



Research article



Graphene oxide as inhibitor on the hydrolysis of fats under simulated in vitro duodenal conditions

Alberto Fernández-Núñez^a, Jamal EL Haskouri^a, Pedro Amorós^a, Jose V. Ros-Lis^{b,*}^a Institut de Ciència dels Materials (ICMUV), Universitat de València, c/ Catedrático José Beltrán 2, Paterna, 46980, Valencia, Spain^b REDOLi Research Group, Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València, Universitat de València. Doctor Moliner 50, Burjassot, Valencia, 46100, Spain

ARTICLE INFO

Keywords:

Graphene oxide (GO)
Pancreatic lipase
Lipids
Fats
Duodenal digestion

ABSTRACT

Obesity is a global pandemic, thus novel developments that reduce the absorption of fats is of interest. We have evaluated the effect of graphene oxide (GO) on the lipase catalyzed hydrolysis of fats (tributyrin, sunflower and olive oil) under simulated duodenal conditions. Results indicate that the presence of GO in the digestion mixture can inhibit lipase activity up to a 90% of the initial reaction rate, and this inhibition lasts even during 2 h of digestion. The inhibition mechanism seems non competitive and could be opposite to the effect of bile salts, although the direct interaction between GO and the enzyme cannot be discarded. The inhibition is found also in alimentary fats suggesting that GO could be a strong inhibitor for fat hydrolysis.

1. Introduction

Triglycerides are the main components of fats and oils in our diet. Not only are one of the main sources of energy, but they act as transporters of non-polar substances such as fat-soluble vitamins [1]. The hydrolysis of fats has a direct effect on their digestibility and absorption. According to the World Health Organization (WHO), obesity is one of the current pandemics in both developed and developing countries. Fat digestion in the human gastrointestinal tract is a dynamic and continuous process involving various steps. It begins in the mouth with chewing and salivation, that despite its short time (0.5–2 min) has a dilution effect due to the saliva, the mechanical forces restructure the emulsions, and the hormone cholecystokinin is segregated. However, up to date, there is no solid proof of human lingual lipase activity. The process continues in the stomach, in which a 10–25% of the hydrolysis is produced catalyzed by the human gastric lipase, an extremophilic enzyme since it remains stable above pH 1. Finally, the triglycerides reach the duodenum and the small intestine, where most of the hydrolysis occurs [2]. Also, the ingestion of fats is related with cholesterol. Elevated plasma cholesterol levels are causally linked to increased cardiovascular morbidity and mortality. Several intervention trials support the clinical benefits of pharmacological cholesterol synthesis inhibition [3].

The relevance of the influence of lipase in the digestion and health has boosted the development of solutions able to modulate (increase or inhibit) the activity of this key enzyme [4,5]. We can highlight the use of tetrahydrolipstatin (Orlistat ©) as inhibitor of pancreatic lipase by modification of the active site, thereby reducing the hydrolysis of triglycerides and the cholesterol absorption and plasma levels [3,6]. Other compounds, such as alginates [7], peptides [8], or certain plants [9], have also been used to modify the lipase activity.

* Corresponding author.

E-mail address: J.Vicente.Ros@uv.es (J.V. Ros-Lis).

<https://doi.org/10.1016/j.heliyon.2024.e28624>

Received 5 October 2023; Received in revised form 21 March 2024; Accepted 21 March 2024

Available online 22 March 2024

2405-8440/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC license (<http://creativecommons.org/licenses/by-nc/4.0/>).

The immobilization of lipases in nanomaterials has been an approach widely used to improve the stability, pH and temperature range, recovery and reutilization, use in organic solvents and increase of the activity [10]. Typically, in the enzyme-nanomaterial composites prepared through supramolecular interactions the loading of the material with the lipase takes several hours and is produced in a “clean” solution containing the enzyme and some salts only [11]. By contrast, the interaction of lipase with nanomaterials in a few minutes and complex environments, such as those produced in the digestion have been barely studied. Plain silica materials and functionalized with alkyl chains can modulate the lipase activity and their effect was dependent on the type of material and the functional groups. It was observed that the porosity inhibits the enzyme activity, however, a maximum reduction of a 25% in the lipase activity was found [12].

Another nanomaterial that has attracted high interest in the recent years graphene oxide (GO). It is a two-dimensional structure composed of a single layer of sp^2 hybridized carbon atoms, but the partial oxidation adds alcohol and ketone groups [13]. This functional groups decrease the tendency to form aggregates and hydrophobicity, and improves the potential to form supramolecular interactions with biomolecules, such as nucleic acids, peptides, and aromatic chemical compounds [14]. Excellent dispersion in a variety of solvent media, including water, makes GO an excellent carrier for lipase immobilization and GO-lipase compounds have been prepared previously mainly for sensing and catalytic applications, usually in combination with other materials [15,16].

Departing from the known interaction of graphene oxide with the lipase through supramolecular interactions, and their 2D structure including coordinating groups like the bile salts, we hypothesize that this type of materials can be of interest to modulate the hydrolysis of fats even in complex media such as duodenal conditions.

2. Materials and methods

2.1. Reagents and materials

GO and chemical reagents were purchased from Sigma-Aldrich and used without further purification. Lipase From porcine pancreas Type II, with a WM of 120 kDa was purchased from Sigma-Aldrich. Sunflower and olive oils were purchased from a local supermarket. Lipase solutions were prepared just before its use and maintained in a bath ice during the full experiment. The duodenal digestion juices were prepared in the lab following reported procedures [17].

2.2. Characterization of the graphene oxide

The particle size and Z-potential and particle size of GO were determined using a Malvern Mastersizer 2000 and Malvern Nanosizer ZS.

2.3. Measurement of lipase activity

2.3.1. pH-stat

The influence of the materials in the lipolysis under in vitro digestion process was measured using lipase assay protocols using the pH-stat method created by the INFOGEST Network—WG4 Lipases and lipid digestion [18]. All the measurements were performed by triplicate using the Metrohm Tiamo 902 Titrando instrument. To perform the assay, 14.5 mL of duodenal digestion juice was mixed with 0.5 mL of tributyrin and 1 mL of GO with diverse concentrations (from 0 to 4 mg of GO/mL). Also, hydrolysis studies have been performed using sunflower and olive oils to explore the effect of GO on the hydrolysis of alimentary fats. The system was thermostated to 37 °C and the pH set at 8.0. An amount of 100 μ L of the enzyme stock solution (1 mg lipase/mL) was added and the consumption rate of NaOH measured over 5 min or 2 h. The activity of the enzyme in solution (A_s) (U/mL), the relative activity and the % of inhibition in the presence of the material were calculated with Eqs (1)–(3) respectively. x being the μ mole of NaOH per minute, v the μ L of the enzyme solution added in the pH-stat vessel, A_{mat} the value of A_s in presence of the material and A_{cont} the value of A_s in absence of the material.

$$A_s = (x \times 1000) / v \quad (1)$$

$$\text{Relative activity (\%)} = 100 \times A_{mat} / A_{cont} \quad (2)$$

$$\% \text{ Inhibition} = 100 - \text{Relative activity (\%)} \quad (3)$$

2.3.2. NMR studies

For the NMR studies, departing from the same solutions of pH-stat, the digestion was allowed to progress for 2 h to simulate a full duodenal digestion process. The sample preparation was done following reported procedures [19]. ^1H NMR spectra were recorded in a 300 MHz Bruker AvanceIII 300 NMR spectrometer. The degree of hydrolysis was calculated with the quotients between the areas of the signals at 5.12 ppm (glycerol) and 2.10, 1.45, and 0.8 ppm (alkyl chain) in presence and absence of GO and Eqs (4) and (5) being Q_{GO} and Q_{TB} the values of the quotients for extraction after the hydrolysis in presence and absence of GO respectively.

$$\text{Relative activity (\%)} = \frac{Q_{GO} - Q_{TB}}{Q_{TB}} * 100 \quad (4)$$

$$\text{Inhibition (\%)} = 100 - \text{Relative activity (\%)} \quad (5)$$

2.3.3. Data analysis

The kinetic analysis and the determination of the main parameters (v_{\max} , K_m , and K_i) were executed using the IBM SPSS statistics software using a non linear regression of the substrate and inhibitor concentration, and the initial reaction rate.

3. Results

3.1. Effect of GO in the initial reaction rate

The initial reaction rate and the % of inhibition was determined using the pH-stat technique. It is based in a volumetric titration to neutralize the acids. Previously to the activity tests and considering a possible buffering activity of GO that could interfere the results, we performed a volumetric acid-base titration at diverse pHs of a solution of GO in water. We observed no constant pH with the NaOH addition at pH 8, thus the buffering activity of GO at this pH can be discarded. Particle size (hydrodynamic radius) and Z-potential of the GO was measured with values of 4.0 μm and -38.9 mV respectively.

As can be seen in Fig. 1, GO is a strong inhibitor of the lipase mediated hydrolysis of fats. The inhibition is concentration dependent and varies among a 30% for the most diluted concentration (9.4 ppm) and an 83% for the most concentrated one (250 ppm) (Fig. 1). The inhibition is significant ($p < 0.05$) for all the concentrations, thus GO can be considered a potential modulator of enzyme activity, even at a concentration as low as 10 ppm.

Bile salts, such as NaTC, are secreted by the gall bladder and the absence of bile salts can lead to digestive disorders. They enhance colloidal stability of lipid droplets and solubilize the digestion products in water, being fundamental for the digestion and epithelial absorption of free fatty acids in the intestine [20]. The inhibitory effect of GO in absence of bile salt is more pronounced at low concentrations (Fig. 1). At 9.4 ppm of GO an inhibition of a 65% is found in absence of bile salts in comparison with the 29% found when the bile salts are present. As the concentration increases, an inhibition of 84 % was found for 25 ppm of GO suggesting an almost complete lipase inhibition.

To gain insight into the inhibitory mechanism of GO, the same experiment was performed, but the GO was added to the enzyme solution instead of to the fat and duodenal juices mixture. The values obtained were similar, suggesting that the order of addition of the reagents is not relevant, so in presence as in absence of bile salt. Also, we used the Lineweaver-Burk plot to determine the type of inhibition. As can be seen in Fig. 2, the linear fitting in presence of inhibitor crosses the non-inhibited fitting in the X axis. Thus, at first glance we can classify the type of inhibition as non-competitive since the value of K_m is maintained, but v_{\max} decreases. In the absence of an inhibitor, K_m and v_{\max} values of 0.016 ± 0.004 M and 7.3 ± 0.7 U/mL ($\bar{x} \pm SD$) respectively were calculated. In the presence of the inhibitor, the adjusted values were 0.015 ± 0.010 M and 5.2 ± 0.4 U/mL for K_m and v_{\max} respectively. Therefore, there are no significant differences in the value of K_m , but v_{\max} undergoes a significant reduction ($p < 0.05$). From these data a value of $K_i = 63 \pm 6$ ppm was determined.

Finally, we evaluated the inhibitory activity of GO in the hydrolysis of sunflower and olive oils in presence of bile salts to determine the effect in a real alimentary fat. 94 ppm of GO was used since at this concentration it is found an intermediate inhibition. An inhibition of the 50 ± 10 % and 70 ± 4 % were found for sunflower and olive oil respectively, similar to the value obtained for tributyrin. It suggests that the effect of GO in tributyrin could be extrapolated to long chain fats and that GO can be an effective inhibitor for the lipase mediated hydrolysis of alimentary fats.

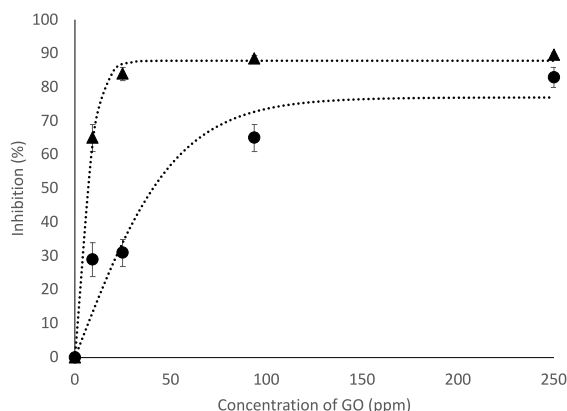


Fig. 1. Percentage inhibition of lipase in the initial reaction rate for different concentrations of GO in presence (circle) or absence (triangle) of bile salt (NaTC). Dashed lines have been added to illustrate the tendency only. Error bars represent standard deviation.

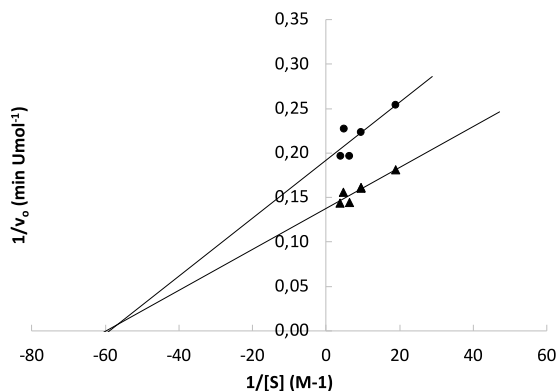


Fig. 2. Lineweaver-Burk plot in absence (triangles) and 25 ppm of GO (circles). The straight lines correspond to the linear fitting.

3.2. Effect of GO in a full duodenal digestion

For the determination of the hydrolysis rate in a 2 h period corresponding to the typical time of a full duodenal digestion, the pH-stat technique was used too. The experiments were developed both, in presence and absence of taurodeoxicholate and the main results summarized in Fig. 3. As can be seen, in presence of bile salts at 2h is observed a lower inhibition in comparison with the initial rate. Conversely, this effect is the opposite in absence of bile salts, with a 78% of inhibition for 9.4 ppm of GO. Again, in all the cases, the hypothesis that the hydrolysis rate differs from 0 is statistically validated ($p < 0.05$).

However, it has been pointed out that the accuracy of the pH-stat titration technique to quantify the fatty acids released during lipid digestion depends on several factors and could not offer reliable results in complex matrixes [21,22]. We have therefore used the NMR technique to validate the results of hydrolysis measured with the pH-Stat at long time. Table 1 summarizes and compares the results obtained with both techniques. As can be seen, there are no significant differences between NMR and pH-Stat for any of the concentrations. When the two-factor ANOVA test (GO concentration and technique of analysis) was applied to the full set of data, it was observed that the influence of the GO concentration in the % of inhibition was significant ($p < 0.01$), but both techniques offered the same results ($p = 0.91$). Duncan Test offered three groups: (1) 0.15 and 0.4, (2) 1.5 and (3) 4.0. Thus, NMR confirm the possible use of the pH-Stat to determine the degree of fat hydrolysis even for 2 h. The inhibitory concentration (IC_{50}) at 2h was calculated in presence of bile salts, obtaining a value of 172 ppm of GO.

4. Discussion

Results indicate that GO can modulate the lipase activity under duodenal conditions. The inhibition reaches values close to a 80% in presence of bile salts. This effect depends not only in the concentration of GO, but also in the reaction time and the presence of bile salts. As the concentration of GO increases the lipase loses its activity, but even concentrations as low as 9.4 ppm of GO can induce significant inhibitions. We have not found GO induced inhibitions higher than 90%. This suggest that the enzyme maintain a residual

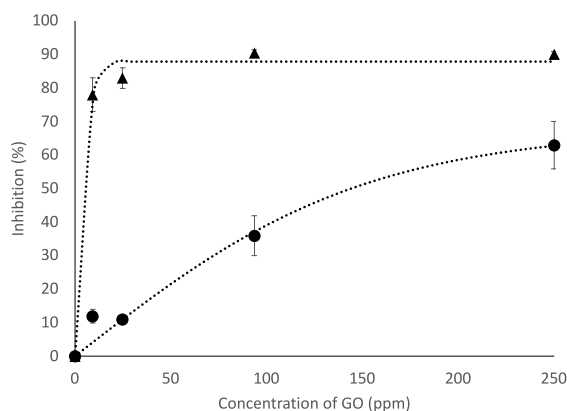


Fig. 3. Percentage of inhibition of lipase at 2 h for different concentrations of GO in the presence (circle) or absence (triangle) of bile salt (NaTC). Dashed lines have been added to illustrate the tendency only. Error bars represent standard deviation.

Table 1
Percentage of inhibition of lipase at 2 h measured using the pH-Stat and NMR techniques.

Concentration of GO (ppm)	Inhibition (%) ($\bar{x} \pm SD$)	
	pH-Stat	NMR
9.4	12.1 \pm 1.2	9.4 \pm 0.8
25	11.4 \pm 0.7	10.1 \pm 1.2
94	36 \pm 6	31 \pm 3
250	63 \pm 8	72 \pm 3

activity in presence of GO or the existence of minor alternative pathways for the hydrolysis of fats. The direct hydrolysis of fats catalyzed by GO can be discarded due to the lack of correlation with the concentration and the necessity of temperatures above 50 °C [23]. The remaining of a certain lipase activity in presence of GO would be supported by the lower values of inhibition found after 2 h of reaction in comparison with the initial reaction rate.

The mechanism of inhibition is unclear and excess the objective of the present communication. When the enzyme approaches an apolar medium, its tertiary structure changes and the active center is exposed. The influence of interfaces in the lipase activity implies that aspects such as the size and surface properties of particles could have a great influence on the enzymatic activity [24]. However, considering that in absence of taurodeoxycholate, even concentrations as low as 9.4 ppm of GO can induce an almost complete inhibition, GO could act in the hydrolysis process with an effect contrary to bile salts. It would interfere in the emulsion of the fats, in the approach of the lipase to the fat globules or the extraction of the free fatty acids to form micelles and allow the progress of the reaction [25].

The direct interaction of GO with lipase cannot be discarded, in fact it has been reported that GO can act as an enzyme inhibitor of α -Chymotrypsin thanks to the presence of both hydrophobic aromatic groups and hydrophilic carbonyl groups on the surface of GO sheets, that generate a fast partially reversible interaction [26]. Also, myricetin, a polyphenol, shows lipase inhibition at a molecular level. It forms aggregates in aqueous medium and under simplified intestinal condition that inhibit lipase by a sequestering mechanism as well [27]. However, some differences can be found between our system and previous reports about GO-lipase interaction. The presence of multiple functional moieties on the surface of graphene oxide facilitates successful attachment of lipase simply by stirring during several hours [28], but we observe the effect in a few minutes. The adsorption could change the enzyme to a state with an opened catalytic cavity, even in the absence of direct interaction of the active site with the GO [29,30]. GO can induce changes in the tertiary structure of lipase while maintaining the secondary structure increasing its activity, but our graphene oxide has not been partially reduced to increase the hydrophobicity and only a minor activation could be expected from GO [31,32]. Lipases from *Thermomyces lanuginosa* and *Alcaligenes* sp. immobilized on GO showed a reduction in the lipase activity up to a 60% for some substrates. This change was accompanied by a lower α -helical content on adsorbed lipase and changes in the lipase secondary structure [33]. A similar effect could be found in our case. We have found that GO induces a non competitive inhibition. Thus, GO would bind equally well to the enzyme whether or not it is bound the substrate. It would not change the apparent binding affinity of the catalyst for the substrate, but it would induce a conformation change that modifies the activity.

A priori, GO has no specific reception centers or molecular functional units able to act as inhibitors in the active center. Thus, we do not expect that GO act a specific inhibitor. However, we cannot discard that other digestive enzymes present a behavior different to lipase. The characteristics of the enzymes vary, the substrate is different, and the hydrolysis of fats is an interfacial process. Further research would be needed to evaluate the specificity.

GO shows a strong inhibitory activity, but before a dietary application it should be evaluated its toxicity upon ingestion. The data for this type of material is limited and sometimes contradictory. Furthermore, not only the concentration, but also the origin and preparation of the material can influence its toxicity [34]. Recently the internalisation of GO in mammalian cells has been reviewed by Dabrowsky et al. [35]. It seems that phagocytosis and endocytosis are the main mechanisms of GO cell internalisation. The internalisation depends on several factors such as size, surface properties, and it can be biotransformed in a biological environment by biomolecules or enzymes present in biological fluids. GO does not enter A549 cells and has no cytotoxicity although it can cause a dose dependent and GO size related oxidative stress and induce a slight loss of cell viability at high concentration [36]. GO has shown toxic effects in zebrafish embryos at concentrations over 50.000 ppm [37], rupture of blood cells at concentrations greater than 25 ppm [38], cell death and decreased adhesion of cells in dermal fibroblast cells in concentrations greater than 0.4 mg [39], and 15 ppm of 100 nm GO induced significant alterations in the gene expression level and the mitochondrial activity [40]. In vivo experiments in mice are scarce. The intraperitoneal administration of 5 mg GO/kg for five days caused oxidative stress and liver inflammation, whereas, at the brain level, GO did not affect neuronal cells [41].

In general, the toxicity experiments were performed by direct exposition of the cells to GO or injection, using GO in the nanometer scales instead of micrometric GO, and the concentration was higher than in our case. Also, none of them explored the effect of ingestion of GO, a key issue considering that materials in the micron range, such as our GO (4 μ m), would not tend to be assimilated through the intestine. Thus, further experiments would be necessary before determining the toxicity of GO by ingestion previously to its application.

In combination with the toxicity studies, before its application, future work should include the study of the effect of graphene oxide in the absorption of other substances such as liposoluble relevant substances (i.e. vitamins), the exploration of a wider set of edible fats, the effect of the full gastrointestinal tract, further studies of the nanomaterial-enzyme-fat interaction, or mice tests.

5. Conclusions

GO has demonstrated its potential as inhibitor of lipase under duodenal conditions. The inhibition is almost complete (90%) and lasts during the full duodenal digestion process (2h). Amounts of GO as low as 9.4 ppm offer significant reduction in the fat hydrolysis. The mechanism of action seems contrary to the bile salts, and kinetic studies suggest a non-competitive inhibition between the enzyme and the GO. The inhibition is effective also with alimentary fats such as sunflower and olive oil.

Data availability statement

Data associated with this study has not been deposited into a publicly available repository because it was included in the article.

CRediT authorship contribution statement

Alberto Fernández-Núñez: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jamal EL Haskouri:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pedro Amorós:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jose V. Ros-Lis:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jose V. Ros-Lis reports financial support was provided by Ministry of Science, Innovation, and Universities. Jose V. Ros-Lis reports financial support was provided by Generalitat Valenciana.

Acknowledgments

This research was funded by the Spanish Ministerio de Ciencia, Innovación y Universidades, grant number PID2021-126304OB-C43 funded by MCIN/AEI/10.13039/501100011033 and by “ERDF A way of making Europe”; the AGROALNEXT programme supported by MCIN with funding from European Union NextGenerationEU (PRTR-C17.I1) and by Generalitat grant number EUA-GROALNEXT/2022/065; and by Generalitat Valenciana and European Union, Next Generation EU grant number INVEST/2022/406.

References

- [1] P. Borel, O. Dangles, R.E. Kopec, Fat-soluble vitamin and phytochemical metabolites: production, gastrointestinal absorption, and health effects, *Prog. Lipid Res.* 90 (2023) 101220.
- [2] R. Marcos, S. Infantes-García, H.E. Verkempinck, F. Carrière, M.E. Hendrickx, T. Grauwet, Pre-duodenal lipid digestion of emulsions: relevance, colloidal aspects and mechanistic insight, *Food Res. Int.* 168 (2023) 112785.
- [3] J. Erdmann, F. Lippl, G. Klose, V. Schusdziarra, Cholesterol lowering effect of dietary weight loss and orlistat treatment – efficacy and limitations, *Aliment. Pharmacol. Ther.* 19 (11) (2004) 1173–1179.
- [4] T.T. Liu, X.-T. Liu, Q.-Y. Chen, Y. Shi, Y., lipase inhibitors for obesity: a review, *Biomed. Pharmacother.* 128 (2020) 110314.
- [5] A. Kumar, S. Chauhan, Pancreatic lipase inhibitors: the road voyaged and successes, *Life Sci.* 271 (2021) 119115.
- [6] S. Henness, M. Perry, Orlistat, a review of its use in the management of obesity, *Drugs* 66 (2006) 1625–1656.
- [7] M.D. Wilcox, I.A. Brownlee, J.C. Richardson, P.W. Dettmar, J.P. Pearson, The modulation of pancreatic lipase activity by alginates, *Food Chem.* 146 (2014) 479–484.
- [8] M. Nayebhashemi, S. Enayati, M. Zahmatkesh, H. Madanchi, S. Saberi, E. Mostafavi, E.M. Ardakani, M. Azizi, V. Khalaj, V., Surface display of pancreatic lipase inhibitor peptides by engineered *Saccharomyces boulardii*: potential as an anti-obesity probiotic, *J. Funct. Foods* 102 (2023) 105458.
- [9] J.-Y. Kim, Y.-S. Lee, E.-J. Park, H.-J. Lee, Honeysuckle Berry, *Caerulea L. Lonicera*, Inhibits lipase activity and modulates the gut microbiota in high-fat diet-fed mice, *Molecules* 27 (15) (2022) 4731.
- [10] M. Bilal, C.D. Fernandes, T. Mehmood, F. Nadeem, Q. Tabassam, L.F.R. Ferreira, Immobilized lipases-based nano-biocatalytic systems — a versatile platform with incredible biotechnological potential, *Int. J. Biol. Macromol.* 175 (2021) 108–122.
- [11] L.W. Yao, F.S.A. Khan, N.M. Mubarak, R.R. Karri, M. Khalid, R. Walvekar, E.C. Abdullah, S.A. Mazari, A. Ahmad, M.H. Dehghani, Insight into immobilization efficiency of Lipase enzyme as a biocatalyst on the graphene oxide for adsorption of Azo dyes from industrial wastewater effluent, *J. Mol. Liq.* 354 (2022) 118849.
- [12] S. Muñoz-Pina, P. Amorós, J.E. Haskouri, A. Andrés, J.V. Ros-Lis, Use of silica based materials as modulators of the lipase catalyzed hydrolysis of fats under simulated duodenal conditions, *Nanomaterials* 10 (2020) 1927.
- [13] L. Sun, Structure and synthesis of graphene oxide, *Chin. J. Chem. Eng.* 27 (10) (2019) 2251–2260.
- [14] F. Zhang, S. Li, Q. Zhang, J. Liu, S. Zeng, M. Liu, D. Sun, Adsorption of different types of surfactants on graphene oxide, *J. Mol. Liq.* 276 (2019) 338–346.
- [15] M.E. Mahmoud, R.M. El-Sharkawy, G.A.A. Ibrahim, A novel bionanocomposite from doped lipase enzyme into magnetic graphene oxide-immobilized-cellulose for efficient removal of methylene blue and malachite green dyes, *J. Mol. Liq.* 369 (2022) 120676.
- [16] J.B. Thakkar, D.J. Aghera, B. Trivedi, C.R. Prabha, Design and characterization of a biosensor with lipase immobilized nanoparticles in polymer film for the detection of triglycerides, *Int. J. Biol. Macromol.* 229 (2023) 136–145.
- [17] C.H.M. Versantvoort, A.G. Oomen, E. Van De Kamp, C.J.M. Rempelberg, A.J.A.M. Sips, Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food, *Food Chem. Toxicol.* 43 (1) (2005) 31–40.
- [18] M. Minekus, M. Alming, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D.J. McClements, O. Ménard, I. Recio, C.N. Santos, R.P. Singh, G.

- E. Vegarud, M.S.J. Wickham, W. Weitschies, A. Brodkorb, A standardised static in vitro digestion method suitable for food – an international consensus, *Food Funct.* 5 (6) (2014) 1113–1124.
- [19] B. Nieva-Echevarría, E. Goicoechea, M.J. Manzano, M.D. Guillén, Usefulness of ¹H NMR in assessing the extent of lipid digestion, *Food Chem.* 179 (2015) 182–190.
- [20] P. Joyce, C.P. Whithy, C.A. Prestidge, Nanostructuring biomaterials with specific activities towards digestive enzymes for controlled gastrointestinal absorption of lipophilic bioactive molecules, *Adv. Colloid Interface Sci.* 237 (2016) 52–75.
- [21] S.J. Hur, E.A. Decker, D.J. McClements, Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during in vitro digestion, *Food Chem.* 114 (1) (2009) 253–262.
- [22] B. Nieva-Echevarría, E. Goicoechea, M.J. Manzano, M.D. Guillén, A method based on ¹H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures, *Food Res. Int.* 66 (2014) 379–387.
- [23] S.-N. Trinh, L.-H. Tran, C. Lee, S.-H. Jang, Lipid hydrolysis catalyzed by graphene oxide, *Bull. Kor. Chem. Soc.* 38 (145) (2017) 1455.
- [24] R.D. Schmid, R. Verger, Lipases: interfacial enzymes with attractive applications, *Angew. Chem. Int. Ed.* 37 (1998) 1608–1633.
- [25] A. Macierzanka, A. Torcello-Gómez, C. Jungnickel, J. Maldonado-Valderrama, Bile salts in digestion and transport of lipids, *Adv. Colloid Interface Sci.* 274 (2019) 102045.
- [26] M. De, S.S. Chou, V.P. Dravid, Graphene oxide as an enzyme inhibitor: modulation of activity of α -chymotrypsin, *J. Am. Chem. Soc.* 133 (2011) 17524–17527.
- [27] A.-S. Bustos, A. Hakansson, J.A. Linares-Pastén, L. Nilsson, Interaction between myricetin aggregates and lipase under simplified intestinal conditions, *Foods* 9 (2020) 777.
- [28] S. Hermanová, M. Zarevúcká, D. Bouša, M. Mikulics, Z. Sofer, Lipase enzymes on graphene oxide support for high-efficiency biocatalysis, *Appl. Mater. Today* 5 (2016) 200–208.
- [29] R.A. Silva, M.L. Souza, G.D. Bloisi, P. Corio, DFS Petri, Bioconjugation of lipase and cholesterol oxidase with graphene or graphene oxide, *J. Nano Res.* 17 (2015) 187.
- [30] O. Kalji, Y. Sefidbakht, A.M. Nesterenko, V. Uskoković, S.-O. Ranaei-Siadat, Colloidal graphene oxide enhances the activity of a lipase and protects it from oxidative damage: insights from physicochemical and molecular dynamics investigations, *J. Colloid Interface Sci.* 567 (2020) 285–299.
- [31] W. Zhuang, X. Quan, Z. Wang, W. Zhou, P. Yang, L. Ge, B. Villacorta Hernandez, J. Wu, M. Li, J. Zhou, C. Zhu, H. Ying, Interfacial microenvironment for lipase immobilization: regulating the heterogeneity of graphene oxide, *Chem. Eng. J.* 394 (2020) 125038.
- [32] M. Mathesh, B. Luan, T.O. Akanbi, J.K. Weber, J. Liu, C.J. Barrow, R. Zhou, W. Yang, Opening lids: modulation of lipase immobilization by graphene oxides, *ACS Catal.* 6 (7) (2016) 4760–4768.
- [33] R.B. Tejaswini, T.R.B. Ramakrishna, T.D. Ashton, S.N. Marshall, T.D. Nalder, W. Yang, C.J. Barrow, Effect of triton X-100 on the activity and selectivity of lipase immobilized on chemically reduced graphene oxides, *Langmuir* 37 (30) (2021) 9202–9214.
- [34] A.M. Dimiev, S. Eglér (Eds.), *Graphene Oxide: Fundamentals and Applications*, Wiley, 2016.
- [35] B. Dąbrowski, A. Zuchowska, Z. Brzózka, Graphene oxide internalization into mammalian cells – a review, *Colloids Surf. B Biointerfaces* 221 (2023) 112998.
- [36] Y. Chang, S.-T. Yang, J.-H. Liu, E. Dong, Y. Wang, A. Cao, Y. Liu, H. Wang, In vitro toxicity evaluation of graphene oxide on A549 cells, *Toxicol. Lett.* 200 (2011) 201–210.
- [37] L. Chen, P. Hu, L. Zhang, S. Huang, L. Luo, C. Huang, Toxicity of graphene oxide and multi-walled carbon nanotubes against human cells and zebrafish, *Sci. China Chem.* 55 (10) (2012) 2209–2216.
- [38] K.H. Liao, Y.S. Lin, C.W. Macosko, C.L. Haynes, Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts, *ACS Appl. Mater. Interfaces* 3 (7) (2011) 2607–2615.
- [39] K. Wang, J. Ruan, H. Song, J. Zhang, Y. Wo, S. Guo, D. Cui, Biocompatibility of graphene oxide, *Nanoscale Res. Lett.* 6 (1) (2011) 1–8.
- [40] M.S. Hashemi, S. Gharbia, S. Jafarnejad-Farsangi, Z. Ansari-Asl, A.S. Dezfuli, Secondary toxic effect of graphene oxide and graphene quantum dots alters the expression of miR-21 and miR-29a in human cell lines, *Toxicol. Vitro* 65 (2020) 104796.
- [41] A. Rhazouani, H. Gamrani, S. Ed-Day, K. Lafhal, S. Boulbaroud, L. Gebrati, N. Fdil, F. Aziz, Sub-acute toxicity of graphene oxide (GO) nanoparticles in male mice after intraperitoneal injection: behavioral study and histopathological evaluation, *Food Chem. Toxicol.* 171 (2023) 113553.