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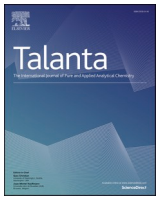

Spectroscopic determination of hydrophobic adulterant tadalafil by aptasensor based ellipsometry

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ABSTRACT

Tadalafil is one of the selective phosphodiesterase type 5 inhibitors (PDE5) and serves as the active compound in drugs used for the treatment of erectile dysfunction. These PDE5 inhibitors are prescribed under medical supervision. However, cases of adulteration of dietary supplements with PDE5 inhibitors or their unapproved analogs have been reported worldwide. The presence of the PDE5 inhibitors in such supplements poses a serious health risk to consumers, particularly when combined with certain nitrate-containing drugs, as their toxic effects have not been thoroughly assessed and may result in unpredictable adverse reactions. Therefore, it is crucial to detect adulteration in these dietary supplements. However, current methods for PDE5 inhibitor detection rely on time-consuming and expensive analytical techniques, although they are sensitive. In this study, we propose an aptasensor based on ellipsometry for the detection of PDE5 inhibitors. To enhance the detection specificity for PDE5 inhibitors, we designed an aptamer with a hydrophobic pocket that incorporates a guanine base-rich region and a three-way junction. This design is particularly effective considering the poor aqueous solubility of PDE5 inhibitors. Preliminary results demonstrate that tadalafil detection in various media can be achieved within the range of 1–2000 ng/mL. The limit of detection for the active compound of tadalafil is as low as 1.82 ng/mL.

1. Introduction

Recently, the market for food supplements aimed at improving human diet and health has grown tremendously, becoming increasingly popular [1]. However, this popularity has also resulted in a rise in illicit food adulteration. Synthetic drugs are frequently illegally added to herbal food supplements or even commonly consumed food products [2–4]. Unfortunately, inadequate regulations and easy accessibility of these adulterants pose a significant risk to consumers [5].

Phosphodiesterase type 5 (PDE-5) inhibitor agents are prescribed for the treatment of erectile dysfunction [6]. Tadalafil is one of the three selective phosphodiesterase type 5 (PDE5) inhibitors [7]. Among the PDE-5 inhibitors, tadalafil differs significantly from sildenafil and vardenafil in terms of chemical structure and selectivity, and it exhibits a wide range of therapeutic benefits [8]. Tadalafil has poor aqueous solubility, but exhibits good absorption properties [9]. Additionally, as a member of PDE-5 inhibitors, it is associated with significant clinical side effects, including physical aches and pains, and visual or hearing impairment [10]. It is also known to result in highly variable blood levels, and inconsistent clinical response [11]. Moreover, when taken in combination with certain nitrate-containing drugs, it can cause a severe drop in blood pressure, leading to serious side effects, loss of consciousness, and even death [12–14].

More than 46 analogs of PDE-5 inhibitors have been identified as adulterants in health supplements, functional foods, and energy drinks [15–18]. Furthermore, new PDE-5 inhibitor analogs are constantly being developed and illicitly used as adulterants, further raising health risks. Therefore, precise analytical methods for the detection of PDE-5 inhibitors in foods and supplements are necessary.

Established methods, such as chromatography either alone or in combination with mass spectrometry, have demonstrated their success in detecting PDE-5 inhibitors as adulterants [19–23]. These methods are indeed accurate and sensitive; however, they have certain drawbacks such as being expensive, complex, requiring trained operators and a large amount of solvents, and may not be suitable for rapid screening purposes. Hence, there is a need to develop screening methods that are specific, sensitive, accurate, and cost-effective [24,25]. Several techniques based on immunological principles have been reported in the literature for the screening of PDE-5 inhibitors in foodstuffs [26,27]. Additionally, various analytical techniques have been employed for the

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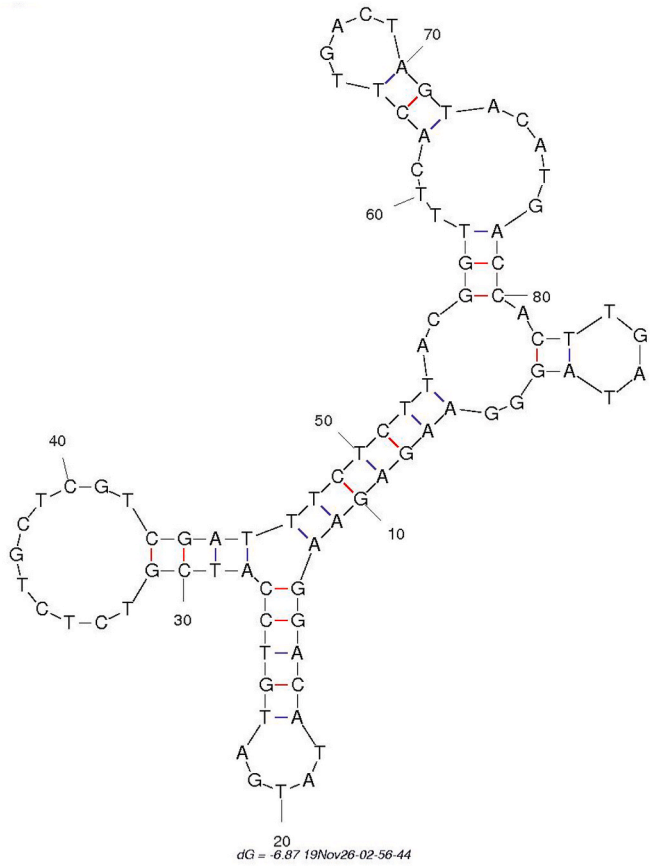

Fig. 1. – Anti-tadalafil (antiTAD) aptamer used in this study. The structure was modeled using the "mfold server" [47].

quantification of tadalafil in pharmaceutical analysis, including HPLC with fluorescence [28], GC-MS [29], and immunoassay [30]. Electrochemical detection of sildenafil and vardenafil has also been reported [31,32].

Aptamers, which are typically single-stranded oligonucleotides produced through the *in vitro* selection process [33,34], exhibit unique properties similar to immuno-based approaches due to their impressive recognition capabilities. In this study, we propose the use of attenuated total internal reflection (AIR) spectroscopic ellipsometry (SE) method to enhance sensitivity, as aptamers contribute to the selectivity of the sensor. Spectroscopic ellipsometry is a non-destructive, simple, and sensitive tool for detecting various target molecules [35,36]. We also propose the utilization of an AIR-SE platform for tadalafil detection. AIR-SE is highly sensitive to changes in dielectric constants at the coupler and dielectric medium interface and, when combined with surface plasmon resonance (SPR) conditions, can achieve a low limit of detection (LOD) in the picomolar range [37].

There are several challenges associated with the detection of tadalafil using aptamers, particularly in terms of selectivity due to its hydrophobic nature. To address this issue, the use of three-way junctions (3WJ) in DNA aptamers has been proposed for identifying hydrophobic molecules. The non-overlapping base pairs at the end of the DNA double helix form a hydrophobic surface that has an affinity for hydrophobic molecules. It has been observed that when the single helix structures are connected in a three-way arrangement, a hydrophobic pocket is created. This was initially reported based on the observation that aromatic compounds, such as hydrophobic drugs, tend to bind at the points where the double helix branches intersect [38]. An aptamer with a 3WJ linkage specific to deoxycholic acid has been developed and reported through SELEX [39]. Such linkages have been reported to exhibit relatively high selectivity towards hydrophobic molecules [40]. The behavior of the hydrophobic pocket can be adjusted by using different fluorophore groups, leading to the development of aptamers with varying affinities for different hydrophobic molecules [41]. Stojanovic and his group have reported the development of nonspecific hydrophobic receptors and derivatives inspired by nucleic acid-based 3WJ structures, particularly during aptamer development studies for cocaine [41–43]. In an article published by Yang et al. [44], both unmodified and modified nucleic acid aptamers with 3WJ and quadruple (4WJ) linkages were developed for selective recognition of urinary steroids. The initial report, based on Lu et al.'s study [45], indicates that the exposed aromatic surface of the unstacked base pairs in the 3WJ junctions forms a lipophilic cavity with a diameter of approximately 11 Å, enabling the entrapment of hydrophobic molecules.

Consequently, in this study, we propose a 3WJ aptasensor for detecting tadalafil in adulterated food samples. This study represents the first report of utilizing AIR-SE under SPR conditions for tadalafil detection in adulterated foodstuffs using aptamer-based sensors.

2. Experimental

2.1. General

Tadalafil (TAD), surface modification agents, buffer solutions, interferent molecules such as vardenafil (VAR) and sildenafil (SIL), along with other chemicals used in this study, were purchased from local representatives of Merck, Sigma-Aldrich, or Riedel companies. Commercial product samples purchased from the local market were referred to using codes instead of their actual brand names. Phosphate buffer saline (PBS) at pH 7.4 (0.01 M) contained 0.8% NaCl (w/v) or additionally Tween 20 (0.05% v/v). All solutions were prepared using ultrapure water (HumanPower, 1+, 18.2 MΩ.cm, S. Korea). Thiol modified aptamer sequences were purchased from TIB Molbiol (Germany) and used as-is. The 3WJ aptamer (i.e. anti-tadalafil aptamer), 5'-SH-TAGG GAAGAG AAGGACATAT GATGTCCATC GTCTCTGCTC GTCGATTTCTC TTACGGTTTCA CTTGACTAGTA CATGACCACTTGA- 3' (Fig. 1) were selected from the literature [46].

The SIL and TAD working standards for HPLC (Shimadzu Corp., Japan) analysis were prepared freshly. Methanol, acetonitrile, ammonium acetate, and water for HPLC were of analytical grade and were purchased from local representatives of the aforementioned companies. An Optosense S2000 model spectroscopic ellipsometer (USA) with manual goniometer was used for thickness measurements and tadalafil sensing applications. AIR-SE measurements were conducted using a flow cell and an SPR coupler assembly. Surface cleaning was performed using a UV-ozone cleaner (Bioforce, USA). All measurements were conducted in an air-conditioned room at 22 ± 1 °C. Unless otherwise stated, experiments/measurements were performed in at least. Results were reported as the arithmetic mean of the measurement, with 1σ.

2.2. Immobilization and AIR-SE measurements under SPR conditions

Thiol- Au surface interaction was used to immobilize 5'-SH modified aptamers in PBS buffer onto Au coated BK7 glass slides. The duration and probe concentration for immobilization were optimized. 6-mercapto-1-hexanol (MCH) was also attached to the aptamer immobilized surface to prevent nonspecific interactions. The aptamer and MCH immobilizations were done according to the previous studies described elsewhere [48,49]. A mini-channel flow-cell and a prism coupler were used to meet the requirements for SPR. The SPR conditions on 50 nm Au coating were set at a wavelength of 530 nm wavelength and 65° angle of incidence for this AIR-SE assembly [50]. Solutions were injected to flow-cell which was already positioned under the incident beam, using a peristaltic pump which was operated at 5 μL/min. The real-time interaction between the anti-TAD aptamer immobilized on Au-surface and TAD molecules in the buffer, as well as extracted real-samples taken into buffer solution, was monitored using ellipsometric angles delta (Δ) and

psi (Ψ).

2.3. Precision, accuracy, selectivity, and real-sample tests

The precision and accuracy of the developed method were determined as follows. Intra-day precision measurements were performed on 5 independent series on the same day, and inter-day precision measurements were conducted over 5 consecutive days using 5 samples in each series were performed. Non-specific interactions were also evaluated using possible interferent molecules such as VAR and SIL to assess the selectivity and reliability of the proposed method. Although there are molecularly similar interferents to TAD, for the purposes of this study, it was preferred to investigate the interaction effects of two well-known PDE5 inhibitors that are likely to be present in adulterated food, in addition to TAD. The effects of possible interfering materials on the sensor performance were investigated by preparing mixtures of 1000 ng/mL SIL or VAR and 100 ng/mL TAD solutions. The detection performance of TAD in real-samples was also investigated using commercial energy drink, chocolate, Turkish delight, and a traditional Turkish theriaca called Mesir theriaca. For real-sample applications, TAD was spiked into test samples that are not suspect to adulteration and prepared according to previously reported methods [51,52]. Spiked samples containing various amounts of TAD were then extracted using a methanol/acetonitrile mixture (10:90) and injected into the flow cell by adjusting the final concentration using a buffer solution. For this purpose, 5 g (or 5 mL) of the real sample was dissolved in the mixture using a vortex mixer for 10 min. After that, the solution was filtered with 0.25 μm syringe filters. The samples were then analyzed using the AIR-SE method described above.

Furthermore, commercial energy drink samples from two different brands and chocolate samples from two different brands, suspected of tadalafil adulteration and purchased from the local market, were used for real-sample applications. The concentrations of adulterants in these products were also determined using HPLC following the procedure described elsewhere [53]. The preparation of mobile phases and HPLC samples followed the procedures outlined in the mentioned report. The analysis was conducted using a UV–Vis detector and a C-18 column. The mobile phase ratio was adjusted to 50:50, and the flow rate was maintained at 1 mL/min. For detection, the wavelengths of 295 nm and 245 nm were employed for SIL and TAD, respectively.

3. Results and discussion

3.1. Immobilization of anti-TAD probes

First, –SH modified anti-TAD aptamer probe immobilization conditions were optimized using ellipsometric thickness measurements before and after immobilization. The multilayer modeling was performed using built-in software where psi (Ψ) and delta (Δ) data have been used to determine ellipsometric thickness. The ellipsometric model parameters included a multilayer model, with refractive indices for ambient air, the organic layer (n = 1.46), Au layer (50 nm), Cr layer (5 nm), and BK7 substrate. The ellipsometric thickness for the interaction of 0.5 μM anti-TAD was evaluated over a duration of 10–60 min. The results indicated that the surface thickness increased upon binding of anti-TAD to the Au-surface and reached a steady-state at after approximately 50–60 min of interaction under specified conditions. Therefore, a 60-min immobilization time was chosen and deemed sufficient for anti-TAD immobilization steps in this study. To ensure optimal surface coverage of the probe, various concentrations of anti-TAD (ranging from 0.1 to 5 μM in buffer) were immobilized on the Au-surface for 60 min. Based on the optimization, the concentration of anti-TAD chosen for sufficient immobilization was 1.2 μM at room temperature, for 60 min. The conditions for immobilizing the blocking agent were selected based on previous studies, where the immobilization parameters were quite similar [54].

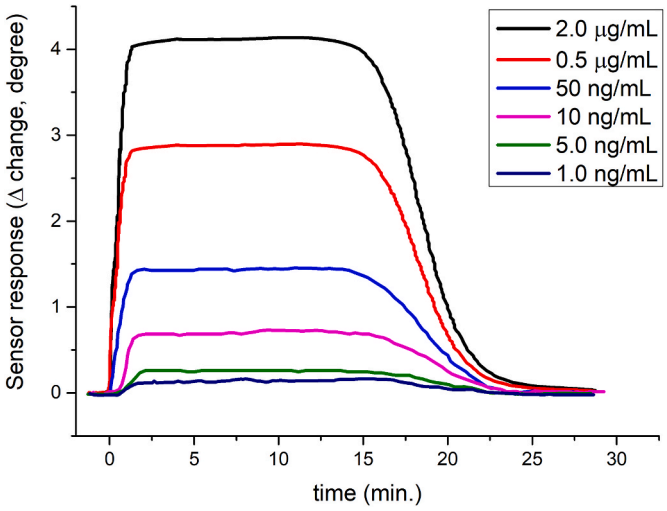

Fig. 2. – Real-time AIR-SE data for anti-TAD/TAD interaction.

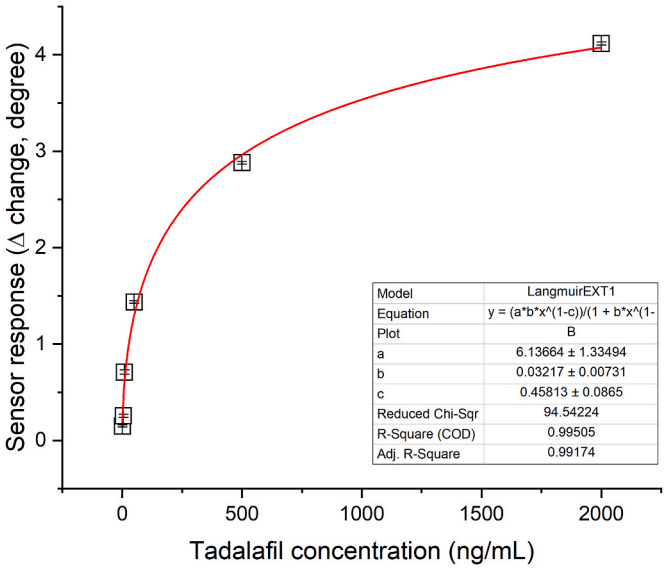

Fig. 3. – Calibration curve for AIR-SE-based TAD aptasensor fitted to the Langmuir model, and its statistical fitting results.

3.2. Analytical performance of AIR-SE sensor

Tadalafil solutions in the buffer were prepared at concentrations ranging from 1 ng/mL to 2000 ng/mL. The interaction between the surface-immobilized anti-TAD probe and TAD was monitored as previously described. At a specific angle and wavelength, the Δ parameter exhibited a shift to lower degrees upon binding or deposition of molecules on the sensor surface. This shift occurred because the dielectric function of the Au surface-analytic media at SPR conditions underwent significant changes due to the aptamer-analyte interaction. The real-time relative changes in Δ during the TAD/anti-TAD interaction are presented in Fig. 2. A relatively high affinity was observed at higher TAD concentrations, while the interaction was less favorable at lower TAD concentrations. However, it can be concluded that the interaction between TAD molecules and anti-TAD probes is more favorable when compared to our previous studies [48,49,54].

Then, the calibration curve was plotted using the Δ values at the plateau (after 5 min). The AIR-SE sensor calibration curve is presented in Fig. 3. The calibration curve was fitted to the modified Langmuir model: $\Delta = (a * b * [TAD]^{(1-c)})/(1 + b * [TAD]^{(1-c)})$.

Table 1
The analytical performance of the AIR-SE tadalafil sensor ($n = 6$).

Analytical characteristic	Values
Working rangeng/mL	1–2000
Regression equation	$\Delta = (a * b * [TAD]^{(1-c)})/(1 + b * [TAD]^{(1-c)})$.
Coefficient a/standard error, ±	6.137/1.335
Coefficient b/standard error, ±	0.032/0.007
Coefficient c/standard error, ±	0.458/0.087
R^2	0.995
LOD, ng/mL (S/N = 3)	1.82
LOQ, ng/mL	5.46

Table 2
Precision and accuracy test results of the developed method for TAD (N = 5).

Added TAD, ng/mL	Intra-day			Inter-day[a]		
	Found Value, ng/mL	RSD %	Accuracy %	Found Value, ng/mL	RSD %	Accuracy %
10	9.87 ± 0.11	1.10	+1.30	9.76 ± 0.35	3.59	−2.40
100	103.7 ± 2.2	2.1	+3.7	104.2 ± 4.6	4.4	−4.2

[a] Five consecutive days.

Table 3
The effects of sildenafil (SIL) and vardenafil (VAR) on the Δ% change of the signal acquired from 100 ng/mL tadalafil.

Interferents added	Concentration (ng/mL)	Δ signal change upon interferent addition (%)
SIL	1000	+9.3 ± 2.3
VAR	1000	+9.7 ± 3.1

It was observed that the sensor response of the analyte at different concentrations resulted in a logarithmic calibration curve. This indicates that the rate of binding has decreased with the amount of free anti-TAD sites on the surface. Additionally, due to the determination coefficient being 0.99 in fitting to the modified Langmuir model, it can be said that the interaction between the aptamer probe and TAD molecules likely occurred in single or multiple regions. The presence of hydrophobic pockets in these regions is quite promising. The calibration curve equation, variable coefficients, and their corresponding standard error values are reported in Table 1. The calculated limit of detection (LOD) was 1.82 ng/mL using a signal-to-noise (S/N) ratio of 3, with the highest standard deviation observed in the study ($\sigma = 0.0871$).

3.3. Precision, accuracy, selectivity, and real-sample tests

The precision and accuracy of the proposed method were evaluated for 10 and 100 ng/mL TAD standards. Intra-day performance evaluation involved 5 independent series of the assay, with 5 measurements performed for each series on 5 consecutive days. The precision, expressed as relative standard deviations, and the accuracies are presented in Table 2. The precision ranged from 1.1% to 4.4%, while the accuracy ranged from −4.2% to +2.1%. The proposed sensor and developed method exhibited good precision and accuracy for intra-day measurements. Although the inter-day precision and accuracies were slightly lower than the intra-day values, they still fell within an acceptable range (<5%). Based on both inter-day and intra-day results, it can be concluded that the proposed method is relatively accurate and precise.

The selectivity of the aptasensor was assessed using potential interferent molecules vardenafil (VAR) and sildenafil (SIL). These molecules are common PDE-5 inhibitors used for the same purpose and are often found as adulterants. The interferents were mixed with TAD, with the final solution concentration of the interferents being 10 times higher than TAD (i.e., 100 ng/mL TAD vs. 1000 ng/mL VAR and SIL). The specificity of the sensor was determined by calculating the relative signal shift as a percentage of sensor response using TAD and nonspecific molecules. These calculations were based on ten replications, and the results were presented as the mean signal change and standard deviation. The relative sensor response change upon the addition of interferents was +9.3% ± 2.3% for 1000 ng/mL SIL and +9.7% ± 3.1% for 1000 ng/mL VAR (Table 3). A positive bias was observed in both cases, which may be due to an available but not favorable interaction between the interferents and the anti-TAD aptamer. This relatively low interference (approximately 10%) resulting from the differences in hydrophobicity of the interfering molecules suggests that the sensor can be used for preliminary screening of adulterated food samples.

Table 4
Analytical recovery of the proposed sensor in real samples (N = 5).

Samples	Spiked amount (ng/mL)	Detected amount (ng/mL)	Recovery (%)
Energy drink	10	9.55	95.5
	100	103.8	103.8
Chocolate	10	10.45	104.5
	100	100.3	100.3
Turkish delight	10	9.61	96.1
	100	94.8	94.8
Mesir theriaca[a]	10	10.96	109.6
	100	107.6	107.6

[a] Mesir theriaca is a traditional Turkish sweet that originated from spicy preparations.

Table 5
Real commercial sample test comparison for TAD and their recovery results (N = 3).

Samples[a]		Detected amount (ng/mL for energy drink or ng/mg for chocolate bar)		
		AIR-SE	HPLC	Accuracy (%)
Energy drink (330 mL)	Brand-E1	283.2 ± 3.1	272.3 ± 3.0	4.0
	Brand-E2	54.2 ± 1.9	54.6 ± 3.0	0.7
Chocolate (100 g)	Brand-C1	ND	ND**	–
	Brand-C2	251.5 ± 3.6	243.6 ± 2.5	3.2

[a] 124 ng/mg SIL detected by HPLC.

Analytical sensor performance on the real samples was evaluated by adding a known amount of TAD to four potential adulteration food candidates (Table 4). The spiked amount of TAD standard was selected to be within the low- and mid-range of the calibration curve, specifically 10 and 100 ng/mL. In the energy drink sample, the recovery percentages for 100 and 10 ng/mL TAD were 103.8% and 95.5%, respectively. Similar results were observed for the other real samples. However, in the Turkish delight sample at 100 ng/mL TAD, as well as in the Mesir theriaca sample at both TAD concentrations, the recoveries deviated higher than ±5%. This deviation may be due to the complexity of the food matrices and/or other non-specific interactions between the analyte media and the anti-TAD aptamer. To address this, more specific aptamers can be used or the surface properties of the sensor can be improved. Nonetheless, these results were considered satisfactory as the recoveries fell within the acceptable limit for the other real samples used in this study (i.e., <±5%).

The AIR-SE aptasensor used for tadalafil detection was also employed to detect possible adulteration in commercial energy drink (330 mL) and chocolate (100 g) samples. With the aim in mind, food samples were acquired, specifically targeting those with a high probability of adulteration, taking into account their packaging and brand

Table 6
Comparison of different methods for the detection of TAD.

Recognition Element	Sensor Type	Range (ng/mL)	LOD (ng/mL)	Selectivity	References
Reduced graphene oxide functionalized β-Cyclodextrin	Electrochemical	38.9–3.9 × 10^5	17.5	Sildenafil, Vardenafil, oxalic, citric, and ascorbic acid	[55]
Polyclonal Ab	Immuno-chromatographic	0.71–141.99	0.71	A group of tadalafil analogs	[56]
β-Cyclodextrin functionalized Au@SiC nanohybrids	Electrochemical	3.89–3.9 × 10^4	0.97	Sildenafil and vardenafil	[57]
Core-shell molecularly imprinted polymers on magnetic nanoparticles	HPLC-UV	98–1960	16.9	Sildenafil and vardenafil	[30]
3-way junction aptamer	AIR-SE	1–2000	1.82	Sildenafil and vardenafil	This study

names. HPLC was used as the reference method, and accuracy was calculated based on the HPLC results. The results for two energy drink brands (Brand-E1 and E2) suspected of PDE-5 inhibitor adulteration were positive (Table 5). The calculated total tadalafil amount in Brand-E1 was approximately 100 μg, while in Brand-E2 it was approximately 20 μg. Both values are significantly below the therapeutic range (i.e., 20 mg/tablet). However, for the tadalafil-positive chocolate sample (Brand-C2), approximately 25 mg of total tadalafil was detected in 100 g of chocolate bar. Additionally, for the second sample positive for a PDE-5 inhibitor (Brand-C1), approximately 12.5 mg of SIL was detected per 100 g of chocolate bar. It was somewhat surprising to find therapeutic levels in the adulterated food products, but this was expected considering their higher market prices (ten times more expensive than a standard chocolate bar). The performance of the proposed AIR-SE aptasensor exhibited a level of accuracy that can be deemed comparable to that of the HPLC method, thereby showcasing its efficacy and reliability in tadalafil detection.

4. Conclusion

In this research study, we have successfully developed and presented a highly sensitive and reliable ellipsometry-based aptasensor for the detection of TAD in adulterated food samples. The performance of the proposed sensor was extensively evaluated in terms of selectivity and reliability using various potential interferents as well as real samples including energy drink, chocolate, Turkish delight, and Mesir theriaca. The analytical performance of the TAD sensor demonstrated satisfactory results in terms of selectivity, sensitivity, accuracy, and its applicability to real samples.

The limit of detection (LOD) achieved by this method, 1.82 ng/mL, was lower compared to previously reported methods (as shown in Table 6). The working range of 1 ng/mL to 2000 ng/mL was also deemed satisfactory and comparable to other methods. Additionally, the sensor exhibited high accuracy and precision, with values exceeding 95% for both intra-day and inter-day tests. The specificity of the sensor was relatively high, as evidenced by the sensor response deviation of less than 10% observed under controlled conditions where tenfold interferent molecules were measured simultaneously. These findings were further validated through real-sample tests.

While the recoveries for energy drink, chocolate, and Turkish delight samples fell within acceptable limits, there was a notable positive bias in the recovery of Mesir theriaca. These results suggest that there is room for improvement in the analytical performance of the proposed sensor, which could be achieved by employing more specific aptamers and implementing appropriate modifications to the sensor surface.

Author statement

İlknur Üstündağ: Conceptualization, Methodology, Validation, Investigation, Resources, Data Curation, Writing- Review & Editing, **Mustafa Oguzhan Caglayan:** Conceptualization, Methodology, Resources, Writing – Original Draft, Writing- Review & Editing, Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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