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Amperometric biosensor based on horseradish peroxidase for biogenic amine determinations in biological samples

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Abstract

An amperometric biosensor for total biogenic amine determinations, using a carbon paste electrode modified with horseradish peroxidase (HRP) enzyme is described. The HRP immobilization on graphite was made using bovine serum albumin, carbodiimide and glutaraldehyde. The biosensor response was optimized using serotonin and it presented the best performance in 0.1 mol l^{-1} phosphate buffer (pH = 7.0) containing 10 μmol l^{-1} of hydrogen peroxide. Under optimized operational conditions at −50 mV versus SCE, a linear response range from 40 to 470 ng ml^{-1} was obtained. The detection limit was 17 ng ml^{-1} and the response time was 0.5 s. The proposed sensor presented a stable response during 4 h under continuous monitoring. The difference of the response between six sensor preparations was <2%. The sensor was applied in the determination of total biogenic amines (neurotransmitters) in rat blood samples with success, obtaining a recovery average of 102%.

Keywords: Neurotransmitters; Biogenic amines; Serotonin; Amperometric biosensor; HRP enzyme; Modified electrodes

1. Introduction

Among several different species of clinical interest, neurotransmitters are the most investigated compounds. Neurotransmitters are compounds that have a very important role in the nervous system, because they are the key to communication between neurons. Biogenic amines, including catecholamines such as dopamine, norepinephrine, epinephrine and indoleamines such as serotonin, are neurotransmitters that have special roles in neuroscience. Dopamine (DA) is related to Parkinson's disease [1,2]. Epinephrine (EP) and norepinephrine (NE) have been a special interest in the origin of neurological tumors [3,4]. Serotonin (5-HT, 5-hydroxytryptamine) plays an important role in a variety of physiological functions (sleep regulation) and pathological states (psychiatric disorders, depression, mental retardation, infantile autism, etc.) [5]. Medical and pharmaceutical research has focused on this matter in order to clarify the mechanisms and reasons for several neurological disorders, with the aim to open possibilities of pharmacological treatments in a disturbed system.

Many techniques have been developed and improved for detection and quantification of biogenic amines in biological fluids and nervous tissues, including fluorimetry [3], thin layer chromatography with fiber optic detection [6] and capillary electrophoresis [7,8]. Nowadays, high-performance liquid chromatography (HPLC) with fluorimetric detection [9–11], electrochemical detection [12–14] or combined fluorimetric and electrochemical detection [15], has been applied for neurotransmitter determinations. However, all these methods require sample pre-treatment and long analysis times, making them inadequate for routine work [16]. In addition, some of these methods present poor selectivity and/or sensitivity. Thus, the development of quick, efficient, sensitive, rapid and low cost methodology for these very important analytes is still important.

Electrochemical determination of biogenic amines using chemically modified electrodes (CME), such as carbon fiber electrodes [17,18], gold electrodes (modified with host–guest

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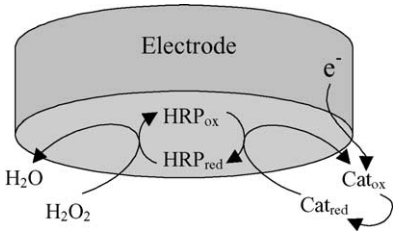
complexes of fullerenes) [19], graphite reinforced by carbon [20], glassy carbon [21–23], copper-plated screen-printed electrodes [24] and carbon paste (modified with iron and copper phtalocyanines) electrodes [25,26], have been reported in the literature, as a good and cheap alternative for the traditional methods.

However, another attractive alternative for biogenic amine determinations are the biosensors, which combine biological recognition through enzyme specificity with construction simplicity. Biosensors, especially the amperometric ones, have been the most successful and still have the most promising future for practical application. In this sense, biosensors for biogenic amines determinations have been reported in the literature based on diamine oxidase (DAO) [27], DAO and HRP [27], and pea seedlings amine oxidase [28] enzymes, for applications in seafood, such as fish and its derivative products.

Among amperometric biosensors, those based on modified carbon paste have been extensively described, in which the carbon paste is a suitable matrix for enzyme immobilization, principally due to the simplicity of the bulk modification, allowing the stabilization of enzymes in the paste, and the possibility of surface renovation [29–31]. Based on this, carbon paste amperometric biosensors become, in potential, a practical tool for rapid and cheap biogenic amine determinations.

Horseradish peroxidase (HRP) has been widely used in the construction of biosensors for phenolic and catecholic compound determinations, since these compounds improve the rate of electron transfer between the electrode and the immobilized enzyme [32–35]. When peroxidase is immobilized on an electrode surface, the oxidized form of the enzyme, which is formed in the reaction with peroxide (its natural substrate), can be reduced to its native form by direct [33,36–39] and/or mediated electron transfer. In the mediated electron transfer mechanism [33,36,37,40,41] (Scheme 1), electron donor compounds, such as catechols are used, which act as electron mediator in the system. This reaction converts catechols to quinones species, which are electroactive and can be electrochemically reduced on the electrode surface. The generated reduction current is proportional to the concentration of catecholic species in solution [32,40,42]. Based on these principles, a lot of phenolic, catecholic and other electron donor substances have been determined by HRP biosensors [32,34,36,37,40,42–45]. However, even though biogenic amines, such as catecholamines and serotonin, are potential analytes to be determined with HRP biosensors, no results have been described in the literature. Based on this context, this work reports biosensor construction with HRP immobilized in a modified carbon paste, to evaluate its potential and performance as a neurotransmitter detector; and their application in the total biogenic amines determination in rat blood plasma samples expressed in term of serotonin.



Scheme 1. Mechanism of mediated electron transfer at a modified carbon paste electrode. Cat_{red} and Cat_{ox} are the reduced and oxidized forms of the catecholic compounds, respectively.

2. Experimental

2.1. Reagents and solutions

All chemicals used were analytical reagent grade. Monopotassium phosphate (KH_2PO_4), 30% (w/v) hydrogen peroxide, disodium phosphate (Na_2HPO_4) and sodium hydroxide were acquired from Synth, São Paulo, Brazil. Serotonin, dopamine, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenosulfonate (CDI), piperazine-*N*-*N*′-bis[2-ethanesulfonic acid] (Pipes), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes), tris-hydroxymethylaminoethane (Tris), bovine serum albumin (BSA) and the HRP lyophilized enzyme (290 U/mg solid) were acquired from Sigma, St. Louis, USA. Epinephrine, norepinephrine, graphite powder (GP) and mineral oil were purchased from Aldrich, Milwaukee, USA. Sodium acetate and acetic acid were supplied by Nuclear, São Paulo, Brazil. A 50% (w/v) glutaraldehyde (Gl) was purchased from Fluka Chemie AG, Buchs, Switzerland.

The solutions were prepared with water purified in a Milli-Q Millipore system and the actual pH of the buffer solutions were determined with a model 350 Corning pH/Ion Analyzer.

2.2. Biosensors construction

For HRP immobilization, first GP activation with CDI was carried out, to obtain the GP-CDI material [46]. For this, 300 mg of GP was mixed with 4.2 mg of CDI and 300 μl of acetate buffer (pH 4.8; 0.05 M), reacting for 2 h at 25 °C. The material obtained (GP-CDI) was washed and allowed to dry for 4 h at room temperature. Then, 40 mg of the GP-CDI material were mixing with 250 μl of 290 U/ml HRP aqueous solution (equivalent to weight 0.25 mg of HRP lyophilized enzyme), 6 μl of 5% (w/v) glutaraldehyde (corresponding to 0.30 mg of Gl) and 160 μl of 2.5 mg ml^{-1} BSA solution prepared in phosphate buffer (pH 7.0; 0.1 M), corresponding to 0.4 mg of BSA, allowing the reaction to proceed for 16 h at 4 °C. After this time the obtained material was dried.

For the modified paste preparation, 30 μl of mineral oil was added to the dried material and mixed until obtaining a homogeneous paste. The biosensor was constructed by putting the modified carbon paste into a cavity of a platinum foil, with a geometrical area of 0.07 cm^2, fixed into a glass tube at 1 mm depth.

For the preparation of an unmodified carbon paste firstly 40 mg of the GP were mixing with 400 μl of phosphate buffer (pH 7.0; 0.1 M). Before dried, this material was mixed with 30 μl of mineral oil for the paste preparation, which was putting into a cavity of a platinum foil, with a geometrical area of 0.07 cm^2, fixed into a glass tube at 1 mm depth, in the same way of the modified carbon paste.

2.3. Amperometric measurements

The amperometric measurements were carried out with a potentiostat (PGSTAT30, Autolab Echo Chemie, Utrecht, Netherlands), using an electrochemical cell with three electrodes: a saturated calomel electrode (SCE) as reference, a Pt wire as auxiliary and the biosensor as working electrode.

The measurements were carried out in 5.00 ml of buffer solution containing H_2O_2 (natural peroxidase substrate) and applying an adequate potential. Initially the current was continuously monitored until it reaches the steady state. After that, additions of standard solution of biogenic amines were made into the buffer solution, which was stirred for a few seconds, in order to homogenize the solution before current monitoring.

The voltammetric measurements were carried out using current–time measurements at potentiostatic conditions, in order to investigate the redox potentials of the four biogenic amines (5-HT, DA, EP, NE) in unmodified carbon paste electrodes. For each compound the paste was exchanged, because there was the electrode surface passivation. Voltammetric measurements were carried out in phosphate buffer (pH 7.0; 0.1 M), containing 1.0 mM of each compound in a scan rate of 10 mV/s.

2.4. Sample preparation

The plasma of the rat blood samples, were supplied by Prof. J.A.R. Gontijo, from Nucleo de Medicina e Cirurgia Experimental of FCM-UNICAMP. These samples were directly used without any previous treatment in the biosensor application.

3. Results and discussions

3.1. Optimization of the paste component amounts in the biosensor preparation

First, the amount of the HRP enzyme in the paste preparation was optimized. Fig. 1 shows that the biosensor response is practically independent of the enzyme quantity for amounts higher than 0.6% of HRP in the paste. Thus, based on this result, pastes containing this percentage of HRP were prepared to perform further experiments.

In spite of the CDI be able to introduce a strong bond between enzyme and electrode, by covalent bonding with carboxylic groups, the possibility of the enzyme active site to contain these groups, and the immobilization to occur through them is viable, could diminish the enzyme activity, supplying biosensors with low sensitivities. The use of stabilizers in the paste preparation is very important, in order to supply more robust biosensor. Thus, in this work the use of Gl in the immobilization procedure promotes a good enzyme reticulation, and BSA supplies an ambient more similar to the natural ones. Considering these aspects, exhaustive studies of adequate amounts of Gl and BSA were carried out, in order to obtain optimum condition for the carbon paste preparation.

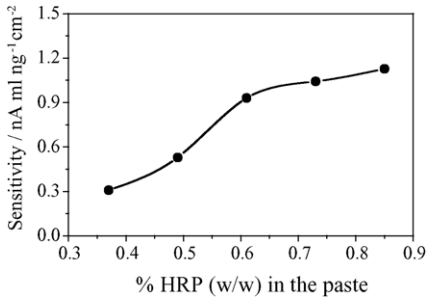


Fig. 1. Influence of the HRP amount on biosensor sensitivity for serotonin determinations. Measurements carried out in phosphate buffer (pH 7.0; 0.1 M) containing 10 μM H_2O_2 and applying −50 mV vs. SCE. Concentration range between 40 and 470 ng ml^{-1} of serotonin.

Results of the study for optimization of the glutaraldehyde (Gl) amount in the paste preparation are listed in Table 1. These pastes were prepared using 0.4 mg of BSA. It can be observed that when 0.7% (w/w) of Gl is used in the paste preparation, a better response for the biosensor is observed, decreasing for higher amounts of Gl. This behavior can be explained considering that a dependent effect occurs between the paste components, specifically between Gl and BSA. As a consequence, in a determined Gl/BSA mass ratio, the response presented by the biosensor will be the best. However, if this ratio was changed (for Gl amounts lower or higher than 0.7%) the sensor response became worst. Thus, Gl amount of 0.7% was chosen for pastes preparation to perform further optimization studies.

Finally, the BSA amount in the paste preparation was optimized, and the results are shown in Table 2. It can be observed that, when 1.0% of BSA (w/w) is used in the paste preparation, the best results are obtained, considering that with this

Table 1
Influence of the amount of glutaraldehyde on the biosensor current density (Δj) for 213 ng ml^{-1} (1 μM) of serotonin

Gl (%, w/w) in the paste	Δj (nA cm^{-2})
0.4	84 ± 2[a]
0.6	125 ± 1[a]
0.7	179 ± 2[a]
0.9	174 ± 2[a]
1.1	126 ± 4[a]
1.3	95 ± 3[a]

[a] Measurements carried out in phosphate buffer (pH 7.0; 0.1 M), containing 10 μM H_2O_2, applying −50 mV vs. SCE. Standard deviation for three replicates.

Table 2
Influence of the BSA amount on the biosensor response, for 213 ng ml^{-1} of serotonin

BSA (%, w/w) in the paste	Δj (nA cm^{-2})
0.5	99 ± 2[a]
0.7	170 ± 2[a]
1.0	180 ± 3[a]
1.2	138 ± 4[a]
1.5	64 ± 1[a]

[a] Measurements carried out in phosphate buffer (pH 7.0; 0.1 M), containing 10 μM H_2O_2, applying −50 mV vs. SCE. Standard deviation for three replicates.

BSA amount, the optimum Gl/BSA mass ratio is reached. A decrease in the signal is observed with the addition of higher amounts of BSA. This result can be explained due to the increase of the diffusion resistance to an unfavorable and too rigid enzyme fixation, or, yet, to a displacement of HRP by BSA on the graphite surface [47].

3.2. Influence of the applied potential

In the investigation of the applied potential effect on the biosensor response for serotonin, it was observed that the response increases for more negative potentials (data not shown). However, it is known that for potentials more negative than −150 mV a slow irreversible deactivation of HRP occurs [38]. Thus, in order to avoid HRP deactivation, a potential of −50 mV was fixed for further experiments, which allowed obtaining satisfactory results. For more positive potential a significant decrease in the signal was observed.

3.3. Influence of pH, buffer and its concentration

The evaluation of pH effect on the biosensor response showed an optimum pH at 7.0 in 0.1 M phosphate buffer solution (Fig. 2). Experiments carried out in different buffer solutions (Tris, Hepes, Pipes and phosphate), in concentration of 0.10 M indicated that phosphate buffer gives the best response. Finally, the influence of the phosphate buffer concentration on the biosensor response showed that the best result was obtained in a concentration of 0.10 M. Thus, these optimal conditions were used for the subsequent studies.

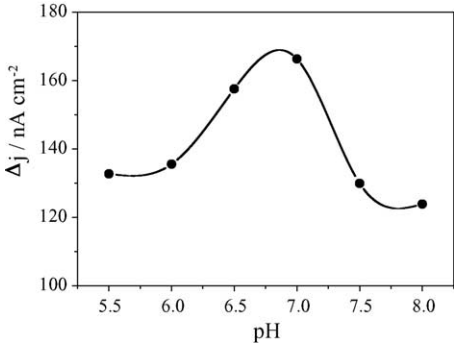


Fig. 2. Response profile for the biosensor in phosphate buffer solutions with different pH values. Applied potential of −50 mV vs. SCE, in phosphate buffer (pH 7.0; 0.1 M) containing 213 ng ml^{-1} of serotonin and 10 μM of H_2O_2.

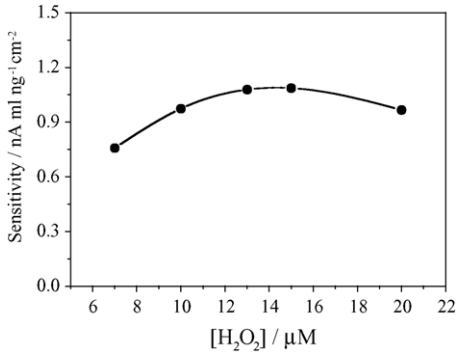


Fig. 3. H_2O_2 concentration effect on the sensitivity of the proposed biosensor. Applied potential of −50 mV vs. SCE, in phosphate buffer (pH 7.0; 0.1 M), in the concentration range of serotonin from 40 to 470 ng ml^{-1}.

3.4. Hydrogen peroxide dependence

Hydrogen peroxide is the natural substrate of the peroxidase enzyme and its presence is essential for the biosensor response. Thus, the effect of H_2O_2 concentration on biosensor performance was investigated, in terms of sensitivity, linear response range and lifetime. This last parameter is very important because it is known that high peroxide amounts can cause enzyme inactivation [48], decreasing the biosensor lifetime. It could be observed in Fig. 3 that, as the H_2O_2 concentration is increased, the biosensor sensitivity is increased, reaching higher values between 13 and 15 μM H_2O_2. However, a concentration of 10 μM H_2O_2 was chosen, which is enough to get good results and also avoids enzyme inactivation.

It is important to emphasize that when unmodified carbon paste electrodes were tested in the catecholamines detection in presence of H_2O_2, under same optimized conditions of the proposed biosensor, practically no signal were obtained (data not shown). This result eliminates the possibility that the H_2O_2 can be oxidizing these analytes without any other mediator.

3.5. Biosensor characteristics

In the optimized conditions the proposed biosensor showed a linear response range from 40 up to 470 ng ml^{-1} of serotonin, which can be expressed according to the following equation:

$$\Delta j\,(\text{nA cm}^{-2}) = 0.01\,(\pm 0.01) + 0.95\,(\pm 0.01) C_{\text{5-HT}}\,(\text{ng ml}^{-1}) \quad (1)$$

with a correlation coefficient of 0.9998 for $n = 10$. This response range is very similar to those showed by L.C. Daws et al., who described a carbon fiber electrode, coated with a

Nafion film, for in vivo chronoamperometric measurements of the serotonin in rat [49]. The detection limit of 17 ng ml^{-1} was calculated from a signal/noise ratio equal to 3, this detection limit is much better than the other biosensor for serotonin determination where the amine oxidases enzyme was used [50]. These excellent characteristics qualify this proposed biosensor for serotonin determination in most real samples.

The response time, considering the time to reach 100% of the signal, was shorter than 1 s. Repeatability in the sensor construction was evaluated by preparing six sensors and determining the sensitivity obtained for each one. The repeatability expressed as the relative standard deviation (r.s.d.) was lower than 2%. This result indicates a very good repeatability in sensor construction.

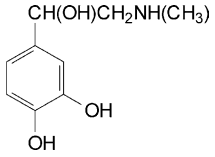
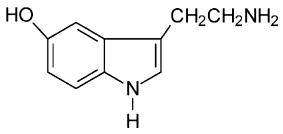
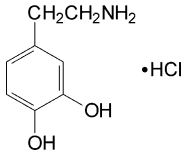
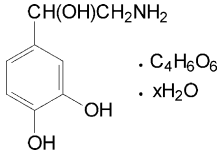
Under optimized conditions the proposed sensor presented good stability, at least during 4 h of continuous use, making possible in a next step the coupling of this proposed biosensor in a flow system.

The influence of the interfering species on biosensor response is another important parameter that was considered in order to the biosensor application in biological samples. In this sense, the interference of reducing metabolites such as ascorbic and uric acids was investigated. Since these species can be directly oxidized on the electrode surface or still react with the H_2O_2 necessary for obtaining the signal, decreasing the biosensor response. The studies demonstrated that the ascorbic and uric acids do not interfere in the serotonin determination in a 1:4 molar ratio (interfering:serotonin).

3.6. Response for other neurotransmitters

Although the biosensor response was optimized in function of the serotonin, it is obvious that the proposed biosensor can detect others cathecolic and monophenolic compounds (Scheme 1). However, it is known that the sensitivity for the monophenols obtained with HRP based biosensor is lower than those obtained with diphenolic systems [32,35]. Among the cathecolic compounds, the catecholamines, such as dopamine, norepinephrine and epinephrine can be detected. Thus, in the case of an analysis of a real sample this biosensor supplied the total amount of neurotransmitter based on biogenic amines (5-HT, DA, NE and EP) contained in the sample and that can be detected by the biosensor. In these sense, the biosensor characteristics for biogenic amines besides serotonin, such as DA, NE and EP, were studied in detail. Table 3 shows the analytical parameters for the evaluated neurotransmitters. The best responses in terms of sensitivity, and consequently selectivity, were obtained with dopamine and epinephrine, followed by serotonin and norepinephrine, respectively. The same responses were expected for DA, EP and NE, since their chemical structures are very similar. This behavior can be explained considering the redox potentials of these compounds in an unmodified carbon paste electrode. The order is DA < EP < NE < 5-HT (Table 3). Thus, compounds with lower redox potential should be more easily and rapidly oxidized, allowing better responses according to the biosensor mechanism. On the other hand, the better response was obtained for serotonin when compared with norepinephrine,

Table 3
Analytical parameters of the biosensor for various biogenic amines

Biogenic amine	Structure	Redox potential[a]	Linear range (ng ml^{-1})	Sensitivity (nA ml ng^{-1} cm^{-1})	Detection limit (ng ml^{-1})
Epinephrine		242	35–405	1.23 ± 0.03 (r = 0.9977; n = 10)	14
Serotonin		350	40–470	0.95 ± 0.02 (r = 0.9998; n = 10)	17
Dopamine		190	38–420	1.23 ± 0.02 (r = 0.9997; n = 10)	19
Norepinephrine		306	60–705	0.71 ± 0.01 (r = 0.9995; n = 10)	33

[a] Potential values in mV vs. SCE, obtained using an unmodified carbon paste electrode in phosphate buffer (pH 7.0; 0.1 M).

Table 4
Values of total biogenic amines, in term of serotonin, obtained with the proposed biosensor, through standard addition method for seven rat blood samples

Sample number	Serotonin concentration (ng ml^{-1})
01	44 ± 2[a]
02	68 ± 6[a]
03	44 ± 4[a]
04	ND
05	47 ± 6[a]
06	ND
07	ND

[a] Standard deviation for three replicates. ND: non-determined, values lower than quantification limit.

even though the serotonin oxidation potential is higher than for norepinephrine. It could be explained considering the ionic pair formed with tartarate, making difficult the interaction with HRP active site.

It is important to emphasize that in spite of the biogenic amines are electroactives on unmodified carbon paste electrode, under the optimized conditions for the proposed biosensor, no responses were obtained for any of these analytes in unmodified electrodes (data not shown). On the other hand, it is known that in unmodified electrodes exists a high probability of electrode surface passivation through polymerization by intermediate phenolic derivatives, which is minimized or eliminated when chemically modified electrodes are used, mainly those containing enzymes.

3.7. Application in biological samples

The proposed biosensor was tested in the determination of the total biogenic amines in the blood. Thus, Table 4 shows the results obtained in the biosensor application for biogenic amines determination, in term of serotonin, obtained through the standard addition method in the serum samples. In order to evaluate the matrix effect Table 5 shows the results obtained in the recovery experiments of seven different samples. The results obtained suggest that this proposed biosensor can be applied very well in biological samples with no significant influence of the matrix. Even for samples 4, 6 and 7 that the serotonin was in very low concentration (in the pg ml^{-1} level) being non-detected by the proposed sensor, the recoveries were very good giving an average recovery of 101 ± 3%.

Table 5
Recovery % obtained with the biosensor after serotonin addition (125 ng ml^{-1}) in each rat blood samples

Sample number	Recovery value
01	105 ± 2[a]
02	103 ± 7[a]
03	97 ± 6[a]
04	104 ± 3[a]
05	105 ± 4[a]
06	96 ± 2[a]
07	102 ± 2[a]

[a] Standard deviation for three replicates.

The biosensor presented a linear response range for serotonin determination in the range between 40 and 470 ng ml^{-1} of serotonin. Considering that in human blood the serotonin level is around 76 ng ml^{-1} for healthy subject [19], thus it could be also employed for serotonin determination in human blood samples.

4. Conclusion

This work reports an easy biosensor construction for neurotransmitter determination, modifying carbon paste with BSA, glutaraldehyde and CDI. The biosensor construction is highly reproducible, allowing to obtain biosensors with very similar sensitivities (r.s.d. < 2%). The biosensor presented a linear response range between 40 and 470 ng ml^{-1} of serotonin, with a very good sensitivity, making possible the determination of neurotransmitters in blood samples, and opening the possibilities for carry out serotonin and/or total biogenic amines quantification in human blood samples. In this sense, the proposed biosensor offer a good alternative to the existing methods, allowing carried out rapid, simples and low cost analysis with no pre-treatment of the sample.

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