Journal of Functional Foods 82 (2021) 104497

Contents lists available at ScienceDirect

Journal of Functional Foods

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

INFOGEST inter-laboratory recommendations for assaying gastric and pancreatic lipases activities prior to *in vitro* digestion studies

Myriam M.L. Grundy [a,*], Evan Abrahamse [b,c], Annette Almgren [d], Marie Alminger [d], Ana Andres [e], Renata M.C. Ariëns [f], Shanna Bastiaan-Net [f], Claire Bourlieu-Lacanal [g,h], André Brodkorb [i], Maria R. Bronze [j,k,l], Irene Comi [m], Leslie Couëdelo [n], Didier Dupont [g], Annie Durand [o], Sedef N. El [p], Tara Grauwet [q], Christine Heerup [r], Ana Heredia [e], Marcos R. Infantes Garcia [q], Christian Jungnickel [s], Ilona E. Kłosowska-Chomiczewska [s], Marion Létisse [o], Adam Macierzanka [s], Alan R. Mackie [t], David J. McClements [u], Olivia Menard [g], Anne Meynier [v], Marie-Caroline Michalski [o], Ana-Isabel Mulet-Cabero [i,w], Anette Mullertz [r], Francina M. Payeras Perelló [x], Irene Peinado [e], Mélina Robert [h], Sébastien Secouard [x], Ana T. Serra [j,k], Sandra D. Silva [j], Gabriel Thomassen [c], Cecilia Tullberg [d], Ingrid Undeland [d], Carole Vaysse [n], Gerd E. Vegarud [m], Sarah H.E. Verkempinck [q], Michelle Viau [v], Mostafa Zahir [y], Ruojie Zhang [u], Frédéric Carrière [z]

[a] *PEGASE, INRAE, Institut Agro, 35590 Saint Gilles, France*
[b] *Danone Nutricia Research, 3584CT Utrecht, the Netherlands*
[c] *Laboratory of Food Chemistry, Wageningen University, the Netherlands*
[d] *Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-41296 Göteborg, Sweden*
[e] *Research Institute of Food Engineering for Development, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain*
[f] *Wageningen Food & Biobased Research, Wageningen University and Research, 6708 WG Wageningen, the Netherlands*
[g] *STLO Science et Technologie du Lait et de l'oeuf, INRAE – Institut Agro, 35000 Rennes, France*
[h] *INRAE, Montpellier SupAgro, CIRAD, Université de Montpellier, IATE, 2 place Pierre-Viala, bâtiment 33, F-34060 Montpellier, France*
[i] *Department of Food Chemistry and Technology, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork P61 C996, Ireland*
[j] *iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal*
[k] *Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal*
[l] *iMED, Faculdade de Farmácia da Universidade de Lisboa, 1649-003 Lisboa, Portugal*
[m] *Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences (NMBU), P.O. Box 5003, NO-1432 Ås, Norway*
[n] *ITERG, Université de Bordeaux, F-33076 Bordeaux, France*
[o] *CarMeN, U1060 INSERM INRA 1397 CARMEN, Hôpital Lyon Sud - Secteur 2, Bâtiment CENS ELI-2D, 165 Chemin du Grand Revoyet, F – 69310 Pierre Benite, France*
[p] *Department of Food Engineering, Ege University, 35100 Izmir, Turkey*
[q] *Laboratory of Food Technology, Leuven Food Science and Nutrition Research Center (LFoRCe), Department of Microbial and Molecular Systems (M^2S), KU Leuven, Kasteelpark Arenberg 22 box 2457, 3001 Heverlee, Belgium*
[r] *Bioneer:FARMA, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen, Denmark*
[s] *Department of Colloid and Lipid Science, Gdańsk University of Technology, 11/12 Gabriela Narutowicza Street 80-233 Gdańsk, Poland*
[t] *School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK*
[u] *Biopolymers and Colloids Laboratory, Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA*
[v] *UR1268 BIA (Biopolymères Interactions Assemblages), INRAE, F-44300 Nantes, France*
[w] *Food Innovation and Health, Quadram Institute Bioscience, Norwich Research Park, Colney, Norwich NR4 7UA, UK*
[x] *Inovation & Development Centre, Fresenius Kabi, 61352 Bad Homburg, Germany*
[y] *Food Quality and Design, Wageningen University and Research Centre, 6708 WG Wageningen, the Netherlands*
[z] *Aix-Marseille Université, CNRS, UMR 7282, 31 chemin Joseph Aiguier, F-13402 Marseille Cedex 9, France*

Abbreviations: API, active pharmaceutical ingredient; BSA, bovine serum albumin; CEH, carboxyl ester hydrolase; CV, coefficient of variation; FA, fatty acid; FFA, free fatty acids; GI, gastrointestinal; GL, gastric lipase; NaTDC, sodium taurodeoxycholate; PL, pancreatic lipase; sPLA2, secreted pancreatic phospholipase A2; PLRP2, pancreatic lipase-related protein 2; PLS, Partial Least Squares; RGE, rabbit gastric extract; $SA_{Pancreatin}$, specific activity of pancreatin; SA_{RGE}, specific activity of RGE; TAG, triacylglycerols; Tris, Trisaminomethane; USP, United States Pharmacopeia; VIP, variable importance for the projection.

* Corresponding author at: PEGASE, INRAE, Institut Agro, 35590 Saint Gilles, France.

https://doi.org/10.1016/j.jff.2021.104497
Received 10 February 2021; Received in revised form 6 April 2021; Accepted 18 April 2021
Available online 10 May 2021
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ARTICLE INFO

Keywords:
Lipases
Lipolysis
Enzyme activity
Titration method
Inhibitor
INFOGEST

ABSTRACT

In vitro digestion studies often use animal digestive enzyme extracts as substitutes of human gastric and pancreatic secretions. Pancreatin from porcine origin is thus commonly used to provide relevant pancreatic enzymes such as proteases, amylase and lipase. Rabbit gastric extracts (RGE) have been recently introduced to provide gastric lipase in addition to pepsin. Before preparing simulated gastric and pancreatic extracts with targeted enzyme activities as described in *in vitro* digestion protocols, it is important to determine the activities of enzyme preparations using validated methods. The purpose of this inter-laboratory study within the INFOGEST network was to test the repeatability and reproducibility of lipase assays using the pH-stat technique for measuring the activities of gastric and pancreatic lipases from various sources. Twenty-one laboratories having different pH-stat devices received the same protocol with identical batches of RGE and two pancreatin sources. Lipase assays were performed using tributyrin as a substrate and three different amounts (50, 100 and 200 μg) of each enzyme preparation. The repeatability results within individual laboratories were satisfactory with coefficients of variation (CVs) ranging from 4 to 8% regardless of the enzyme amount tested. However, the inter-laboratory variability was high (CV > 15%) compared to existing standards for bioanalytical assays. We identified and weighted the contributions to inter-laboratory variability of several parameters associated with the various pH-stat equipment used in this study (e.g. reaction vessel volume and shape, stirring mode and rate, burette volume for the automated delivery of sodium hydroxide). Based on this, we established recommendations for improving the reproducibility of lipase assays using the pH-stat technique. Defining accurate and complete recommendations on how to correctly quantify activity levels of enzyme preparations is a gateway to promising comparison of *in vitro* data obtained from different laboratories following the same *in vitro* digestion protocol.

1. Introduction

In vitro models simulating gastrointestinal (GI) digestion are widely used as a tool to study, under well-controlled conditions, a wide range of food items without the constraints associated with human trials, e.g. ethics, cost, time, toxicity, limitation in the number of food products that can be tested or samples that can be collected, issues on variation among individuals (Hur, Lim, Decker, & McClements, 2011; Guerra et al., 2012; Minekus et al., 2014; Bornhorst, Gouseti, Wickham, & Bakalis, 2016). *In vitro* digestion models are based on the use of digestive enzyme preparations, often from animal sources, as substitutes of salivary, gastric and pancreatic secretions. Pancreatin from porcine origin is most commonly used to provide relevant pancreatic enzymes such as proteases, amylase and lipases (PL), while human salivary α-amylase can be used for preparing simulated salivary fluid (Minekus et al., 2014). More recently, rabbit gastric extract (RGE) has been introduced to provide gastric lipase (GL) in addition to pepsin in simulated gastric fluid (Brodkorb et al., 2019). Prior to performing *in vitro* digestion experiments, it is essential to assess the activities of these enzyme preparations in order to use enzyme concentrations that mimic physiological conditions, are consistent between experiments and enable comparison. With that in mind, standard protocols have been established based on *in vivo* data collected during the digestion of test meals, especially within the INFOGEST network, an international and multidisciplinary consortium of researchers from more than 35 countries (http://www.cost-infogest.eu). Within INFOGEST, a subgroup (WG4) is collaborating and sharing expertise in the field of lipid digestion phenomena and digestive lipases. One of its main tasks is to validate and standardize lipase assays for GL and PL, the two main digestive enzymes involved in triacylglycerols (TAG) digestion (Bakala N'Goma, Amara, Dridi, Jannin, & Carrière, 2012). Briefly, TAG, are the main dietary lipids (~90%) in the human diet and GL and PL are the most important enzymes in TAG hydrolysis.

A major difficulty in assaying the activity of these enzymes resides in the preparation of their substrate that is insoluble in water and forms a distinct liquid phase dispersed in water. Appropriate substrate emulsions have to be prepared to allow lipase adsorption and activity at the oil–water interface. This is essential because most lipases are activated at this interface by adapting their structure (lid opening) and access to their catalytic site (Mateos-Diaz et al., 2017). This is the main reason why many lipase assays with micellar or monomeric substrates rather than emulsions are not specific or sensitive enough for GL and PL activity determination. This is the case with most chromogenic and fluorogenic substrates that have been developed so far (Beisson, Tiss, Rivière, & Verger, 2000). In some assays with fluorescent or UV-absorbing (Beisson et al., 1999; Serveau-Avesque, Verger, Rodriguez, & Abousalham, 2013; Ulker et al., 2016), TAG substrates have been developed to allow high throughput assays using microtiter plates. They are adapted for studying purified enzymes but have not been validated with complex samples of digestion mixtures. Therefore, lipase assays using TAG emulsions are still the most specific and sensitive option. Sensitivity is due firstly to the high specific activities of lipases on emulsified TAG (several hundred to thousand international units (U) per mg of pure enzyme; 1 U = 1 μmole of fatty acid (FA) release per minute) and secondly, to the acid-base titration of the reaction products, i.e. FA, that can be performed using the pH-stat technique (Beisson et al., 2000). Various types of oils with short, medium and long chain FAs or their mixture can be used. Olive oil has been used as a reference substrate since decades, and it is recommended in most pharmacopeia assays of PL (see Pancrelipase and pancreatin monographs from European and United States Pharmacopeia (USP), (United States Pharmacopeia. (2018), 2018; European Pharmacopoeia, 2019)). Nevertheless, using long chain TAG as substrate requires the use of emulsifiers such as gum arabic to form a stable emulsion (Tiss, Carrière, & Verger, 2001). Moreover, long chain FAs have a pKa close to 7.6 (Benzonana, 1968; Bakala-N'Goma et al., 2015) and their full titration requires alkaline pH conditions. The USP pancrelipase assay is thus performed at pH 9, a pH value that is far different from the physiological conditions of the GI tract (Bakala N'Goma et al., 2012). Back-titration assays can be performed with the release of FAs by the lipase at a physiological pH prior to FA full titration at pH 9, but these assays are time consuming and not as precise as the direct titration of FAs (Bakala-N'Goma et al., 2015). Because of all these drawbacks of long chain FA substrates, the use of a synthetic short chain oil, such as tributyrin, was introduced in 1970 by Erlanson and Borgström for the determination of lipase activity of pancreatic juice and small intestinal content (Erlanson & Borgstrom, 1970). Using tributyrin has several advantages: i) it readily forms a fine emulsion under mechanical stirring and the addition of emulsifiers is not necessary; ii) butyric acid (pKa = 4.55) released from tributyrin can be directly titrated at the physiological pH of the small intestine and even at acidic pH found in gastric contents using a correction factor (Carrière, Barrowman, Verger, & Laugier, 1993); iii) the lipolysis products of tributyrin are soluble in water and do not accumulate at the oil–water interface like long chain lipolysis products that can subsequently inhibit lipase activity. Although some researchers were hesitant to use this non-natural substrate, tributyrin has progressively become a standard substrate in many lipase assays, including GL assay, because it allows the

measurement of enzymatic activity at an acidic pH close to the optimum pH of activity (Gargouri et al., 1986; Carrière et al., 1993). This has made it possible to use a single reference substrate for both GL and PL adapted to and optimized for of the reaction conditions for each. GL is thus assayed in the presence of bile salts (2 mM sodium taurodeoxycholate (NaTDC)) and bovine serum albumin (BSA, 1.5 μM), whose roles are to decrease the interfacial tension at the tributyrin-water interface to avoid interfacial denaturation of the enzyme and thereby reach optimum conditions of activity (Gargouri, Piéroni, Lowe, Sarda, & Verger, 1986). PL activity is measured in the presence of a supramicellar concentration of bile salts (4 mM NaTDC) and of colipase, the specific PL cofactor that allows lipase anchoring at the oil–water interface in the presence of bile salts (Borgström, 1975). In both assays, the free fatty acids (FFAs) released upon tributyrin hydrolysis by the lipase can be titrated with sodium hydroxide (NaOH) using the pH-stat technique, i.e. the titration of FFAs at a constant pH value (5.5 for GL and 8 for PL).

Besides lipase assays for the characterization of enzyme preparations, the pH-stat technique has also been used for measuring protein and lipid digestion *in vitro* (Beisson et al., 2000; Pinsirodom & Parkin, 2001; Zangenberg, Mullertz, Kristensen, & Hovgaard, 2001; Gilham & Lehner, 2005; Li & McClements, 2010; Williams et al., 2012; Minekus et al., 2014; Chatzidaki, Mateos-Diaz, Leal-Calderon, Xenakis, & Carrière, 2016; Mat, Cattenoz, Souchon, Michon, & Le Feunteun, 2018; Mat, Souchon, Michon, & Le Feunteun, 2020). One has to be cautious, however, with the use of the pH-stat technique for these applications for the reasons previously mentioned (high pKa and poor titration of long chain FAs without back-titration) and also the strong buffering effects of some food components that can interfere with the titration.

One of the advantages of implementing the pH-stat technique in a food digestion laboratory is that it permits the measurement of activities of other lipolytic enzymes such as pancreatic lipase-related protein 2 (PLRP2), pancreatic carboxyl ester hydrolase (CEH) and pancreatic phospholipase A2 (sPLA2) using various lipid substrates from which FAs can be released and titrated (phospholipids, galactolipids, cholesterol and vitamin esters, synthetic esters) (Fernandez et al., 2008; Fernandez et al., 2010; Salhi et al., 2020). These assays are however not considered as part of this work.

The main objectives of this multicentre study were: i) to test the repeatability and reproducibility of established GL and PL assays using the pH-stat technique; ii) to identify critical parameters for improving inter-laboratory variability; iii) to investigate the use of boronic acid as lipase inhibitor to block lipolysis. For this purpose, 21 laboratory members of the INFOGEST network involved in the WG4 group received three sources of lipases (one GL and two PLs) and measured their activities following the same protocol but using different types of pH-stat equipment.

2. Materials and methods

2.1. Materials and equipment

Trisaminomethane (Tris), sodium chloride (NaCl), calcium chloride ($CaCl_2{\cdot}2H_2O$), NaTDC, NaOH, glyceryl tributyrate (tributyrin, ≥99%; #T8626 from Sigma-Aldrich), BSA (98%; #A7030 from Sigma-Aldrich), and 4-bromophenylboronic acid (#B75956 from Sigma-Aldrich) were purchased by each lab.

Fig. 1 gives a schematic overview of the principle behind the pH-stat technique and of the different reaction vessel shapes as well as the stirring devices used by the 21 laboratories. To take part in this study, the participants were required to have at their disposal a pH-stat device equipped with a thermostated reaction vessel, a mechanical stirrer (propeller or magnetic stirrer), an automated system for 0.1 N NaOH delivery, and a pH electrode adapted for viscous/oily dispersions, ideally with a sleeve diaphragm.

2.2. Lipase sources

The 21 laboratories were supplied with three sources of lipases (one GL and two PLs), each from a single batch. Lyophilized RGE (#RGE25-100MG) were a generous gift from Lipolytech S.A. (Marseille, France) and contained 2.5% w/w of rabbit GL. One source of PL was porcine pancreatic extract (pancreatin) purchased from Sigma Aldrich (#P7545; 8 × USP specification); this sample is referred to as Sigma pancreatin in the manuscript. Nordmark Arzneimittel GmbH & Co KG (Uetersen, Germany) provided the second source of PL, referred to as Nordmark pancreatin (#N0066397, lipase activity ≥ 80,000 Ph. Eur. U/g). Both pancreatin sources contained colipase, at a colipase to lipase molar ratio estimated at around 1.5 (Salhi et al., 2020), in other words a colipase amount that is sufficient to measure PL activity in the presence of bile salts without adding exogeneous colipase.

2.3. Enzyme solution preparations

Dispersion of each enzyme powder (1 mg/mL) was prepared by dissolving 10 mg of enzyme powder in 10 mL of cold ultrapure deionised water initially stored on ice, vortexed for 30 s and kept on ice until the assay was performed.

The step-by-step protocol as communicated to the laboratories can be found in Supplementary Data (see *Protocol for preparing enzymes stock solutions for lipase assays*).

It is worth noting that pancreatic extracts are not completely soluble in water and some enzyme activity (around 10%) remains associated to insoluble solid particles. We therefore recommended using this enzyme dispersion without prior filtration of the insoluble material and after homogenous mixing using a vortex. Also, it was requested to the participants to perform the assay as soon as possible after preparing the enzyme solution since RGE and the pancreatin sources contain active proteases that can rapidly degrade the lipases, especially in pancreatin. Three different volumes (50, 100 and 200 μL) of each dispersion were used for the lipase assays, i.e. 50, 100 and 200 μg of enzyme preparation per assay.

2.4. GL activity assay

The assay solution (pH 5) in which the substrate (tributyrin) emulsion was formed contained 1.5 μM of BSA, 150 mM of NaCl and 2 mM of NaTDC, as optimized and described by Moreau et al. (Moreau, Gargouri, Lecat, Junien, & Verger, 1988) for rabbit GL and by Gargouri et al for human GL (Gargouri et al., 1986). The step-by-step protocol received by the laboratories can be found in the Supplementary Data (see *Protocol for the assay of gastric lipase in RGE*). The substrate emulsion was prepared directly in the pH-stat vessel thermostated at 37 °C while mixing 14.5 mL of the assay solution and 0.5 mL of tributyrin. After the temperature was stabilized, the pH was adjusted to 5.5 and the pH-stat regulation started to keep this pH value constant. After recording the baseline and ensuring it was stable over time, the sample to be titrated (50, 100 or 200 μL of the enzyme stock solution, or 100 μL of the enzyme-inhibitor solution (when GL inhibition is tested; see *Section 2.7*)) was added and NaOH (0.1 N) delivery was recorded as a function of time.

Depending on the equipment used (regulation rate) and enzyme activity, the rate of NaOH delivery may not immediately progress linearly (presence of a lag time; Fig. 2) and therefore recording of NaOH delivery was recommended for at least 5 min.

GL activity (A_{GL}) was then estimated from the rate of NaOH delivery. Since butyric acid is not fully ionized and titrable at pH 5.5, a correction factor of 1.12 was applied to obtain the full activity from equation (Eq. (1)):

$$A_{GL} = (V_{NaOH}/(t_2 - t_1)) \times N \times 1.12 \quad (\mu mol/min) \tag{1}$$

where A_{GL} is the rate of NaOH delivery in μmole per minute or enzyme

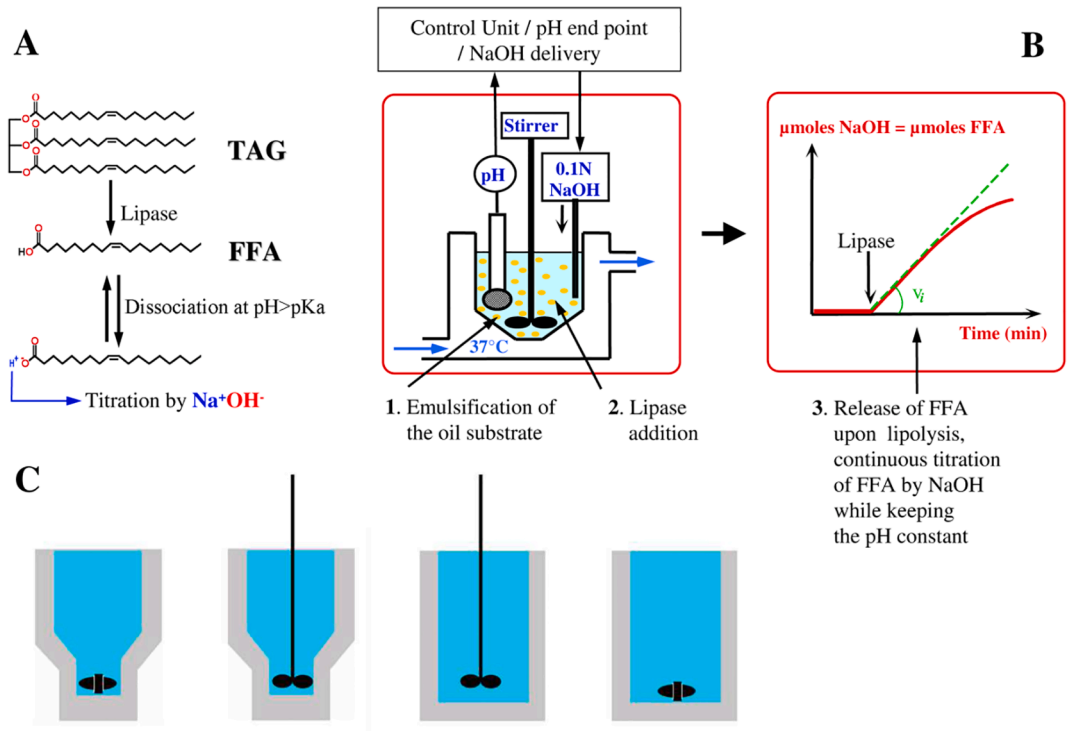

Fig. 1. *Principle of lipase assay using the pH-stat technique.* (A) Triacylglycerol (TAG) is the substrate of lipase (EC3.1.3.1 triacylglycerol hydrolase) that catalyses the release of free fatty acids (FFAs) from TAG. FFAs can be titrated by NaOH. (B) Schematic illustration of a pH-stat device, equipped with a thermostated reaction vessel, in which the substrate emulsion is formed by mechanical stirring; a pH-electrode connected to a pH-meter and a control unit for the automated delivery of NaOH by a burette. Every time the pH decreases due to the release of FFAs, NaOH is delivered to keep the pH constant at a pre-determined endpoint value. The delivery of NaOH (µmoles) is recorded as a function of time (min) and lipase activity is expressed in µmoles NaOH (or FFAs titrated) per min, with 1 international unit (U) equal to 1 µmol FFA/min. (C) Different geometries of reaction vessel (conical or cylindrical) and stirring devices (magnetic stirrer or propeller) available at the laboratories involved in the study.

activity expressed in international units (U) (1 U = 1 µmol of butyric acid released per minute), V_{NaOH} is the volume of NaOH delivered, in µL, between times t_2 and t_1 and N is the titre of NaOH (0.1 N).

In a next step, the specific activity of RGE (SA_{RGE}) was calculated from Eq. (2):

$$SA_{RGE} = (A_{GL} \times 1000)/(C \times v) \quad (U/mg) \tag{2}$$

where SA_{RGE} is expressed in U per mg of powder, C is the concentration (mg/mL) of powder in the enzyme solution and v is the volume of enzyme solution added in µL.

To note: While biochemists usually express enzyme specific activity in IU per mg of proteins, it is a common practice to express activities of pharmaceutical enzyme preparations in IU per mass of material powder and these activities are the reference for further use of these preparations. Since protein amounts in commercial pancreatin preparations are rarely provided, it is more convenient to express specific activities in IU per mg for researchers performing *in vitro* digestion experiments.

For each RGE concentration tested, three repeats were performed using a fresh preparation for each repeat.

2.5. PL activity assay

The assay conditions for the measurement of PL activity were identical to those described in the Supplementary Materials section of the INFOGEST protocol (Minekus et al., 2014; Brodkorb et al., 2019). They were based on the original work by Erlanson and Borgström (Erlanson & Borgstrom, 1970) who introduced the use of tributyrin as substrate for

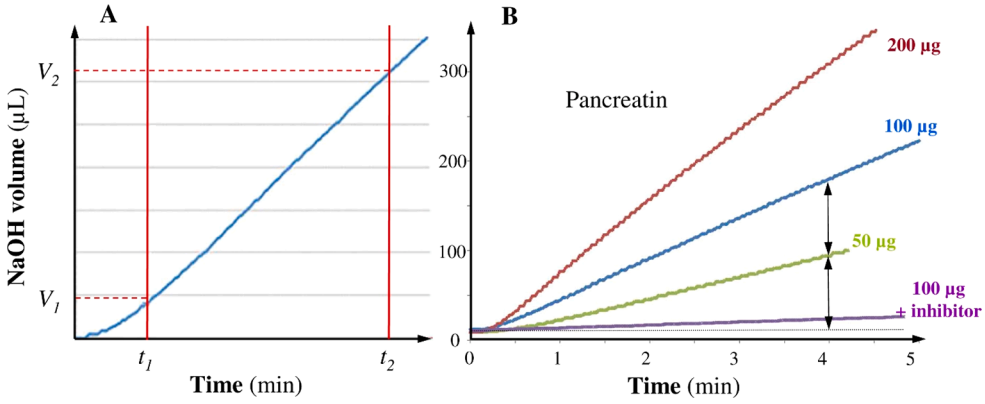

Fig. 2. Typical titration curves showing the delivery of NaOH (mL) as a function of time (min) in the course of a lipase assay with a pH-stat device. (A) Typical titration curve with a lag time showing the time period (around 5 min) during which the volume of NaOH delivered with time should be measured. (B) Assay of PL activity with increasing amounts of pancreatin (50, 100 and 200 µg) showing the proportional increase in NaOH delivery rate, as well as the reduced rate measured in presence of the inhibitor 4-bromophenyl boronic acid.

PL and have been established as standard conditions for measuring PL activity in complex mixtures such as pancreatin (Salhi et al., 2020) or intestinal contents (Carrière et al., 1993). The assay solution contained 0.3 mM of Tris, 150 mM of NaCl, 2 mM of $CaCl_2$, and 4 mM of NaTDC. The step-by-step protocol received by the laboratories can be found in Supplementary Data (see *Protocol for the assay of pancreatic lipase in pancreatin*). The substrate emulsion was prepared directly in the pH-stat vessel thermostated at 37 °C while mixing 14.5 mL of the assay solution and 0.5 mL of tributyrin. After the temperature was stabilized, the pH was adjusted to 8 and the pH-stat regulation started to keep this pH value constant. After recording the baseline and ensuring it was stable with time, the sample to be titrated (50 μL, 100 μL or 200 μL of the enzyme stock solution, or 100 μL of the enzyme-inhibitor solution (when PL inhibition is tested; see Section 2.7)) was added and NaOH (0.1 N) delivery was recorded as a function of time.

PL activity (A_{PL}) was estimated from the rate of NaOH delivery. Since butyric acid is fully ionized at pH 8, no correction factor was required and activity was obtained from Eq. (3):

$$A_{PL} = (\Delta V_{NaOH}/(t_2 - t_1)) \times N \quad (\mu mol/min) \tag{3}$$

where A_{PL} is the rate of NaOH delivery in μmoles per minute or enzyme activity expressed in international units (U) per milliliter (1 U = 1 μmol of butyric acid released per minute), V_{NaOH} is the volume of NaOH delivered, in μL, between times t_2 and t_1 and N is the titre of NaOH (0.1 N).

Next, the specific activity of pancreatin ($SA_{Pancreatin}$) was calculated from Eq. (4):

$$SA_{pancreatin} = (A_{PL} \times 1000)/(C \times v) \quad (U/mg) \tag{4}$$

where $SA_{Pancreatin}$ is expressed in U per mg of powder, C is the concentration (mg/mL) of powder in the enzyme solution and v is the volume of enzyme solution added in μL.

For each concentration of pancreatin (Sigma or Nordmark) tested, three repeats were performed using a fresh preparation for each repeat.

2.6. Inhibition of GL and PL by boronic acid

Lipase inhibition was performed by pre-mixing the RGE or the pancreatin sources with boronic acid. A solution of 4-bromophenylboronic acid (1 M) was prepared in methanol (Williams et al., 2012) and 5 μL of this stock solution was added to 1 mL of the enzyme stock solution at 1 mg powder per mL, prepared as described in Section 2.4. The mixture was vortexed and incubated at room temperature for 10 min before performing the assays of residual lipase activity as described in Sections 2.5 and 2.6.

For the RGE, higher amounts of boronic acid were also tested using 10, 25 and 50 μL of the 1 M stock solution. The assays, for each amount of boronic acid tested, and the negative control (methanol only) were performed in triplicate.

To note: Efficient and full inhibition of purified lipases usually requires the addition of surfactants like bile salts or lipids because they favour conformational changes giving inhibitor access to the lipase active site. However, it is not an absolute requirement with crude enzyme extracts in which lipases are mixed with other proteins and residual lipids.

2.7. Statistical analysis

The data of enzyme activity for the 3 sources of lipases were analysed using SPSS version 24.0 and the coefficients of variation (CVs) calculated. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines were used to define and assess the parameters of precision and evaluate the magnitude of variation among the activity assessment within and between labs (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2005). For all tests, the significance level was set at $P < 0.05$ (2 tailed). All data are expressed as means ± SD. Differences between enzyme preparation concentrations, for a same enzyme source, were analysed by Student's paired *t*-test.

For each enzyme preparation and concentration tested, 63 measurements of lipase activity were performed (three replicates for 21 laboratories) and analysed using the Shapiro-Wilk implementation in XLSTAT. Dixon test for outliers was performed on the average CV values for each set of 3 replicate measurements. Each laboratory was compared for the average CV of all replicates. Partial Least Squares (PLS) regression was used to determine the variables (vessel shape, burette volume, stirring mode, i.e. propeller or magnetic stirrer, and stirring speed) that have the most significant influence on the lipase activity (Tobias, 1995). For cross validation, the jackknife (leave-one-out; LOO) was used. The variables were centred and reduced prior to modelling. The variable importance for the projection (VIP) scores was determined from the following equation (5), according to Farrés et al. (Farrés, Platikanov, Tsakovski, & Tauler, 2015).

$$VIPj = \sqrt{\frac{\sum_{f=1}^{F} w_{jf}^2 \cdot SSY_f \cdot J}{SSY_{total} \cdot F}} \tag{5}$$

where w_{jf} is the weight value for j variable and f component and SSY_f is the sum of squares of explained variance for the f^{th} component and J number of x variables. SSY_{total} is the total sum of squares explained of the dependent variable and F is the total number of components.

3. Results

3.1. Overview of the equipment used

Instruments from 8 different brands and/or models were used by the 21 laboratories: Titrino (code 1; n = 3) and Titrando (code 2; n = 10) from Methrom, Mettler Toledo (code 3; n = 2), Radiometer Copenhagen Meterlab (code 4; n = 1), Kyoto Electronics Manufacturing (code 5; n = 2), Orion (code 6; n = 1), Dasgip Eppendorf (code 7; n = 1) and Cerko Lab System (code 8; n = 1). Conical reaction vessels (n = 14) were more widely found than cylindrical vessels (n = 7), and a magnetic stirrer (n = 14) more than a propeller (n = 7). Diversity was also observed in the maximum volume (ranging from 25 up to 200 mL) of the reaction vessel and the maximum volume (5, 10 or 20 mL) of the automated burette used for NaOH delivery. An automated burette works like a syringe with a piston. One laboratory (lab 16) used however a Dasgip Eppendorf bioreactor system instead of a classical pH-stat. This device had no burette and was based on a different principle (pump) for NaOH delivery.

3.2. Specific activities of the lipase preparations

The 21 laboratories involved measured the lipase activities, with tributyrin as a substrate, of a single batch of RGE as well as a single batch of each of the two different porcine pancreatin sources from Sigma and Nordmark, respectively. Assays were performed in triplicate using 3 different amounts of enzyme preparation in each case. Only one laboratory (lab 5) deviated from the average CV for all repeated replicates and was identified as a statistically significant outlier with the Dixon test having the highest mean CV of 14.2%. The values of this lab were therefore removed from the mean calculation and the data presented below. Table 1 shows the average (mean ± SD) of the specific activities obtained for each amount of enzyme preparation tested. For RGE and Sigma pancreatin the mean specific activities obtained for the three amounts tested (50, 100 and 200 μg) did not vary with the amount of enzyme (Fig. 3), which confirmed that these amounts of lipase were in the range allowed to measure a constant specific activity. This is a requirement for enzyme assays indicating that the substrate is in

sufficient excess to fully saturate enzyme active sites. Therefore, specific activities of GL in RGE and PL in Sigma pancreatin could be averaged from the three amounts tested and were 22.3 ± 7.1 (n = 63) and 65.9 ± 15.3 (n = 63) U/mg, respectively (**Table S1** in Supplementary Data). These specific activities were slightly lower than those expected from the suppliers' information which indicated 25 U/mg for RGE powder and between 80 and 240 U/mg for Sigma pancreatin (as deduced from USP units and the equivalence with units determined using tributyrin as substrate).

For Nordmark pancreatin, the specific activities determined with 50 and 100 μg of the enzyme preparation were both close to 140 U/mg (Table 1), but the specific activity measured with the third amount tested, i.e. 200 μg, was 1ower (124.8 ± 34.8 U/mg). This decrease in the specific activity at the highest concentration tested indicates that this amount was outside the range allowed for measuring a constant specific activity. Therefore, only the values obtained with the two lowest concentrations were considered for estimating the average specific activity of Nordmark pancreatin (140.9 U/mg, value obtained from data for 50 and 100 μg only). This activity was 2.14-fold higher than the specific activity of the Sigma pancreatin, as expected from the information provided by the supplier.

From the measured specific activities and the known specific activities of pure GL and PL (with tributyrin as a substrate), we could estimate how much active enzymes were present in the preparations (Table 2). The amount of GL in RGE was found to be 18.3 ± 6.0 μg/mg, while the amounts of PL in Sigma and Nordmark pancreatin sources were 8.2 ± 1.9 and 17.6 ± 3.7 μg/mg, respectively. We could thus estimate how much equivalents of pure enzymes were used in the assays when testing RGE (0.9–3.8 μg GL) and pancreatin sources (0.4 –3.5 μg PL). Since GL and PL have molecular masses of around 50 kDa and the reaction volume was 15 mL, the enzyme concentrations in the reaction vessel ranged from 1.2 to 5 nM for GL and from 0.6 to 4.7 nM for PL (Table 2).

The intra-laboratory CVs were overall acceptable with mean values <10% (Table 1**),** regardless the enzyme source. On the other hand, the inter-laboratory variabilities were too large (>21%) to reach international standards for bioanalytical method validation (see ICH M10 guidelines as well as guidance for bioanalytical methods established by the Food and Drug Administration (Food and Drug Administration, 2018) and the European Medicines Agency (European Medicines Agency, 2015), according to which CV values should not exceed 15%. The inter-laboratory variability was not reduced by removing data from the outlier laboratory. We therefore searched for the potential sources of variability among the laboratories involved. Since all laboratories were provided with the same detailed protocol, including the reagents to be used, and received lipase samples from the same batches, special attention was given to the various pH-stat devices that were used and their respective characteristics (see **Table S2** in *Supplementary Data*).

Table 1
Specific activities (U/mg of powder) measured with various amounts of each lipase source with tributyrin as a substrate.

Enzyme source	*Amount of powder tested (μg)*	*Specific activity (U/mg)**	*Mean Intra-laboratory CV %*	*Inter-laboratory CV %*
Lipolytech RGE	50	22.3 ± 5.3	8	24
	100	22.0 ± 4.7	5	21
	200	22.6 ± 10.4	4	46
	All amounts	22.3 ± 7.1	5	32
Sigma pancreatin	50	66.0 ± 15.7	8	24
	100	65.1 ± 17.1	8	26
	200	66.5 ± 13.7	6	21
	All amounts	65.9 ± 15.3	6	23
Nordmark pancreatin	50	140.1 ± 30.2	7	22
	100	141.7 ± 30.1	5	21
	200	124.8 ± 34.8	5	28
	50 + 100	140.9 ± 29.8	5	21

Values are means ± SD (n = 63; in triplicate by 21 laboratories for each amount tested) and coefficients of variation (CVs in %) were estimated for individual and for all laboratories involved. For complete data sets, see Tables S1 (data from individual laboratories with intra-laboratory CV%) and S2 (mean values for each enzyme preparation and amount for each laboratory, with inter-laboratory CV %) in Supplementary Data.

* Constant specific activity in the linear range.

3.3. Search for pH-stat devices' characteristics responsible for the variabilities observed

All laboratories involved provided characteristics of the pH-stat device they used. Characteristics considered as variables in the following PLS analysis were: the maximum volume (mL) of the reaction vessel, the vessel shape (conical or cylindrical), the stirring mode (propeller vs magnetic stirrer), the stirring speed (rpm) and the maximum burette volume (mL) for the delivery of NaOH (except for one instrument for which NaOH delivery was not based on the use of a burette).

PLS regression showed that certain experimental variables resulted in higher CVs than others (Fig. 4**A**). It could be seen that only two variables had a VIP score larger than 1 (considered statistically significant), namely the conical and cylindrical shapes of the reaction vessel. Analysis of the contribution of each variable showed either a reduction or an increase of the CV for a particular laboratory (Fig. 4**B**). The conical vessel was associated with the lowest CV, whereas the cylindrical vessel conversely resulted in the highest CV. This may be attributed to a better mixing in the conical vessel. This mixing effect is also reflected in the effect of the volume of the vessel in which the assay was conducted. Indeed, a positive correlation between vessel volume and CV is shown in Fig. 4**B**, i.e. the larger the volume the larger the CV. Interestingly, a higher stirring speed also resulted in higher CVs, which may be due to changes in the emulsion droplets size and specific surface accessible for lipase adsorption and activity. A larger burette volume also resulted in a larger CV, probably because a larger volume of NaOH is delivered per actuated motion of the burette piston and regulation of titration is not as fine as with a smaller burette. The impact of the stirrer type was the least important. The instrument type was not included in the PLS analysis, assuming that all relevant instrument parameters were sufficiently covered by the variables previously mentioned.

Besides the PLS analysis, we also plotted the variations in lipase specific activities of the three enzyme preparations as a function of the identified variables. Fig. 5 shows trends for the variables related to hydrodynamics of the reaction mixture, and thus the formation of the substrate emulsion. The highest specific activities were measured with the smallest reaction vessel volume (Fig. 5**A**) and the highest stirring rates (Fig. 5**C**), which is in agreement with the correlations deduced from the PLS regression. Concerning the vessel shape variables that were considered as the most influent according to PLS (Fig. 5**B**), no significant differences were observed between the mean values for all enzyme preparations ($P < 0.001$ for RGE, Sigma and Nordmark pancreatins). However, the most active enzyme preparation, Nordmark pancreatin, showed the highest (+8%) specific activity in the conical vessels. Dispersion of data (SD) around the mean was also reduced with conical vessels compared to cylindrical vessels and this was observed for all three enzyme preparations. Concerning the stirring mode, there was a tendency for measuring higher activities of RGE and Nordmark pancreatin with a propeller than with a magnetic stirrer (Fig. 5**D**), which was not highlighted by the PLS analysis.

Fig. 6 displays the effect of the burette volume, with higher specific activities measured with the smallest burette volumes, especially for the Nordmark pancreatin.

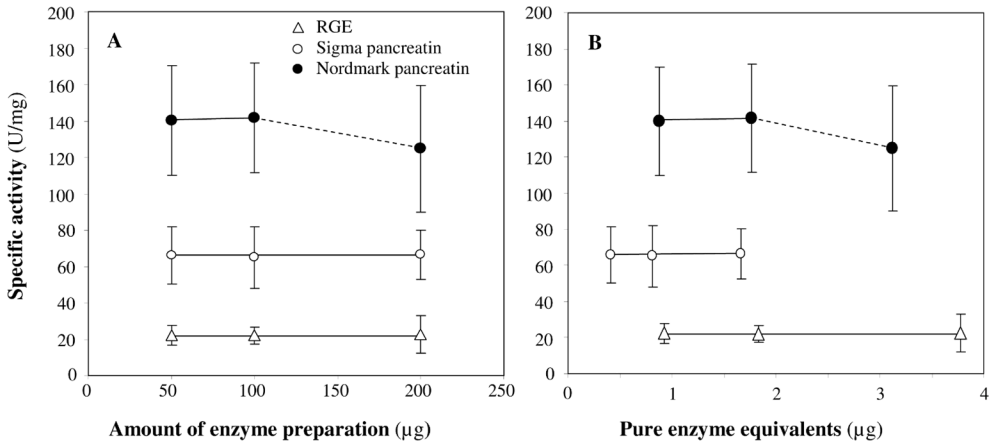

Fig. 3. *Specific activities of the lipases sources as a function of the amount of enzyme preparation used in the assay.* (A). Three different amounts (50, 100 and 200 µg) were tested for each enzyme preparation. (B) Representation of the same specific activities as a function of the pure enzyme equivalents (µg) present in these enzyme preparations.

Table 2
Estimation of active lipase amounts (µg/mg of powder) in RGE and pancreatins with tributyrin as a substrate.

Enzyme source	*Amount of powder tested (µg)*	*Specific activity (U/mg)**	*Lipase amounts in the powder (µg/mg)*	*Lipase amounts in the assay (µg)*	*Lipase concentration in the assay (nM)*
Lipolytech RGE	50	22.3 ± 5.4	18.6 ± 4.5	0.9 ± 0.1	1.2
	100	22.0 ± 4.8	18.3 ± 4.0	1.8 ± 0.1	2.4
	200	22.6 ± 10.5	18.8 ± 8.8	3.8 ± 0.2	5.0
	All amounts	22.3 ± 7.1	18.3 ± 6.0		
Sigma pancreatin	50	66.0 ± 15.8	8.3 ± 2.0	0.4 ± 0.1	0.6
	100	65.1 ± 17.2	8.1 ± 2.2	0.8 ± 0.1	1.1
	200	66.5 ± 13.8	8.3 ± 1.7	1.7 ± 0.1	2.2
	All amounts	65.9 ± 15.4	8.2 ± 1.9		
Nordmark pancreatin	50	140.1 ± 30.3	17.5 ± 3.8	0.9 ± 0.5	1.2
	100	141.7 ± 30.2	17.7 ± 3.8	1.8 ± 0.5	2.4
	200	124.8 ± 34.9	out of range	3.5 ± 0.5	4.7
	50 + 100	140.9 ± 29.9	17.6 ± 3.7		

This estimation is based on the determination of specific activities (U/mg of powder) measured for each enzyme preparation and the known specific activities of rabbit GL (1200 U/mg of pure enzyme) (Moreau et al., 1988) and porcine PL (8000 U/mg of pure enzyme) (Tuvignon et al., 2008).

* Constant specific activity in the linear range.

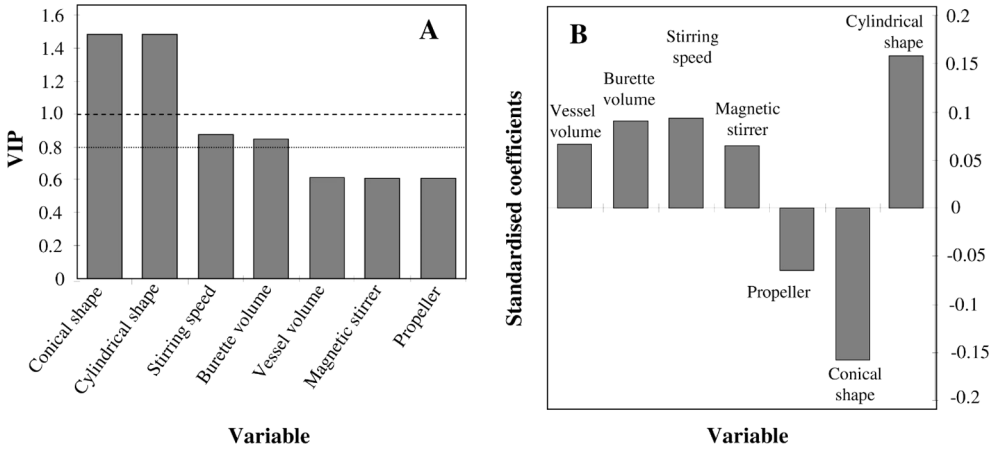

Fig. 4. *Variable importance for the projection (VIP).* (A) Comparison of VIPs influencing the performance of the test. It can be seen that only one variable had a VIP larger than 1 (considered statistically significant), namely the shape of the reactor vessel (conical and cylindrical). (B) Analysis of the contribution of each variable showing either a reduction of the coefficient of variation (CV) for a particular laboratory (typically vessels with conical shape) or an increase in the CV for a particular laboratory (typically vessels with cylindrical shape).

3.4. Inhibition of lipases by 4-bromophenylboronic acid (series 1, n = 21)

All 21 laboratories were involved in a first series of inhibition assays performed with 100 µg of each enzyme preparation (after incubation 10 min) with a fixed amount of inhibitor (Table 3). The specific activities of the two pancreatin sources were significantly reduced ($P < 0.0001$) when they were mixed with 4-bromophenylboronic acid, with only 8% and 6% of residual activity for Sigma and Nordmark pancreatins, respectively. On the other hand, 34% of gastric lipase from the RGE was still active when using the same quantity of the inhibitor.

Inhibition assays of gastric lipase were therefore repeated with higher amounts of boronic acid (10, 25 and 50 µL of a 1 M solution) in a second series of experiments involving only 8 laboratories out of the 21 (Table 4). This set of laboratories was based on their availability only.

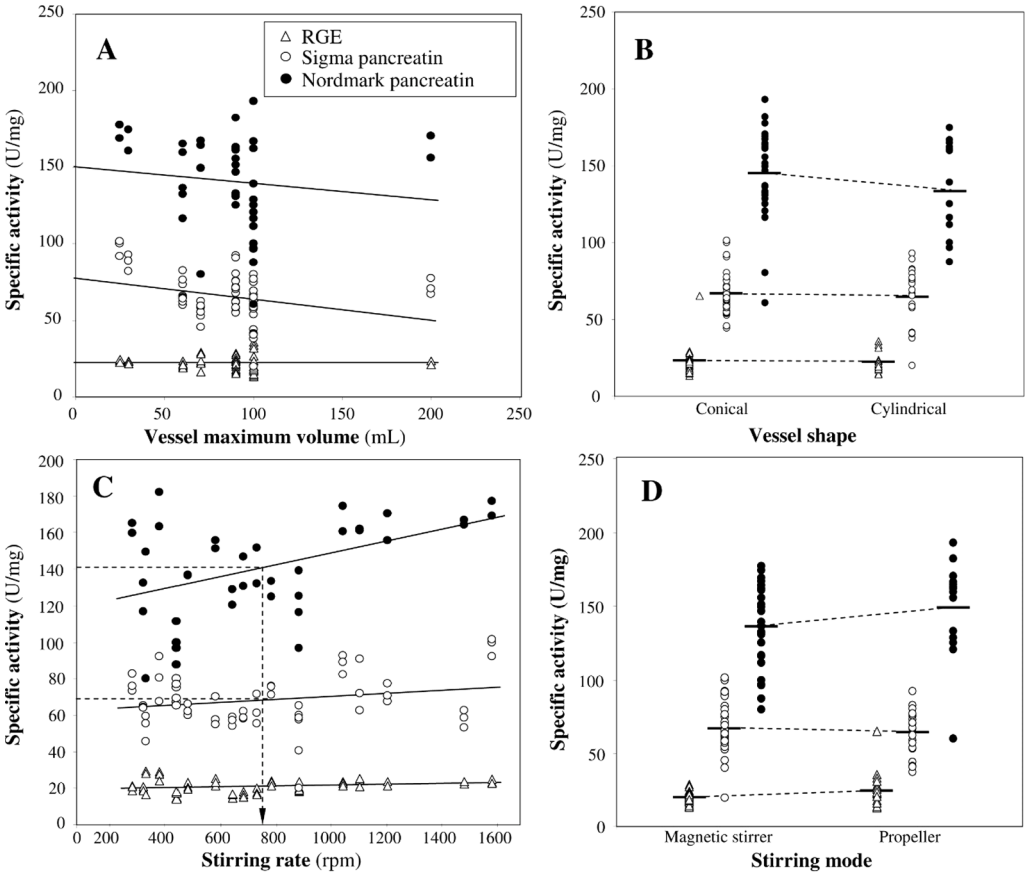

Fig. 5. *Impact of pH-stat devices' characteristics on lipase activity measurement.* This Figure illustrates the variations in lipase specific activities of the three enzyme preparations (RGE (50, 100 and 200 μg), Sigma pancreatin (50, 100 and 200 μg), and Nordmark pancreatin (50 and 100 μg)) with several characteristic parameters of the pH-stat devices used in the various laboratories, namely (A) the reaction vessel maximum volume, (B) the vessel shape (conical or cylindrical), (C) the stirring rate, and (D) the stirrer type (propeller or magnetic stirrer). Each dot corresponds to the mean specific activity (n = 3) measured by one laboratory for each amount of enzyme preparation. In panels A and C, lines indicating the trends were obtained by linear regression.

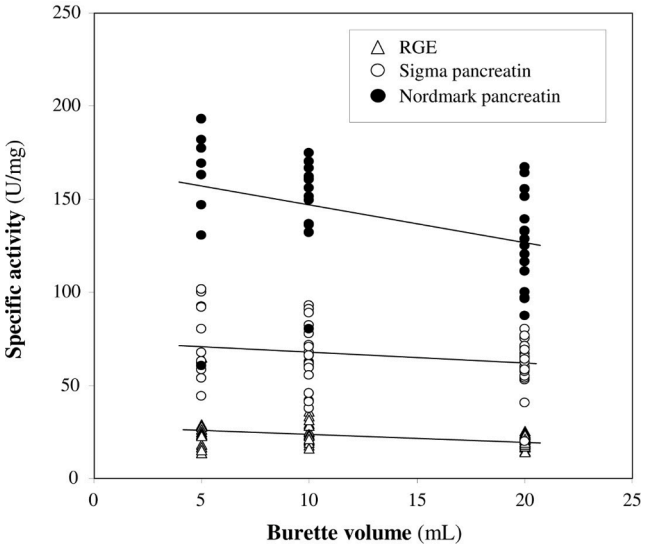

Fig. 6. *Variations in lipase activities with the maximum volume of the burette for NaOH delivery.* Each dot corresponds to the mean specific activity (n = 3) measured by one laboratory for each amount of enzyme preparation: 50, 100 and 200 μg for RGE, 50, 100 and 200 μg for Sigma pancreatin, 50 and 100 μg for Nordmark pancreatin. Lines indicating the trends were obtained by linear regression.

Addition of 50 μL of inhibitor solution, equivalent to an extremely high inhibitor to lipase molar ratio of 135,100 resulted in a residual activity of 12 ± 8%, which was still higher than the residual activities measured with the two pancreatin sources using a 10-fold lower inhibitor to lipase molar ratio of 13,500.

3.5. Repetition of GL assay using RGE (series 2, n = 8)

The second series of inhibition assays (n = 8) also repeated the assay of RGE specific activity using 100 μg of enzyme preparation as controls without the inhibitors (methanol only). These control assays were performed in the presence of a small amount of methanol (5–50 μL), the solvent used for boronic acid solubilisation, added to the total reaction volume of 15 mL. The presence of methanol had no effect on the mean specific activity of RGE (Table 5). Therefore, this second series tested the repeatability and reproducibility of 100 μg RGE assay on two occasions for 8 laboratories. The number of repeats per laboratory was, however, higher in the second series (n = 12) than in the first series (n = 3) because 3 repeats were performed for each of the four inhibitor amounts tested. No significant difference was observed between the two series ($P < 0.001$). The mean intra-laboratory CV% was increased from 4 (first series, n = 21) to 10% (second series, n = 8) likely due to a higher number of repeats performed by each laboratory, but the repeatability remained acceptable (<15%). Interestingly, the inter-laboratory variability was much improved with a CV% of 10% in the second series versus 18% in the first series. In addition, this subgroup of 8 laboratories had already a better reproducibility than the whole group of 21 laboratories (CV of 18 vs 21%) in the first series. The possible reasons for this improvement are analysed in the following discussion section.

Table 3
Residual specific activities of the lipase sources mixed with 4-bromophenylboronic acid.

Enzyme source		Specific activity (U/mg)*	Coefficient of variation (%)	Residual activity (%)	Inhibitor to lipase molar ratio
Lipolytech RGE	*Control*	22.0 ± 4.7	21	34	13,500
	Inhibitor	7.5 ± 0.9	12		
Sigma pancreatin	*Control*	65.1 ± 17.1	26	8	30,300
	Inhibitor	5.5 ± 1.0	19		
Nordmark pancreatin	*Control*	141.7 ± 30.1	21	6	14,300
	Inhibitor	8.9 ± 0.9	10		

Incubations were performed a room temperature for 10 min, after mixing 5 μL of a 4-bromophenylboronic acid solution (1 M) in methanol with 1 mL of the enzyme stock solution at 1 mg/mL. One hundred μL of this mixture was then used for measuring residual lipase activity. See the Result section for the estimation of the inhibitor to lipase molar ratio. Specific activity values (U/mg of powder) are means ± SD. The 21 laboratories were involved. See all individual data in Table S1 of Supplementary Data.

* Constant specific activity in the linear range.

Table 4
Residual specific activity (%) of the GL in RGE incubated with various amounts of 4-bromophenylboronic acid.

Volume of inhibitor solution (μL)		Specific activity (U/mg)*	Coefficient of variation (%)	Residual activity (%)	Inhibitor to lipase molar ratio
5	*Control*	19.5 ± 3.3	17	44 ± 11	13,500
	Inhibitor	8.4 ± 1.9	23		
10	*Control*	19.8 ± 3.8	19	33 ± 11	27,000
	Inhibitor	6.3 ± 1.9	29		
25	*Control*	19.3 ± 3.1	16	17 ± 7	67,500
	Inhibitor	3.1 ± 1.0	30		
50	*Control*	17.9 ± 3.5	20	12 ± 6	135,100
	Inhibitor	2.0 ± 1.1	54		

A 1 M solution of 4-bromophenylboronic acid was prepared in methanol and 5, 10, 25 or 50 μL of this solution (5, 10, 25 or 50 μmoles) were mixed with 1 mL of RGE solution at 1 mg powder per mL (containing 18.3 μg or 0.37 nmoles of GL). Eight laboratories out of 21 were involved in this part of the study. See all individual data in Table S3 of Supplementary Data.

* Constant specific activity in the linear range.

Table 5
Specific activity of 100 μg RGE (means ± SD) measured in two successive series by 8 laboratories.

Assay series	*Specific activity (U/mg)*	*Mean Intra-laboratory CV(%)*	*Inter-laboratory CV (%)*
Series 1- All labs	22.0 ± 4.7	5	21
Series 1–8 labs	21.6 ± 3.9	4	18
Series 2–8 labs	19.1 ± 3.1	10	10

Numbers of assay repeats in each laboratory were n = 3 assays in series 1 and n = 12 assays in series 2. Coefficient of variations (CVs in %) were estimated for individual and for all laboratories involved. For complete data sets, see Tables S1 and S3 in Supplementary Data.

4. Discussion

4.1. GL and PL assays using tributyrin as substrate

This study was performed to assess the repeatability and reproducibility of GL and PL assays that are recommended by the INFOGEST network to characterize lipase preparations prior to *in vitro* GI digestion studies. These assays were developed many years ago (Erlanson & Borgstrom, 1970; Gargouri et al., 1986; Moreau et al., 1988) and are considered as well optimized regarding reagents of the assay solution, temperature, pH and substrate. Tributyrin readily forms a fine emulsion upon mechanical stirring with a high specific surface area ensuring that all lipase molecules are adsorbed at the lipid-water interface and are available to form an enzyme-substrate complex. Indeed, lipase activity correlates with lipase adsorption (Benzonana & Desnuelle, 1965). A stationary state with a linear release of FFA over time is reached rapidly after launching the reaction (Fig. 2). Lipase assays with tributyrin are highly sensitive based on the high specific activities measured with purified lipases (1200 U/mg for rabbit (Moreau et al., 1988) and human (Gargouri et al., 1986) GL; 8000 U/mg for human and porcine PL (Tuvignon et al., 2008)) under optimized assay conditions. These specific activities can also be expressed as molecular turnover number or catalytic constant (k_{cat}; mole of substrate hydrolysed per mole of enzyme per unit time), which gives values of 1000 and 6670 s^{-1} for GL and PL, respectively, and places these enzymes in the upper range of all enzyme activities (Schomburg et al., 2017; Smejkal & Kakumanu, 2019). GL and PL assays with tributyrin have been used to characterise crude enzyme preparation (Sternby & Nilsson, 1997; Salhi et al., 2020) and purified enzymes (Borgström, 1975; Gargouri et al., 1986), as well as to assay lipase activities in biological samples collected from the GI tract (biopsies (Moreau, Laugier, Gargouri, Ferrato, & Verger, 1988), digestive juices (Erlanson & Borgstrom, 1970; Ville, Carrière, Renou, & Laugier, 2002), gastric and intestinal contents (Erlanson & Borgstrom, 1970; Carrière et al., 1993)). They have also been used for estimating GL and PL concentrations in GI tract contents and secretory outputs in the course of several clinical trials (Sternby, Nilsson, Melin, & Borgström, 1991; Carrière et al., 1993; Borovicka et al., 1997; Carrière et al., 2001; Renou et al., 2001; Sternby, Hartmann, Borgstrom, & Nilsson, 2002; Carrière et al., 2005; Roman et al., 2007), because it is possible to convert enzyme activity (U/mL) into concentration of active enzyme (mg/mL), knowing the specific activity (U/mg) of pure enzymes. In some clinical studies, such as S245.2.003 (sponsored by Solvay Pharmaceuticals GmbH), PL assay using tributyrin as substrate has been validated according to international guidelines for bioanalytical assays (ICH Guideline Q2A, Validation of Analytical Procedures: Definitions and Terminology, March 1995 and ICH Guideline Q2B, Validation of Analytical Procedures: Methodology, May 1997) and approved by the US Food and Drug Administration (Frédéric Carrière's personal communication). The specificity and precision of the PL assays were therefore considered as acceptable, with definition of lower and upper limits of quantification.

The use of both GL and PL assays have become more widely spread in recent years due to their recommendation in the protocols defined by the INFOGEST network (Minekus et al., 2014; Brodkorb et al., 2019). Because of this larger use, some discrepancies between laboratories have appeared, especially when identical batches of enzyme preparation were tested by different laboratories. It was therefore important to identify the variables that could explain these differences, although precise protocols describing the assay conditions had been provided (Minekus et al., 2014; Brodkorb et al., 2019). In laboratories familiar with pH-stat assays of lipase activities, it is well known that different results can be obtained using pH-stat devices from different brands/models. This is assumed to be caused by differences in reaction vessels (shape and volume) and stirring modes, because these parameters influence the formation of the substrate emulsion and thus, the specific surface

available for lipase adsorption and activity at the oil–water interface. Some laboratories have, for instance, introduced correction factors to compare results obtained with different types of equipment or by different collaborators. This is not satisfactory since the result of an assay should not depend on the equipment utilised to be considered robust and widely applicable. A water-insoluble substrate, emulsified in the reaction vessel, can create this dependency and therefore, protocols should also include recommendations about the parameters of the pH-stat equipment to be used. This was the main objective of the ring trial described in this study and designed by the INFOGEST working group on lipid digestion and digestive lipases (WG4).

4.2. Choosing the right amount of enzyme preparation for the assay

Another potential source of variability in all enzyme assays are the respective quantities of enzyme and substrate used per assay. One has to check that these amounts fall within the range in which the activity is proportional to the amount of enzyme, i.e. constant specific activity. This is usually ensured by using a large excess of substrate versus enzyme to saturate enzyme active sites in the reaction. In this study, we used enzyme preparations containing micrograms of enzymes, and lipase concentrations in the assay reaction ranged from around 1 to 5 nM (Table 2). We used 0.5 mL of tributyrin as a substrate in a 15 mL reaction volume, which corresponds to an apparent concentration of 113 mM (if that substrate was not insoluble in water). Substrate to enzyme molar excess was very large and exceeded 10^7. Having such a high molar excess is however not always sufficient to ensure a constant specific activity of lipases in a large range of enzyme concentrations. Indeed, due to their peculiar mode of action involving enzyme adsorption at the lipid-water interface, the adsorption of various amounts of lipase can change the interfacial properties and thus lipase activity, as would do any surface active agent (Aloulou et al., 2006; Delorme et al., 2011) or other proteins present in complex enzyme preparations such as pancreatin and RGE. This is the reason why a linear relationship between the amount of lipase and lipase activity (or constant specific activity) is usually observed in a very narrow range of concentrations when assaying lipases with an emulsified TAG substrate. For instance, a previous validation of the PL assay has shown that a constant specific activity could only be measured using 0.5–3.0 μg of pure PL (Frédéric Carrière's personal communication).

In the current work, we had preliminary information on lipase contents in the pancreatin and RGE, and the laboratories involved in the ring trial were asked to perform assays with three different quantities of enzyme preparations (50, 100 or 200 μg) containing suitable amounts of lipases. This was confirmed by the 21 laboratories involved that obtained consistent average specific activities of 22.3 and 65.9 U/mg for RGE and Sigma pancreatin, respectively, regardless the amount of enzyme preparation tested (Fig. 3 **and** Table 1). With Nordmark pancreatin, a highly active source of pancreatic enzymes used as an active pharmaceutical ingredient (API) in drug products for the treatment of exocrine pancreatic insufficiency, the highest amount tested (200 μg) contained 3.5 μg of active PL and specific activity decreased (−13%) compared to the specific activities estimated with 50 and 100 μg (Fig. 3). These assays have therefore confirmed the range of enzyme preparation amounts to be tested and demonstrated that using too much enzyme in the current assay conditions can result in an underestimation of the specific activity. This effect might be drastically amplified when using higher quantities of enzyme. When assaying the lipase activity of a new or unknown batch of enzyme preparation, we therefore recommend starting with 50 μg and then double the amount (100 μg) to check if the activity is doubled while having a constant specific activity. Following such a procedure will ensure further adaptation of assay conditions in the future if enzyme manufacturers are providing preparations with different lipase enrichments.

4.3. Optimal pH-stat equipment parameters

This study confirmed that the mixing conditions in the pH-stat vessel are critical to obtain accurate and reproducible lipase activities. This is influenced by the shape and size of the reaction vessel, the stirrer type and the stirring rate (Figs. 1, 4 and 5). Using a reaction vessel with a conical shape was the most important parameter influencing the inter-laboratory variability. In the second series of RGE assays involving only 8 laboratories out of the 21, most of these laboratories (7 out of 8) had a pH-stat instrument equipped with a conical vessel (see **Table S4** of Supplementary Data) and reproducibility was improved compared to the first series of assays (Table 5). The maximum volume of the reaction vessel had a limited influence (Fig. 5**A**) but we recommend using a thermostated conical vessel with a 70 mL maximum capacity, which is a rather standard model on the market. The stirrer type (propeller vs magnetic bar) also had only a minor effect, although the highest activity of the most active enzyme preparation (Nordmark pancreatin) was recorded with the propeller (Fig. 5**D**). It is generally recommended to use a propeller because it allows a better monitoring of the stirring rate with viscous reaction mixtures. It does not seem to be the case here, but since the pH-stat equipment can also be used for running *in vitro* digestions of various food systems, we still recommend the propeller as the best option. Specific activities were found to increase with the stirring rate (Fig. 5**C**) as intuitively expected. However, it is worth noting that the rates provided by the laboratories were often rough estimations of rates found in the instruction manuals. Indeed, most pH-stat instruments are equipped with stirrers having a few pre-set rates and it is not possible to finely adjust the stirring rate. If possible, we would recommend using a stirring rate between 700 and 800 rpm, a range in which the specific activities measured with pancreatins are close to the mean specific activities measured by all laboratories (Fig. 5**C**). The last variable of importance was the volume of the burette used for the automated delivery of NaOH, with higher specific activities recorded with the smallest burettes (Fig. 6). We assume this is due to the precision of NaOH delivery to keep the pH of the system at the set value (pH 5.5 for GL and pH 8 for PL), the smallest burettes being the most precise at adding small volumes of NaOH into the reaction vessel. In this ring trial, the smallest burettes had a volume of 5 mL and we recommend using this volume although smaller burettes of 2 mL would be even better, as for instance with the old pH-stat instruments manufactured by Radiometer (TTT80). Indeed, the latest generation of pH-stat equipment on the market may not be the most appropriate instruments for measuring lipase activities. These instruments have been developed for classical acid/base titration and not enzyme assays during which the rate of acid release varies with enzyme activity. Ideally, it should be possible to adjust the rate of NaOH delivery in the course of the titration, which is necessary for systems having lag times or changes in enzyme activity in the course of the reactions such as when testing lipase inhibitors.

In conclusion, it is recommended to use a pH-stat device equipped with a conical vessel of 70 mL maximal capacity, a propeller stirrer (stirring rate between 700 and 800 rpm) and a burette of 5 mL for NaOH delivery.

4.4. The lipase inhibitor 4-bromophenyl boronic acid is not an efficient inhibitor of GL

Boronic acids were shown to be PL inhibitors by Guarner in 1980 (Garner, 1980). More recently, 4-bromophenyl boronic acid was chosen as PL inhibitor to stop lipolysis in samples collected from the digestion of lipid-based formulations (Williams et al., 2012). This blocks lipolysis at given times during digestion prior to the analysis of lipolysis products and avoids the evolution of these products into other lipase substrates like diacyl- and monoacylglycerols. By analogy, it was proposed to use this inhibitor and a similar protocol to stop lipolysis in samples collected from food digestion by PL. We confirmed here that 4-bromophenyl boronic acid significantly inhibits PL present in Sigma and Nordmark

pancreatins, with low residual activities of 6–8% after 10 min of incubation with an inhibitor to lipase molar ratio of at least 14,300 (Table 3). For the sake of comparison, a similar inhibition of porcine PL by the lipase inhibitor Orlistat (also called tetrahydrolipstatin; THL) is achieved using an inhibitor to lipase molar ratio of 10,000 (Gargouri, Chahinian, Moreau, Ransac, & Verger, 1991). Inhibition of PL by THL was however shown to be reversible during the assay of residual PL activity (Tiss, Lengsfeld, Carrière, & Verger, 2009) and therefore 4-bromophenyl boronic acid is currently recommended for blocking lipolysis in samples containing PL.

While THL also acts as an inhibitor of GL, there was no report in the literature that 4-bromophenyl boronic acid could also inhibit GL. We therefore tested the inhibition of GL present in RGE using the same protocol as for PL in pancreatin. An inhibitor to lipase molar ratio of 13,500 was first used, leading to a high residual activity of 34% (Table 3). Even after increasing the inhibitor to lipase molar ratio up to 135,100, residual GL activity remained high (12 ± 6%; Table 4 **and** Fig. 7**A**). 4-Bromophenyl boronic is therefore a weak inhibitor of GL, especially compared to THL that fully blocks GL from RGE at an inhibitor to lipase molar ratio of 2,000 (Fig. 7**B**) (Gargouri et al., 1991). Half-inhibition of GL is achieved with inhibitor to lipase molar ratio of around 10,000 with 4-bromophenyl boronic compared to approximately 300 with THL.

In conclusion, it is not recommended to use 4-bromophenyl boronic for GL inhibition but rather THL, an inhibitor very efficient in blocking gastric lipase and intragastric lipolysis (Carrière et al., 2001).

5. Conclusion

The step-by-step detailed protocols for GL and PL activity assays provided to the laboratories involved in this ring trial allowed each laboratory to precisely assess the specific activities of enzyme preparation with a good repeatability. Further recommendations were required concerning the characteristics of the pH-stat instruments in order to improve the reproducibility of the assays among the laboratories involved. These suggestions are now available, especially concerning the type of reaction vessel (conical with a maximal capacity of 70 mL) to be used, and as a consequence a better reproducibility was observed in the second series of experiments. In addition, it is recommended to use 4-bromophenyl boronic for PL inhibition, however, for GL inhibition THL should be used. The results of this study improve the GL and PL assays associated with the INFOGEST protocols of *in vitro* GI digestion (Minekus et al., 2014; Brodkorb et al., 2019; Mulet-Cabero et al., 2020). Before running an *in vitro* lipid digestion, lipase activities have to be verified for each new batch of digestive enzyme preparation, or if the source has been stored for an extended period of time, to ensure the targeted activity is reached. The new recommendations presented here will contribute to a better quality control of enzyme preparations.

6. Ethics statements

The research did not include any human subjects and animal experiments.

CRediT authorship contribution statement

Myriam M.L. Grundy: Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration. **Evan Abrahamse:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Annette Almgren:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Marie Alminger:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ana Andres:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Renata M.C. Ariëns:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Shanna Bastiaan-Net:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Claire Bourlieu-Lacanal:** Investigation, Data curation, Formal analysis, Writing - review & editing. **André Brodkorb:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Maria R. Bronze:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Irene Comi:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Leslie Couëdelo:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Didier Dupont:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Annie Durand:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Sedef N. El:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Tara Grauwet:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Christine Heerup:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ana Heredia:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Marcos R. Infantes Garcia:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Christian Jungnickel:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ilona E. Kłosowska-Chomiczewska:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Marion Létisse:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Adam Macierzanka:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Alan R. Mackie:** Investigation, Data curation, Formal analysis, Writing - review & editing. **David J. McClements:** Investigation, Data

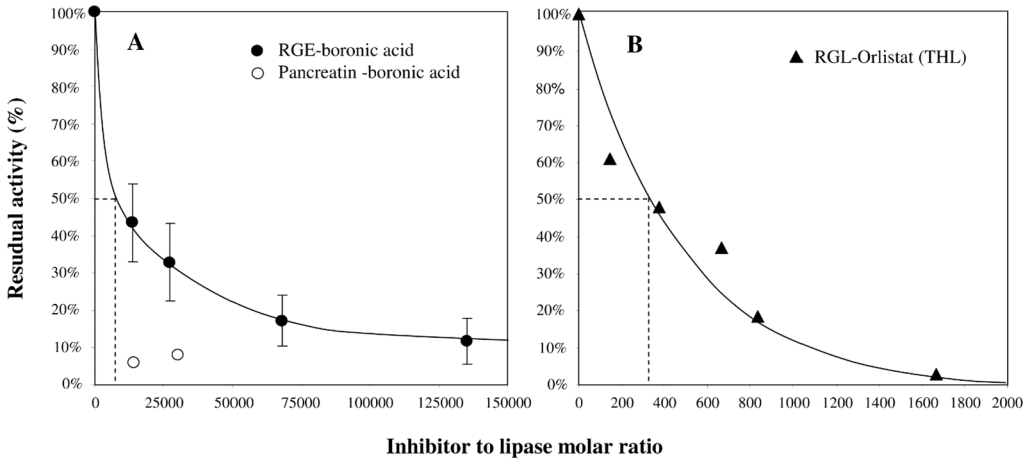

Fig. 7. Inhibition of gastric lipase after mixing RGE with 4-bromophenylboronic acid (A) and comparison with rabbit gastric lipase (RGL) inhibition by Orlistat (tetrahydrolipstatin, THL). Panel B was adapted from Gargouri et al. (Gargouri et al., 1991).

curation, Formal analysis, Writing - review & editing. **Olivia Menard:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Anne Meynier:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Marie-Caroline Michalski:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ana-Isabel Mulet-Cabero:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Anette Mullertz:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Francina M. Payeras Perelló:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Irene Peinado:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Mélina Robert:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Sébastien Secouard:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ana T. Serra:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Sandra D. Silva:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Gabriel Thomassen:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Cecilia Tullberg:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ingrid Undeland:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Carole Vaysse:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Gerd E. Vegarud:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Sarah H.E. Verkempinck:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Michelle Viau:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Mostafa Zahir:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ruojie Zhang:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Frédéric Carrière:** Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

Rabbit gastric extract is available commercially from Lipolytech, a start-up company founded by a researcher, Dr Sawsan Amara, who had previously worked in F. Carrière's (co-author of this paper) group. Apart from this, the authors declare no conflict of interest.

Acknowledgements

The authors thank the European Commission COST Action FA1005 INFOGEST and INRAE for financing the meetings and conferences that enable the 21 laboratories to network and organise the work presented in this article. We also acknowledge the 'Healthy and Safe Food System (KB-37)' knowledge base program of the Dutch Ministry of Agriculture, Nature and Food Quality (LNV), with grant number KB-37-001-007 for funding of this work performed at Wageningen Food & Biobased Research. We are grateful to Dr Sawsan Amara (CEO of Lipolytech SA., Marseille, France) for her generous gift of RGE and to Drs Jan Lüdemann, Anja Jensen, Olaf Friedrich and Claus Middelberg from Nordmark Arzneimittel GmbH & Co. KG (Uetersen, Germany) for their generous gift of Nordmark pancreatin. Finally, we thank Drs Maria Fátima Cabral and Judite Costa from iBET for their technical support and discussion.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104497.

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