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Research Article

Colorimetric paper-based device for rapid screening of orlistat in weight loss supplements

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Abstract

The colorimetric paper-based device using lipase inhibition assay was developed for rapid and visual detection of orlistat in weight loss supplements. The paper device with five circular detection zones was simply fabricated from filter paper No 1 using a low-cost paper craft puncher with a design of a flower-like shape. The enzymatic reaction on the detection zone employed a small volume of a substrate, α-naphthyl acetate, a sample, and a lipase enzyme. After incubation, the Fast Blue B solution was used as a chromogenic reagent. The decrease in purple color can be observed by the naked eye, in the presence of orlistat. Under optimized conditions, the paper device showed satisfactory sensitivity and selectivity. The device was applied for rapid screening of orlistat in weight loss supplements and the results agreed with those obtained from TLC analysis.

Keywords

paper-based device, orlistat, lipase inhibition, weight loss supplements

Introduction

Overweight and obesity are recognized as the most common metabolic disease worldwide. Anti-obesity drugs and alternative treatments such as herbal slimming formulations and weight loss supplements may be used as part of a weight-control program. One of the approved weight management medications by the Food and Drug Administration (FDA) in many countries is orlistat, a semisynthetic derivative of lipstatin. It prevents the absorption of fats from the human diet by acting as a potent and specific inhibitor of intestinal lipase. The use of orlistat seems to have beneficial effects on blood pressure and carbohydrate metabolism. However, several mild-to-moderate gastrointestinal adverse effects, such as oily stools, diarrhea, abdominal pain, and fecal spotting, and a few cases of serious hepatic adverse effects have been reported (European Medicines Agency 2012). The absorption of certain fat-soluble vitamins (such as A, D, and E) and many drugs (such as warfarin, amiodarone, ciclosporin, and thyroxine as well as fat-soluble vitamins) can be affected by orlistat, affecting their bioavailability and effectiveness (Liu et al. 2020)

Owing to its potential use for the treatment of overweight and obesity, orlistat is reported to be one of the undeclared, unapproved pharmaceutical ingredients found in weight loss supplements. Being unaware of taking the drug ingredient in dietary supplements, overuse, interactions with other medications, or with underlying health conditions could cause serious adverse health effects (Calahan et al. 2016; Tucker et al. 2018). The detection of drug adulteration in dietary supplements is therefore important. Various analytical approaches for the detec-

tion of synthetic adulterants in herbal medicine based on chromatographic and spectroscopic techniques such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) and diode array detectors (DAD), ion mobility spectrometry (IMS), high-performance, thin-layer chromatography (HPTLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), nuclear magnetic resonance (NMR) analysis, and hyphenated-mass spectrometric techniques are still widely used (Pratiwi et al. 2021). These methods are sensitive and selective but require expensive lab instruments and the expertise of an analyst. HPLC and TLC have been reported for the determination of orlistat in pharmaceutical formulations and herbal products (Ahamad et al. 2016; Joshi et al. 2017; Fathy et al. 2019).

Recently, the paper-based analytical device has received considerable attention as a tool for drug analysis. This device offers several advantages, such as simplicity, low cost, rapid analysis, low consumption of reagents and samples, and portable analytical tools capable of on-site drug screening (Novian et al. 2020; Pratiwi et al. 2021). A paper-based device has been developed for the determination of adulterated drugs, dexamethasone and prednisolone in traditional medicine, and sibutramine in slimming products (Primpray et al. 2019; Karamahito et al. 2021). The most widely used method for detecting substances on paper is the colorimetric method based on rapid chemical and enzymatic-based reactions (Garnier and Then 2013). Color visualizations of orlistat on TLC plates with universal chemical staining reagents such as phosphomolybdic acid and *p*-anisaldehyde and the enzymatic colorimetric detection of orlistat based on its potent and specific lipase inhibitory activity were reported (Hassan 2012; Bayineni et al. 2014; Tang et al. 2016; Fathy et al. 2019). In this study, a low-cost colorimetric paper-based device for rapid screening of orlistat based on the enzymatic inhibitory reaction was developed. The color change upon the enzyme-substrate reaction with and without orlistat on the paper device is observed and captured using a scanner and analyzed by the image analysis software.

Materials and methods

Chemicals and reagents

Lipase from porcine pancreas (L3126), orlistat (O4139), tris (hydroxymethyl) aminomethane (Tris, 154563), Fast Blue B Salt (FBB, FD9805), calcium chloride, hydrochloric acid, caffeine and quercetin were all obtained from Sigma-Aldrich (St Louis, MO, USA). α-Naphthyl acetate (A17482) was obtained from Alfa Aesar (Lancashire, United Kingdom). 50 mM Tris-HCl-$CaCl_2$ buffer pH 7.5 was prepared from 50 mM tris (hydroxymethyl) aminomethane with 20 mM $CaCl_2$ in water. The pH of the solution was adjusted to pH 7.5 with hydrochloric acid. Herbal extracts were provided through the courtesy of Specialty Natural Products Co., Ltd. (Chonburi, Thailand) and Science Innovative Product Co., Ltd. (Nakhon Ratchasima, Thailand). Various brands of weight loss supplements were purchased either through the Internet or from local stores in Bangkok, Thailand. Analytical-grade reagents and deionized water from Merck Millipore Simplicity Water Purification Systems (Darmstadt, Germany) were used throughout this experiment. Whatman No. 1 (W1), No. 3 (W3), and No. 4 (W4) filter papers were purchased from GH Healthcare (Buckinghamshire, United Kingdom). Silica gel G60 F254 aluminium plate, 0.25-mm thickness, was purchased from Merck (Darmstadt, Germany). All solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Preparation of enzyme solutions and reagents

A concentrated stock lipase solution at 50 mg/mL in 50 mM Tris-HCl-$CaCl_2$ buffer pH7.5 was prepared according to the previously reported method and kept at -20 °C (Gil-Rodríguez and Beresford 2020). The enzyme substrate, α-naphthyl acetate, was prepared in ethanol and the chromogenic agent, FBB solution, was prepared in deionized water. For the optimization study, an aliquot amount of lipase stock solution was diluted with Tris-HCl-$CaCl_2$ buffer to the working solution at 2.5, 5 and 10 mg/mL; 0.75, 1.0 and 1.5 mg/mL of α-naphthyl acetate and 0.5 and 1.5 mg/mL of FBB solution were prepared.

Preparation of orlistat solution

An appropriate quantity of standard orlistat was accurately weighed and dissolved in ethanol to obtain a 5 mg/mL stock solution. The stock solution was further diluted with ethanol to obtain a series of working solutions of 0.00008–1.2 mg/mL and stored at 4 °C, before analysis.

Fabrication and optimization of paper-based device

The paper device was simply fabricated by using a low-cost paper craft puncher with a design of a flower-like shape with five circular detection zones and one for a chromogenic reagent reservoir at the center. Each detection zone was with a diameter of 7 mm and connected to the reagent reservoir through a hydrophilic channel (3 mm × 1.5 mm). For paper optimization, W1, W3, and W4 filter papers were used. The experiments were carried out by placing the paper device on a transparent plastic sheet and adding 3 µL of 1.5 mg/mL α-naphthyl acetate into five detection zones, followed by air-drying at room temperature to remove ethanol. Then, 3 µL of 5 mg/mL lipase solution was subsequently added. The substrate and enzyme solution were all absorbed on the paper at each detection zone. The paper device was then placed into a laboratory-made humidity chamber and incubated at 37 ± 2 °C for 20 min, allowing the enzyme and substrate reaction to take place. After incubation, 45 µL of 1.0 mg/mL FBB solution was

added in the center zone of W1 and W4 and 80 µL to W3 paper, simultaneously filling the FBB solution in all detection zones through the capillary force. Color intensity on the paper was captured from the bottom layer by a digital scanner (Brother DCP1610W). The scanned images were saved as a joint photographic experts group (JPEG) file and processed by UN-SCAN-IT (Silk Scientific, Inc., USA) using Dot Blot Analysis in grayscale color mode. Circular segment values of width and height, 76 × 76, were applied to each detection zone. The color intensity in terms of pixels (± the standard deviation) was calculated from 5 independent measurements. The paper type which showed the best color intensity was used for further studies.

The optimization experiments including lipase concentrations of 2.5, 5 and 10 mg/mL, α-naphthyl acetate concentrations of 0.75, 1.0 and 1.5 mg/mL, and FBB concentrations of 0.5 and 1.0 mg/mL were carried out. For each case, 3 µL of ethanol was used as a blank and loaded at the detection zone before adding the enzyme. After incubation, the color intensity was determined by UN-SCAN-IT in terms of pixels. The cases which showed good color intensity were further investigated by loading 3 µL of 0.02 mg/mL orlistat at the detection zone. The color intensity was measured and the relative percentage change between I_{ol} and I_b, where I_{ol} and I_b are the intensity of the orlistat signal and the blank signal, respectively, was calculated (rel. $\Delta I\% = (I_{ol}\text{-}I_b/I_b)*100$). Three replicates were performed for each experiment.

Operation of the paper-based device

The case showing good color intensity with the optimal relative change of the orlistat signal in comparison to the blank was employed as follows. The paper was placed on a transparent plastic sheet and 3 µL of 1.5 mg/mL of α-naphthyl acetate was first pipetted into the detection zone. The paper was left to dry at room temperature for 5 min. Then, 3 µL of ethanol or 3 µL of orlistat solutions at concentrations of 0.00008–1.2 mg/mL was loaded into each circular zone of the paper, followed by air-dryig at room temperature for 5 minutes to remove ethanol. 3 µL of 5 mg/mL lipase solution was subsequently added. The paper device was incubated at 37 ± 2 °C for 20 min. After incubation, 45 µL of 1.0 mg/mL FBB solution was added to the center zone and the paper device was scanned within 5 minutes. The measurement of the color intensity was performed by digital image analysis using UN-SCAN-IT. At each concentration, the color intensity data in terms of pixels were averaged from measurements of four detection zones. The curve between the averaged color intensity and the concentrations was fitted by the AAT Bioquest website program (https://www.aatbio.com/tools/ic50-calculator) to determine the IC_{50}, the concentration of orlistat that inhibited 50% of the lipase activity. Three replicates were performed from three sets of paper devices.

The performance of the paper device for the detection of orlistat in the real sample was investigated by analyzing the orlistat-free sample spiked with orlistat at various concentrations (0.0003, 0.001, 0.02, 0.3, 1 mg/mL). The color intensity of the non-spiked (blank) and spiked samples was measured and expressed in terms of the color intensity difference between I_{blank} and I_{spiked} ($\Delta I = I_{blank} - I_{spiked}$), where I_{blank} and I_{spiked} are the color intensity of the orlistat-free sample and the orlistat-spiked sample, respectively. A spiked sample at a concentration of 0.02 mg/mL was further subjected to the recovery study. The color intensity was measured and interpolated in the calibration curve constructed from the matrix-matched standards at a concentration range of 0.00008–1.2 mg/mL. By using a four-parameter logistic regression model created by the AAT Bioquest website program, the amount of orlistat in the spiked samples was determined. The percent recoveries (± the standard deviation) were calculated from six replicates.

The repeatability (intra-day precision) and the intermediate (inter-day) precision of the paper device was performed by determining the relative percentage change (rel. ΔI%) of color intensity of the orlistat-spiked sample at 0.002 and 0.02 mg/mL as compared to the non-spiked sample. The percent relative standard deviation (%RSD) values for repeatability and intermediate precision were calculated.

To study the selectivity of the proposed method, natural components and extracts reported for lipase inhibitory activity (Lunagariya et al. 2014; Liu et al. 2020), i.e. caffeine, quercetin, and natural extracts found in a dietary supplement for fat blocker, i.e. cactus extract, capsicum extract, garcinia extract and white kidney bean extract were investigated. Caffeine and quercetin were dissolved in ethanol at 0.4 mM (a 100-fold concentration of IC_{50} of orlistat). The extract powder was prepared by sonicating in ethanol at 50 mg/mL for 30 min and the clear supernatant was subjected to the paper device according to the proposed method. The color intensity values were measured and the relative percentage change (rel. ΔI%) of color intensity was calculated.

Detection of orlistat in weight loss supplement

Four different weight loss supplements and an orlistat (120 mg) capsule were used for the analysis. The weighed contents (~250–500 mg) of one single dose (one tablet or one capsule) of each weight loss supplement were placed in a 25 mL volumetric flask, then made to the volume with ethanol. The mixture was sonicated for 30 min. A 100 µL aliquot of a clear sample was diluted to 1 mL with ethanol and directly applied to the paper device. The color intensity values were measured and the rel. ΔI% was calculated for each sample. The samples with a significant reduction in color intensity on the paper device were further subjected to TLC analysis using a previously published method with small modification (Joshi 2017). A silica gel 60 F254 TLC plate and a hexane-ethyl acetate (7:3, *v/v*) were employed and 0.5% w/v phosphomolybdic reagent was used for spot detection.

Results and discussion

A low-cost colorimetric paper-based device for the detection of orlistat based on a lipase inhibition assay was developed. In this study, α-naphthyl acetate was used as the enzyme substrate because it is inexpensive and can be catalyzed by lipase to yield α-naphthol, which can subsequently react with FBB to give a distinct purple-colored diazonium dye (Fig. 1) (Hassan 2012). In the presence of orlistat, a potent lipase inhibitor, the reduction of purple color intensity was observed by the naked eye and with image analysis software.

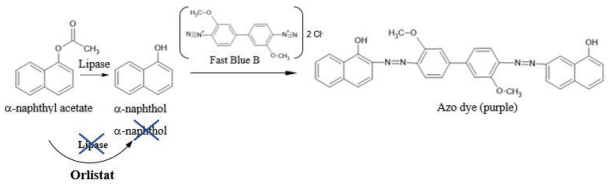


Figure 1. Reaction of lipase with α-naphthyl acetate and the subsequent formation of the purple color with Fast Blue B.

The effect of the paper type on the observation of color intensity was studied. In general, the same grades of filter and chromatography paper produced comparable results in terms of signal intensity and uniformity (Evans et al. 2014). The filter paper grades W1, W3 and W4 were utilized in the optimization study because they were less expensive than the chromatography paper. Each paper grade has unique characteristics, especially the thickness. The thickest filter paper is W3 while W1 and W4 are characterized by similar thicknesses and qualitative grades.

The results showed that the thicker paper (W3) yielded poorer color intensity; better intensity was obtained with thinner papers (W1 and W4) (Fig. 2). According to Evans et al. 2014, a reason for this is that the colorimetric detection method is mainly sensitive to the compounds present on the surface of the paper device. Due to being thicker and more opaque, the paper device made from W3 yielded poor color intensity. W1 showed better color intensity and uniformity than W4, possibly due to the smaller pores resulting in slower wicking speed, which increased reaction efficiency and resulted in a more noticeable color observation. Therefore, W1, the most popular and the least expensive filter paper was employed for the following studies.

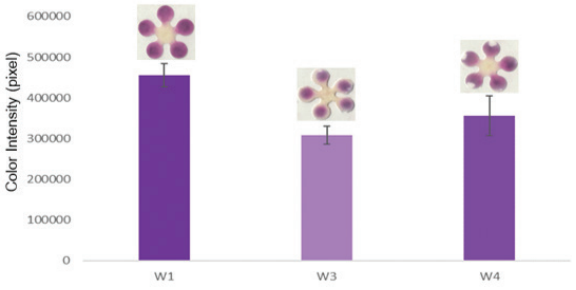


Figure 2. Color intensity of the paper device made from filter paper grades W1, W3 and W4.

The intensity of the purple color in the detection zone was affected by different concentrations of lipase enzyme (2.5, 5 and 10 mg/mL), α-naphthyl acetate (0.75, 1.0 and 1.5 mg/mL), and FBB (0.5 and 1.0 mg/mL). Good color intensity and uniformity were observed with the use of enzyme concentration at 5 and 10 mg/mL, substrate concentration at 1.0 and 1.5 mg/mL, and FBB solution at 0.5 and 1.0 mg/mL (Fig. 3a–c). The cases which showed good color intensity were further investigated with the presence of orlistat at 0.02 mg/mL to observe the change of purple color reduction on the paper device as compared to the case used without orlistat. The results showed that the reduction in purple color in the presence of orlistat was not that obvious when a high concentration of lipase at 10 mg/mL (L10) was employed (Fig. 4). The case with orlistat at low concentrations but using the excess amount of lipase and substrate still produced good purple color intensity. Thus, the optimal conditions for visual detection of orlistat by an enzymatic colorimetric paper-based device were 5 mg/mL lipase, 1.5 mg/mL α-naphthyl acetate, and 1.0 mg/mL FBB solution. A scheme of the procedure for the determination of orlistat using the paper device is shown in Fig. 5.

The performance of the paper-based device was examined against orlistat at various concentrations ranging from 0.00008–1.2 mg/mL. The intensity of the purple color at the detection zone decreased with increasing orlistat concentrations (Fig. 6). As observed by the naked eye, there was little difference in the reduction of purple color intensity at orlistat concentrations from 0.02 to 1.2 mg/mL. The limit of detection (LOD) by visual detection was estimated to be around 0.005 mg/mL. The dose-dependent inhibition responses of orlistat and the semi-log of the data were constructed to determine LOD given by IC_{50} (Jin et al 2020). The calculated LOD (IC_{50}) fitted by the AAT Bioquest website program was found to be 0.0018 ± 0.0003 mg/mL (0.0036 ± 0.0006 mM).

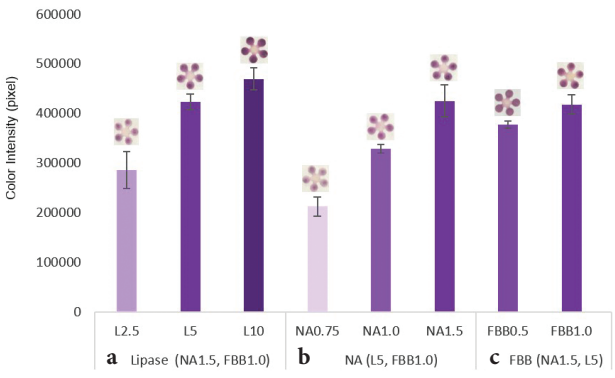
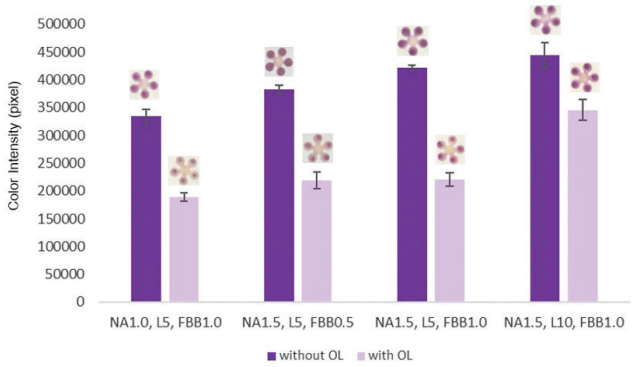


Figure 3. (**a**) Effect of lipase concentrations (2.5, 5 and 10 mg/mL) with 3 µL of 1.5 mg/mL α-naphthyl acetate and 45 µL of 1.0 mg/mL FBB. (**b**) Effect of α-naphthyl acetate (NA) concentrations (0.75, 1.0 and 1.5 mg/mL) with 3 µL of 5 mg/mL lipase and 45 µL of 1.0 mg/mL FBB. (**c**) Effect of FBB concentration (0.5 and 1.0 mg/mL) with 3 µL of 1.5 mg/mL α-naphthyl acetate and 3 µL of 5.0 mg/mL lipase.



Conditions	rel. ΔI% (±SD)
NA1.0, L5, FBB1.0	-43.5 ± 2.9
NA1.5, L5, FBB0.5	-42.8 ± 3.0
NA1.5, L5, FBB1.0	-47.7 ± 3.4
NA1.5, L10, FBB1.0	-22.1 ± 4.8

Figure 4. Color intensity and the relative percentage change (rel. ΔI%) of color intensity with and without orlistat at various conditions on the paper device.

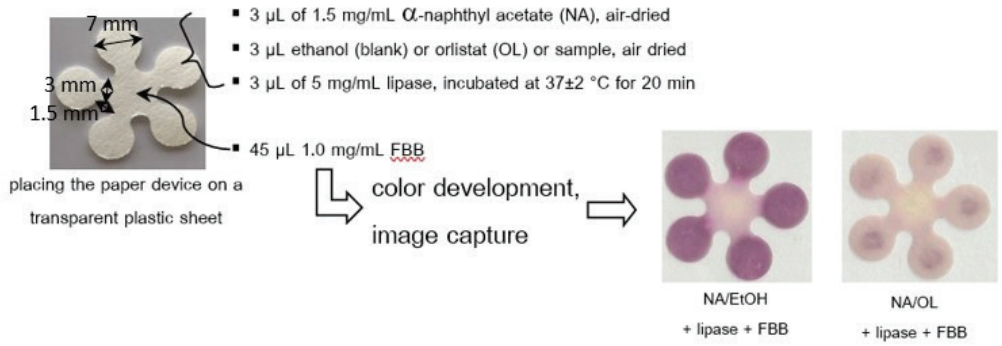
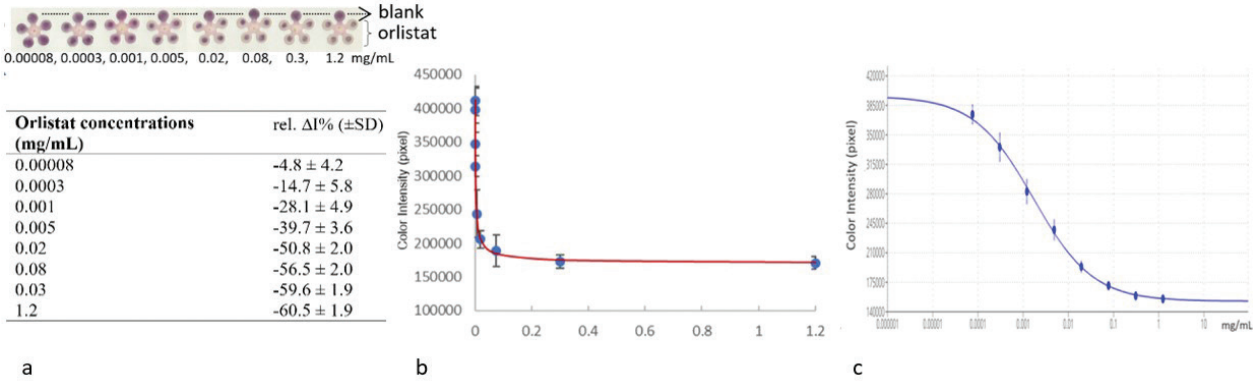


Figure 5. The schematic diagram of paper-based lipase inhibition assay for orlistat detection.



Orlistat concentrations (mg/mL)	rel. ΔI% (±SD)
0.00008	-4.8 ± 4.2
0.0003	-14.7 ± 5.8
0.001	-28.1 ± 4.9
0.005	-39.7 ± 3.6
0.02	-50.8 ± 2.0
0.08	-56.5 ± 2.0
0.03	-59.6 ± 1.9
1.2	-60.5 ± 1.9

Figure 6. a. A representative image of the paper devices and the rel. ΔI% of orlistat signals at various concentrations; **b**. A dose-response curve; **c.** A semi-log plot of the data, fitted by the AAT Bioquest website program to determine IC_{50}. Data are the means (± SD) of three replicates.

Orlistat-spiked samples at varied concentrations were analyzed to assess the ability of the paper device for visual detection of orlistat in real samples. The color intensity difference (ΔI) between the orlistat-free sample and the orlistat-spiked sample showed that the ΔI increased with increasing orlistat concentration (Fig. 7a). The concentrations of orlistat higher than 0.02 mg/mL showed apparent saturation in the reduction of color intensity. This was possible since orlistat irreversibly inhibits lipase, thus, when the orlistat concentration increases, the catalytic site of the enzyme becomes saturated.

A four-parameter logistic regression equation was used to determine the analytical recovery of the spiked sample at a concentration of 0.02 mg/mL, a 10-fold concentration to the calculated LOD value. The percent recovery (±SD) was 96.9 ± 12.5. The color intensity at higher concentrations than 0.02 mg/mL did not decline significantly, resulting in unsuitability for quantitative analysis. However, in terms of qualitative analysis, the reduction of color intensity was clearly observed for the orlistat-spiked sample at 0.002 mg/mL and 0.02 mg/mL on the paper device (Fig. 7b). The detection performance

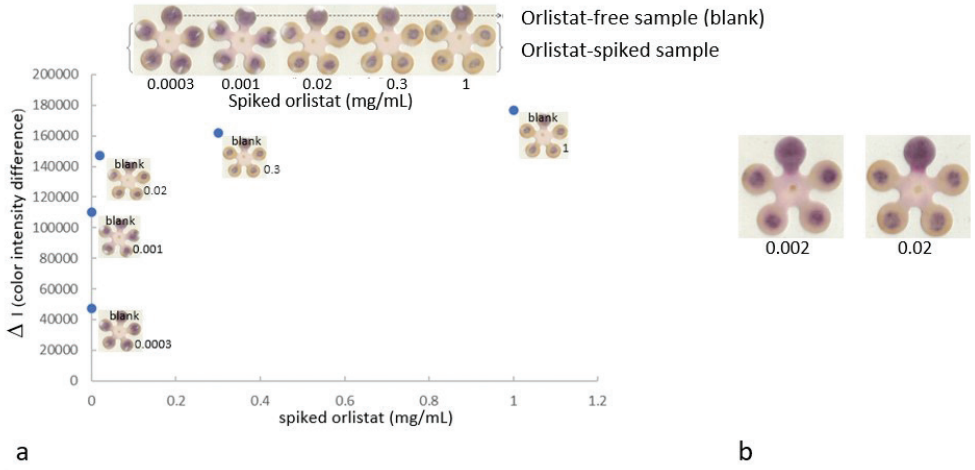


Figure 7. a. The color intensity difference (ΔI) between the orlistat-free sample and the orlistat spiked sample at various concentrations; **b**. A representative image of the paper devices with orlistat spiked sample at 0.002 and 0.02 mg/mL.

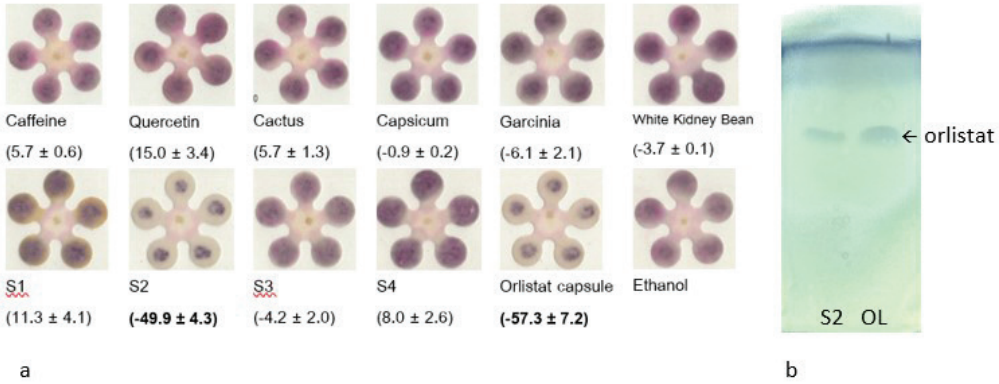


Figure 8. a. A representative image of each sample analysis (with rel. ΔI% ± SD given in parenthesis) on the paper device; **b.** TLC of S2 and orlistat (OL) using 0.5% w/v phosphomolybdic reagent as visualization reagent.

of the device at these concentrations was determined by the intra- and inter-day precision of the orlistat spiked sample. The data showed that rel. ΔI% for intra-day analysis of the orlistat spiked sample at 0.002 and 0.02 mg/mL were -39.6 and -50.9, and for the inter-day were -38.2 and -50.8, respectively. %RSD of rel. ΔI% for intra-day precision of the samples at 0.002 and 0.02 mg/mL were 9.0 and 2.9, and for the inter-day precision were 8.9 and 3.1, respectively. The detection performance with good precision was obtained with the orlistat spiked sample at 0.02 mg/mL, suggesting that the device may be beneficial for the qualitative detection of adulterated orlistat in a real sample.

Known compounds reported for lipase inhibition and herbal extracts commonly found in a fat blocker supplement were tested on the paper device. At the detection zones, no obvious change in the color intensity was observed for caffeine and quercetin and the herbal extracts as compared to the blank (Fig. 8a). All did not inhibit the enzymatic reaction as the orlistat did on the paper device, suggesting the selectivity of the proposed method for orlistat detection.

The paper-based analytical device was applied for rapid detection of lipase inhibitory activity in four different weight loss supplements claimed as fat blockers (S1–S4) and the orlistat capsule. The results show a distinguished purple color intensity reduction for S2 and the orlistat capsule (Fig. 8a). Other samples did not show an apparent decrease in color intensity nor a significant decreasing rel. ΔI%. The results from the paper-based assay revealed that S2 could be adulterated with orlistat. The presence of orlistat in S2 was ensured by TLC analysis, showing a distinctive blue band of orlistat at the R_f value of 0.6 (Fig. 8b).

Conclusion

A simple, minimized reagent consumption and low-cost colorimetric paper-based device for visual detection of orlistat in weight loss supplements was developed. The color detection was based on enzymatic inhibitory reaction. The color change can be easily observed by the naked eye or with image analysis software. The device provided satisfactory sensitivity and selectivity for the qualitative

analysis. Compared with the TLC analysis, the device used in the proposed method could be easily fabricated in the laboratory using widely available, affordable and disposable materials. Furthermore, the method avoided the use of unsafe organic mobile phase and required simple procedures, thus also enabling the portability for in-field analysis and applicable for rapid screening orlistat adulteration and potential lipase inhibitors in weight loss supplements.

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