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Natural Inhibitors of Lipase: Examining Lipolysis in a Single Droplet

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ABSTRACT: Inhibition of lipase activity is one of the approaches to reduced fat intake with nutritional prevention promoting healthier diet. The food industry is very interested in the use of natural extracts, hence reducing the side effects of commercial drugs inhibiting lipolysis. In this work we propose a novel methodology to rapidly assess lipolysis/inhibition in a single droplet by interfacial tension and dilatational elasticity. The evolution of the interfacial tension of lipase in simplified duodenal fluid in the absence and that in the presence of the pharmaceutical drug Xenical are the negative (5 ± 1 mN/m) and positive (9 ± 1 mN/m) controls of the inhibition of lipolysis, respectively. Then, we correlate the inhibition with the reduction of the interfacial activity of lipase and further identify the mode of action of the inhibition based on dilatational response (conformational changes induced in the molecule/blocking of adsorption sites). This work provides new insight into the lipase inhibition mechanism and a rapid methodology to identify the potential of new natural inhibitors.

KEYWORDS: *lipase, natural inhibitor, adsorption, lipolysis, interfacial tension*

INTRODUCTION

Overconsumption of saturated fats, trans fatty acids, and cholesterol increases the susceptibility of individuals to a range of diseases, including cardiovascular disease, diabetes, and obesity.[1] The regulation of fatty acid and triglyceride availability in foods depends on the activity of lipolytic enzymes, pancreatic lipase being the main lipid-digesting enzyme (lipolysis). Thereby, the inhibition of pancreatic lipase is an attractive approach for the discovery of potential antiobesity agents.[2] At present, there are on the market several pharmaceutical compounds that inhibit pancreatic lipase. Namely, Xenical is a lipase competitive substrate that irreversibly binds to the catalytic site of the lipase. Specifically, the inhibition occurs after nucleophilic attack of the β-lactone ring of Xenical and the active site serine residue of lipase. Xenical is a commercial drug that inhibits the hydrolysis of dietary triacylglycerols, hence reducing the production and subsequent intestinal absorption of monoacylglycerols and free fatty acids.[3,4] However, this product presents several adverse effects owing to the presence of undigested lipid in the colon,[5] and therefore the patients currently treated with Xenical cannot lead a normal life. To reduce these adverse side effects, the market is currently looking for natural compounds that slow or prolong lipid digestion rather than block lipid digestion. This would reduce hunger and promote satiety by stimulating satiety hormones secreted by cells in the ileum,[6,7] eventually reducing energy intake and diseases related with lipid ingestion.

Fats from the diet are converted into a coarse oil-in-water emulsion in the mouth, and this emulsion develops through the stomach and/or small intestine to become a more stable emulsion by peristaltic movements of the tract and mixes with surface active molecules from food or secreted by the body, such as bile salts, phospholipids, and proteins.[8−10] As a result, the substrate for lipid digestion in the duodenum (lipolysis) is an oil−water emulsion and lipolysis is, hence, an interfacial process whereby lipids, lipase, colipase, and bile salts compete at the oil−water interface. First, lipases are water-soluble enzymes that facilitate fat metabolism and digestion at the interface. They are acyl hydrolases, which hydrolyze the fats (triacylglycerols) in di- and monoglycerides and one or two fatty acids (amphiphilic products), although under certain circumstances complete hydrolysis into fatty acid and glycerol may occur.[11] Second, colipase is a smaller protein that is less surface active than lipase and promotes lipase activity.[12,13] Finally, bile salts are negatively charged natural amphiphiles produced in the liver and secreted through the bile duct.[14−16] With these premises, the bioavailability of lipids can be controlled by changing the molecular characteristics and altering the emulsion structure (droplet size or interfacial properties),[1,17−19] providing stability against the action of bile salts and lipase/colipase. One approach to affect the bioavailability of lipids is adding components that interfere (inhibit) with the enzymatic action (lipolysis) in different ways, altering the molecule (inhibitors), or blocking the access to the interface.[20]

To assess lipase inhibition it is important to correctly quantify lipolysis first. There are a number of methods available in the literature to assess lipolysis in vitro such as titrometry, spectroscopy, chromatography, radioactivity, interfacial tensiometry, turbidimetry, conductimetry, immunochemistry, microscopy, and, more recently, light scattering and HPLC[21,22]. However, as stated by Beisson et al., there is to date no single universal method of lipase assay, and the choice will depend on the user's own specific requirements.[21] Among current alternatives, pH stat titrator is to date the most used and

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appears very suitable in many cases.[23−25] Still, it has certain limitations as it can be used only within a restricted pH range and measure activities >0.1 (μmol FFA/min).[22]

Because lipolysis is an interfacial process, techniques based on surface tension offer an interesting platform to develop new methods to assess it. However, these have been to date much less used for various reasons.[21] Back in 1990, Piéroni and co-workers developed a method based on monolayers by using a Langmuir film balance to study lipase inhibition by proteins.[26] However, this method offered some disadvantages such as the high amount of material needed and the air−water interface, which can be unrealistic. Then, Nury et al. developed a method to study lipase kinetics study at the oil−water interface with drop tensiometry.[27] A much smaller amount of material was needed and, hence, this method was used later by Flipsen et al. to measure lipase inhibition.[28] Studies so far with this method focus more specifically on the lag time dynamics. Only very recently, there are interesting references using drop tensiometry to relate lipase activity and interfacial pressure also addressing the impact of enzyme−surfactant interactions on lipolysis.[8,29] Another example is the work of Arnold et al., in which oil viscosity and surface tension of poorly soluble drugs is found to affect the quality of the interface and lipolysis kinetics.[3] In this line, here we upgrade current models of lipolysis in a pendant drop to simulate in vitro lipolysis at the oil−water interface by a duodenal simplified simulated fluid. Simulation of the digestion process offers new quantitative data importantly improving the characterization of the system. Specifically, concerning the characterization of lipase inhibitors, most literature works use pH titration and colorimetric methods.[23−25] Thus, the use of pendant drop tensiometry to accurately measure the impact of inhibitors on lipase by simulating in vitro lipolysis is indeed innovative. A main goal of this work is the development of a rapid screening method to assess lipase activity in a single droplet as a screening test. This methodology allows us to screen lipolysis at the oil−water interface and characterize the inhibition reached by different compounds. We have used this new method first to establish the control by using Xenical as a positive control of inhibition. Then, we validate the methodology with two flavonoids of known inhibition potential supplied by our industrial partners Biosearch Life, and finally we apply the proposed methodology to various green tea extracts providing quantitative comparative data on the inhibition. This is important new information because understanding the behavior of lipase inhibitors can provide rational routes to control fat uptake in the diet. After the screening test, application of more complex digestion models should be carried out, but now only on selected systems with proven inhibition potential. This modus operandi provides important reductions in time and costs as recognized by our industrial partners in this work.

MATERIAL AND METHODS

Chemicals. Lipase from porcine pancreas (type II, 100−500 units/mg protein using olive oil was purchased from Sigma-Aldrich (catalog no. L3126), stored at 4 °C, and used as received. Pancreatin from porcine pancreas from Sigma (P3292) was stored at −18 °C and used as received. Highly refined olive oil (Sigma-Aldrich, catalog no. 01514) was purified with Florisil resins (Fluka, 60−10 mesh, catalog no. 46385) prior to use by following the procedure used in previous studies.[15,30,31] Namely, a mixture of oil and Florisil in proportion 2:1 w/w was shaken mildly for 2 h and then centrifuged at 14300 rpm for 30 min in a centrifuge from Kronton instruments (Centrikon T-124). The olive oil mixture was filtered with Millex filters (0.1 μm PDVF) and stored under nitrogen in the dark. The presence of surface-active impurities in oil could interfere with the interfacial tension measurements and invalidate the correlation of interfacial tension measurements with the presence of lipase or lipase/inhibitor at the interface.

The components used in the duodenal media are set according to the standardized in vitro digestion method for food.[32] The duodenal buffer used in all solutions was 2 mM BIS-TRIS (Sigma, 14879), 0.15 M NaCl, and 0.002 M $CaCl_2$, pH 7.0. Body temperature was adjusted to 37 °C with an external temperature control in the measurement equipment. Lipase samples (7.8 g/L) and pancreatin (0.12 g/L) were prepared immediately before use in the duodenal buffer. Inhibitors were added to this mixture and filtered before use with Millex filters (0.1 μm PDVF).

Ultrapure water, cleaned using a Milli-Q water purification system (0.054 μS), was used for the preparation of buffer solutions. All glassware was washed with 10% Micro-90 cleaning solution and exhaustively rinsed with tap water, isopropanol, deionized water, and ultrapure water in this sequence. All other chemicals used were of analytical grade and used as received.

Xenical was bought from a local pharmacy, and the concentration was fixed at 0.22 g/L in methanol (5.2% final concentrations). Flavonoid fractions were obtained by high-pressure liquid chromatography (HPLC) and supplied by Biosearch Life; they were used at the same concentration of 0.22 g/L. Green tea extract (GTE) was also supplied by our industrial partners; it was obtained by hydroalcoholic extraction and then dried by atomization of commercial extract *Camellia sinensis*. This extract has 30% epigallocathechin gallate (EGCG) and 60% cathechin. It was dissolved in duodenal buffer and tested at different final concentrations of 0.4, 0.9, 1.1, 1.8, 2.2, 2.4, 2.6, 3.5, and 4.4 g/L.

Methods. *In Vitro Lipolysis in a Single Droplet.* All measurements were made in the OCTOPUS. This device has been implemented on the basis of the single subphase exchange device, where the normal capillary tip was substituted by an arrangement of two coaxial capillaries, connected each to one of the channels of a specific microinjector, which can operate independently[30] (Spanish patent registration no. P9801626), to achieve a fully automated subphase multiexchange device.[33] The OCTOPUS computer software DINAT-EN has been also fully programmed at the University of Granada. The detection and calculation of surface area and surface tension is based on axisymmetric drop shape analysis (ADSA). The pendant drop is placed on a three-axis micropositioner and is immersed in a glass cuvette filled with the oil phase (Hellma), which is kept in an externally thermostated cell at 37 °C for all of the experiments, simulating body temperature.[34]

Lipolysis of the oil−water interface was measured in two steps: adsorption and desorption. Interfacial tension is recorded at constant interfacial area (20 mm^2) throughout the whole adsorption−desorption process. The dilatational rheology of the interfacial layers is measured by applying an oscillatory perturbation to the interface at the end of each adsorption step by injecting and extracting volume to the drop. The system records the response of the interfacial tension to this area deformation, and the dilatational modulus (E) of the interfacial layer is calculated from this response. In a general case, the dilatational modulus is a complex quantity that contains a real and an imaginary part:

$$E = E' + iE'' = \varepsilon + i\nu\eta \quad (1)$$

E' is the storage modulus that accounts for the elasticity of the interfacial layer (ε), E'' is the loss modulus that accounts for the viscosity (η) of the interfacial layer, and ν is the angular frequency of the applied oscillation. In this work, the applied oscillations in interfacial area were maintained at amplitude values of <5%, to avoid excessive perturbation of the interfacial layer, whereas the measurement frequency (ν) is set to 0.1 Hz. At this relatively high frequency, compared to typical relaxation processes in protein films, the viscous component of the dilatational modulus is very small, and the adsorbed layer is predominantly elastic. Thus, in this study we report values of the dilatational modulus $|E|$ given by eq 1 at a fixed interfacial tension

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at the end of the adsorption step. All curves were recorded in the absence and presence of inhibitor to test the inhibition of lipolysis by different compounds.

In Vitro Lipolysis Using a Colorimetric Method. The inhibition of lipase was measured using lipase colorimetric assay 11821792216 (LIP, Cobas, Roche) containing 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid-(6-methylresorufin) ester as substrate, colipase, and bile salts. Fraction flavonoid-1, fraction flavonoid-2, and Xenical were assayed at different concentrations.

Statistical Analysis. All of the experimental curves have been analyzed with statistical tools in Microsoft Excel to estimate the number of measurements needed depending of the variability of the results as well as the mean values and standard deviations obtained. The number of repetitions in each case was determined depending on the standard deviation obtained: three replicates were made when deviation was found to be <2%, whereas six repetitions were needed when deviations ranged from 2 to 8%. Final values are expressed as mean values of replicates ± standard deviations according to statistical analysis tools.

RESULTS AND DISCUSSION

Method for Assessing Lipase Inhibition. A first step in the study of the inhibition of lipolysis by natural extracts is the development of a methodology, based on interfacial tension measurements, that enables an accurate screening of the samples. To this end, we characterize initially the lipolysis in the absence and presence of a commercial inhibitor, Orlistat (Xenical), as positive and negative control of inhibition, respectively. The inhibiting action of Orlistat is well established because it is known that it inhibits the action of lipase.[4,5] Hence, the model experiments were designed to discern the different in vitro lipolysis profiles of lipase or lipase/Xenical adsorbed layers at oil−water interfaces as negative and positive controls, respectively.

To design a rapid screening method, we envisage a simplified scenario containing only lipase dissolved in duodenal buffer. This is a model system that would need to be validated in a more complex medium in a second phase of evaluation of the extracts. However, the results can be safely used as a rapid test for screening purposes. Prior to removing colipase and bile salts from our systems, we investigated in detail the system,[35] concluding that the presence of colipase accelerates the lipolysis, whereas the presence of bile salts principally affects the solubility of the products of lipolysis as studied in detail in a previous work.[35] Likewise, Labourdenne et al. also demonstrated that the presence of bile salts and colipase does not affect the hydrolysis rates and the binding of pancreatic lipase.[12] Anyway, the results presented here are intended to be treated as a screening test so that application of more complex digestion models is later carried out only on selected systems, thus importantly reducing time and costs.

The model experiment (screening test) consists in measuring the adsorption−desorption profile of lipase/inhibitor and comparing it to the adsorption profile of lipase and lipase/Xenical as negative and positive controls. Adsorption−desorption profiles are measured in an aqueous drop immersed in the oil phase, mimicking the physiological conditions of the duodenum. In the adsorption phase, lipase or lipase/inhibitor adsorbs onto the oil−water interface. The reduction of the interfacial tension is determined by the lipase molecules adsorbed and the lipolysis products at the interface. The lower the interfacial tension, the more lipolysis has occurred. The dilatational modulus of this layer provides additional information on the molecular conformation. The desorption step should offer additional information on the degree of lipolysis reached and should be used only in misleading cases.[30,36] During the desorption, the bulk solution is depleted of material, and soluble products from lipolysis will be eliminated from the bulk/interface, hence increasing the interfacial tension.[34] The dilatational modulus at the end of the desorption process can be confusing owing to surface active residues remaining at the interface after exchange and hence will not be considered.[34]

Negative (Lipase) and Positive (Lipase/Xenical)-Controls: No Inhibition (Negative) and Inhibition (Positive). With these premises we now analyze the adsorption−desorption curves obtained for the controls. First we look into the negative−positive controls and obtain a complete characterization of lipolysis and the inhibitory action of Xenical. Subsequently, we can study the performance of various natural inhibitors by comparison with positive−negative controls.

Figure 1 shows the adsorption−desorption profiles lipase and lipase/Xenical at the oil−water interface (negative−positive control, respectively). The adsorption−desorption profile of pure Xenical at the olive oil−water interface is included here, but will not be generally determined for inhibitors. The values of interfacial tension and dilatational moduli at the end of the adsorption and desorption steps for lipase, Xenical, and lipase/Xenical solutions are displayed in Table 1.

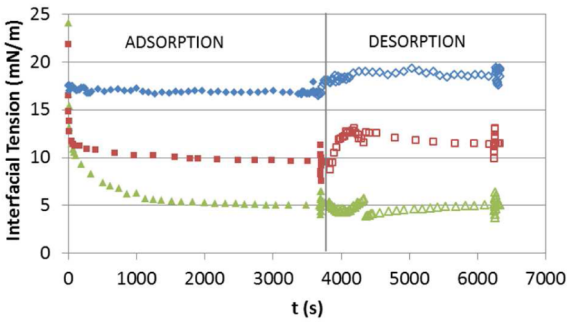

Figure 1. Adsorption (solid)−desorption (open) profiles of Xenical (blue rhombus), lipase-negative control (green triangles), and lipase/Xenical-positive control (red squares) at the olive oil−water interface under duodenal media. Curves are obtained as the mean of at least three replicate measurements (standard deviation < 2%).

Table 1. Interfacial Tension (γ) and Dilatational Elasticity (E) Obtained after 1 h of Adsorption−Desorption in Duodenal Media at the Olive Oil−Water Interface[a]

	adsorption		desorption	
	γ (mN/m)	E (mN/m)	γ (mN/m)	E (mN/m)
lipase	5 ± 1	16 ± 2	5 ± 1	
Xenical	16 ± 1	4 ± 1	17 ± 1	
lipase/Xenical	9 ± 1	25 ± 3	12 ± 1	

[a]Values are the mean of at least three replicate measurements.

The adsorption profile of lipase shows how the interfacial tension decreases immediately and very steeply to 10 mN/m in the first 100 s and then continues to decrease more smoothly to reach a final interfacial tension of 5 mN/m (Figure 1 and Table 1). Hence, lipase adsorbs rapidly onto the oil−water interface, forming a loose monolayer in agreement with previous works.[35] Then, as the adsorption proceeds, adsorbed lipase is able to

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hydrolyze triglycerides from olive oil into mono- and di-unsaturated glycerides and two fatty acids, and this continues to lower the interfacial tension to 5 mN/m, as seen in Figure 1. The adsorption profile of lipase/Xenical mixture is very different (Figure 1). The interfacial tension also decreased immediately and very steeply in the first 100 s, overlapping with the lipase curve, but then reaches a pseudo plateau in which the interfacial tension practically remains constant at around 10 mN/m. This plateau indicates that lipase cannot hydrolyze the triglycerides from olive oil due to the presence of Xenical, and hence the interfacial tension remains constant once the interface has saturated at a much higher interfacial tension. These different profiles illustrate the inhibition of lipolysis in the presence of Xenical and provide the negative and positive controls. Similarly, the dilatational modulus is lower for lipase than for lipase/Xenical (Table 1). This lower value can be attributed to the presence of lipolysis products at the interface due to the lipolysis action of the lipase, preventing the formation of a cohesive network that develops easily when lipolysis is inhibited in the presence of Xenical (Table 1). The adsorption profile of pure Xenical is given here as description of the control, but in general, the adsorption profile of inhibitors is not necessary. As long as the interfacial tension curve of the mixture lipase/inhibitor lies between the negative and positive controls, the interfacial activity of inhibitors is intrinsically considered in the curves. The adsorption profile of pure Xenical shows a low interfacial activity with a rapid decrease of interfacial tension to a plateau at higher interfacial tension values than the controls (16 mN/m) (Figure 1). The low dilatational modulus of pure Xenical (Table 1) confirms the formation of the diluted layer of rigid molecules with low elastic response.

Consider now the desorption. At 3800 s, the subphase of the drop was exchanged by using the OCTOPUS and the desorption process started to record (Figure 1). During the change of subphase, the soluble products of lipolysis are eliminated from the interface as the bulk is depleted of material. Herein, an increase of the interfacial tension implies that molecules desorb from the interface. Again, the desorption profiles of lipase and lipase/Xenical are very different. The desorption profile of pure Xenical shows just a slight increase of the interfacial tension, meaning that few molecules of Xenical desorb upon subphase exchange. Desorption of lipase shows a similar response; the interfacial tension remains unchanged (5 mN/m) during and after the subphase exchange with duodenal buffer, meaning that the lipase and lipolysis products remain adsorbed at the olive oil–water interface.[1] Conversely, we register a clear increase in the interfacial tension after subphase exchange for the lipase/Xenical mixture (Table 1). These values indicate that the interface is depleted of material, suggesting that the complex lipase/Xenical partially desorbs. Desorption of unbound Xenical could also contribute to this increase as seen in the pure Xenical desorption curve. Accordingly, the desorption profile of lipase/Xenical indicates again an inhibition of lipolysis. Maldonado-Valderrama and co-workers[30] showed lipase adsorption and desorption profiles coinciding with our results; lipase is adsorbed into the oil–water interface and is not desorbed, and the elastic modulus is the same at adsorption and desorption processes.[30]

The different adsorption–desorption profiles of lipase and lipase/Xenical at duodenal media provide hence a clear negative–positive control of inhibition. Negative control means no inhibition and is quantified by an interfacial tension of 5 mN/m. Positive control means full inhibition is characterized by 9 mN/m. With this method, the activity of inhibitors can be screened by measuring the interfacial tension after 1 h of adsorption of lipase/inhibitor in duodenal media and comparing with negative (5 mN/m) and positive (9 mN/m) controls. This method is valid for resulting curves contained within the two control curves; curves of this interval should require further analysis of the interfacial activity. A priori, analysis of adsorption, dynamics, and dilatational modulus should serve as a screening method, and desorption should be measured only for misleading cases, thus providing further information.

Lipase Inhibition Potential of Natural Extracts from Flavonoids. The proposed methodology is now validated measuring the degree of inhibition reached by two natural extracts supplied by our industrial partner in this work, Biosearch Life, and with known inhibition properties. Flavonoid-1 and flavonoid-2 are two different HPLC semi-preparative fractions with different inhibition profiles as measured by lipase colorimetric assays. Figure 2 shows the

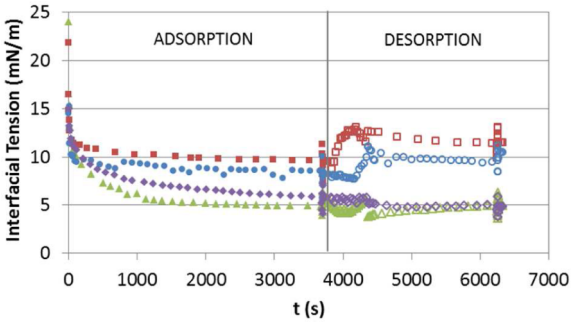

Figure 2. Adsorption (solid)–desorption (open) profiles of lipase/flavonoid-1 (blue circles), lipase/flavonoid-2 (purple rhombuses), negative control-lipase (green triangles), and positive control-lipase/Xenical (red squares) at the olive oil–water interface under duodenal media. Curves are obtained as the mean of at least three replicate measurements (standard deviation < 2%).

inhibition of lipase by the flavonoids and Xenical as a function of concentration. The lipase activity decreased when higher concentrations of inhibitors were used. Flavonoid-1 showed better inhibition than flavonoid-2, whereas lipase activity decreased very rapidly and at lower concentrations for Xenical.

Thus, to validate the methodology proposed, we tested the performance of these flavonoids in our screening test. Figure 3 shows the adsorption–desorption profiles of the two flavoniods in the presence of lipase along with the negative and positive controls. The values of the interfacial tension at the end of each step and the elastic moduli are displayed in Table 2. As explained in the previous section, the proposed methodology consists of first screening the inhibition with the final interfacial values and then looks into dynamics, dilatational moduli, and desorption for details of the inhibition mechanism.

First, it is important to note in Figure 3 that the adsorption–desorption profiles of the tested samples lie between the extreme behaviors of the negative control (no inhibition, lipase) and positive control (inhibition, lipase/Xenical). Then, the relative position of the curves can provide an estimation of the degree of lipolysis reached by the tested products. Accordingly, lipase/flavonoid-1 reaches a higher interfacial tension (7 mN/m), therefore indicating a slightly better inhibition potential

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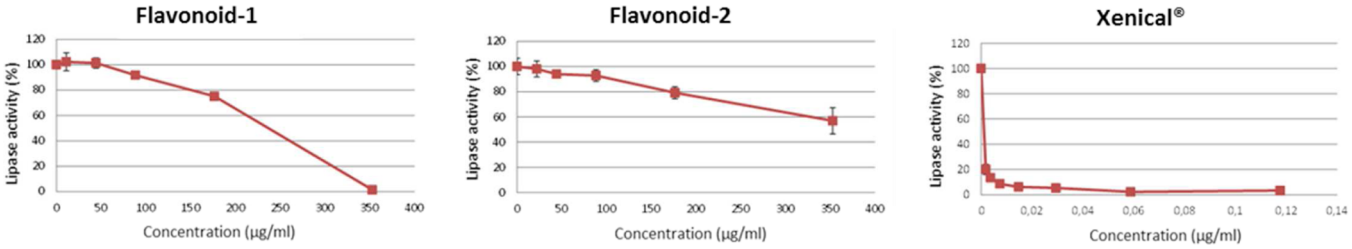

Figure 3. Inhibition of lipase by fraction flavonoid-1, fraction flavonoid-2, and Xenical using lipase colorimetric assay. Values are expressed as percent of lipase activity. Curves are obtained as the mean of at least three replicate measurements.

Table 2. Interfacial Tension (γ) and Dilatational Elasticity (E) Obtained after 1 h of Adsorption−Desorption in Duodenal Media at the Olive Oil−Water Interface[a]

	adsorption		desorption	
	γ (mN/m)	E (mN/m)	γ (mN/m)	E (mN/m)
lipase/flavonoid-1	7 ± 1	10 ± 2	10 ± 1	
lipase/flavonoid-2	6 ± 1	10 ± 3	5 ± 1	

[a]Values are the mean of at least three replicate measurements.

than that of lipase/flavonoid-2 (6 mN/m). This is the first indication of the screening test of inhibition.

After screening the better inhibitor flavonoid-1, we look into the dynamics in more detail. Adsorption of lipase/flavonoid-1 shows a steep decrease followed by a plateau similar to positive control (lipase/Xenical). Differently, lipase/flavonoid-2 shows a dynamic curve more similar to negative control (lipase) with a steep decrease followed by a smooth decrease before plateau. Hence, this confirms the better inhibition potential of flavonoid-1.

Then we evaluate the dilatational moduli of inhibitors flavonoid-1 and flavonoid-2 (Table 2) and again compare with the controls (Table 1). The dilatational moduli of the lipase/flavonoid-1 and lipase/flavonoid-2 adsorbed layers appears different from that of negative and positive controls. The presence of inhibitor results in a decrease of the dilatational elasticity. This indicates that these compounds have an inhibition mechanism different from that of Xenical. Xenical is known to bind covalently to the digestive lipase's active site, but it does not seem to disrupt the lipase molecule. Conversely, the lower dilatational moduli of lipase/flavonoid-1 and lipase/flavonoid-2 indicates that the inhibiting mechanism produces a conformational change of lipase, which implies a loss of cohesiveness of the interfacial layer.

The desorption profiles of lipase/flavonoid-1 and lipase/flavonoid-2 again corroborate the better inhibition achieved by the former compound. Lipase/flavonoid-1 shows an important increase of the interfacial tension after subphase exchange in contrast to the desorption profile of lipase/flavonoid-2, for which the tension remains practically unchanged (Table 2). These results corroborate the better inhibition potential of flavonoid-1.

In view of this analysis, the results obtained for the inhibition reached by flavonoids as measured by calorimetric assays (Figure 2) are fully validated with the interfacial tension (Figure 2). Hence, the proposed methodology provides a rapid screening method for lipase inhibition, and analysis of the curves offers novel information on the inhibition mechanism at a molecular level.

Lipase Inhibition of Green Tea Extract. We now analyze the inhibition potential of natural extracts of GTE provided by our industrial partners, Biosearch Life, with the new methodology proposed. Polyphenols represent the major class for the pancreatic lipase inhibitor.[37,38] They bind noncovalently to the enzyme by polyvalent sites present in them. Previous studies have shown that tea and phenolic components such as catechins from tea have antiobesity and antidiabetic effect in humans, reduce adipose mass in rodent models, and influence lipid digestion in vitro.[37,38] It is thought that green tea consumption can be useful to treat obesity owing to increased thermogenesis and inhibition of pancreatic lipase.[39] In this case, the adsorption profile provided enough information for screening purposes and, hence, desorption profiles are not provided here.

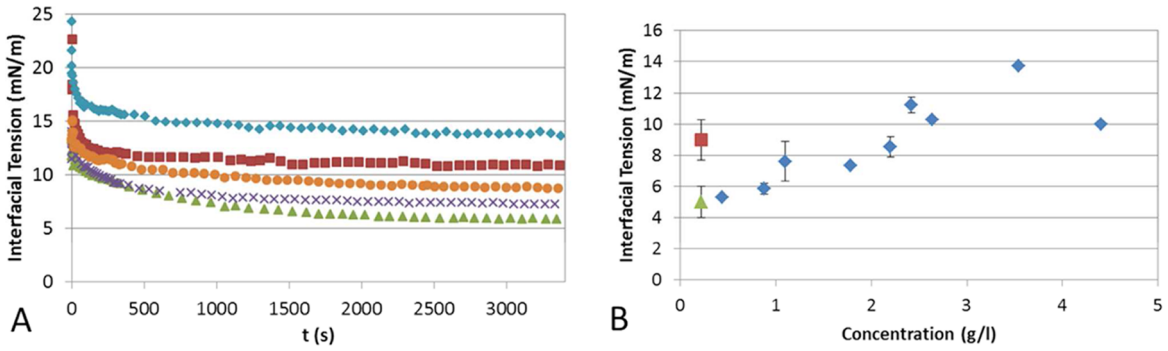

Figure 4. (A) Adsorption profiles of lipase/GTE: 1.8 g/L (purple crosses), 2.2 g/L (orange circles), 3.5 g/L (blue rhombuses), negative control-lipase (green triangles), and positive control-lipase/Xenical (red squares) at the olive oil−water interface under duodenal media. (B) Interfacial tension after 1 h of adsorption: lipase/GTE (blue rhombuses), negative control-lipase (green triangles), and positive control-lipase/Xenical (red squares). The values are obtained as the mean of at least three replicate measurements (standard deviation < 2%).

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Figure 4A shows the adsorption profile of lipase/GTE with various concentrations of extract along with the negative (lipase) and positive (lipase/Xenical) controls. First, we look into the final interfacial tension reached and compare it with negative−positive controls as displayed in Figure 4B. Then we look into the dynamics, and finally we evaluate the dilatational moduli, which provide further information on the inhibition mechanism.

Figure 4B shows the final interfacial tension values of mixtures lipase/GTE in duodenal media after 1 h of adsorption at the olive oil−water interface. The final interfacial tension (inhibition) reached increases with the concentration of GTE, reaching a maximum for 3.5 g/L GTE, above which the interfacial tension (or inhibition) decreases. Hence, the inhibition potential of GTE seems to saturate at a given concentration of GTE in the system. This saturation effect could be due to an excess of inhibitor GTE in the system as compared to the lipase concentration. Also, it is interesting to note here that the numerical value of the interfacial tension at the maximum (14.0 ± 0.2 mN/m) is even higher than that recorded for lipase/Xenical (9 ± 1 mN/m). Nonetheless, we need to consider that the concentration of inhibitor at the maximum of interfacial tension is 16 times higher than in the positive control. Hence, to discern whether we have improved the inhibition of Xenical or not, we need to investigate further.

The dynamic curve obtained for the lower concentration of GTE (1.8 g/L) coincides with that of lipase for the first 400 s and then attains a plateau while the lipase curve continues to decrease (Figure 4A). Hence, we can ensure that 1.8 (g/L) GTE allows adsorption of lipase but then inhibits moderately lipolysis as the interfacial tension attains a plateau above the pure lipase curve (Figure 4A). Similarly, the adsorption profile of 2.2 g/L GTE overlaps with negative control in the first 200 s, where lipase occupies the oil−water interface. After that, the decline is moderated, attaining a plateau; at this point the lipase has been inhibited. The higher concentration of GTE resulted in a higher inhibition of lipolysis. Still, these two concentrations of GTE (1.8 and 2.2 g/L) provided less inhibition than the positive control (attaining lower interfacial tension than lipase/Xenical). Interestingly, by increasing further the concentration (3.5 g/L) the adsorption profile changes drastically. The interfacial tension decreases steeply and attains rapidly a plateau well above the positive control as discussed in Figure 4B. Again, whether this higher interfacial tension value means higher inhibition or saturation of the interface with GTE remains unclear.

Figure 5 shows the dilatational moduli of lipase/GTE mixtures plotted against GTE concentration. The values of the negative and positive controls are also plotted in the graph. Conversely to Xenical, GTE clearly increases the dilatational modulus of lipase at the interface. This is due to the different mode of action of polyphenol-based inhibitors as compared to Xenical. Lipase/Xenical forms a covalent bond after the nucleophilic attack of the catalytic serine residue on the β-lactone group from lipase,[4] which results in a slightly more cohesive layer at the interface. Conversely, lipase/GTE binds by a noncovalent and spontaneous interaction.[12] Specifically, according to Wu et al., a recent study shows that the interaction between EGCG from green tea and lipase occurs through hydrogen bonds and electrostatic interactions, resulting in an alteration of the molecular conformation of lipase, which decreases the enzyme catalytic activity.[40] This conformation could well improve substantially the cohesiveness

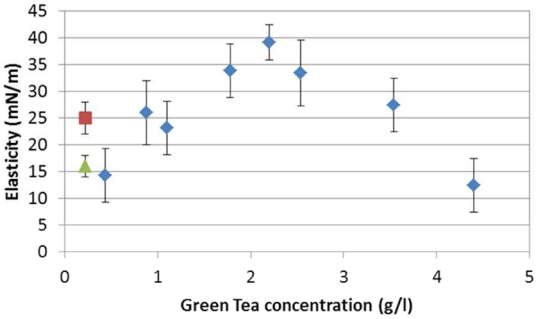

Figure 5. Dilatational elastic modulus of adsorbed layers at the olive oil−water interface under duodenal media after 1 h: lipase/GTE (blue rhombuses), negative control-lipase (green triangles), and positive control-lipase/Xenical (red squares). Values are obtained as the mean of at least three replicate measurements (standard deviation < 2%).

of the interfacial layer. Accordingly, the presence of a maximum in the dilatational modulus is possibly related to a maximum inhibition of lipolysis. Then, the elasticity loss recorded for higher concentrations of GTE possibly implies that the inhibition is less favored or has saturated. A reduction of the dilatational modulus could originate from the presence of unbound GTE at the interface as inferred from the dynamic curve in Figure 4. Accordingly, the dilatational modulus (Figure 5) is the magnitude that provides the concentration of GTE that leads to a maximum inhibition, rather than the maximum interfacial tension (Figure 4). Very few literature works have studied the inhibition of lipase at the interface. Among these studies, Chu and co-workers[14] showed that an air−water interface occupied by digalactosyldiacylglycerol (DGDG) is more resistant to the adsorption of bile salts, colipase, and lipase than dipalmitoylphosphatidylcholine (DPPC).[14] Torcello-Gómez and co-workers[20] compared the effect of two different surfactants, Pluronic F68 and lecithin, on lipolysis and demonstrated that olive oil emulsions stabilized by nonionic surfactant (Pluronic F68) are more resistant to lipolysis than lecithin-stabilized emulsions.[20] This different behavior is owed to the different interfacial structures formed by these surfactants and the ability of Pluronic F68 to inhibit the action of lipase by steric hindrances.[20] Both works found a relationship between the measurement of lipase inhibition and the increase of interfacial tension[14,20] as we propose in this work. However, they did not look into the dilatational modulus of lipolysis.

This work reports a new, accurate, fast, and easily handled, and cheap methodology to measure the activity of lipase adsorbed at olive oil−water interface as a screening test. The mechanism of pancreatic lipase inhibition is related to reduction of interfacial activity in agreement with previous studies.[14,20] However, we also provide dilatational moduli and desorption of lipolysis products contributing crucial magnitudes in the interpretation of the lipase inhibition potential of natural compounds. The acquired knowledge provides new tools for the control of fat metabolism, which is central to health threats such as obesity and diabetes mellitus II and important for the regulation of energy metabolism in general. Understanding the mechanisms influencing lipase catalysis at the interface provides a platform for approaching the much more complex in vivo systems.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

GTE, green tea extract; EGCG, epigallocatechin-3-gallate; DGDG, digalactosyldiacylglycerol; DPPC, dipalmitoylphosphatidylcholine; HPLC, high-pressure liquid chromatography

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