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Analytical Methods

A flexible paper based electrochemical portable biosensor towards recognition of ractopamine as animal feed additive: Low cost diagnostic tool towards food analysis using aptasensor technology

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ABSTRACT

Due to the costly and time-consuming traditional techniques, providing a low-cost, portability and flexibility diagnostic tool with the ability to monitor and detect various animal feed additive is highly demanded. Over the years, paper-based biosensors have emerged as point of care (POC) diagnostic, easy-to-use and miniaturized tools. However, they have been suffered from low sensitivity. Aptamer as appropriate bioreceptor can overcome the most common disadvantage of paper based sensor by increasing selectivity and sensitivity. In this study, a novel paper-based electrochemical aptasensor was successfully developed to detection of ractopamine (RAC). RAC concentration was evaluated using a designed three-electrode paper based biodevice system. Under the optimal experimental conditions, the engineered aptasensor provided good sensitivity and selectivity for the detection of RAC. Using proposed flexible sensor RAC was determined in the range of 0.001 μM to 100 mM which the lower limit of quantitation (LLOQ) was obtained as 0.01 μM. Finally, aptasensor was used to the monitoring of RAC in untreated human plasma specimens which LLOQ and linear range were 0.01 μM and 0.01 μM to 10 mM, respectively. We hope that the exploitation of aptamer in electrochemical paper based sensor will be able to broaden our understanding for developing the application of low-cost and portable biodevices for the sensitive and selective paper-based sensor to identify other chemical and biological compounds.

1. Introduction

Creating conductive paths on heat-sensitive substrates such as polymeric surfaces (Jeong, Song, Lee, Lee, Choi, Son, et al., 2011), textiles (Hu, Pasta, La Mantia, Cui, Jeong, Deshazer, et al., 2010), plastic (Xu, Yang, Jing, Wei, & Han, 2014) and paper (Russo, Ahn, Adams, Duoss, Bernhard, & Lewis, 2011; Tai & Yang, 2011), as a suitable approach to provide low-cost and flexible electronic devices has attracted much attention. Paper substrates have received a great deal of attention due to their flexibility (Hasanzadeh and Shadjou, 2016), disposability, availability and weight (Siegel, Phillips, Dickey, Lu, Suo, & Whitesides, 2010; Yang, Gu, Lin, Yuen, Wong, Xiong, et al., 2011). In addition, these platforms have high adsorption capacity, ability immobilization of biomolecules through chemical groups, easy disposal, capillary action which make them particularly suitable for flexible electronic biodevices (Lee, Mohd-Naim, Tamiya, & Ahmed, 2018; Liu, Su, & Ding, 2016). There are various protocols (Gan et al., 2015a; Gan et al., 2015b; Gan et al., 2018) for the synthesised of silver nanoparticle (AgNPs) and making conductive lines by ink of these NPs, such as inkjet printing, screen printing and direct writing, but the latter method is more user-friendly due to its low cost and simplicity (Farshchi & Hasanzadeh, 2020). A rollerball pen filled with semi-conductive or conductive inks can be used to write electronics on paper (Liu, Shen, Zhang, & Ma, 2021). Silver nano-ink are widely employed indirect printing due to their hefty antioxidant traits and high electrical conductivity (Wu, Li, Ong, Liu, Gardner, & Chiang, 2005). Despite studies to improve the conductivity of silver ink after printing, it still requires a sintering temperature above 200 °C and a high concentration of silver

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nanoparticles to produce high-conductivity printing patterns (Ma, Suh, Kim, Chung, & Baik, 2011; Russo, Ahn, Adams, Duoss, Bernhard, & Lewis, 2011). Given that silver is inherently a precious metal, its high concentration is not cost-effective. On the other hand, as the baking temperature increases, the paper substrates are damaged. Therefore, high baking temperatures limit the use of silver inks on paper substrates and productivity. Hence it is necessary to reduce the baking time and temperature. Despite many efforts to reduce the baking temperature, the migration of silver has become a serious problem in the impact of silver lines (Li, Lu, Chou, & Liu, 2010).

Given the problems mentioned above about Ag nano-inks, more efforts are needed to produce new conductive inks to solve them. Graphene has attracted a lot of attention due to its outstanding thermal, mechanical and electrical properties (Geim & Novoselov, 2010; Li & Kaner, 2008; Wang, Ang, Wang, Tang, Thong, & Loh, 2010). With these unique traits, graphene can be utilized in diverse fields, including energy storage materials (Xu, Wang, Zu, Han, & Wei, 2010), biological and chemical sensors (Wang, Li, Hu, Lin, Li, & Lin, 2010; Shadjou, Hasanzadeh, & Khalilzadeh, 2018), and electronics (Huang, Huang, Liang, Wan, & Chen, 2011; Shin, Hong, & Jang, 2011). Graphene has also attracted the attention of researchers in the production of graphene-based conductive nano-inks due to its high conductivity and current density (Kordasht & Hasanzadeh, 2020). However, the widespread use of pure graphene inks is limited due to their low conductivity. Therefore, to prepare inks with higher conductivity, the advantages of these two materials can be used to reduce the silver concentration and increase the conductivity by mixing graphene and silver nanoparticles. So far, a variety of hybrid conductive nano-inks consisting of carbon-based materials such as graphene quantum dot (GQD) and silver nanoparticle (AgNPs) have been successfully developed and used to make conductive lines (Farschi, Saadati, & Hasanzadeh, 2020; Farshchi, Saadati, & Hasanzadeh, 2020; Saadati, Hassanpour, Bahavarnia, & Hasanzadeh, 2020). The electrical conductivity of a solution-processed Ag film is reduced due to the voids created in the printed Ag film as a result of solvent evaporation and the aggregation of AgNPs (Polavarapu, Manga, Cao, Loh, & Xu, 2011). Carbon-based materials can reduce the negative effects of voids by creating efficient electrical pathways between aggregated silver nanoparticles. However, it has been shown that the overall resistance of electronic devices is significantly affected by the contact resistance between carbon-based material joints (Ma, Suh, Kim, Chung, & Baik, 2011). In addition, polyvinylpyrrolidone (PVP) organic macromolecules are used as stabilizers in ink synthesis. Due to their hydroxyl terminal groups, these groups can act as a relatively gentle reluctance for the controlled kinetic synthesis of AgNPs with a yield of 75% (Hassanpour, Hasanzadeh, Saadati, Shadjou, Soleymani, & Jouyban, 2019).

Paper-based electrochemical aptasensors (Hasanzadeh, Razmi, Mokhtarzadeh, Shadjou, & Mahboob, 2018) are small devices that have received a great deal of attention due to their excellent traits such as high sensitivity, good selectivity, portability and low power consumption. These tools are able to convert the chemical amounts of the target analytes into electrical signals and have a high potential for use in point of care detection due to their ability to detect analytes quantitatively (Su et al., 2015). Paper-based electrochemical aptasensors can be utilized to detect a variety of analytes such as biomarkers, toxins, and chemicals such as RAC.

RAC is a significant member of β-adrenergic agonists used in the treatment of respiratory diseases (Xiao et al., 2015). It can also lead to increased protein accumulation in livestock. Therefore, in order to increase muscle growth, it is illegally added to animal feed as an additive (Duan et al., 2017). By consuming animal meat by humans, RACs accumulated in animal tissue threaten the health of the consumer and lead to side effects such as muscle tremors, headaches and tachycardia (Wang et al., 2016). For these reason, the use of RAC in animal feed is banned in most countries to prevent human health hazards (Brambilla et al., 2000). As a result, the development of diagnostic tools is essential to food safety and limit the illicit use of RAC. Various analytical methods such as HPLC (high-performance liquid chromatography) (Vichapong et al., 2016), enzyme-linked immunoassay (Zhang et al., 2006), LC-MS/MS (liquid chromatography-tandem mass spectrometry) (Blanca et al., 2005), CE (capillary electrophoresis) (Wang et al., 2010) and electrochemical sensors (Roushani et al., 2020; Zhou et al., 2018) have been reported to measure RAC. Although most of these techniques are sensitive and reliable, they have disadvantages such as the need for an experienced operator, long-term analysis, expensive equipment, and accuracy in sample preparation. Electrochemical techniques can be used as a suitable method for measuring RAC due to its advantages such as simplicity, cost-effectiveness, portability, sensitivity and high speed (Vasseghian et al., 2020).

Here, a novel, flexible and easy to use paper-based electrochemical biosensor was developed based on aptamer for in-field monitoring of RAC instead of conventional electrochemical platform. For this purpose, the working and counter electrodes were drawn on the surface of the ivory sheet by directly writing the synthesized conductive inks silver nanoprism-graphene quantum dots (AgNPr/GQDs) through the sampler and the reference electrode was drawn directly by writing the silver ink through pen on paper technology. Graphite ink was used to prepare apta-ink. The designed three-electrode system was utilized as a novel biosensor towards detection and determination of RAC. It seems that the fabricated concept not only provides a novel POC analytical tool but also expands the exploitation of the sensing interface to design other paper based aptasensors for drugs, biomarkers and chemical compounds in real samples.

2. Materials and methods

2.1. Chemicals and reagents

Tris-HCl (hydroxymethyl), polyethylene glycol (PEG), mercaptoethanol (MCH), chitosan, ascorbic acid (AA), arginine (Arg), tyrosine (Tyr), L-proline, cysteine (Cys) and uric acid (UA), glucose were purchased from Merck (Darmstadt, Germany). PVP, $K_4Fe(CN)_6$ (potassium ferrocyanide), $K_3Fe(CN)_6$ (potassium ferricyanide), methylene blue (MB), citric acid (glacial), sodium borohydride ($NaBH_4$, 96%), silver nitrate ($AgNO_3$), hydrogen peroxide (H_2O_2, 30 wt%), sodium hydroxide (NaOH), potassium chloride (KCl), ethanol, hydrochloric acid (HCl) and graphite were obtained from Sigma-Aldrich (Ontario, Canada). T95108 aptamer (5′-SH-AAA AAG TGC GGGC-3′) and RAC were achieved from Takapouzist Co (Iran) and Tocris Bioscience (Bristol, United Kingdom), respectively. Human plasma samples and deionized water were prepared from Blood Transfusion Research Center (Tabriz, Iran) and Ghazi Pharmaceutical Company (Tabriz, Iran), respectively. Ivory sheet paper (Liverpool, English) was obtained from paper mill.

2.2. Equipment

PalmSense 4c (Palm Instruments, Utrecht, The Netherlands) system and designed three-electrode system was utilized for electrochemical measurement by PSTrace 5.3 software. Silver conductive ink pen (Chemtronics, Netherland) was utilized to drawn the reference electrode on the paper. Synthesized ink (graphite ink and AgNPr/GQDs ink) also used for designing counter and working electrode. The field emission scanning electron microscope (FE-SEM, Hitachi-SU8020, Czech) was exploited for investigating surface morphology of inks and surfaces. Transmission electron microscopy (TEM), Adelaide, Australia was applied for studying morphology of prepared inks. In this aptasensor, chronoamperometry (ChA) technique was ran in $t_{equilibrium} = 2$ s, $E_{DC} = 0.05$ V, $t_{interval} = 0.1$ s and $t_{run} = 100$ s. $Fe(CN)_6]^{3-/4-}$ (0.01 M) containing KCl were prepared to the electrochemical measurement at the room temperature.

2.3. Synthesis of AgNPr/GQDs ink

GQDs and AgNPr were synthesized according to our previous works (Farshchi, Saadati, & Hasanzadeh, 2020; Kordasht, Moosavy, Hasanzadeh, Soleymani, & Mokhtarzadeh, 2019). Briefly, 3 mL of PVP media (0.06 g PVP) was mixed with 200 mL of water. Afterward, 4 mL of $AgNO_3$ (0.01 M) solution was gradually appended and stirred strongly. 8 mL of 75 mM trisodium citrate (TSC) solution was slowly added to the above mixture. Following stirring magnetically at high speed, H_2O_2 (960 mL) was slowly added. Subsequently, 3200 μL of 100 mM $NaBH_4$ solution (as a reducing agent) was added to the mixture. Color alternation to deep yellow indicate that formation of small AgNPrs due to $NaBH_4$ addition. After a few moments, the colloidal media color changed to light yellow. It should be noted, the final mixture stirred for 30 min at room temperature for altering color to blue. At the end, developed colloidal mixture was centrifuged for 15 min at 6000 rpm and stored at 4 °C for subsequent use.

In order to synthesis conductive nano ink, 0.013 g of chitosan was first dissolved in 2 mL acetic acid solution (0.1 M). Afterward, 0.2 g of PVP and 0.6 g of graphite powder were added to beginning solution. Then, 2 mL of GQDs which was prepared according to our previous reported (Saadati, Hassanpour, & Hasanzadeh, 2020) magnetically mixed with above mixture and stirred. Then, AgNPr (600 μL) media was added into the upper mixture and stirred for about 10 min. The pervious mixture was incubated for 12 h at 60 °C. After completed incubation, NaOH media (0.12 g dissolved in 400 mL water) was attached to mixture heated at 80 °C. It was centrifuged (30 min at 8000 rpm) and washed three times with distilled water to remove extra reactant. Finally, the resulting solution was converted to hybrid conductive ink by dispersing the AgNPr/GQDs nano ink into deionized water, ethanol and ethylene glycol at a ratio of 6:6:3 (v/v/v) and ultra-sonicating for 30 min synthesis nanoink was used for the pattern of counter electrode on the surface of ivory sheet paper. Counter of electrode was designed on the surface of ivory sheet paper by direct writing of the conductive nano-ink by handwriting.

2.4. Preparation of aptaink

In summary, the mixture of polyethylene glycol (6 wt% in water) and chitosan (0.1 wt% in 0.1 M acetic acid) was papered (with volume ratio of 2:1) for added glucose solution (2 M). Then, methylene blue (0.01 wt %), graphite powder (3 wt%) were added, respectively and dispersed for 30 min at the room temperature. Finally, 10 μL of RAC aptamer (sequence, 5′-AAA AAG TGC GGGC-3′) was added to 100 μL of this conductive solution (1:10) for the preparation of apta-ink sensor and kept at 4 °C for further application. Physically reaction of RAC aptamer with graphite caused improve properties of aptasensor due to excellent advantages of graphite which completely discuss at the introduction. In addition, recognition area of aptamer was free for conjugating with RAC. Furthermore, this matrix could provide excellent substrate for efficient detection of this drug.

2.5. Resistance and conductivity evaluation of synthesized conductive ink (AgNPr/GQDs and Graphite)

To test resistance of synthesized inks, conductive lines were first drawn on ivory sheets. Conductivity was then tested using a battery and a 1.5 V lamp. In this way, the cathode surfaces of the battery were connected to one side of the conductive path and the battery anode was connected to the cathode of the LED lamp. As a result of connecting the lamp anode to the other side of the conductive path, the lamp turned on (Fig. S1 (see supporting information). Furthermore, the flexibility of paper based substrate was evaluated outwards by 90°.

2.6. Biosensor fabrication: barrier free patterned substrate

2.6.1. Treatment of ivory sheet paper by chitosan

For this purpose, the fresh pieces (30 × 20 mm^2) of paper (ivory sheet) were dipped into the 1 mL mixture of chitosan (0.1 wt% in 0.1 M acetic acid) for 3 min at the room temperature. Therefore, the paper put onto piece of cotton wool to remove excess of chitosan solution. The chitosan decorated ivory sheets were dried at room temperature. Hence, chitosan-coated the reagent ink pen was directly brought into contact with chitosan-coated paper to obtain spot-patterned sensor. We have noticed that chitosan, a natured bio-polymer, is well documented candidate as an immobilization support with excellent biocompatibility for paper based biosensing application. According to recent studies various type of biomolecules could be adsorbed on chitosan modified paper can wash-off effectively. Therefore, the reaction product would not migrate out of pattern after introduction of analytes because the biological elements could remain at the initial location on chitosan coated paper. In this case, the patterned signal readout could be acquired even without the help with hydrophobic boundaries.

The three-electrode system (Fig. S2, See supporting information) was prepared by writing directly on the surface of the ivory sheets previously impregnated with chitosan solution. The reference electrode was prepared by writing silver ink using commercial conductive pens. Synthetic AgNPr/GQDs ink and apta-ink prepared with graphite ink were utilized to preparation of working electrode. The counter electrode was also prepared using AgNPr/GQDs ink.

2.6.2. Preparation of Ag/AgCl reference electrodes

Initially, silver conductive ink with line size of 20 mm was directly written on chitosan treated ivory sheet (Fig. 1). For the preparation of Ag/AgCl reference electrode, Ag pattern created on the surface of paper were dipped into KCl + HCl (0.1 M + 0.1 M) solution. Therefore, the potential is swept from −0.1 to 1 V of 4 segments at the scan rate of 0.1 V/s. This process was completely carried out by CV technique ($t_{equilibrium}$ = 2 s, E_{begin} = −1 V, $E_{vertex1}$ = +1 V, $E_{vertex2}$ = −1 V, E_{step} = +1 V, scan rate = 0.05 V/s, number of scan = 4).

After pattering of reference electrode, 30 μL of AgNPr/GQDs ink (as a high conductive and current density ink) for each ivory sheet was immediately drawn on the surface of the chitosan treated paper using pen-on-paper strategy as counter electrode (line size of 20 mm). It should be noted that developed surface was bucked at 60 °C for 5 min. Afterward, 5 μL of mixer of T95108 aptamer (sequence, 5′ SH-AAA AAG TGC GGGC-3′) and graphite ink (1:10) was incubated on the sensing zone of chitosan treated ivory sheet modified AgNPr/GQDs ink for 3 h at room temperature (d = 3 mm). Then, 5 μL of MCH (0.1 M) was dropped on the sensing area for the 30 min at the 25 °C for blocking non-specific active sites of electrode. Finally, 5 μL of RAC was dropped on the modification sensing zone for efficient conjugation with apta-ink (scheme 1).

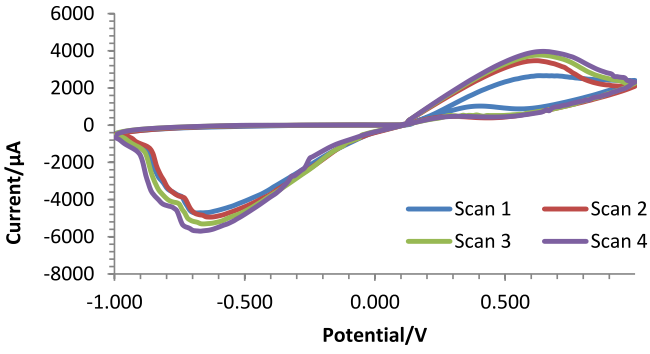
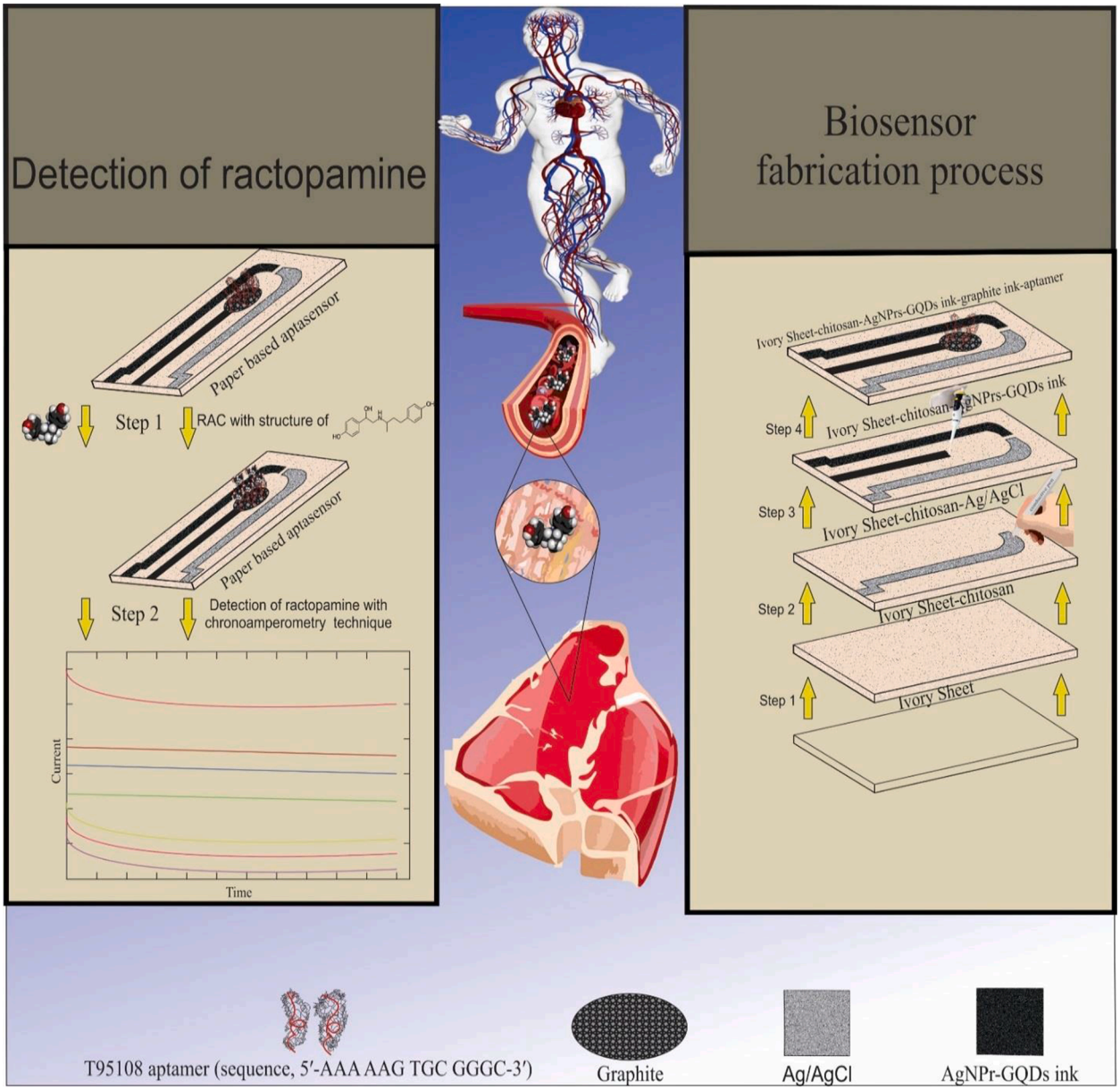


Fig. 1. CVs of chitosan-treated ivory sheet in the solution of KCl/HCl (0.1 M, 0.1 M) solution in the potential range of −1 to 1 V at the scan rate of 0.1 V/s, number of cycle = 4.



Scheme 1. The schematic representation of developed paper based aptasensor for detection of RAC.

3. Result and discussion

3.1. Characterizations

3.1.1. Characterizations of bulk AgNPr/GQDs and AgNPr/GQDs modified ivory sheet

In order to investigation binding of AgNPs on to graphite sheets and AgNPs-GQDs nano-ink synthesis mechanism, TEM images were recorded. Based on obtained results (Fig. 2 (A-H), several dispersed spherical AgNPs were coated on the surface of graphite sheets.

The FE-SEM images of AgNPr/GQDs ink were demonstrated in Fig. S3(A-I) (see supporting information) for studying uniformity of ink. The small-sized AgNPs with the spherical uniform structure were successfully decorated on the surface of graphite sheets. As can be seen, in AgNPr/GQDs dense dye inks, compact Ag prisms have decorated graphite plates. It should also be noted that there is no accumulation of nanoparticles in AgNPr/GQDs nanoparticles in solvent (water/ethanol/ethylene glycol). Therefore, FE-SEM images conformed result of TEM images.

The Fig. S4 (A-I) (see supporting information) shown FE-SEM images of AgNPr/GQDs ink on the surface of ivory sheet which provide appropriate information about morphology of the electrodes surface.

3.1.2. Characterizations of graphite ink and apta-ink

FE-SEM images of bulk graphite ink, bulk apta-ink and apta-ink on the superficies of chitosan-treated ivory sheet was indicated in Fig. S5 (see supporting information). As shown in Fig. S5(A-K), the clearly uniform solution was constructed due to bending of the N—H and stretching O—H, N—H, C—O, C—H and C═O in the ink mixture. Therefore, it is revealed that graphite ink acts as an appropriate and efficient host for stabling of T95108 aptamer.

3.2. Electrochemical evolution of sensor fabricated steps

The fabricated steps of designed paper based aptasensor were investigated by using square wave voltammetry (SWV) and chronoamperometry (ChA) techniques in the presence of $[Fe(CN)_6]^{3-/4-}$ solution (Fig. 3). Three types of substrate were drawn in the sensing zone

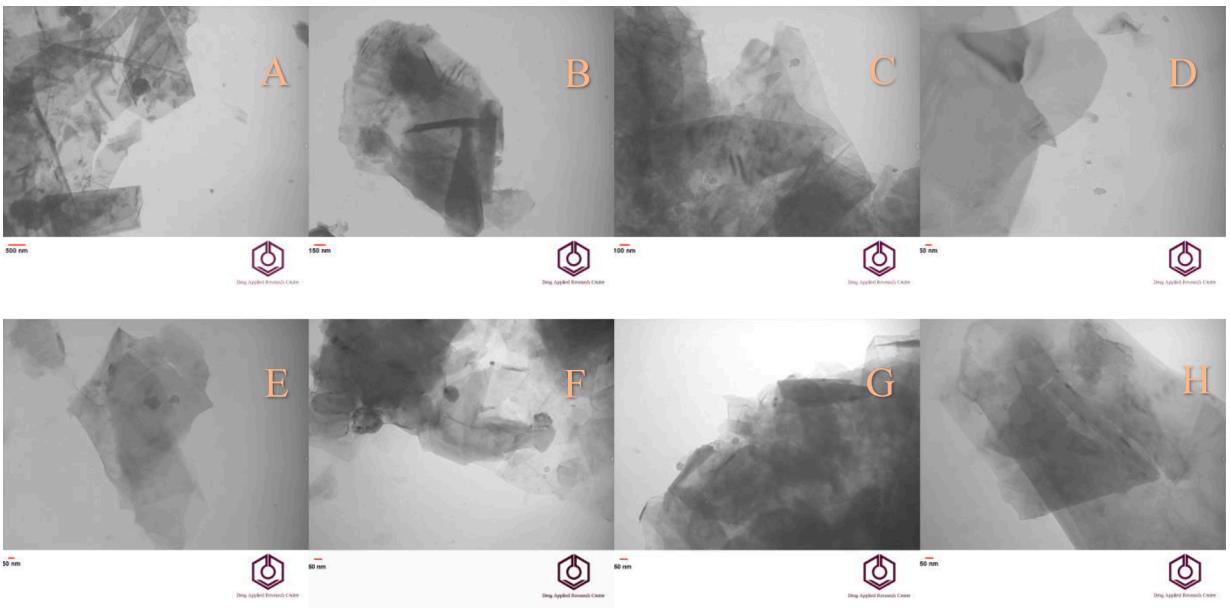


Fig. 2. (A-H) TEM images of AgNPr/GQDs in different magnification.

including Gr ink, Gr ink-aptamer (apta-ink) and Gr ink-aptamer-RAC for evaluating performance of aptasensor. Thus, the results of both techniques were similar. According to the obtained results, the peak current in the presence of ferrocyanide was obtained using a paper electrode prepared with graphite ink is 190.7 μA. However, as a result of the combination of aptamer with ink, the peak current increased to 402 μA, which seems to be due to the presence of two inks on the structure of sensor and increased electron transfer by it. Hence, it showed that aptamer has been favorably bonded with Gr ink. Increasing ractopamine and its interaction with aptamer lead to a decrease in peak current to 98.4 μA. Therefore, proposed aptasensor was obey from signal-off system.

3.3. Analytical performance

In the present concept, ChA as a sensitive electrochemical technique were used for determination of RAC using flexible paper based aptasensor. Fig. 4A shown ChAs of chitosan treated ivory sheet modified by AgNPr-GQDs-Apta-ink in various concentration of RAC (0.001 μM-100 mM) in the presence of $Fe(CN)^{-3/-4}$. As result, 5 μL of different level of RAC was dropped casting on the sensing area of substrate and incubated for 30 min at room temperature. Then, 5 μL of DW was dropped on the working for washing non-interaction RAC. As shown in calibration plots (Fig. 4 (B-C)), with a decrease in the concentration of RAC, the oxidation peak current of sensor was decreased. According to the obtained results, linear range of proposed aptasensor is 0.001 μM–100 mM which LLOQ is 0.001 μM. Also, regression equation was obtained as $I_p = -26.69C_{Rac} + 201$ ($R^2 = 0.925$). Analytical performance of proposed aptasensor was compared with some other reported of biosensor and summarized in Table 1. Comparison of different types of electrochemical biosensor for determination of RAC indicated that there is not report for the paper based electrochemical biosensing of RAC. According to our best knowledge, proposed aptasensor is the first platform based on paper for determination of RAC.

Although various types of electrochemical biosensors based on different kind of electrode such as screen printed carbon electrode, glassy carbon electrode and gold electrode were developed for the determination of RAC, there is not paper based aptasensor for the detection of RAC till now. Comparison sensitivity of different electrochemical electrode substrate demonstrated that the sensitivity of paper based biosensor is lower than other types of biosensors. Also, this paper based aptasensor is more efficient due to excellent advantages including portability, flexibility and low-cost (He et al., 2017; Shen & He, 2007; Wang et al., 2013; Wang et al., 2015).

3.4. Real samples analysis

The applicability of fabricated paper based aptasensor in complex biological fluids was investigated by incubating 5 μL of mixture (1:1 of plasma and RAC) in sensing zone for 30 min at room temperature. ChA recorded curved (Fig. 5A and B) revealed that the oxidation peak had directly relationship with concentration of RAC. Moreover, the LLOQ and linear range of fabricated aptasensor were 0.01 μM and 0.01 μM–10 mM, respectively. Under the optimum circumstance, the calibration curve was attained by plotting the peak current versus the concentration of RAC with regression equation $I_p = -27.82C_{RAC} + 133$. Comparison of result of real samples and standard samples showed that the prepared aptasensor was more efficient in standard sample.

Three different concentrations of RAC was monitored by a proposed aptasensor. Reliability was further evaluated by recovery experiments in real samples using the standard spiking experiment. The concentrations of RAC were determined using the developed method described above and calculated by using the linear equation. The result is shown in Table 2. The recoveries ranged from 89 to 99.98%. This method can be used to evaluation of RAC in real food samples.

3.5. Selectivity of aptasensor

The specificity of aptasensor was exanimated by incubating some interfering agents such as Pro, UA, Arg, Tyr, Cys, AA instead of RAC. This important factor demonstrated ability of T95108 aptamer as a bio-recognition element on the aptasensor's structure to yield a quantifiable response selectively for the target molecule (RAC). Based on Fig. S6(A-B) (see supporting information), the current response of RAC is more than other targets, except AA and UA. AA and UA have higher peak current than RAC. Hence, the selectivity of developed paper based aptasensor was acceptable. So, AA and UA as electroactive molecules and has little interfere on the performance of aptasensor for the detection of RAC in real samples.

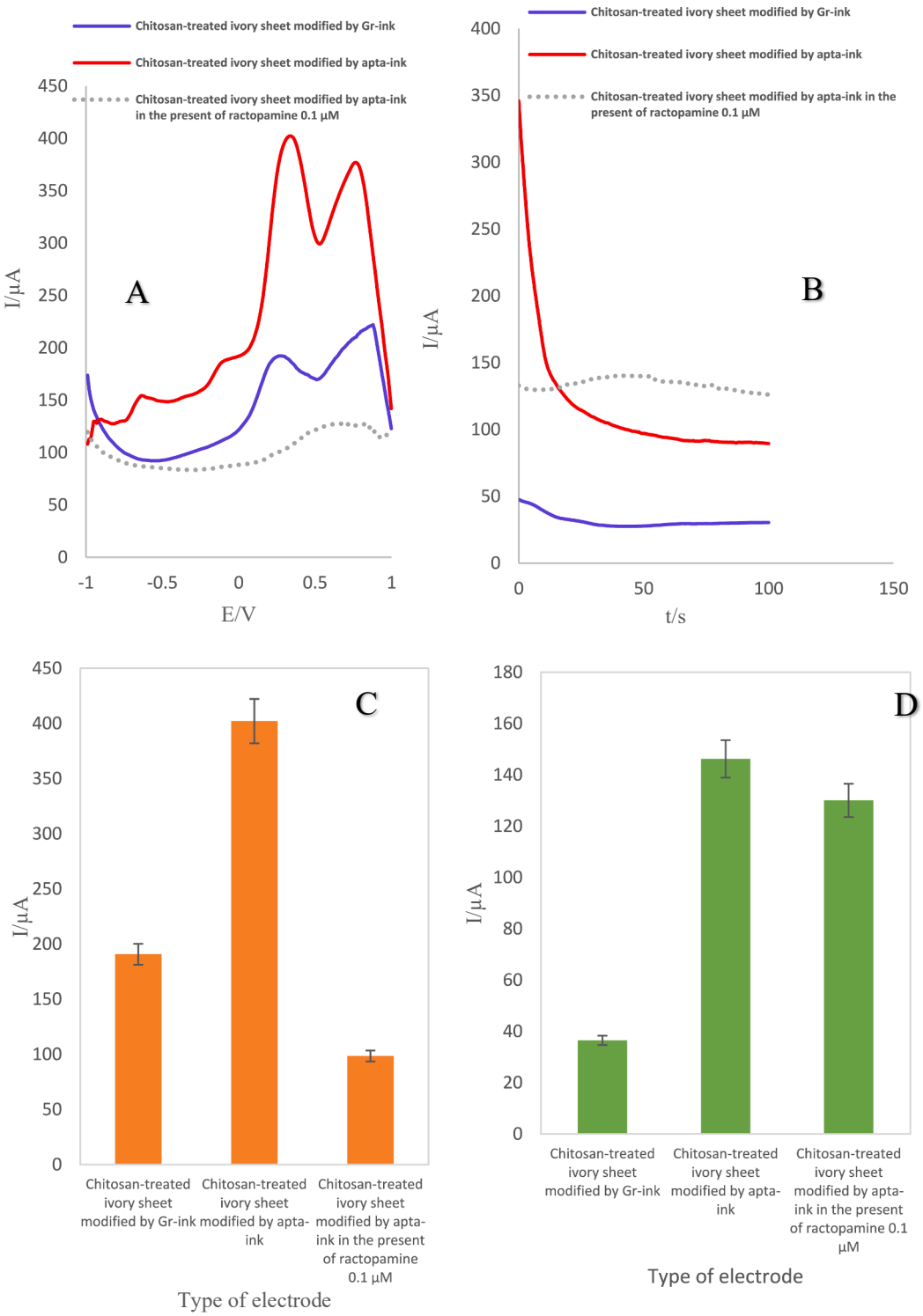


Fig. 3. (A, B) SWVs and ChAs of chitosan-treated ivory sheet modified by Gr-ink, chitosan-treated ivory sheet modified by apta-ink, chitosan-treated ivory sheet modified by apta-ink in the presence of 0.1 µM RAC in the solution of 0.1 $Fe(CN)^{3-/4-}$. (C, D) Histogram of peak current versus type of electrode (SD = 2.04, n = 3).

3.6. Aptasensor stability

The intraday stability of fabricated aptasensor as great importance factor for point-of-care diagnostic applications was evaluated in three days. As shown in Fig. S7 (see supporting information), developed aptasensor has acceptable stability which related to high stability of nano-ink and use of chitosan for chemical binding of inks to the ivory sheet as substrate. Therefore, prepared paper based aptasensor was stable enough for detection of RAC in three days.

Besides sensitivity and selectivity, the reproducibility of the RAC aptasensor was evaluated. Five different electrodes were conducted independently for 100 µM of RAC. Four repetitive measurements of the aptasensor yielded a reproducible ChAs signal with a relative standard deviation (RSD) of 2.41% which represented a satisfactory acceptable precision and good reproducibility of the aptasensor. To study the repeatability effect of the proposed aptasensor, current signal of ChAs

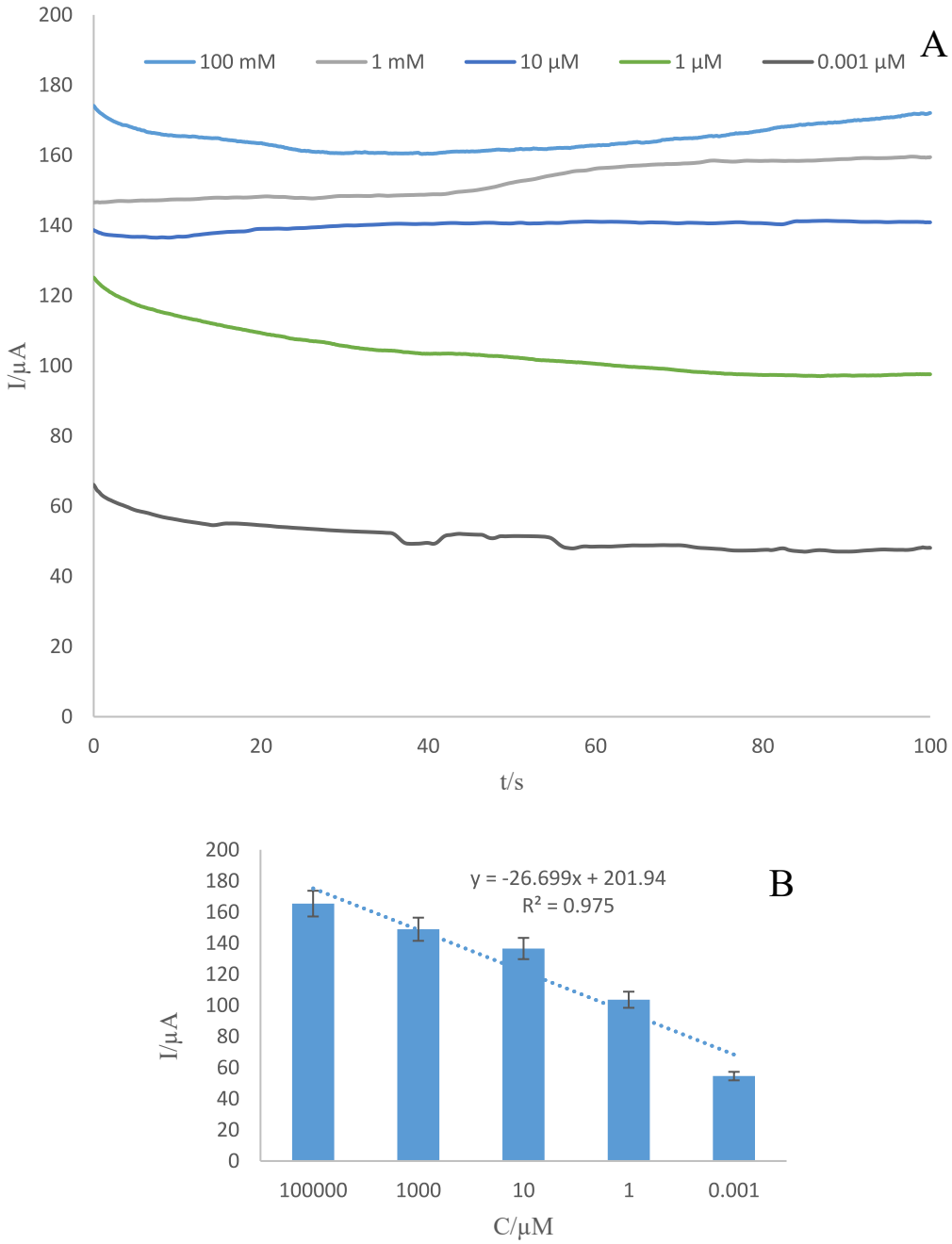


Fig. 4. (A) ChAs of aptasensor in different concentration of RAC (100,000–0.001 µM) (B) Calibration curve of aptasensor (SD = 1.96, n = 3).

related to a modified electrode which was incubated with 100 µM of RAC was recorded and this manner was repeated four times. According to the obtained results, the resulted data are close together and illustrates the relatively high repeatability of the developed aptasensor.

4. Conclusion

As a result, a three-electrode platform paper based aptasensor using direct writing of ink on the barrier-free substrate was designed. For this purpose, three types of inks were used: i- AgNPr/GQDs ink to prepare the working and counter electrode, ii- Gr ink to prepare apta-ink and iii- commercial pen containing Ag ink. The electrical conductivity and resistance of the synthesized inks on different surfaces were evaluated using LED lamps and ohmmeters. Engineered aptasensor was used to electrochemically detection of RAC. The electrochemical behavior of aptasensor was evaluated using chronoamperometric technique. The methylene blue in apta-ink acts as an electron transfer interface and improves the electrochemical detection of RAC. The results revealed that with increasing the concentration of RAC, electrochemical signal of the aptasensor was decreased which linear range was 0.001 µM to 100 mM with LLOQ of 0.001 µM. Constructed aptasensor was also used to measurement of RAC in human plasma specimens. According to the proposed protocol, the use of conductive inks for the manufacture of paper-based aptasensors plays a critical and special role. Although various types of electrochemical biosensor based on different substrate have been developed for detection of RAC, we focused on excellent advantages flexibility, availability, disposability and weight of paper based substrate which could create a novel, low-cost and simple amperometric aptasensor for the monitoring of RAC. It worth to noted that the simple process of synthesizing inks and low-cost materials leads

Table 1
Recent electrochemical biosensors for detection of RAC based on various substrates.

Assay method	Detection method	Electrode	Biorecognition element	Used materials	LOD [a] and LLOQ [b]	Dynamic range	Ref
Electrochemical	Impedance	Gold electrode	Antibody	Chitosan-AuNPs	6.7×10^{-15} M (LOD)	0.01–5 ng•mL^{-1}	(He, Guo, Song, Zhang, Wang, Peng, et al., 2017)
	Voltammetric	Gold electrode	Antibody	Reduced oxide graphene and Cu/Cu_2O nanocrystal	7.5 pg mL^{-1} (LOD)	0.1 –10 ng mL^{-1}	(Wang, Kang, Guo, Fang, He, Jia, et al., 2015)
	Impedance	Glassy carbon electrode	Aptamer	AgNPs/QDs @GQD	330×10^{-18} M (LOD)	1×10^{-15} to 901.4×10^{-9} M	(Roushani, Ghanbarzadeh, Shahdost-Fard, Sahraei, & Soheyli, 2020)
	Voltammetric	Glassy carbon electrode	Antibody	Agarose hydrogel films	–	1–1000 ng/mL	(Shen & He, 2007)
	Voltammetric	Screen-printed carbon electrode	Antibody	Reduced graphene oxide and silver–palladium alloy nanoparticles	1.52 pg mL^{-1} (LOD)	0.01–100 ng mL^{-1}	(Wang, Zhang, Li, Du, Ma, Wu, et al., 2013)
	Amperometric	Paper based	Aptamer	Graphite ink and silver nanoprism ink	0.01 μM (LLOQ)	0.001–100 mM	This work

[a] LOD; Limit of detection.
[b] LLOQ; Low limit of quantification.

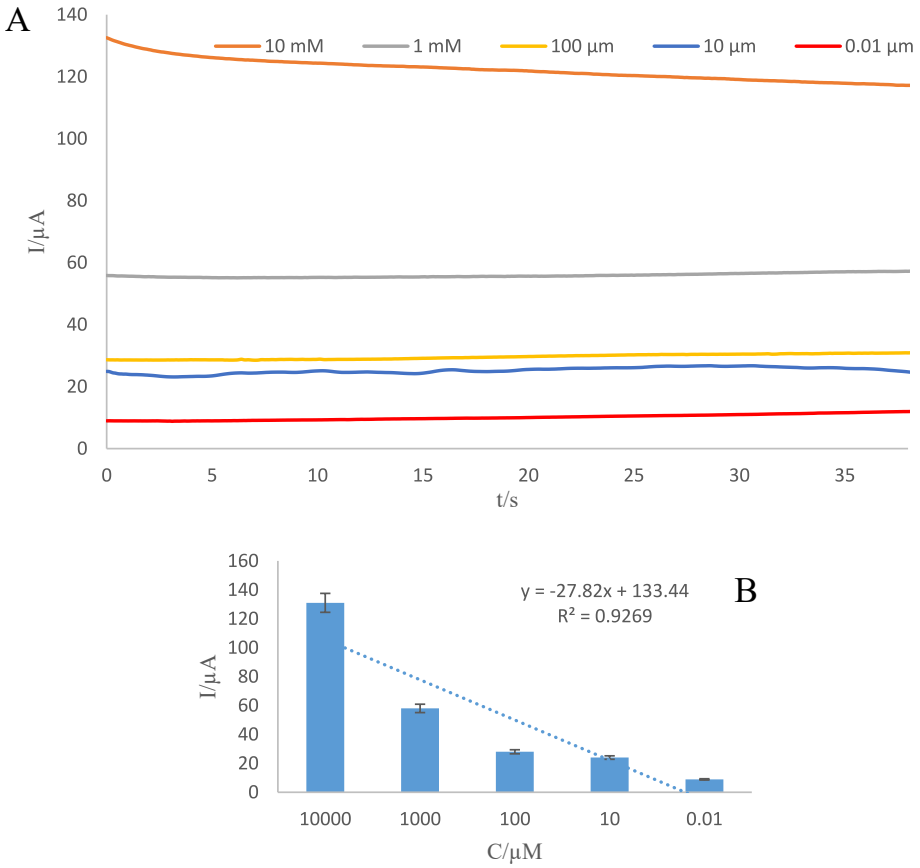


Fig. 5. (A) CHA records of aptasensor in various concentration of RAC in biological fluids (10,000, 1000, 100, 10, and 0.01 μM) (B) Calibration curve of aptasensor. (SD = 1.87, n = 3).

Table 2
Real measurements of RAC at different concentrations in real samples.

Type of sample	Added/μM	Found/μM	Recovery (%)
RAC in human plasma samples	0.01	0.0088	88
	100	101	101
	10,000	9998	99.98

to cost-effective aptasensors. We hope that, the application of aptamer in electrochemical paper based sensor will be able to broaden our understanding for developing the application of low-cost and portable biodevices for the sensitive and selective paper-based sensor to identify other chemical and biological compounds.

Ethical issues

This study was ethically approved (Grant No. 68635) by Tabriz University of Medical Sciences.

CRediT authorship contribution statement

Houman Kholafazad Kordasht: Investigation, Formal analysis, Validation, Writing - original draft. **Arezoo Saadati:** Investigation, Formal analysis, Validation, Writing - original draft. **Mohammad Hasanzadeh:** Conceptualization, Supervision, Investigation, Formal analysis, Validation, Writing - review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131411.

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