RESEARCH ARTICLE

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Fluorescence polarisation for high-throughput screening of adulterated food products via phosphodiesterase 5 (PDE5) inhibition assay

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Abstract

The surge in the consumption of food products containing herbal aphrodisiacs has driven their widespread adulteration. A rapid screening strategy is, therefore, warranted to curb this problem. This study established an enzyme inhibition assay to screen phosphodiesterase 5 (PDE5) inhibitors as adulterants in selected food products. Fluorescein-labelled cyclic-3',5'-guanosine monophosphate was utilised as substrates for the PDE5A1 enzyme, aided by the presence of nanoparticle phosphate-binding beads on their fluorescence polarisation. The sample preparation was optimised to improve the enzyme inhibition efficiency and applied to calculate the threshold values of six blank food matrices. The assay was validated using sildenafil, producing an IC_{50} of 4.2 nM. The applicability of the assay procedure was demonstrated by screening 55 distinct food samples. The results were subsequently verified using confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis. Altogether, 49 samples inhibited the PDE5 enzyme above the threshold values (75.7%–105.5%) and were registered as potentially adulterated samples. The remaining six samples were marked as non-adulterated with percentage inhibition below the threshold values (-3.3%–18.2%). The LC-HRMS analysis agreed with the assay results for all food products except for the instant coffee premix (ICP) samples. False-positive results were obtained for the ICP samples at 32% (8/25), due to possible PDE5 inhibition by caffeine. Contrarily, all other food samples were found to produce 0% (0/30) false-positive or false-negative results. The broad-based assay, established via a simple mix-incubate-read format, exhibited promising potential for high-throughput screening of PDE5 inhibitors in various food products, except those with naturally-occurring phosphodiesterase inhibitors such as caffeine.

Keywords: PDE5 inhibitors, adulterated food, enzyme inhibition assay, high-throughput screening, fluorescence polarisation

INTRODUCTION

The immense success of sildenafil, vardenafil, and tadalafil has since led to the massive influx of adulterated herbal remedies into the market, typically labelled to contain herbal aphrodisiacs with claims to enhance male sexual performance. These adulterated products are frequently marketed as herbal medicines and dietary supplements; and advertised as all-natural, without any side-effects[1-3]. However, in recent years, the trend has shifted towards food products as they are not heavily regulated compared to those in pharmaceutical dosage forms[4]. These food products can be easily purchased through drugstores, supermarkets, convenience stores, herbal shops, restaurants, electronic commerce platforms, and black markets[5]. Most of them, unfortunately, were found to be adulterated with phosphodiesterase 5 (PDE5) inhibitors and their analogues[6,7]. The widespread adulteration has sparked an elevated food safety and public health concerns, as consumers are often unaware of the risks associated with the consumption of such products[8].

PDE5 inhibitors are generally synthesised to mimic the structure of the purine ring of cyclic-3',5'-guanosine monophosphate (cGMP)[9]. Due to the structural similarities, these drugs competitively bind to the catalytic domain of PDE5 enzyme, subsequently inhibiting the cGMP degradation; thus, enhancing the effects of nitric oxide. The series of events sustain cGMP levels and prolong penile erection[10]. PDE5 enzyme, on the contrary, acts through a negative feedback control mechanism in the corpus cavernosum. It degrades cGMP to the inactive 5'-guanosine monophosphate (GMP),

resulting in penile detumescence[11,12]. Based on the penile erection mechanism, the differences between cGMP (substrate) and GMP (product) levels may indicate the presence or absence of PDE5 inhibitors.

At present, several analytical methods have been utilised to determine PDE5 inhibitors in various matrices[13-16]. More commonly, liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) has proven to be invaluable in the analysis of PDE5 inhibitors[17,18]. However, LC-MS/MS, in general, demands higher operational and maintenance costs with limited sample throughput. It also requires experienced users to operate the analytical instrument and interpret the MS data[19]. Therefore, a rapid, simple, and cheap screening test is warranted to discriminate adulterated from the non-adulterated food products, particularly those with PDE5 inhibitors and their analogues.

Only a few rapid screening tests have been proposed to discriminate PDE5 inhibitors in adulterated or counterfeit products based on colour changes[20] and immunochromatographic assay[21-23]. However, both of these techniques are targeted for a distinct PDE5 inhibitor or a group of PDE5 inhibitors, making them limited for broad-based screening. A study has recently proposed a broad-based screening of PDE5 inhibitors in herbal dietary supplements via the PDE5 inhibition assay[24]. The assay utilised fluorescence intensity measurements of tetramethyl rhodamine-labelled cGMP in the presence of zirconyl chloride octahydrate as a quenching agent. This study, however, was not validated using real samples and the need to measure the fluorescence intensity at seven points over a length of time may not be well-suited for high-throughput screening.

Herein, a broad-based enzyme inhibition assay was established via a simple mix-incubate-read format to rapidly screen PDE5 inhibitors, found as adulterants in selected food products. A PDE5-specific cGMP fused with a fluorescein fluorophore via a 9-atom spacer moiety, known as FAM-cGMP, was utilised as substrates for the human recombinant PDE5A1 enzyme. The substrate depletion and the product formation from the PDE5 enzyme activity were measured using their molecular movements and rotations, aided by the presence of nanoparticle phosphate-binding beads on their fluorescence polarisation (FP). The assay was optimised and validated to improve the enzyme inhibition efficiency and to certify the robustness of the assay performance, respectively. Altogether, 55 distinct food samples were submitted to the PDE5 inhibition assay, and the results were subsequently verified using confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis. To the best of our knowledge, this is the first study utilising the FP technique to rapidly screen PDE5 inhibitors as adulterants in food products via PDE5 inhibition assay. This paper also highlighted the advantages as well as the shortcomings encountered in detecting the adulterated food products.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

The PDE5A1 assay kit (Catalogue No. 60351) was purchased from BPS Bioscience Inc. (San Diego, CA, United States). It comprised the following: human recombinant PDE5A1 enzyme (PDE5 enzyme) 0.36 mg/mL (Catalogue No. 60050), fluorescein-labelled cGMP substrate (FAM-cGMP) 20 µM (Catalogue No. 60201), phosphodiesterase (PDE) assay buffer (Catalogue No. 60393), PDE binding agent (Catalogue No. 60390), PDE binding agent diluent for cGMP (Catalogue No. 60392),

and Greiner 384-well microtiter plate (black, low binding, flat bottom) with a clear lid. The vendor for sildenafil certified reference material was TLC Pharmaceutical Standards Ltd. (Aurora, Ontario, Canada); while Sigma Aldrich Pty Ltd. (Castle Hill, NSW, Australia) supplied the dimethyl sulfoxide (DMSO) of analytical grade.

2.2. Standard solution preparation

The stock solution of sildenafil was prepared at 1 mM in DMSO and stored at 4°C in the dark. To validate the PDE5 inhibition assay, different concentration solutions of sildenafil ranging from 0.01–100 μM were prepared from the stock solution, serially diluted in DMSO. Each of these solutions was further diluted at 10-fold in PDE assay buffer before being submitted to the assay, producing a final 100-fold dilution of sildenafil in each microtiter plate well.

2.3. Sample collection and storage

A total of 50 distinct food samples were obtained from Malaysia (48 samples) and Australia (2 samples), in the form of instant coffee premix (ICP, 25 samples), powdered drink mix (PDM, 16 samples), honey (HNY, 4 samples), jelly (JLY, 2 samples), hard candy (HCD, 2 samples), and chewing gum (CWG, 1 sample). These suspected adulterated food products were selected based on the brand names, label claims, images, herbal ingredients, or advertising materials with connotations of male sexual performance. The Pharmacy Enforcement Division, Ministry of Health Malaysia, kindly donated two-thirds of these samples, which were confiscated by the pharmacy enforcement officers at the international airport (5 samples) and international seaport (10 samples), as well as from routine market surveillance activities (19 samples). The rest of the samples were purchased from various

electronic commerce platforms established in Malaysia (14 samples) and Australia (2 samples). The samples were kept in separate plastic zip-lock bags and stored in an airtight container in the dark. Blank matrices of each food products, free from any analyte of interests, were sourced from a local supermarket in Australia and used to establish the threshold value of PDE5 inhibition for adulterated food products. The compositions of each of the blank food matrices are outlined in Table S1 (supporting information) based on the products' label. Due to the small sample size of PDM, HNY, JLY, HCD, and CWG, their blank matrices were included as additional samples providing a total of 55 food samples.

2.4. Sample preparation

The initial weight of each sample was recorded based on the recommended daily intake on its label. These samples were divided into group A (ICP, PDM, and HNY; with average recommended daily intake of >5 mg) and group B (JLY, HCD, and CWG; with average recommended daily intake of <5 mg). The samples in group A were taken directly from their sachets, while samples from group B were initially homogenised with mortar and pestle. For PDE5 inhibition assay, 50 mg of group A samples or 10 mg of group B samples were weighed in a polypropylene tube and then extracted with 5 mL of DMSO via 1-min vortex mixing, 20-min sonication and 5-min centrifugation at 2500 × *g*, successively. Using a 0.22 mm PTFE syringe filter, the upper layer was filtered and diluted for enzyme inhibition assay with the PDE assay buffer at 10-fold dilution, yielding a final 100-fold dilution of samples in each microtiter plate well. The blank matrices were given the same treatment as the steps described above.

2.5. PDE5 inhibition assay protocol

Table 1 outlines the schematic three-step protocol of the PDE5 inhibition assay. The PDE5 inhibition assay established in this study was adapted and modified according to the manufacturer's instruction[25]. Initially, the stock solutions of the PDE5 enzyme and FAM-cGMP substrate were respectively diluted with PDE assay buffer to produce 10 pg/µL and 200 nM working solutions. The reagents, PDE5 inhibitors, and samples solutions were pipetted into each well of the microtiter plate according to step 1. Subsequently, each assay; comprised of blank, substrate control, positive control, and sample analysis; was covered with the microtiter plate's lid and incubated at room temperature for an hour. Step 2 involved the addition of 50 µL PDE binding agent into each well, initially diluted 100-fold with PDE binding agent diluent. The mixtures were covered with the microtiter plate's lid and incubated at room temperature for 20 min with slow shaking before submitting it to FP measurements via Tecan Infinite M1000 Pro plate reader (Tecan Group Limited, Switzerland) in step 3. The wavelength of the FP was set within 5 nm bandwidth for excitation at 470 nm, and within 20 nm bandwidth for emission at 528 nm. The gain and Z-position values were automatically calculated from the positive control well. The calibration was performed from the substrate control well by correcting the G-factor to achieve a fixed value of 22 mP. The readings were captured at 10 flashes with a settling time of 500 ms. All assays were performed in triplicate wells, and the results obtained were automatically subtracted with the blank well readings. The well-to-well precision was expressed as a percentage of the FP readings' coefficient of variation (%CV) with an acceptable value of <20%.

2.6. LC-HRMS analysis

The confirmatory LC-HRMS analysis was employed to verify the findings of the PDE5 inhibition assay using an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent Technologies 6510 quadrupole time-of-flight mass spectrometry (QTOF-MS) according to the previous literature[26-28].

2.7. Data analysis

The Tecan i-control software version 1.11.1.0 automatically calculated all the FP values. The differences between the parallel and the perpendicular emission light intensities, normalised by the total fluorescence emission intensity of the excitation light plane, generated the absolute FP value based on Eq. 1 and represented in millipolarisation (mP) unit[29].

$$FP\ (mP) = \frac{\mathrm{I}_{\parallel} - \mathrm{I}_{\perp}}{\mathrm{I}_{\parallel} + \mathrm{I}_{\perp}} \times 1000 \qquad \text{Eq. 1}$$

where $I_{\parallel}$ is the parallel emission light intensities; $I_{\perp}$ is the perpendicular emission light intensities.

The substrate control and the positive control theoretically produced 0% and 100% enzyme-substrate activity, respectively. Therefore, the percentage of PDE5 enzyme activity of a given sample or PDE5 inhibitor can be determined using Eq. 2.

$$\%\ of\ PDE5\ activity = \frac{FP_{SPL} - FP_{SUB}}{FP_{POS} - FP_{SUB}} \times 100\% \qquad \text{Eq. 2}$$

where FP_{SPL} is fluorescence polarisation of a sample; FP_{SUB} is fluorescence polarisation of substrate control; FP_{POS} is fluorescence polarisation of positive control.

The percentage of PDE5 inhibition was calculated based on Eq. 3.

$$\%\ of\ PDE5\ inhibition = 100 - \%\ of\ PDE5\ activity \quad \text{Eq. 3}$$

The threshold value of PDE5 inhibition ($T_{inhibition}$) was calculated via the 99.7% normal distribution rule for each food products using Eq. 4[30,31]. All of these values were calculated using Microsoft (Redmond, WA, USA) Excel 2016 Microsoft Office.

$$T_{inhibition} = \mu + 3\sigma \quad \text{Eq. 4}$$

where μ is the average % of PDE5 inhibition; σ is the standard deviation.

The calculated percentages of PDE5 activity of sildenafil versus their concentrations were plotted into a concentration-response inhibition curve using Prism GraphPad software version 8.0.1 by GraphPad Software Inc. (San Diego, CA, United States), and then fitted into a non-linear regression model of $\log_{10}$ (inhibitor) versus response (variable slope, four parameters) in Eq. 5. The non-linear regression data transformation automatically generated the half-maximal inhibitory concentration (IC_{50}) of sildenafil via the symmetrical sigmoidal curve model.

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(Log\ IC_{50} - X) \times Hill\ Slope}} \quad \text{Eq. 5}$$

where X is $\log_{10}$ [inhibitor] concentration; Y is % of PDE5 activity; *Top* is maximum % of PDE5 activity; *Bottom* is minimum % of PDE5 activity.

3. RESULTS AND DISCUSSION

3.1. PDE5 inhibition assay scheme

The high-throughput screening of PDE5 inhibitors in food products was established via the PDE5 inhibition assay. This bioactivity-based assay utilises an FP technique to screen PDE5 inhibitors such as sildenafil (Fig. 1A), by competing with FAM-cGMP to bind to the catalytic domain of PDE5 enzyme (Fig. 1B)[9,11]. Therefore, the assay provides a broad-based screening for multiple PDE5 inhibitors that is non-targeted for a distinct inhibitor or a group of inhibitors, which is helpful to tackle the proliferation of novel analogues, deliberately added into various food products. The assay utilises a PDE5-specific cGMP substrate fused with a fluorescein fluorophore via a 9-atom spacer moiety, known as FAM-cGMP (Fig. 1C). The cGMP plays a pivotal role in the mechanism of penile erection[32].

Fig. 2 presents the schematic illustration of the PDE5 inhibition assay using FP technique. The enzymatic reaction initiation by the PDE5 enzyme, hydrolysed the phosphodiester bond of FAM-cGMP (substrate) to produce the inactive FAM-GMP (product) over a length of time. Once the incubation period ended, a PDE binding agent composed of nanoparticle beads is added to the assay to selectively bind the phosphate group of the FAM-GMP, consequently increasing its size. As a result, the FAM-cGMP and the FAM-GMP are distinguishable using FP based on the differences in their molecular weight. The low molecular weight FAM-cGMP (small, unbound molecule) produces a rapid rotational movement when excited with polarised light, generating low FP readings via depolarised light emission. Contrarily, the high molecular weight FAM-GMP-bead complex (large, bound molecule) rotates slowly during excitation with polarised light, continuing its polarisation with high FP readings.

The adulteration of food products with PDE5 inhibitors can initially be suspected with low FP readings as their presence blocks the hydrolysis of FAM-cGMP to FAM-GMP. However, to undoubtedly discriminate adulterated from the non-adulterated food products, these FP readings are transformed into the percentage of PDE5 inhibition and then compared with the threshold values obtained for each blank food matrix. The established PDE5 inhibition assay via FP is based on a simple and automation-friendly[33] mix-incubate-read format to screen PDE5 inhibitors in food products. Therefore, the demand for multiple readings over a length of time to monitor the enzymatic reaction progress was eliminated, and thus fitted the assay for high-throughput screening.

3.2. Optimisation of sample preparation

The quality of sample preparation is one of the pivotal factors in determining the success of an enzyme inhibition assay. Therefore, it is crucial to prepare the samples into a form that is compatible with the assay procedure. Food products that claimed to enhance male sexual performance may contain an array of PDE5 inhibitors with diverse chemical structures, often exhibiting different inhibitory effects depending on their concentrations. Besides, the information on the potency of almost all unapproved PDE5 inhibitor analogues remains scarce in the literature. Due to these ambiguities, the sample preparation procedure in this study was established based on sildenafil as a representative adulterant, within its recommended dose of 25–100 mg.

The weight of different types of food products was initially assessed to determine the appropriate ratio of the sample and the adulterant (sildenafil) to produce optimal PDE5 inhibition. The sample weight was then fixed based on these findings for a specific

group of food products as detailed in Section 2.4. Ideally, the selected sample weight should produce an acceptable sensitivity via inhibitory potency at the lowest level of adulterant, avoiding false-negative results, while, at the same time, preventing oversaturation of the enzyme at the highest adulterant's level. The detailed calculation for samples in group A (ICP, PDM, and HNY) and group B (JLY, HCD, and CWG) are provided in Excel Spreadsheets S1 and S2 (supporting information), respectively, based on the minimum and maximum dose of sildenafil. The effect of interferences, particularly from the matrix components of the food products, should also be ascertained to ensure reliable assay performance; thus, avoiding false-positive results.

A threshold value of PDE5 inhibition was established for each blank matrices of the food products to discriminate adulterated from the non-adulterated samples. The threshold value used in this study represents the percentage of PDE5 inhibition at which the likelihood of obtaining a false-positive result from a blank, non-adulterated food product is <0.3% using the 99.7% normal distribution rule[30,31]. Adulterated food samples are qualified by their average PDE5 inhibition above the threshold value, while those below the threshold are categorised as non-adulterated samples. Each of the obtained threshold values was respectively assigned for a specific food product, as displayed in Fig. 3.

The threshold values revealed low PDE5 inhibition within 4.0%–25.8% for all blank matrices of the food products, as expected from any non-adulterated sample, except for the blank ICP. The blank ICP produced an average of 91.0% PDE5 inhibition with a calculated threshold value of 94.3%. The selected blank matrix of the ICP was pre-

determined by LC-HRMS analysis to be free from any PDE5 inhibitors and was expected to produce a low PDE5 inhibition. Here, the matrix components may play a significant role in the observed outcome. Given the ambiguities of the ICP sample matrix which could comprise of either caffeinated or decaffeinated coffee, a decaffeinated blank ICP was also tested, yielding a calculated threshold value of 48.1% to be used to qualify adulterated ICP samples.

3.3. Assay validation

The concentrations of enzyme and substrate are fixed at 4 pg/µL and 100 nM, respectively, for each reaction, based on the manufacturer's recommendation. The specific activity of the human recombinant PDE5A1 enzyme is 3100 U/µg, where 1 U represents the amount of enzyme that converts a picomole of cGMP to GMP per min. The specific activity assay exhibited a linear relationship between the PDE5 enzyme concentration and its activity based on the detection of GMP using a malachite green reagent. The molecular weight and purity of the PDE5 enzyme were determined using 4%–20% SDS-PAGE, visualised using Coomassie staining[34].

Apart from monitoring its biological and pharmacological relevance, the PDE5 inhibition assay is validated to certify the robustness of the assay performance. The validation also served to ensure that all the reagents supplied are working as described by its manufacturer. Therefore, an established PDE5 inhibitor, i.e. sildenafil, was chosen, serving as a reference for the enzyme inhibition. Furthermore, sildenafil is the most frequently detected adulterant, reported in many countries worldwide[26,28,35-37].

Fig. 4 displays the concentration-response plot of sildenafil using the PDE5 inhibition assay. The sigmoidal curve shows that sildenafil inhibits the PDE5 activity down to a minimum level where the response remained unchanged. The curve conformed to a classic symmetrical sigmoidal shape, as typically observed from any concentration-response plot of an enzyme inhibitor[38,39]. The obtained data fitted well to the regression model in Eq. 5 with a coefficient of determination (R^2) of 0.9915. Sildenafil inhibits the PDE5 enzyme with an IC_{50} of 4.2 nM. The IC_{50} value is the concentration of an inhibitor required to reduce the enzyme activity by 50%, typically attributed to the potency of an inhibitor[40]. The IC_{50} value of sildenafil obtained from the PDE5 inhibition assay is comparable to those reported from previous studies ranging from 3.5–6.6 nM[41-45].

Nevertheless, the published IC_{50} values of sildenafil may vary significantly, depending on the source and purity of the PDE5 enzyme; the type and concentration of substrate; as well as the selected assay procedure[40,46]. The well-to-well precisions of sample analysis were also acceptable with %CV within 0.7%–12.6%. These findings served to validate the overall assay performance, including the recommended concentrations of the PDE5 enzyme and its substrate. The enzyme reaction scheme thus complies with the Michaelis-Menten kinetic model[47].

3.4. Analysis of PDE5 inhibitors in food products

Altogether, the 55 distinct food samples were screened using the PDE5 inhibition assay and subsequently verified via the confirmatory LC-HRMS analysis. The primary goal of the enzyme inhibition assay is to rapidly discriminate adulterated from the non-adulterated food products through the presence of PDE5 inhibitors. Tables 2 to 4

summarise the results of the PDE5 inhibition assay, including the confirmatory LC-HRMS analysis. Collectively, 49 samples inhibited the PDE5 enzyme with percentage inhibition within 75.7%–105.5%, significantly above the threshold values, and were registered as potentially adulterated samples. In contrast, only six food samples were marked as non-adulterated with PDE5 inhibition ranged from -3.3% to 18.2%, notably below the threshold values. Altogether, 17 samples produced average PDE5 inhibitions exceeding 100%, which may have been contributed by the high concentrations of PDE5 inhibitors in the sample solutions. In these circumstances, the enzyme may have reached a saturation point within the one hour incubation period where no more inhibitors could bind to the catalytic domain of the PDE5 enzyme[47]. As the PDE5 inhibition assay is based on a simple mix-incubate-read format, the need to monitor the enzymatic reaction progress over a length of time was eliminated, making the saturation point insignificant to the assay outcomes.

The PDE5 inhibition assay results, however, were not in full agreement with the confirmatory LC-HRMS analysis, particularly those of the ICP samples in Table 2, which were reported previously[26]. The LC-HRMS analysis resulted in 41 positive samples, with nine distinct PDE5 inhibitors identified via targeted analysis, while another four inhibitors detected by suspected-target screening. Sildenafil again dominated the top list of PDE5 inhibitors found as adulterants in male sexual performance products, as previously mentioned in Section 3.3. It was identified as a sole adulterant in 5 samples and also found in combination with other PDE5 inhibitors in 14 samples. Its popularity is often linked to the accessibility and low cost of raw materials to obtain or synthesise sildenafil[48].

Other PDE5 inhibitors identified via the LC-HRMS targeted analysis included: tadalafil (16 samples); thiodimethylsildenafil and thiosildenafil (13 samples each); dimethylsildenafil (10 samples); desmethylcarbodenafil, propoxyphenyl-thiodimethylsildenafil, and propoxyphenyl-thiohydroxyhomosildenafil (2 samples each); and vardenafil (1 sample). The LC-HRMS analysis additionally detected hydroxythiohomosildenafil (2 samples); and 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, nortadalafil, and propoxyphenyl-dimethylsildenafil (1 sample each) via the suspected-target screening. In addition to the one adulterant per sample composition, these adulterants were also found in combination with each other, where each adulterated sample contains as many as five distinct PDE5 inhibitors.

The confirmatory LC-HRMS analysis verified the findings of the PDE5 inhibition assay for all food products in Tables 3 and 4, concluded as true-positive (24 samples) and true-negative (6 samples), except for the ICP samples. The blank ICP had previously produced significant PDE5 inhibition during the establishment of the threshold value in Section 3.2. Coincidently, the decaffeinated blank ICP also moderately inhibits the PDE5 enzyme. Both of these blank ICPs were pre-determined by LC-HRMS analysis to be free from any PDE5 inhibitors. Matrix components of the ICPs may, therefore, play a significant role in inhibiting the PDE5 enzyme, which resulted in false-positive outcomes of 8 ICP samples. Zero false-negative results indicated an acceptable sensitivity of the established assay procedure for the purpose of non-targeted screening.

Coffee is well known to contain caffeine, a central nervous system stimulant, making it the most widely consumed psychoactive substance worldwide[49,50]. Caffeine possesses a chemical structure similar to those of the purine ring of cGMP and cyclic-3',5'-adenosine monophosphate (cAMP). The heterocyclic ring structure of caffeine is also comparable to those of the pyrazolopyrimidine-7-one ring of sildenafil and imidazotriazine-4-one ring of vardenafil. Due to the structural similarities, caffeine may be expected to exhibit the same inhibitory effects as the PDE5 inhibitors. In fact, caffeine is one of the earliest PDE inhibitors, discovered through the bronchodilating effects of coffee[51]. These initial findings suggest that caffeine may act as a non-selective PDE inhibitor, demonstrated by its inhibitory effects on diverse PDE families[52].

The literature about the role of caffeine on selective inhibition of the PDE5 enzyme in human, however, remained scarce. Nonetheless, a couple of studies have demonstrated the up-regulation of cGMP by caffeine, through relaxation of the penile erectile tissue of rabbits[53] and rats[54]. A recent study via computational approach had also predicted the PDE5 inhibition potential of caffeine[55]. Furthermore, data from the National Health and Nutrition Examination Survey from the United States' male respondents revealed a lower incidence of erectile dysfunction with increased caffeine consumptions[56,57]. All of these findings, although via limited evidence, suggest the existence of PDE5 inhibition by caffeine.

Coffee is, therefore, not a suitable matrix for the PDE5 inhibition assay. Other naturally-occurring non-selective PDE inhibitors such as theophylline from tea and theobromine from cocoa[9] may exhibit similar findings. Thus, these kinds of food

products should be analysed with caution or excluded altogether from the PDE5 inhibition assay. It is also worth noting that the adulteration of food products with caffeine is currently on the rise[36]. Accordingly, producing definite evidence of food adulteration would be a challenging task for samples with naturally-occurring PDE inhibitors.

CONCLUSION

A PDE5 inhibition assay was optimised and validated for high-throughput screening of PDE5 inhibitors, found as adulterants in food products. The three-step assay protocol, recorded via FP measurements, relied on a simple mix-incubate-read format, that is automation-friendly. Data interpretation is straightforward, discriminating adulterated food samples based on their PDE5 inhibition above the pre-determined threshold value. Altogether, the 55 distinct food samples, preliminarily screened via the PDE5 inhibition assay, registered 49 potentially adulterated samples, while the remaining six samples were marked as non-adulterated. The assay results were then verified using the LC-HRMS via targeted analysis and suspected-target screening. The confirmatory LC-HRMS analysis was in agreement with the PDE5 inhibition assay results for all food products except for the ICP samples. These findings indicated false-positive results from 8 ICP samples (out of 25 ICP samples in total, or 32%), possibly due to the PDE5 inhibition activity of caffeine present in the sample matrix. The established assay procedure is, therefore, not suitable for certain types of food products such as ICP and those with the presence of naturally-occurring PDE inhibitors. The PDE5 inhibition assay nevertheless has shown promising potential to rapidly screen PDE5 inhibitors as adulterants in other types of food products. A two-tier screening strategy via rapid and confirmatory tests would enhance performance and productivity, where

the adulterated samples from the PDE5 inhibition assay can be credibly marked as priority for confirmatory analysis.

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Table 1: Schematic three-step protocol of the phosphodiesterase 5 (PDE5) inhibition assay. (adapted and modified from BPS Bioscience Inc.[25])

Reagents	Blank	Substrate control	Positive control	Sample analysis
FAM-cGMP (200 nM)	-	12.5 µL	12.5 µL	12.5 µL
PDE assay buffer	22.5 µL	10.0 µL	-	-
PDE5 inhibitors/ samples	-	-	-	2.5 µL
10% DMSO in PDE assay buffer	2.5 µL	2.5 µL	2.5 µL	-
PDE5 enzyme (10 pg/ µL)	-	-	10.0 µL	10.0 µL
Step 1	Pipette into each microtiter plate well			
	↓	↓	↓	↓
	Total reagents in each microtiter plate well (25 µL)			
	↓			
	Incubate at room temperature for an hour			
Step 2	↓			
	Pipette 50.0 µL diluted binding agent into each microtiter plate well			
	↓			
	Incubate at room temperature for 20 min with slow shaking			
Step 3	↓			
	Measure the fluorescent polarisation (excitation at 470 ± 5 nm and emission 528 ± 20 nm)			
	↓			
	Calculate the percentage of inhibition			

(Abbreviations: FAM-cGMP, fluorescein-labelled cyclic-3',5'-guanosine monophosphate; PDE, phosphodiesterase; DMSO, dimethyl sulfoxide)

Table 2: Phosphodiesterase 5 (PDE5) inhibition assay results and confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis[26] of instant coffee premix (ICP) samples.

Sample	Average percentage of PDE5 inhibition (outcome)	Well-to-well precision (%CV)	PDE5 inhibitors detected from the confirmatory LC-HRMS analysis	Conclusion
ICP001*	99.7% (adulterated)	9.3%	Desmethylcarbodenafil	True-positive
ICP002*	94.2% (adulterated)	9.4%	Thiosildenafil, Hydroxythiohomo-sildenafil**	True-positive
ICP003*	91.6% (adulterated)	1.9%	Dimethylsildenafil, propoxyphenyl-thiodimethylsildenafil, thiodimethylsildenafil	True-positive
ICP004*	102.6% (adulterated)	7.9%	Sildenafil, tadalafil	True-positive
ICP005*	92.9% (adulterated)	8.6%	3,5-dimethylpiperazinyl-dithiodesmethyl-carbodenafil**	True-positive
ICP006*	91.9% (adulterated)	3.8%	Hydroxythiohomo-sildenafil**	True-positive
ICP007*	100.0% (adulterated)	12.0%	Sildenafil	True-positive
ICP008*	88.1% (adulterated)	1.7%	Not detected	False-positive
ICP009*	90.3% (adulterated)	11.3%	Not detected	False-positive
ICP010*	87.7% (adulterated)	4.4%	Not detected	False-positive
ICP011*	92.6% (adulterated)	1.9%	Not detected	False-positive
ICP012*	100.6% (adulterated)	2.7%	Sildenafil	True-positive
ICP013*	96.8% (adulterated)	6.0%	Sildenafil	True-positive
ICP014*	91.0% (adulterated)	1.8%	Not detected	False-positive
ICP015*	101.0% (adulterated)	12.6%	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
ICP016*	85.5% (adulterated)	7.2%	Not detected	False-positive
ICP017*	93.9% (adulterated)	8.2%	Desmethylcarbodenafil	True-positive

ICP018*	92.6% (adulterated)	3.9%	Not detected	False-positive
ICP019*	101.3% (adulterated)	5.6%	Dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
ICP020*	101.3% (adulterated)	2.8%	Sildenafil, tadalafil, propoxyphenyl-sildenafil	True-positive
ICP021*	98.4% (adulterated)	4.9%	Tadalafil	True-positive
ICP022*	99.4% (adulterated)	9.2%	Sildenafil	True-positive
ICP023*	83.9% (adulterated)	1.5%	Not detected	False-positive
ICP024*	98.7% (adulterated)	2.5%	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
ICP025*	101.3% (adulterated)	7.4%	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive

Note:
*LC-HRMS data published in Mohd Yusop et al.[26]
**suspected-target analytes

Table 3: Phosphodiesterase 5 (PDE5) inhibition assay results and confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis of powdered drink mix (PDM), honey (HNY), jelly (JLY), hard candy (HCD), and chewing gum (CWG) samples.

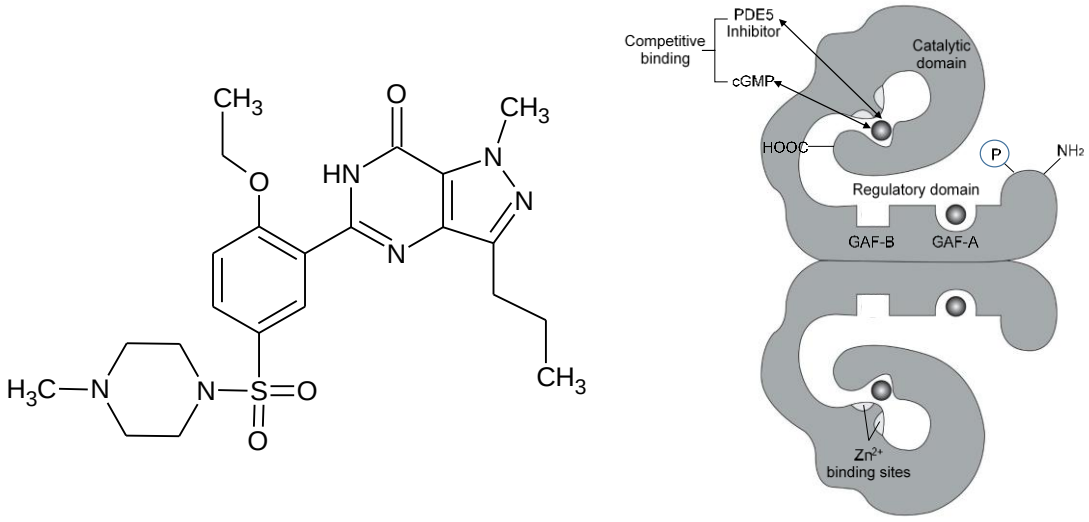
Sample	Average percentage of PDE5 inhibition (outcome)	Well-to-well precision (%CV)	PDE5 inhibitors detected from the confirmatory LC-HRMS analysis	Conclusion
PDM001	105.5% (adulterated)	8.2%	Sildenafil, tadalafil	True-positive
PDM002	82.9% (adulterated)	6.4%	Propoxyphenyl-thiohydroxyhomo-sildenafil	True-positive
PDM003	102.8% (adulterated)	12.4%	Tadalafil, thiosildenafil, thiodimethylsildenafil	True-positive
PDM004	102.8% (adulterated)	2.8%	Tadalafil	True-positive
PDM005	103.9% (adulterated)	7.8%	Tadalafil	True-positive
PDM006	89.5% (adulterated)	5.4%	Thiosildenafil, thiodimethylsildenafil, propoxyphenyl-thiohydroxyhomo-sildenafil	True-positive
PDM007	98.9% (adulterated)	6.7%	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
PDM008	96.1% (adulterated)	10.3%	Tadalafil	True-positive
PDM009	-3.3% (non-adulterated)	0.7%	Not detected	True-negative
PDM010	101.7% (adulterated)	9.5%	Sildenafil, tadalafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
PDM011	94.5% (adulterated)	6.0%	Tadalafil, thiodimethylsildenafil	True-positive
PDM012	102.2% (adulterated)	5.6%	Sildenafil, tadalafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
PDM013	102.2% (adulterated)	2.8%	Sildenafil, tadalafil	True-positive
PDM014	103.3% (adulterated)	5.0%	Sildenafil, tadalafil	True-positive
PDM015	100.6% (adulterated)	2.7%	Dimethylsildenafil, thiodimethylsildenafil	True-positive

PDM016	103.3% (adulterated)	5.0%	Tadalafil	True-positive
HNY001	91.2% (adulterated)	5.6%	Sildenafil, thiosildenafil	True-positive
HNY002	95.6% (adulterated)	2.3%	Sildenafil, thiosildenafil	True-positive
HNY003	75.7% (adulterated)	4.2%	Tadalafil	True-positive
HNY004	92.3% (adulterated)	5.7%	Dimethylsildenafil, thiodimethylsildenafil, propoxyphenyl-thiodimethylsildenafil, propoxyphenyl-dimethylsildenafil**	True-positive
JLY001	91.7% (adulterated)	3.7%	Vardenafil	True-positive
JLY002	100.6% (adulterated)	2.7%	Sildenafil	True-positive
HCD001	99.4% (adulterated)	5.2%	Tadalafil	True-positive
HCD002	85.1% (adulterated)	3.2%	Nortadalafil**	True-positive
CWG001	77.3% (adulterated)	1.6%	Sildenafil, thiosildenafil	True-positive

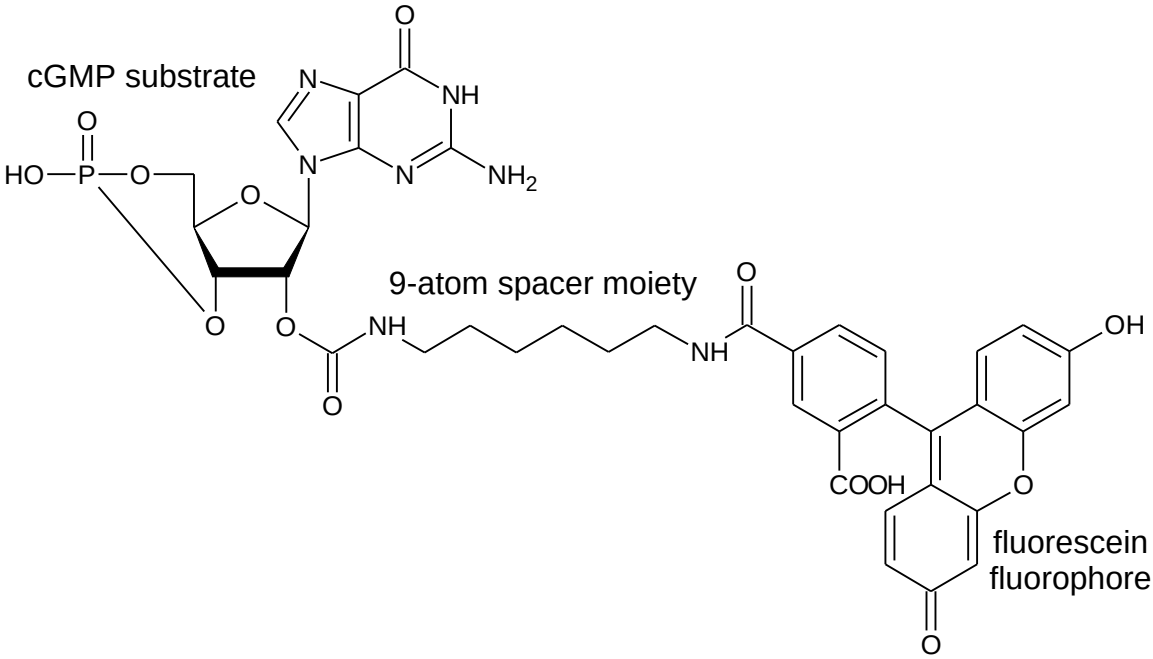
Note:
**suspected-target analytes

Table 4: Phosphodiesterase 5 (PDE5) inhibition assay results and confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis of the blank (BL) food matrices comprising powdered drink mix (PDM), honey (HNY), jelly (JLY), hard candy (HCD), and chewing gum (CWG) included as additional samples.

Sample	Average percentage of PDE5 inhibition (outcome)	Well-to-well precision (%CV)	PDE5 inhibitors detected from the confirmatory LC-HRMS analysis	Conclusion
BL-PDM	1.1% (non-adulterated)	0.7%	Not detected	True-negative
BL-HNY	1.1% (non-adulterated)	1.9%	Not detected	True-negative
BL-JLY	13.8% (non-adulterated)	2.3%	Not detected	True-negative
BL-HCD	6.6% (non-adulterated)	2.0%	Not detected	True-negative
BL-CWG	18.2% (non-adulterated)	2.1%	Not detected	True-negative



(A) **(B)**



(C)

Fig. 1: The structure of (A) sildenafil; (B) phosphodiesterase 5 (PDE5) enzyme; and (C) fluorescein-labelled cyclic-3',5'-guanosine monophosphate (FAM-cGMP) substrate.

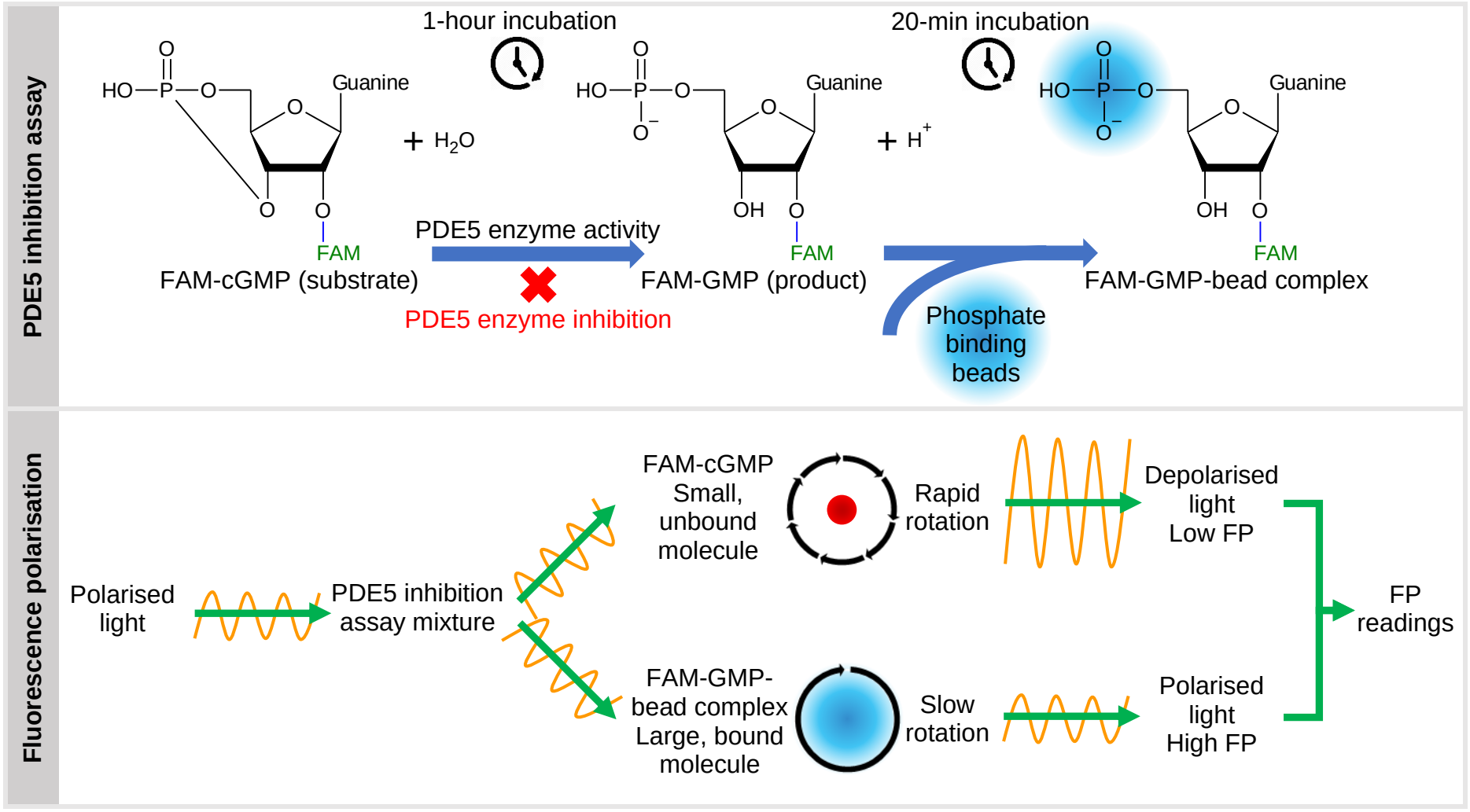


Fig. 2: Phosphodiesterase 5 (PDE5) inhibition assay scheme using fluorescence polarisation (FP) technique. (adapted and modified from BPS Bioscience Inc.[24]) (Abbreviations: FAM-cGMP, fluorescein-labelled cyclic-3',5'-guanosine monophosphate; FAM-GMP, fluorescein-labelled 5'-guanosine monophosphate) [Colour figure can be viewed at wileyonlinelibrary.com]

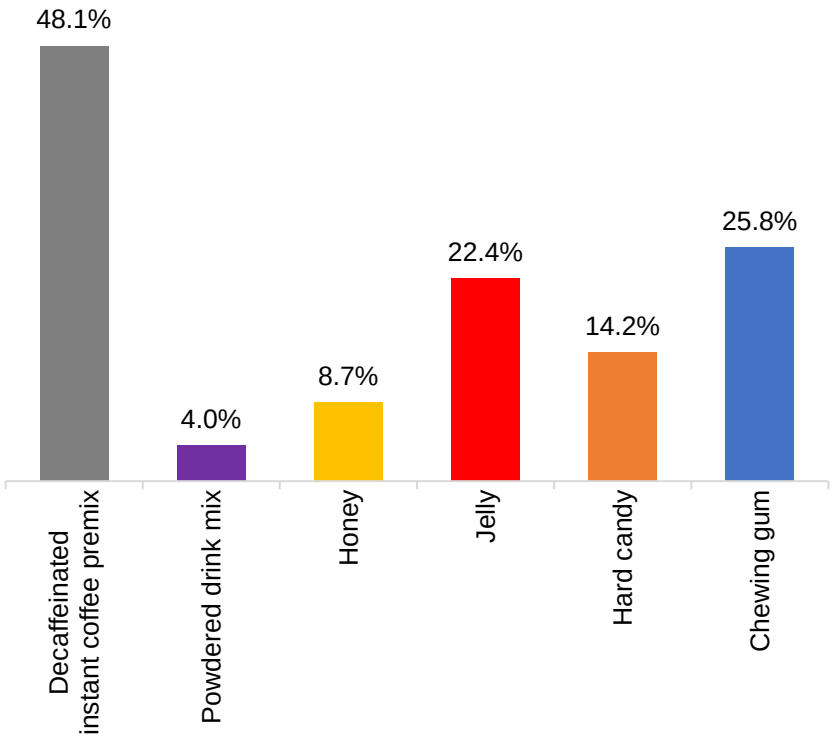


Fig. 3: Threshold values for each blank matrices of the food product.

[Colour figure can be viewed at wileyonlinelibrary.com]

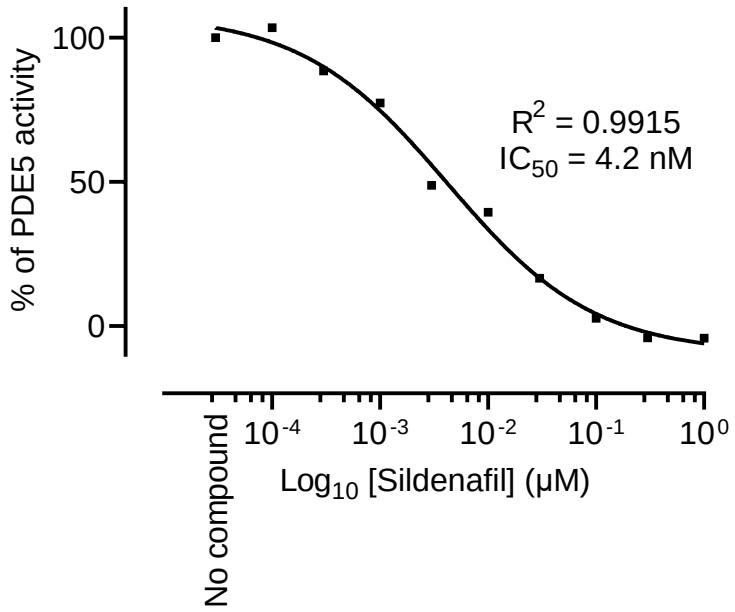


Fig. 4: The concentration-response plot of sildenafil. (Abbreviations: PDE5, phosphodiesterase 5; R^2, coefficient of determination; IC_{50}, half-maximal inhibitory concentration; Log_{10}, logarithm with base 10)