, 295, 309, 315, 317, 338, 323	23	6.8
Versaw et al., 1989 **(SS, EP, C)**	15, 16, 22, 40, 73, 88, 107, 128, 136, 158, 165, 168, 171, 182, 188, 199, 205, 218, 272, 313, 333	21	6.2
Winkler and Stuckmann, 1979 (Including Pera et al., 2006) **(SS, EP, C)**	24, 95, 108, 110, 115, 120, 133, 149, 175, 185, 196, 207, 209, 239, 265, 273, 276, 308, 327, 303	20	5.9
Borlongan, 1990 **(NS, EP, T)**	25, 41, 42, 57, 74, 75, 76, 77, 102, 131, 162, 164, 189, 204, 227, 287, 304, 325	18	5.3
Gawlicka et al., 2000 **(SS, K, C)**	35, 37, 82, 92, 104, 138, 160, 183, 195, 201, 228, 293, 180, 194	14	4.1
Cherry and Crandall, 1932 **(NS, EP, T)**	51, 52, 121, 143, 146, 212, 213, 222, 254, 269, 280	11	3.2
Spinreact, (2017) (including Couto et al., 2016) **(SS, K, C)**	167, 169, 216, 220, 221, 257, 259, 316, 329, 335	10	2.9
Albro et al., 1985 **(SS, EP, C)**	30, 79, 127, 129, 200, 206, 224, 264, 312	9	2.7
Gjellesvik et al., 1992 **(SS, K, C)**	3, 161, 219, 252, 260, 294, 319, 320	8	2.4
Roberts, 1985 (including Izquierdo and Henderson, 1998, that cited Roberts, 1985) **(SS, EP, F)**	55, 64, 148, 170, 242	5	1.5
Santigosa et al., 2011 **(SS, K, C)**	28, 68, 330, 331	4	1.2
Cusabio; Shanghai Jianlai Industry Co., Ltd; Shanghai Lengton Biological **(Elisa)**	87, 93, 274, 286	4	1.2
Sæle et al., 2010 (Cited Murray et al., 2003, that cited Iijima et al., 1998) **(SS, K, C)**	78, 126, 192, 245	4	1.2
Markweg et al., 1995 (including Hlophe et al., 2014) **(SS, K, C)**	60, 122, 253, 326	4	1.2
Sigma kit **(NS, EP, C)**	118, 174, 263	3	0.9
Biovision kit K722–100 **(NS, EP, C)**	151, 278, 328	3	0.9
Worthington, 1988 (according to SIGMA) **(NS,EP, T)**	33, 34, 155	3	0.9
Shihabi and Bishop, 1971 **(NS, K, Tb)**	36, 186, 229	3	0.9
Rotllant et al., 2008 **(SS, EP, F)**	130, 314	2	0.6
Mckellar and Cholette, 1986 **(SS,EP, C)**	59, 271	2	0.6
Bionik-Canada kit **(not found, could be SS, K, C)**	202, 203	2	0.6
Ismat et al., 2013 **(NS, EP, T)**	214, 337	2	0.6
King, 1965 **(NS, EP, T)**	89, 90	2	0.6
Zamani et al., 2009 **(NS, EP, T)**	61, 62	2	0.6
Bülow and Mosbach, 1987 **(SS, K, C)**	250, 306	2	0.6
Beijing Sino UK, 2023 **(Not found, could be SS, K, C)**	4, 98	2	0.6
Brockman, 1981 **(NS, EP, T)**	261, 321	2	0.6
Faulk et al., 2007 **(SS, K, C)**	48	1	0.3

(Continued)

TABLE 1 Continued

Reference studies	Studies	Number of studies	%
Seligman and Nachlas, 1963 **(SS, EP, C)**	100	1	0.3
Bioclin, 2023 kit (k025) **(SS, EP, C)**	81	1	0.3
Pars Azmoon kit **(Not found)**	154	1	0.3
Nolasco-Soria et al., 2018 **(SS, EP, C)**	54	1	0.3
Zahran et al., 2014, that used QUANTICHROM Kit **(SS, EP, C)**	243	1	0.3
Ogunbiyi and Okon, 1976 **(NS, EP, T)**	163	1	0.3
Mustafa et al., 2016 **(NS, EP, T)**	178	1	0.3
Gotthilf, 1974 **(NS, EP, T)**	277	1	0.3
Wang et al., 2019 **(NS, EP, C)**	70	1	0.3
Tietz and Fiereck, 1966 **(NS, EP, T)**	336	1	0.3
Linfield et al., 1984 **(NS, EP, T)**	198	1	0.3
Mahadik et al., 2002 **(SS, EP, C)**	58	1	0.3
Metin and Akpinar, 2000 that cited Winkler and Stuckmann, 1979 **(SS, K, C)**	85	1	0.3
Murashita et al., 2008 (that cited Albro et al., 1985) **(SS, K, C)**	270	1	0.3
Didinen et al., 2014 **(Digestion agar test)**	132	1	0.3
Cited by authors of studies, but the cited authors do not use or define a specific lipase method (Liu et al., 2017a; Kofuji et al., 2006; Jiang et al., 2016; Liu et al., 2017b; Kwon and Rhee, 1986; Kofuji et al., 2006; Pavasovic et al., 2006; Wang et al., 2013; Zhao et al., 2015) or the authors of the studies do not describe a lipase method. **(Unknown)**	49, 96, 113, 141, 145, 241, 256, 281, 324, 339	10	2.9
Nind	21, 32, 38, 39, 45, 65, 66, 116, 117, 123, 211, 217, 247, 255, 298	15	4.4

Nind, not indicated; NS, natural substrate (triglyceride); SS, synthetic substrate; EP, end point; K, kinetic; C, color; F, fluorescence; T, titration; Tb, turbidimetric.
Values reported are based on a total of 339 retrieved articles on this topic published in the last 5 years, between 2016 and 2020 (Search chain: "lipase AND fish"; Web of Science Core Collection™; Thomson Reuters™).

a cupric acetate pyridine reagent for colorimetric free-fatty-acid determination was used by Mustafa et al. (2016) and Wang et al. (2019).

In contrast, at the microplate level, the method most used by the authors is that of a commercial kit. Unfortunately, for obvious reasons, the actual composition and concentration of the reactants are still being determined, which limits their practical utility (for example, if the study requires that the concentrations of substrate or developer be varied). This also applies to all other commercial kits used by the authors (Table 1).

The methods used to quantify fish digestive tract lipase were revised in the 339 studies retrieved from the systematic bibliographic search (Table 1). Without considering commercial kits, the method that was most used by the authors is that of Iijima et al. (Iijima et al., 1998, a modified method of Albro et al., 1985). The original method was explicitly described by Albro et al. (1985) as follows:

"A mixture of 0.4 M Tris-HCI (pH 7.4) at 37°C (200 ml), 116 mM sodium taurocholate (pH 7.4) (70 ml), 14 mM p-nitrophenyl myristate in ethylene glycol monomethyl ether (20 ml), and water (210 ml) was made fresh daily. Each assay tube received 5 ml of this mixture and was equilibrated at 37°C. Enzyme solution (5-100 µl as appropriate) was added and the solution was swirled at 200 rpm for from 5 to 15 min at 37°C. A 0.2 ml aliquot of incubation mixture was added to 2.8 ml of 0.014 M aqueous NaOH in a cuvette and the absorbance at 410 nm was determined relative to a procedure blank."

In the reaction mixture used by Albro et al. (1985), the final concentrations of the buffer, sodium taurocholate, and *p*-NPM substrate were 160 mM, 16.2 mM, and 0.56 mM, respectively. In contrast, Iijima et al. (1998) reduced the mixture to one-tenth of its original volume, the bile salt concentration by a factor of 3 (but changed to sodium cholate), and the temperature to 30°C. However, they increased the pH of the reaction and used a stopper-extractor to separate the colored phase of the reaction by centrifugation. The Iijima et al. method was explicitly described as follows:

"Each assay (0.5 ml) contained 0.53 mM p-nitrophenyl myristate, 0.25 mM 2-methoxyethanol, 5 mM sodium cholate, and 0.25 M Tris-HCl (pH 9.0). Typically, 5-10 µl of enzyme solution was added to the substrate solution. Incubation was carried out for 15 min at 30°C, and the reaction was terminated by adding 0.7 ml of acetone/n-heptane (5:2, v/v). The reaction mixture was vigorously mixed and centrifuged at 6,080 × g for 2 min. The absorbance at 405

nm in the resulting lower aqueous layer (0.5 ml) was measured. The extinction coefficient of p-nitrophenol was 16,500 M^{-1} cm^{-1} per liter at pH 9.0."

The *p*-nitrophenyl substrates with short fatty acids, such as acetate, should be avoided due to their non-specific hydrolysis (De Caro et al., 1988).

As reported by Nolasco-Soria et al. (2018), many authors have modified the original method developed by Albro et al. (1985) for assessing lipase activity, by using different reaction volumes (Gjellesvik et al., 1992), microplates instead of test tubes or kinetic techniques (Sæle et al., 2010, which cited Murray et al., 2003), or including Triton X-100 in the reaction mixture (Gawlicka et al., 2000, which cited German et al., 2004; Murashita et al., 2008, which cited Albro et al., 1985), with all of them using *p*-nitrophenyl myristate as a substrate (Table 1). Therefore, these authors are part of the most cited group. Winkler and Stuckmann (1979) previously developed a similar method using *p*-nitrophenyl palmitate as a substrate after it was modified by Markweg et al. (1995) (including Mahadik et al., 2002; Pera et al., 2006; Hlophe et al., 2014). Metin and Akpinar (Metin and Akpinar, 2000, who cited Winkler and Stuckmann, 1979) used *p*-nitrophenyl acetate as a substrate, as did Bülow and Mosbach (1987). The main disadvantage of some of the abovementioned methods is the requirement for organic solvents (toxic for users) or artificial detergents such as Triton X-100 (lipase inhibitor, in accordance with Aryee et al., 2007 and Nolasco-Soria et al., 2018) to clarify the reaction mixture before absorbance readings can be taken. Faulk et al. (2007) reported a kinetic method at the microplate level, using *p*-nitrophenyl caproate as a substrate (Table 1). The basis of the above methods is the quantification of the moles of *p*-nitrophenol released by the lipase hydrolysis of (the ester bond) the synthetic substrate.

The second most used method by the authors is that of Bier (Bier, 1955, including Furné et al., 2005, who cited Bier, 1955), which is very similar to the method previously developed by Cherry and Crandall (1932). According to Bier (1955), the assay protocol is as follows:

"In a 125-ml Erlenmeyer flask are mixed 10 ml of the freshly prepared PVA (polyvinyl alcohol)-emulsified substrate, 5 ml of buffer (McIlvaine), and 5 ml of the enzyme preparation to be tested. The mixture is shaken gently and incubated for 4 hours at 37° with constant shaking. At the end of the incubation time, 30 ml of a 1:1 alcohol-acetone solution is added to stop the reaction and break the emulsion. Phenolphthalein indicator is added, and the solution is titrated with 0.05 N NaOH".

The basis of the method is the quantification of hydroxide moles, which are required to neutralize the protons released by the hydrolysis of ester bonds on olive oil substrate and alkalize the reaction to the change of the phenolphthalein color.

The third most used method by the authors is that of Versaw et al. (1989) (which is a modified version of the method of Mckellar and Cholette, 1986), using β-naphthyl caprylate (BNC) as a substrate. The original method was explicitly described by Versaw et al. (1989) as follows:

"The incubation mixture of the modified procedure, identical to that of Mckellar and Cholette (1986) in containing 0.2 ml 200 mM Na-T, 1.8 ml 50 mM BES buffer (pH 7.2), and 0.05ml lipase source, was equilibrated to 40°C in a water bath. Then 0.02 ml 200 mM BNC in DMSO was added and the mixture was incubated for 30 min. After incubation, the color reaction was produced by the addition of 0.02 ml Fast Blue BB salt (100 mM in DMSO) followed by an additional 5 min incubation at 40°C. The reaction was stopped with 0.2 ml 0.72N TCA. In McKellar and Cholette's method, 5 ml ethyl acetate was added, the sample was mixed, centrifuged, and the ethyl acetate layer read for absorbance at 540 nm. At this juncture in our modification, 2.71 ml of a 1:l ethanol (95%)/ethyl acetate (v/v) mixture was added for a final volume of 5 ml. The addition of the mixed solvent produced a clarified sample which was read for absorbance."

Previously, Seligman and Nachlas (1963) reported an end point method using β-naphthyl laurate. Furthermore, more recently, Nolasco-Soria et al. (2018) published a new method, with a new stopper-clarifier reagent at the microplate level. The basis of this method is quantifying the moles of β-naphthol released by the lipase hydrolysis of the ester bond of the synthetic substrate (β-naphthyl caprylate).

Other techniques for lipase activity, according to the method cited by the authors (Table 1), have a lower use (3% or lower, each), including colorimetric, fluorometric, and enzyme-linked immunosorbent assay (ELISA) methods.

The incomplete definition of the methods and the use of concatenated citations with other references make it difficult to know how lipase activity was measured. In addition, the variations or adjustments made by different authors to a lipase method cited as a reference make comparison difficult, even when the same method and author are cited, which requires a standardization effort, considering the following.

2.2.1 Assay volume

The volumes among the only 55 studies (16.2%) that explicitly reported reaction ranged from 70 µL to 25,000 µL. The remaining studies (n = 284, 83.8%) did not report the trial volume, so it is assumed that they used the one reported in the reference cited by the authors (Table 2). By type of method, the assay volumes were between 100 µl and 7,290 µl for colorimetric, between 100 µl and 3,300 µl for fluorometric, and between 2,100 µl and 31,000 µl for titrimetric procedures.

2.2.2 Type of substrate

The type of substrate used to measure lipase activity is highly relevant, mainly when synthetic substrates are used with a single associated fatty acid and different chain lengths (Nolasco-Soria et al., 2018). Thus, regarding lipase substrate, 175 studies (51.6%) explicitly reported the substrate; most of them used either *p*-nitrophenyl myristate (16.2%) or olive oil (13.6%). The rest of the studies (n = 164, 48.4%) did not report the substrate in the reaction mixture, so it is assumed that they used the substrate reported in the reference cited by the authors (Table 3). Only 67 studies (19.8%) explicitly reported the substrate concentration, ranging from 0.03 mM to 50 mM (1,666 times difference). By type of substrate, the concentrations were as follows: olive oil, 1% to 40%; other authors

TABLE 2 Assay volumes used for the determination of lipase activity.

Vol (µL)	References	%
70	312	0.3
100	109, 148	0.6
110	264	0.3
150	174	0.3
180	68	0.3
205	161	0.3
210	48	0.3
225	219	0.3
237	122	0.3
250	167, 335	0.6
265	66, 130, 314	0.9
390	200	0.3
500	67, 84, 266, 305	1.2
505	282	0.3
1,000	30, 79, 303	0.9
1,080	250	0.3
1,200	258	0.3
1,205	6, 29	0.6
1,267	179, 180	0.6
1,270	327	0.3
1,497	128	0.3
1,500	206	0.3
2,000	92	0.3
2,050	235	0.3
2,650	255	0.3
3,000	69	0.3
3,040	242	0.3
3,060	170	0.3
3,100	55	0.3
5,000	88, 172, 232, 298, 313, 338	1.8
6,000	214, 337	0.6
6,500	212	0.3
7,000	213	0.3
7,500	217	0.3
8,520	281	0.3
9,500	51	0.3
10,580	65	0.3
25,000	244	0.3

(Continued)

TABLE 2 Continued

Nind, not indicated. References are listed in Online Resource 1.
Thirty one thousand µL, 0.3%, according to the study by Gotthilf (1974); 20,000 µL, 1.5%, according to the study by Bier (1955); 17,500 µL, 0.3%, according to the study by Tietz and Fiereck (1966); 16,500 µL, 0.3%, according to the study by Linfield et al. (1984); 15,100 µL, 0.6%, according to the study by Worthington (1988); 9,500 µL, 2.4%, according to the study by Cherry and Crandall (1932); 8,000 µL, 0.9%, according to the studies by King (1965) and Seligman and Nachlas (1963); 7,290 µL, 0.6%, according to the study by Mckellar and Cholette (1986); 6,000 µL, 0.3%, according to the study by Wang et al. (2019); 5,100 µL, 0.9%, in accordance with Albro et al. (1985); 5,000 µL, 9.1%, according to the studies by Furnè et al. (2005) and Versaw et al. (1989); 4,000 µL, 5.0%, according to the study by Borlongan (1990), including Yanbo and Zirong (2006), which cited Borlongan (1990); 3,300 µL, 0.3%, in accordance with instructions for the Bioclin kit; 3,100 µL, 0.9%, according to the study by Shihabi and Bishop (1971); 3,000 µL, 0.3%, according to the study by Metin and Akpinar (2000); 2,500 µL, 5.0%, according to the study by Winkler and Stuckmann (1979), including Aryee et al. (2007), which cited Winkler and Stuckmann (1979); 2,100 µL, 0.6%, according to the study by Brockman (1981); 2,000 µL, 0.3%, according to the study by Ogunbiyi and Okon (1976); 1,500 µL, 0.3%, according to the study by Murashita et al. (2008); 1,270 µL, 0.3%, in accordance with instructions for the Pars Azmoon kit; 1,100 µL, 0.3%, according to the study by Mahadik et al. (2002); 1,000 µL, 2.7%, according to the study by Gjellesvik et al. (1992) and Santigosa et al. (2011); 500 µL, 0.3%, according to the study by Iijima et al. (1998); and 390–100 µL, at microplate level, 28.3%, according to the studies by Roberts (1985); Sæle et al. (2010); Mustafa et al. (2016), and Nolasco-Soria et al. (2018), and the Sigma kit, BioVision kit, Cusabio kit, Spinreact (2017) (including Couto et al., 2016), and Nanjing kit (including Verduin et al., 1973; Habte-Tsion et al., 2013; Wang et al., 2017; Chen et al., 2018; Cai et al., 2020). The rest of the studies did not report the reaction volume used (14.7%).

reported molarity from 0.1 mM to 100 mM but did not report which of the triglycerides of those found in olive oil (Boskou et al., 2006) was taken as the calculation basis. For *p*-nitrophenyl substrates (butyrate, caproate, and myristate), 0.04 mM to 50 mM. For 4-methylumbelliferyl substrates (butyrate, heptanoate, octanoate, and oleate), 0.03 mM to 50 mM. For β-naphthyl caprylate, 0.49 mM to 200 mM. For DDGR (1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester), 0.12 mM. In addition, an excess of substrate in the reaction mixture must be present during the entire reaction time (according to the Michaelis–Menten law) to determine lipase activity.

2.2.3 Reaction buffer

Regarding the reaction buffer, different solutions and concentrations were reported (Table 4). In particular, most authors who reported this information used a Tris buffer (16.2%) or phosphate buffer (6.2%). Unfortunately, 75.3% of the studies did not explicitly report the buffer employed, so it is assumed that they used the buffer reported in the reference cited by the authors. Most buffers have been used correctly depending on their working pH (Mohan, 2003), and the experiments in most of the reported studies were performed at an alkaline pH of between 7 and 9. If the working pH is between 7 and 9, the use of Tris-HCl buffer at a concentration between 20 mM and 50 mM is recommended. Regardless of the use of ions reported explicitly by authors, only 1.2% of the studies used Na^{+} (32 nm to 1000 mM), and 2.1% used Ca^{+2} (0.05 mM). As reported by Nolasco-Soria (2020), the requirement for NaCl and divalent ions, such as $CaCl_2$, has to be previously determined, in this case for lipase activity in fish.

TABLE 3 Substrates used for the determination of lipase activity.

Substrate	References	%
p-nitrophenyl myristate (β-nitrophenyl myristate, one study)	3, 25, 26, 29, 30, 31, 32, 35, 54, 56, 67, 71, 78, 80, 82, 84, 86, 91, 104, 109, 125, 126, 129, 139, 147, 152, 156, 160, 179, 180, 183, 192, 195, 200, 201, 206, 211, 219, 224, 226, 228, 245, 258, 264, 266, 279, 293, 297, 299, 300, 305, 310, 311, 312, 322	16.2
Olive oil	21, 33, 34, 36, 41, 42, 51, 57, 65, 69, 74, 75, 76, 85, 89, 90, 102, 131, 132, 140, 143, 146, 155, 164, 172, 189, 197, 204, 212, 213, 214, 217, 222, 227, 232, 244, 261, 269, 277, 281, 287, 309, 321, 325, 337, 338	13.6
p-nitrophenyl palmitate	24, 45, 58, 92, 110, 115, 120, 122, 185, 207, 209, 239, 253, 276, 298, 303, 327	5.0
DDGR (1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester)	28, 46, 68, 123, 167, 191, 202, 203, 208, 220, 221, 257, 259, 330, 331, 335	4.7
β-naphthyl caprylate (α-naphthyl caprylate, one study)	22, 73, 88, 128, 158, 165, 168, 171, 199, 255, 272, 313, 333,	3.8
4-methylumbelliferyl butyrate	55, 64, 66, 130, 148, 314, 336	2.1
Triglycerides (Nind)	186, 187, 193, 241, 278, 328	1.8
p-nitrophenyl caproate	48, 116, 252, 260	1.2
4-methylumbelliferyl heptanoate	38, 117, 170	0.9
p-nitrophenyl acetate	79, 273	0.6
p-nitrophenyl caprylate (octanoate)	161	0.3
p-nitrophenyl butyrate	250	0.3
p-nitrophenyl substrate (Nind)	196	0.3
p-nitrophenyl phosphate	4	0.3
Liposomal substrate (Nind)	242	0.3
NA, Densitometry SDS-PAGE	39	0.3
Nind		48.4

Studies are listed in the Supporting Information.
Nind, not indicated. Therefore, it is assumed that they used the substrate reported by the reference cited by these authors: DGGR (1,2-o-dilauryl-racglycero-3-glutaric acid-(6′-methylresorufin) ester), 21.5%, in accordance with instructions for the Nanjing kit and Spinreact (2017); olive oil, 9.1%, according to the studies by Cherry and Crandall (1932); Bier (1955); Shihabi and Bishop (1971); Ogunbiyi and Okon (1976); Linfield et al. (1984); Borlongan (1990); Furné et al. (2005); Zamani et al. (2009); Mustafa et al. (2016), and Wang et al. (2019); p-nitrophenyl miristate, 5.0%, according to the studies by Albro et al. (1985); Gjellesvik et al. (1992); Iijima et al. (1998); Gawlicka et al. (2000), and Murashita et al. (2008); β-Naphthyl caprylate, 3.2%, according to the studies by Mckellar and Cholette (1986) and Versaw et al. (1989); p-nitrophenyl palmitate, 2.7%, according to the studies by Winkler and Stuckmann (1979) and Markweg et al. (1995); ELISA method, 1.2%, in accordance with instructions for the Cusabio kit, Shanghai Jianglai Industry Co., Ltd kit; Triglycerides, 1.2%, in accordance with instructions for the Sigma kit, Biovision kit; p-nitrophenyl (phosphate, acetate, propionate, butyrate, caprylate), 0.6%, according to the study by Bülow and Mosbach (1987); β-Naphthyl laurate, 0.3%, according to the study by Seligman and Nachlas (1963), Beijing Sino UK, (2023) kit; 2-3-dimercapto-1-propanol tributyrate-dithionitrobenzoic, DTNB, 0.3%, in accordance with instructions for the Bioclin, (2023); Nind, 3.3%.

2.2.4 Reaction temperature

As is the case for all enzymes, the reaction temperature directly influences the reaction rate of lipases. Therefore, if there are differences in the incubation temperature (Table 5) in the methods used by the authors, it is complicated to compare the lipase units reported among the studies, even using the same method. In addition, it is advisable to measure lipase activity under the physiological or culture temperature conditions of the fish under study. Notably, 13.6% of the studies reported a reaction temperature higher than 35°C. However, if we consider the temperature used by references cited by the authors, the percentage of studies using temperatures from 35°C to 60°C, which are unusual for fish, rises to 59.7%. Likewise, the optimal temperature determined in the laboratory *in vitro* tests should not be used to measure lipase activity in fish. It is recommended to use a temperature of 25°C, which is closer to the ecophysiological temperature, at least for temperate-water fish (Gisbert et al., 2018).

2.2.5 Incubation time

Incubation time varies from 1 min to 60 min for synthetic substrates and 2 h to 24 h for natural substrates (oils) (Table 5). It is recommended that the incubation time for lipase determination should be from 5 min to 30 min (Nolasco-Soria et al., 2018) to have a fast and practical method.

2.2.6 Reaction stopper

For end point protocols regarding the method for stopping lipase reaction, the used chemicals are shown in Table 6. The foundation of the stopper is considered adequate. However, those based on acidification (acetic acid) or alkalinization (Na_2CO_3, NaOH, and Tris) of the medium have their limitations if the enzymes are active at those pH values, particularly if they are alkaline. It is recommended not to use organic solvents to avoid user exposure. One way to avoid stoppers is to use kinetic methods to measure lipase activity.

TABLE 4 Lipase reaction buffers.

Buffer	Studies	%
Ammonium bicarbonate, 24 mM (pH 7.8)-Triton (0.5%)	30, 79, 129, 160, 206, 224, 228, 312	2.4
McIlvaine buffer, 100 mM citric acid and 200 mM bisodium phosphate (pH 8.0)	69, 85, 172, 232, 338	1.5
Phosphate, 25 mM–200 mM (pH 7.0, 7,5, 7.8, 8.0)	21, 38, 51, 66, 89, 90, 117, 122, 130, 143, 146, 170, 212, 214, 222, 244, 281, 314, 327, 337	5.9
Phosphate,? mM (pH 7.0)-Triton X-100 (0.2%)	336	0.3
Tris, 1 mM–250 mM (pH 7.5, 7.7, 8.0, 8.3, 9.0)	55, 56, 65, 67, 71, 91, 123, 148, 213, 217, 226, 242, 255, 311, 313	4.4
Tris (50, 100 mM, pH 8.5)-Triton X-100 (0.4, 0.5%)	92, 200	0.6
Tris, 20 mM (pH 8.0)-Triton X-100 (0.01%)-NaCl (150 mM)	264	0.3
Tris, 20.5 mM (pH 8.3)-Taurodeoxycholate (3.6 mM)-Deoxycholate (0.9 mM)-Tartrate (0.8 mM)-CaCl2 (0.05 mM)-Mannitol (30 mM)-Colipase (1 mg/mL)	68, 330, 331	0.9
Tris, 200, 500 mM (pH 7.4)-Sodium taurocholate (6 mM)-NaCl (100 mM)	48, 161, 219, 252, 260	1.5
Tris, 25 mM (pH 7.5)-sodium cholate (5.2 mM)	32, 211	0.6
Tris, 0.25, 25 mM (pH 9.0)-sodium cholate (5 mM)-2-methoxyethanol (0.25 mM)	26, 29, 31, 84, 86, 125, 152, 179, 180, 258, 266, 297, 299, 300, 305, 322	4.7
Tris, 250 mM (pH 7.5)-Deoxycholic acid (5.2 mM)-ethanol (10 mM)	109	0.3
Tris, 50 mM (pH 7.2)-sodium taurocholate (100 mM)	88, 128, 158, 165, 168, 199, 272, 333	2.4
Tris (50 mM) pH 8.0-Ethanol (4%), (1 mL)	250	0.3
Tris, 50 mM (pH 8.0)-Tween 80 (0.4%)	303	0.3
NaCl (32 mM)-Na Taucholate (20 mM), 4.4 mL, pH adjusted to 9.0 final volume 5.0 mL	277	0.3

Studies are listed in the Supporting Information.
Unfortunately, 73.5% of the studies did not specifically report the buffer employed; so it is assumed that they used the buffer reported by the reference cited by these authors: ammonium bicarbonate (24 mM, pH 7.8)-Triton X-100 (0.5%), 2.9%, according to the study by Gawlicka et al. (2000); ammonium bicarbonate (24 mM, pH 8.5)-sodium deoxycholate (7.5 mM)-Triton X-100 (0.5%), 0.3%, according to the study by Murashita et al. (2008); ammonium bicarbonate (25 mM, pH 7.8)-Sodium cholate (125 mM)-NaCl (37.5 mM), 1.2%, according to the study by Sæle et al. (2010); BES (5 mM, 50 mM, pH 7.2), 4.1%, according to the studies by Mckellar and Cholette (1986) and Versaw et al. (1989); Good´s buffer (40 mM, pH 8)-taurodeoxycholate (3.4 mM)-deoxycholate (2.6 mM)-$CaCl_2$ (12 mM)-colipase (1mg/L), 0.3%, in accordance with instructions for the Pars Azmoon kit; McIlvaine buffer (0.1 M citric acid and 0.2 M bisodium phosphate, pH 8.0), 5%–6%, according to the studies by Bier (1955) and Furné et al. (2005); Phosphate buffer (? mM, pH 8.0)-Triton X-100 (10%), 0.3%, according to the study by Mustafa et al. (2016); phosphate buffer (0.33 M, pH 7.0), 1.5%, according to the study by Cherry and Crandall (1932); phosphate buffer (100 mM, pH 7.0)-Triton X-100 (0.4%), 0.3%, according to the study by Pera et al. (2006); phosphate-citrate buffer (20 mM, pH 7.5), 0.3%, according to the study by Wang et al. (2019); phosphate buffer (70 mM, pH 7.8)-acetonitrile (1%)-ethanol(4%), 0.3%, according to the study by Bülow and Mosbach (1987); Sorensen phosphate buffer (50 mM, pH 8.0), 5.0%, according to the study by Winkler and Stuckmann, 1979; Tris-HCl (400 mM, pH 7.4); Tris (10–400 mM, pH 7.4, 7.5, 9.0, 10.0), 10.6%, according to the studies by Albro et al. (1985); Roberts (1985), instructions for the Bioclin, (2023), and the study by Borlongan (1990); Tris (20.5 mM, pH 8.3)-taurodeoxicolate (3.6 mM)-deoxicolate (0.9 mM)-tartrate (0.8 mM)-$CaCl_2$ (0.05 mM)-mannitol (30 mM)-colipase (1 mg/L), 0.3%, according to the study by Santigosa et al. (2011); Tris (25 mM, pH 8.8)-deoxycholate (15 mM), 0.3%, according to the study by Shihabi and Bishop (1971); Tris (40 mM, pH 8.3)-desoxycholate (1.8 mM)-taurodesoxycholate (7.2 mM)-colipase (1 mg/mL), 0.3%, in accordance with instructions for the Spinreact (2017); Tris (100–150 mM, pH 8.0, 8.5)-taurocholate (11–15 mM), 0.3%, according to the studies by Gjellesvik et al. (1992) and Nolasco-Soria et al. (2018); Tris (2 mM, pH 6.5)-taurodeoxycholate (6 mM)-NaCl (150 mM)-$CaCl_2$ (1 mM)-NaN_3 (0.3 mM), 0.3%, according to the study by Brockman (1981); veronal buffer (100 mM, pH 7.4)-taurocolate (1.48 mg/mL), 0.3%, according to the study by Seligman and Nachlas (1963).

TABLE 5 Reaction temperature used for the determination of lipase activity.

Temperature (°C) (min or h)	Studies	%
4°C (24 h)	213	0.3
15°C (30 min)	277	0.3
15°C (2 h)	179, 180	0.6
17°C (? min)	194	0.3
19°C (? min)	32	0.3
20°C (20 min)	123	0.3
24°C (24 h)	269	0.3
25°C (15 min)	26	0.3

(Continued)

TABLE 5 Continued

Temperature (°C) (min or h)	Studies	%
>25°C or 37°C (? min)	191, 208, 278, 328	1.2
>25°C (? min)	33, 34, 35, 108, 183, 195, 199, 211, 252	2.7
>25°C (2 min)	161	0.3
>25°C (10 min)	54, 313	0.6
>25°C (15 min)	84	0.3
>25°C (25 min)	128	0.3
>25°C (30 min)	78, 79, 126, 129, 192, 245, 272	2.1
>25°C (45 min)	206	0.3
>27°C (24 h)	143	0.3
>30°C (? min)	48, 116, 321	0.9
>30°C (15 min)	6, 29, 67, 71, 152, 226, 258, 266, 282, 299, 305, 322	3.5
>30°C (30 min)	31, 125, 160, 228, 279, 281, 300	2.1
>30°C (60 min)	219	0.3
>30°C (24 h)	89, 90	0.6
>35°C (15 min)	170	0.3
>35°C (30 min)	65, 217	0.6
>37°C (? min)	58, 81, 118, 124, 186, 193, 207, 220, 246, 251, 257, 267, 276	3.8
>37°C (1 min, 2–3 min, 7 min)	174, 200, 235	0.9
>37°C (10 min)	45, 55, 148, 168, 187, 297	1.8
>37°C (15 min)	92, 298	0.6
>37°C (30 min)	214, 255, 337	0.9
>37°C (2 h)	264	0.3
>37°C (4 h)	69, 85, 172, 232, 338	1.5
>37°C (24 h)	51, 146, 212	0.9
>40°C (? min)	115, 120, 239	0.9
>40°C (15 min)	244	0.3
>40°C (30 min)	88	0.3
>60°C (? min)	24	0.3
>60°C (30 min)	327	0.3
>Nind °C (4 min, 5 min, 15 min, 20 min, 30 min, 45 min)	1, 30, 109, 165, 224, 250, 303, 312, 333	2.7
>Nind		65.8

Nind, not indicated. Therefore it is assumed that they used the temperature and incubation time reported by the reference cited by these authors: 25°C (20 min), 0.3%, according to the study by Brockman (1981); 30°C (15 min), 4.7%, according to the study by Iijima et al. (1998); 37°C (? min), 2.7%, according to the study by Cherry and Crandall (1932) and Pera et al. (2006); 37°C (1 min –5 min), 21.0%, in accordance with instructions for the Nanjing kit and Pars Azmoon kit, Shihabi and Bishop (1971) and Murashita et al. (2008); 37°C (4 min –15 min), 2.7%, according to the study by Albro et al. (1985) and instructions for the Spinreact (2017); 37°C (10 min –20 min), 5.6%, according to the study by Markweg et al. (1995); Santigosa et al. (2011); Winkler and Stuckmann (1979), and Zahran et al. (2014); 37°C (40 min), 0.3%, according to the study by Mustafa et al. (2016); 37°C (60 min –90 min), 0.6%, in accordance with instructions for the Biovision kit and Sigma kit; 37°C (3 h–6 h), 10.9%, according to the study by Bier (1955); Seligman and Nachlas (1963); Tietz and Fiereck (1966), and Borlongan (1990); 37°C (24 h), 0.3%, according to the study by Ogunbiyi and Okon (1976); 40°C (15 min), 0.3%, according to the study by Wang et al. (2019); 40°C (30 min), 4.4%, according to the study by Mckellar and Cholette (1986). The remaining studies (12.1%) did not report it.

2.2.7 Wavelength

Only 106 studies (31.3%) reported the wavelength used for absorbance or fluorescence measurements. The wavelengths used are shown in Table 7. In the case of colorimetric methods that use *p*-Nitrophenyl series substrates, the wavelengths reported (400 nm–415 nm) by the authors to measure *p*-nitrophenol (*p*-NP) are close to the maximum absorption peak (Iijima et al., 1998). However, to correctly calculate the *p*-NP moles released by lipase activity, it is necessary to make a *p*-NP standard curve at the corresponding wavelength and pH. The same situation occurs in methods using β-

TABLE 6 Reaction stopper used for the determination of lipase activity.

Reaction stopper/substrate	Studies	%
Acetic acid/olive oil	214, 337	0.6
Alcohol (ethanol), 95% olive oil	51, 65, 143, 146, 212, 213, 217, 244	2.4
Alcohol (ethanol)–acetone solution (1:1)/olive oil	69, 85, 172, 232, 338	1.5
HCl (? N)-Isooctane (1:6)/olive oil	281	0.3
Acetone-n heptane (5:2)/*p*-NPM	6, 26, 29, 31, 67, 84, 125, 152, 179, 180, 258, 266, 279, 282, 297, 299, 300, 305, 322	5.6
NaOH (10, 25 mM)/*p*-NPM	30, 129, 160, 206, 224, 228, 312	2.1
Na_2CO_3 (100 mM)/*p*-NPM	264	0.3
Na_2CO_3 (1000 mM)/*p*-NPP	327	0.3
TCA (0.72 N)/β-NC	88, 128, 165, 168, 255, 272, 298, 313, 333	2.7
Tris (1.0 M, 4°C, pH 7.5)/MUB	55, 148, 170	0.9
Nind or NA	Rest of the studies (283)	83.5

p-NPM, p-nitrophenyl miristate; p-NPP, p-nitrophenyl palmitate; β-NC, β-naphthyl caprylate; MUB, 4-methylumbelliferyl butyrate.
Nind, not indicated; NA, not applicable. References are listed in Online Resource 1.
The remaining 283 studies (68.5%) did not specifically report the use of a stopper, so it is assumed that they used the conditions reported by the reference cited by these authors. Acetone (20 M)/2-3-dimercapto-1-propanol tributyrate, 0.3%, in accordance with instructions for the Bioclin, (2023) kit acetone-ethanol (1:1, v/v)/olive oil or coconut oil, 5.9%, according to the study by Bier (1955); Ogunbiyi and Okon (1976); Linfield et al. (1984), and Furné et al. (2005); Acetone-n-heptane (5:2, v/v)/p-NPM, 5.6%, according to Iijima et al. (1998); alcohol (ethanol) (95%)/olive oil, 8.3%, according to the study by Cherry and Crandall (1932); King (1965); Tietz and Fiereck (1966), and Borlongan (1990); alcohol (ethanol) (95%)-HCl (6M) (6:1, v/v), 0.3%, according to the study by Wang et al. (2019); NaOH (14 mM)/p-NPM, 0.9%, according to Albro et al. (1985); TCA (0.72 N)/β-NC, 4.7%, according to the studies by Mckellar and Cholette (1986) and Versaw et al. (1989); TCA (2.0%)-SDS (12.5%) stopper-clarification reagent, 0.3%, according to the study by Nolasco-Soria et al. (2018); Tris (1M, pH 7.5, cold)/MUB, 0.6%, according to the study by Roberts, (1985). The remaining 50.9% did not report it, or it was not applicable.

naphthyl series substrates, such as β-naphthyl caprylate (Versaw et al., 1989). In the case of the quantification of MU (fluorometric method), the wavelengths reported by the authors are close to those recommended (Roberts, 1985) for excitation and emission. Nevertheless, the fluorometric unit calculation requires the construction of a standard curve for MU under the same experimental conditions. Unfortunately, many studies did not provide specific information on the wavelength for measuring absorbance or fluorescence of the assay mixture, so it is assumed that they used the wavelength reported in the reference cited by the authors (see Table 7).

2.2.8 Molar extinction coefficient

Only 3.0% of the studies reported molar extinction coefficients (MEC; $M^{-1}cm^{-1}$) for lipase quantification (Table 8). Another 3.8% used a standard curve (*p*-nitrophenol or β-naphthol standard curve), but without reporting a MEC or curve equation. The remaining 316 studies did not report the MEC used, so it is assumed that they used the MEC or calculation procedure reported in the references cited by the authors. The construction method of the standard curve (including pH) must be reported, in addition to the equation of the curve obtained, or the MEC used, for the calculation of the lipase units.

2.2.9 Lipase unit definition

Finally, only 118 studies (34.8%) reported the lipase unit definition. Of the studies that used *p*-nitrophenyl series substrates, only four used absorbance units (at 400 nm or 410 nm). Fortunately, 54 studies used unit expressions relative to moles of hydrolyzed substrate or generated product. Regarding the studies that used β-naphthyl series substrates, eight expressed the units in weight, and only two used unit expressions relative to moles of product generated per unit of time. The 36 studies that used lipid substrates expressed the lipase units in moles of product (fatty acids or glycerol) generated per unit of time (Table 9). The remaining 221 studies (65.2%) did not report the lipase unit explicitly used, so it is assumed that they used the lipase unit definition reported in the reference cited by the authors. The high variability or lack thereof, including the lipase unit definition, makes any comparison difficult. As examples of the variability of the reported results on lipase activity, Table 10 compares lipase units for similar samples and methods.

3 Discussion

According to Brockman (2013), lipases are water-soluble proteins, as ester hydrolases act on non-polar, water-insoluble ester substrates. According to BRENDA (2022), the lipases are classified with EC 3.1.1.3—triacylglycerol lipase. Pirahanchi and Sharma (2022) report that lipases break triglycerides into free fatty acids and glycerol by hydrolyzing the bonds of the latter. Lipases are present in pancreatic secretions for the digestion of dietary fats in the intestine. The natural foods (preys) that fish consume have high lipid contents in oils (rich in triacylglycerols). For example, lipids can account for two-thirds of the total weight of high-latitude zooplankton. In the case of artificial feeds used in aquaculture, fish oil was the most commonly used ingredient (Tocher, 2003; Qiu et al., 2017), but now the most common is vegetable oils (soybean, rapeseed, palm, corn, and sunflower) (Rombenso, 2018), with concentrations of up to 300g/kg dietary DM (Sargent and Tacon, 1999). The digestive capacity of fish will depend on the lipase

TABLE 7 Wavelength used for the determination of lipase activity.

Wavelength in nm/ substrate type	Studies	%
400/*p*-NP substrates	48, 116, 219, 252	1.2
404/*p*-NP substrates	78, 126, 192	0.9
405/*p*-NP substrates	6, 25, 26, 29, 30, 31, 35, 54, 67, 84, 104, 109, 125, 129, 152, 160, 179, 180, 183, 195, 200, 201, 206, 224, 228, 250, 258, 266, 270, 279, 282, 293, 297, 298, 299, 300, 305, 306, 312, 322	11.8
405/DGGR substrate	154, 169	0.6
410/*p*-NP substrates	24, 45, 92, 110, 115, 120, 122, 185, 209, 239, 265, 303, 327	3.8
410/2-3-dimercapto-1-propanol tributyrate	81	0.3
415/*p*-NP substrates	161, 264	0.6
420/Olive oil or Nind glyceride substrate	36, 193, 235	0.9
510/β-NC substrate	255	0.3
540/β-NC substrate	88, 107, 128, 165, 168, 199, 272, 313, 333	2.7
540/α-NC substrate	171	0.3
570/Nind triglyceride substrate	174, 328	0.6
570/DGGR substrate	191, 208	0.6
580/DGGR substrate	46, 123, 167, 220, 221, 257, 259, 330, 331, 335	2.9
714–715/olive oil substrate	70, 281	0.6
450 (emission), 355 (excitation)/ MUH substrate	38	0.3
450 (emission), 365 (excitation)/ MUB substrate	64, 170, 242	0.9
450 (emission), 380 (excitation)/ MUB substrate	55, 148	0.6
460 (emission), 365 (excitation)/ MUB substrate	66, 117, 130, 314, 336	1.5
Nind or NA	Rest of the studies (233)	68.7

p-NP, p-nitrophenyl; β-NC, β-naphthyl caprylate; α-NC, α-naphthyl caprylate; DGGR, 1,2-o-dilauryl-racglycero-3-glutaric acid-(6′-methylresorufin) ester; MUB, 4-methylubelliferyl butyrate; MUH, 4-methylubelliferyl heptanoate; TG, triglyceride.

Nind, not indicated; NA, not applicable. References are listed in Online Resource 1.

The remaining 233 studies (68.7%) did not report specifically the wavelength used, so it is assumed that they used the conditions (wavelength in nm/substrate type) reported by the reference cited by these authors. 340/olive oil, 0.6%, according to the study Shihabi and Bishop (1971); 404–405/p-NP substrates, 7.4%, according to the studies by Gjellesvik et al. (1992); Iijima et al. (1998); Pera et al. (2006), and Sæle et al. (2010); 410/p-NP substrates, 4.4%, according to the studies by Albro et al. (1985); Markweg et al. (1995); Mahadik et al. (2002), and Winkler and Stuckmann (1979); 412/dimercaptopropanol tributyrate, 0.3%, according to the study by Zahran et al. (2014); 540/β-NC, 4.1%, according to the studies by Seligman and Nachlas (1963); Mckellar and Cholette (1986), and Versaw et al. (1989); 570/TG, 1.2%, in accordance with instructions for the Biovision kit and Sigma kit; 580/DGGR, 22.1%, according to the study by Santigosa et al. (2011), and instructions for the Nanjing kit and Spinreact (2017); 655/olive oil, 0.3%, according to the study by Mustafa et al. (2016). The remaining 28.6% did not report it, or it was not applicable.

units that are synthesized in the pancreas and secreted into the intestine to digest dietary lipids. Considering the lipase definition, the crude extract hydrolytic activity on triglycerides (preferably natural ones) must be demonstrated, in agreement with Cherry and Crandall (1932).

The titrimetric methods (Cherry and Crandall, 1932) or pH stat (Kurtovic et al., 2009; Espinosa-Chaurand and Nolasco-Soria, 2019) using oil substrates can be considered tedious, as they require a large reaction volumen and their step-by-step replicate análisis is time-intensive. Because of that, the use of more practical spectrophotometric (colorimetric) methods has been preferred, as these require a smaller reaction volume and can measure the hydrolytic activity on the synthetic substrates (such as *p*-NPM or β-NC) of several samples simultaneously. However, as evidenced in this review, the variability of the methods makes comparative studies of lipase activity in fish very difficult to conduct. Given that the methods with *p*-NPM (Iijima et al., 1998) and β-NC (Versaw et al., 1989) have been recognized as sufficient to determine the presence of lipase activity and its quantification, it is considered that they can continue to be used, due to their practicality in being able to evaluate a large number of samples at the same time. However, applying the hydrolysis method of natural oils is also recommended as definitive proof of the presence of lipase activity. In all cases, adjustments must be proposed for a practical standardized method that allows comparative studies for the same or different species.

TABLE 8 Molar (or g/L) extinction coefficient or standard curve type used for lipase activity determination.

MEC/substrate type/wavelength in nm	Studies	%
15000/*p*-NP/410 nm	92	0.3
16300/*p*-NP/400 nm	116	0.3
16500/*p*-NP/405 nm	29, 84, 258, 305	1.2
p-nitrophenol standard curve/*p*-NP/405 nm	54, 179, 180	0.9
p-nitrophenol standard curve/*p*-NP/410 nm	24, 115, 185, 239, 327	1.5
p-nitrophenol standard curve/*p*-NP/? nm	32	0.3
0.02/βN-FBBB/540 nm	158, 199	0.6
0.02 (in μg/mL)/βN-FBBB/540 nm	88, 333	0.6
β-Naphthol standard curve/βN/540 nm	128	0.3
Methylumbelliferone standard curve/Methylumbelliferone/540 nm	336	0.3
Lipase (Sigma, 22,980 U/mg solid standard/6′-methylresorufin/580 nm	330, 331	0.6
Nind or NA	Rest of the studies (316)	93.2

p-NP, p-nitrophenol; βN, β-naphthol; βNC, β-naphthyl caprylate; αNC, α-naphthyl caprylate; DGGR, 1,2-o-dilauryl-racglycero-3-glutaric acid-(6′-methylresorufin) ester; MBU, 4-methylubelliferyl butyrate; MUH, 4-methylubelliferyl heptanoate.
Nind, not indicated; NA, not applicable. References are listed in Online Resource 1.
The remaining 316 studies (93.2%) did not specifically report the MEC, so it is assumed that they used the conditions (MEC or standard curve type/substance type/wavelength) reported by the reference cited by these authors. 19,800/p-NP/405 nm, 2.4%, according to the study by Gjellesvik et al. (1992); 16,500/p-NP/405 nm, 9.4%, according to the study by Iijima et al. (1998); 16,300/pNP/400 nm, 0.3%, according to the study by Faulk et al. (2007); 15,000/pNP/410 nm, 4.1%, according to the study by Winkler and Stuckmann (1979); 11,500/p-NP/418 nm, 0.3%, according to the study by Metin and Akpinar (2000); 10,300/p-NP/405 nm, 0.3%, according to the study by Pera et al. (2006); fatty acid standard curve, 0.3%, according to the study by Mustafa et al. (2016); methylumbelliferyl standard curve, 1.5%, according to the study by Roberts (1985) and Izquierdo and Henderson (1998); glycerol standard curve, 1.8%, in accordance with instructions for the Sigma and Biovision kits; lipase standard curve, 23%, in accordance with instructions for the Nanjing kit and study by Santigosa et al. (2011); β-naphthol standard curve, 5.0%, according to the studies by Mckellar and Cholette (1986) and Versaw et al. (1989); 6-methyl resorufin standard curve, 3.2%, in accordance with instructions for the Spinreact (2017) and Pars Azmoon kits. The remaining 40.7% did not report it, or it was not applicable.

TABLE 9 Type of units used for the expression of lipase activity.

Lipase units/substrate	Studies	%
0.001, 0.01, or 0.1 absorbance unit per min/*p*-NP myristate	35	0.3
0.1 absorbance unit per min/*p*-NP caproate	252	0.3
1.0 absorbance unit (400 nm) per minute/*p*-NP myristate	219	0.3
1.0 absorbance unit (405 nm) per min/*p*-NP myristate	270	0.3
1 nmol of hydrolyzed substrate per min/*p*-NP myristate	279	0.3
1 μmol of hydrolyzed *p*-nitrophenyl butyrate per min/*p*-NP butyrate	250	0.3
1 μmol hydrolyzed *p*-nitrophenyl myristate per min/*p*-NP myristate	192	0.3
1 μmol of hydrolyzed substrate per min/*p*-NP myristate or palmitate or olive oil	36, 160, 183, 206, 209, 224, 228, 245, 226, 230, 235, 246, 248, 249, 251, 297, 299, 300, 311, 312, 322	6.2
1 μmol of *p*-nitrophenylpalmitate released per min/*p*-NP palmitate	207	0.3
1 μmol *p*-NP miristate liberated per min/*p*-NP myristate	201	0.3
1 μmol of *ρ*-nitrophenol released per min/p-NP caproate, myristate, or palmitate	24, 26, 30, 31, 32, 48, 54, 67, 78, 84,92, 109, 126, 152, 211, 258, 265, 266, 276, 298, 303, 305, 326	6.8
1 μmol of *ρ*-nitrophenol released per hour/p-NP acetate	273	0.3
1 μmol product per min/*p*-NP myristate	108	0.3
1 μmol per mL per min/*p*-NP palmitate	253	0.3
1 mmol *p*-nitrophenol released per min/p-NP myristate	179, 180	0.6
1 μg β-naphtol per min/β-NC	88, 158, 165, 168, 199, 333	1.8

(Continued)

TABLE 9 Continued

Lipase units/substrate	Studies	%
1 mg β-naphtol released per min/β-NC	272	0.3
1 mg β-naphtol released per min/p-NP myristate?	260	0.3
1 µmol of β-naphtol released per min/β-NC	128, 313	0.6
1 picomol fatty acid released per min/olive oil	277	0.3
1 µmol fatty acid released per min/olive oil	33, 34, 143, 261, 281, 321	1.8
1 µmol fatty acid released per min/*p*-NP myristate	58	0.3
1 µmol fatty acid released per min/Nind substrate	198, 236	0.6
1 µmol substrate liberated per min/Nind triglyceride	186, 187	0.6
1 µmol of fatty acid released per hour/olive oil	65, 69, 85, 172, 197, 309	1.8
1 µ equivalent of fatty acid released from a triglyceride per hour/olive oil	217, 232	0.6
1 µmol of hydrolyzed triglyceride substrate per min/DGGR	114, 124, 137, 193	1.2
1 µmol product generated per min/DGGR	116, 169, 216, 220, 221, 259, 316	2.1
1 µmol of hydrolyzed triglyceride substrate per min/Nind triglyceride	241	0.3
1 µmol of glycerol released from triglycerides per min/Nind triglyceride	118, 263	0.6
1 mmol of glycerol released from triglycerides per min/Nind triglyceride	174	0.3
1 µ equivalent of fatty acids from triacetin per hour/DGGR	330, 331	0.6
1 µmol of methylumbelliferone released per min/4-methylumbelliferyl butirate, octanoate, oleate	336	0.3
1 µmol of TNB (undefined, 2-3-dimercapto-1-propanol-dithionitrobenzoic)? per min/Nind substrate	151	0.3
1 µmol (undefined) per min/Nind substrate	194	0.3
Relative fluorescence units per mg dry weight/4-methylumbelliferyl butyrate	314	0.3
Quantity of 10 mM NaOH required to maintain a constant pH per 4 h (phenolphthalein as indicator)/olive oil	338	0.3
Quantity of 10 mM NaOH required to maintain a constant pH per 24 h (phenolphthalein as indicator)/olive oil	213, 214	0.6
Quantity of 25 mM NaOH required to maintain a constant pH per 18 h (phenolphthalein as indicator)/olive oil	89, 90	0.6
Quantity of 50 mM NaOH required to maintain a constant pH per 30 min (phenolphthalein as indicator)/olive oil	212, 222, 269	0.9
Quantity of 50 mM NaOH required to maintain a constant pH per 6 h (phenolphthalein as indicator)/olive oil	164	0.3
Nind	Rest of the studies (221)	65.2

pNP, p-nitrophenol; β-NC, β-naphthyl caprylate; DGGR, 1,2-o-dilauryl-racglycero-3-glutaric acid-(6′-methylresorufin) ester.
Nind, not indicated; NA, not applicable. References are listed in Online Resource 1.
The remaining 221 studies (65.2%) did not report specifically the units, so it is assumed that they used the unit definition/substrate type reported by the reference cited by these authors: 0.01 mg of β-naphthol liberated in 5 hours/β-naphthyl laurate, 0.3% according to the study by Seligman and Nachlas (1963); 1 µmol of β-naphthol liberated per hour, 0.6%, according to the study by Mckellar and Cholette (1986); 1 nmol of methylumbelliferone released per min/4-methylumbelliferyl hepatanoate, 0.3%, according to the study by Izquierdo and Henderson (1998); 1 nmol of p-nitrophenol released per min/pNPPalmitate, 3.5%, according to the study by Winkler and Stuckmann (1979); 1 µmol fatty acids released per min/olive oil, 0.6%, according to the study by Ismat et al. (2013) and Mustafa et al. (2016); 1 µmol fatty acids released per hour/olive oil, 4.1%, according to the studies by Furné et al. (2005) and Worthington (1988), cited by Zamani et al. (2009); 1 µmol of glycerol released per min/Nind triglyceride, 0.6%, in accordance with instructions for the Biovision kit; 1 µmol triglyceride bonds broken/min/olive oil, 0.3%, according to the study by Shihabi and Bishop (1971); 1 µmol of p-nitrophenol released per min/p-NP myristate, 5.9%, according to the study by Iijima et al. (1998); 1 µmol of substrate per min/Dimercaptopropanol tributyrate, 0.3%, according to the study by Zahran et al. (2014) that used the QuantiChrom Lipase Assay Kit; 1 microequivalent of fatty acids from triacetin per hour/DGGR, 0.6%, according to the study by Santigosa et al. (2011); absorbance units × 21.15 (the latter term incorporates the dilution factors and molar absorbancy of p-nitrophenolate) per min/pNP myristate, 1.5%, according to the study by Albro et al. (1985); quantity (mL) of 50 mM NaOH required to color development using phenolphthalein as indicator, per 4–6 h/olive oil 7.7%, according to the studies by Cherry and Crandall (1932); Bier (1955), and Borlongan (1990). The remaining 38.9% did not report it, or it was not applicable.

The result of the review of the 339 articles taken as a sample reports reaction volumes ranging from 70 µL (Moro et al., 2016) to 25,000 µL (Su et al., 2017). The proposal is to use practical volumes of 200 µL (approximately 0.571 cm of the liquid column of a well of a standard 96-well microplate) for spectrophotometric methods with *p*-NPM and β-NC substrates and only 5 mL for the pH-stat method with natural oils as a substrate (Nolasco-Soria et al., 2018).

Due to the insoluble nature of lipase substrates, lipase measurement methods incorporated a detergent into the reaction mixture (to facilitate substrate solubility or the formation of micelles to multiply the lipid–water surface) or an organic solvent. However, due to the toxic nature of organic solvents, it is recommended not to use them. Triton-X100 must also be avoided due to its potential inhibitory action (Aryee et al., 2007; Nolasco-Soria et al., 2018). Replacing artificial detergents with natural ones, such as bile salts, is recommended. Only 34 of the studies analyzed reported the use of bile salts, including Na cholate (Kenari and Naderi, 2016), Na taurocholate (Frias-Quintana et al., 2016), (Na) taurodeoxycholate (Garcia-Meilan et al., 2016), and (Na) deoxycholate (Weinrauch et al., 2019). Of those bile salts cited, Na cholate was the most used (50%) in the reported cases. The concentration of bile salt used and reported by the authors fluctuated between 1.8 mM and 100 mM: Na cholate (1.8 mM –6 mM, 0.8–2.6 mg/mL), Na taurocholate (5 mM–100 mM, 2.7–53.8 mg/mL), (Na) taurodeoxycholate (3.6 mM, 1.9 mg/mL), and (Na) deoxycholate (5.2 mM, 2.0 mg/mL). It is possible that, in the case of the 100 mM concentration, this refers to the concentration of the reagent used and not to the final concentration in the reaction mixture.

Only four studies reported using 0.8 mM $CaCl_2$ in the lipase reaction mixture. Therefore, the recommendation is to measure the lipase reaction mixture's calcium requirements (0 mM–10 mM).

The working pH for determining lipase activity varied from pH 7 (Blanco et al., 2016) to pH 9 (Guo et al., 2016). It is proposed to use an intermediate pH between these values (pH 8) to determine lipase activity, which brings the working pH closer to the physiological pH of the fish intestine (average pH 7.5, according to Solovyev and Izvekova, 2016). Tris-HCl buffer is recommended at the final concentration in the reaction mixture, which was about 20 mM–30 mM.

The working temperature for determining lipase activity varied from 4°C (Jayant et al., 2018) to 60°C (Hahor et al., 2016). It is proposed to use a temperature of 25°C, which is closer to the ecophysiological temperature for temperate-water fish (Gisbert et al., 2018).

The working wavelength used to measure reaction mixture absorbance for the lipase determination with *p*-NP substrates ranged from 400 nm (Rueda-Lopez et al., 2017) to 410 nm (Hahor et al., 2016). Although the absorbance difference within the 400 nm to 410 nm range is slight, the recommendation is to measure the absorbance at the wavelength according to the absorption spectra of the final lipase reaction mixture. In the case of substrates from the β-N group, the wavelengths to measure the absorbance of the reaction mixture were 510 nm (Pujante et al., 2017) or 540 nm (Frias-Quintana et al., 2016). All the authors used the method of Versaw et al. (1989), who proposed using a wavelength of 540 nm. The β-naphthyl caprylate method is recommended for the first analysis of samples with low activity because of its high sensitivity and noticeable purple color. In the case of fluorometric methods, the wavelength used was 450 nm–460 nm (emission) and 365 nm–380 nm (excitation) for the MBU substrate.

The molar extinction coefficient (MEC) of *p*-NP will vary depending on the wavelength used to measure its absorbance. The few values reported by the authors were 16,300 at 400 nm (Thompson et al., 2019), 16,500 at 405 nm (Ramzanzadeh et al., 2016), and 15,000 at 410 nm (Adeyemi et al., 2020). The recommendation is to build a *p*-NP standard curve (pH 8.0) and to measure the absorbance at 400 nm to obtain the MEC for converting the absorbance to moles of *p*-NP to calculate the lipase units. Something similar occurs with the β-N MEC. Only two studies present the value of 0.02 MEC used (Najera-Arzola et al., 2018; Frias-Quintana et al., 2019). Because the MEC value used by authors is a low number, the expression units should differ from $M^{-1}cm^{-1}$.

The proposal is that lipase units should be expressed in micromoles of the product (*p*-NP or β-N or fatty acid) released per minute. Each mole would represent the hydrolysis of one mole of ester bonds. The significant differences between the reported lipase units (see Table 10) could be due to methodological differences. The proposal is to standardize the temperature, type of substrate, standard curve or MEC, and calculation formulas.

If lipase activity is measured at a mildly acidic pH, extreme care must be taken, as the color generated by either β-N or *p*-NP is negatively affected (color decrease). In contrast, at an alkaline pH, the color is not significantly affected. This forces, where appropriate, the building of a specific standard curve for the working pH.

The measurement of lipase activity using emulsified NaT-olive oil as a substrate in pH stat is a practical method (Nolasco, 2008; Nolasco-Soria et al., 2018) recommended as definitive proof of the presence of lipases, according to Cherry and Crandall (1932). If it is supposed that a pH stat (laboratory equipment) is unavailable (because it is relatively expensive), one option would be to use a high-precision potentiometer (with three or four decimal places) or a standard potentiometer (which is available in most laboratories) instead. The pH of the reaction mixture (containing the oil emulsion) is adjusted to the working pH (for example, 8.0), the enzymatic reagent is added (at pH 8.0), and the pH is adjusted to 8.0 every minute of digestion with NaOH at 10 mM or 20 mM (using repeater pipette), depending on the rate of hydrolysis by the experimental lipases. The consumption of NaOH per minute is recorded, and the micromoles per min of NaOH are calculated to have the micromole of ester bonds hydrolyzed per minute. This method can be used to measure the digestibility of edible oils for the species of interest. For this procedure, it is recommended to determine the digestive capacity by quantifying the total lipase units in the digestive tract (pyloric cecum intestine). In addition, the amount of food oil that the fish consumes in one serving should also be calculated. With these values, 10% of the lipase units can be used as enzymatic reagent and 10% of the equivalent oil of an intake can be used as substrate. In this way, the lipase units of a fish would serve to make 10 replicates of *in vitro* digestibility.

TABLE 10 Comparison of lipase in digestive tract and larvae, reported by authors, in fish.

Conditions (tissue, substrate)	Adjusted units/mg protein or mg dry weight (DW)	Unit definition	Difference in times	Study
Larvae, 4-methylumbelliferyl butyrate*	20,000,000	Relative fluorescence units (RFU)**		66
Larvae, 4-methylumbelliferyl butyrate	1,010	Relative fluorescence units (RFU)**	19,802	314
Larvae, *p*-NPM	2	Nind		125
Larvae, *p*-NPM	0.022	1 µmol of ρ-nitrophenol released per minute	95	31
Juvenile intestine, *p*-NPM	18.2	Nind		86
Juvenile intestine, *p*-NPM	0.017	1 µmol of hydrolyzed substrate per minute	1,070	297
Larval intestine, β-NC	106	1 µmol of β-naphtol released per minute		158
Larval intestine, β-NC***	0.00178	Nind	59,551	22
Juvenile intestine, β-NC	8.43	Nind		73
Juvenile intestine, β-NC	0.029	Nind	295	255
Juvenile intestine/DGGR	1.88	1 µ equivalent of fatty acid from triacetin per hour		28
Juvenile intestine/ DGGR****	0.009	1 µ equivalent of fatty acid from triacetin per hour	209	330
Juvenile digestive tract/ olive oil	211	Amount of 0.025 N NaOH needed to neutralize the fatty acids liberated per 18 hours		89
Juvenile digestive tract/ olive oil	0.190	Amount of 0.025 N NaOH needed to neutralize the fatty acids liberated per 18 hours	1,111	90

*2e+7 RFU/mg DW was converted to 20,000,000 RFU/mg DW, **Units/mg dry weight (DW), ***1.78 mU were converted to 0.00178 U, **** 9.01 mU were coverted to 0.009 U, p-NPM, p-nitrophenyl mirystate, β-NC, β-naphthyl caprylate.

For lipase detection on SDS-PAGE electrophoresis gels, several methods have been used. Agar with emulsified olive oil was placed on the electrophoresis gel to reveal hydrolysis zones using Victoria Blue staining (Villanueva-Gutierrez et al., 2020). The authors also used a 2% agar, 1% oleic acid, and 0.01% phenol red, either with or without 0.01% ox bile salts (in 0.01 M $CaCl_2$), revealing the lipases by the formation of a yellow spot on the agar substrate (González-Félix et al., 2018). Previously, lipase zymograms were obtained using a 4%–30% native gradient PAGE, where β-naphthyl caprylate (200 mM) was copolymerized and revealed in the form of a red spot using Fast Blue BB (100 mM) (Alvarez-González et al., 2008; Nolasco et al., 2011).

Authors should consider using the proposed lipase methods as an alternative to facilitate comparative studies. It is recommended that authors describe their way of calculating lipase activity in detail. Lipase units must be expressed regarding micromoles of hydrolyzed substrate or released product per min. Data on fish, digestive tract, and organ weight must be included. In addition to the specific enzyme activities (U/mg protein), total lipase units are required (lipase units per fish, per gram of fish, per gram of the digestive tract).

A proposal for standardizing lipase methods for fish is required. Because the most used substrate has been *p*-nitrophenyl substrates (mainly myristate) should be considered. It is necessary to experimentally evaluate some environmental and compositional variants of the lipase reaction mixture to ensure that it is safe, sensitive, and practical at the microplate level: (a) the presence of bile salts (type and concentration) as emulsifiers and lipase activity enhancers; (b) the optimum concentration of ions (Ca^{+2} and Na^{+}); (c) the proper wavelength for absorbance readings and the adjustment of absorbance values at 1 cm light path; and (d) the use of the MEC according to the corresponding standard curve (built under the same experimental conditions at which the measurement of lipase activity was carried out). In addition, it is proposed that the lipase method using β-naphthyl substrate (mainly caprylate) be considered, because of its high sensitivity and colorful reaction mixture as a medium carbon-length substrate. The measurement of lipase activity using natural oils as substrates in a small volume is proposed as a definitive test to confirm the presence of lipases in the digestive tract of fish.

4 Conclusions

The most widely used methods to measure lipase activity in cultured fish are currently the colorimetric methods (with p-NPM and BNC as substrates) and the titrimetric method with natural oil (with olive oil as a substrate). However, methodological variability, even for studies that use the same type of methodology, makes it difficult to compare the data obtained. Standardizing current lipase analytical procedures should improve the reliability of comparative

studies of aquaculture fish species. The above also applies to other taxonomic groups of aquacultural interest, such as crustaceans and mollusks.

Author contributions

HNS: Conceptualization, Methodology, Software, Data curation, Investigation, Validation, Formal analysis, Supervision, Resources, Project administration, Visualization, Funding acquisition, Writing—original draft, Writing—review & editing.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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