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ARTICLE

Chromatographic paper-based analytical devices using an oxidized paper substrate

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A novel detection scheme using chromatographic retention was proposed for paper-based analytical devices (PADs). Using a wax printer and an ink-jet printer, the PADs consisted of a straight-flow channel, a circular sample zone, and a band of brilliant green (BG) printed at the connection between the sample zone and the flow channel. When a constant volume of a sample solution was applied to the sample zone, the BG band formed a colored bar along the flow channel that marked the adsorption and desorption of the paper substrate according to the principles of chromatography. If the sample solution contained an anionic complex of boric acid with chromotropic acid, the anionic complex enhanced desorption of the BG from the paper substrate via the formation of an ion-pair with the BG, which resulted in an elongated colored bar. Fundamental equations for the retention behavior of the BG on the PADs were derived using a model based on chromatographic principles and ion-pairing formation. A retardation factor, R_f, was correlated with the concentration of boric acid contained in the sample solutions. To enhance the adsorption of the BG, 2,2,6,6-tetramethylpiperidine 1-oxyl was used to oxidize the paper substrate. The oxidized paper substrate shortened the colored bar of a blank solution and formed a clear boundary for the color. When the analytical conditions were optimized for pH and the concentration of chromotropic acid, the PAD permitted the measurement of boric acid for a concentration ranging from 0.3 to 3 mM. The proposed model was validated when the fitting curve was calculated using the derived equations, which resulted in good agreement with the experimental data.

1. Introduction

The interest in paper-based analytical devices (PADs) has grown rapidly in fields that require point-of-care testing and on-site analysis since 2007 when the fundamental concept was proposed by the Whitesides group.[1] Advantages of the PADs include the low cost of substrates, easy fabrication and operation, disposability, and portability. These characteristics enable the PADs to work more effectively in situations where the availability of instrumentation and operator expertise is limited, as in ordinary home settings and in developing countries. Therefore, the promotion of instrument-free analysis is desirable for PADs.[2]

Different types of detection schemes based on visual judgment have been reported, and these have included time-based,[3–6] distance-based,[7–10] and zone-counting forms of detection.[5,11–13] Time-based detection was demonstrated by the Philips and Zhang groups using enzymatic reactions in which a color change occurred at different times depending on the concentration of the analytes.[3,4] Conversely, Henry's group developed a distance-based PAD that can quantify analytes without capturing images via a scanner or a digital camera. The distance-based PAD determined the amount of an analyte via the distance of the color bar as it evolved on the PAD.[7–10] Zhang's group employed both the measurement of reaction time and a count of the number of detection zones that changed color.[5,6,13] We have also reported titrations with PADs wherein the endpoint can be judged by counting the number of detection zones with the color change of an indicator.[11,12] Counting-based methods are also applicable to a quantitative immunoassay.[14]

Recently, distance-based PADs have been applied to several targets such as lead and antioxidants.[15,16] The mechanisms of the detection schemes used in these PADs are fundamentally based either on the precipitation reaction of a colored product between an analyte and a colorimetric reagent or on immobilized materials that change color via a reaction with analytes. For example, iron ions form an insoluble complex with bathophenanthroline that results in a red color bar the length of which is dependent on the concentration of iron.[8] Likewise, nickel ion forms a red complex with dimethylglyoxime to form a red bar on the channel of a distance-based PAD.[8] In addition, the sensitivity of a distance-based PAD can be improved by introducing a large volume of a sample, as we demonstrated in the determination of iron ions.[17] While the distance-based detection scheme is applicable to several reactions of precipitation formation, other chemical reactions will expand the utility of distance-based PADs.

Here we use chromatographic principles to propose a novel detection scheme for distance-based PADs. A dye was

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deposited at the entrance to a flow channel that led to a sample zone where a constant volume of a blank or sample solution is introduced. The solution transports the dye along the flow channel according to chromatographic principles. When the sample solution contains an analyte that forms a water-soluble ion-pair with the dye, the ion-pair formation weakens the interaction of the dye with the paper, and this results in an elongation of the bar formed by the dye on the paper substrate, which increases as the dye dissolves into the sample solution. We derived fundamental equations for the proposed detection scheme and demonstrated the determination of boric acid in aqueous solutions using the chromatographic PAD. The derived equations fulfilled a fitting curve that was in good agreement with the experimental data.

2. Theory

In the present study, when the band of brilliant green (BG) was deposited at the connection between the sample zone and the flow channel, it could move with the adsorption and desorption of the paper substrate. Thus, the paper substrate and the sample solution acted as the stationary and mobile phases, respectively, in this form of paper chromatography. According to the theory of chromatography, solutes flow through the stationary phase only when they are dissolved in the mobile phase. Therefore, the chromatographic retention of BG is expressed in equation (1).[18]

$$V_{BG} = \frac{[BG]_m}{[BG]_s+[BG]_m} V_0 \qquad (1)$$

In equation (1), V_{BG} and V_0 are the retention volume of BG and the void volume, and $[BG]_s$ and $[BG]_m$ are the concentrations of BG in the stationary phase and the mobile phase, respectively. It should be noted that we omitted the charges of the species in all equations in order to simplify the description. The equilibrium constant of the BG between the stationary and mobile phases is given by equation (2).

$$K_{BG} = \frac{[BG]_s}{[BG]_m} \qquad (2)$$

Then, equation (1) can be rewritten as equation (3).

$$V_{BG} = \frac{1}{1+K_{BG}} V_0 \qquad (3)$$

Therefore, the retardation factor, R_f, for the BG is presented as equation (4).

$$R_f = \frac{V_{BG}}{V_0} = \frac{1}{1+K_{BG}} \qquad (4)$$

In this study, R_f indicates the ratio of the distance traveled by the BG to the distance traveled by the solvent in the flow channel. Therefore, in principle, the volume and flow rate of the sample should have no influence on the R_f. In contrast to standard paper chromatography, here, the BG forms a colored bar instead of a spot because the BG is continuously supplied from the band deposited at the connection between the sample zone and the flow channel.

Conversely, in the presence of an anionic complex, X, which forms a water-soluble ion-pair with the BG, equation (1) can then be rewritten as equation (5).

$$V_{BGX} = \frac{[BG]_m+[BGX]_m}{[BG]_s+[BG]_m+[BGX]_m} V_0 \qquad (5)$$

In equation (5), $[BGX]_m$ is the concentration of the ion-pair BGX in the mobile phase. Here, we assume that BGX has no interaction with the stationary phase (paper) because of a high negative charge, which dictates that the concentration of BGX in the stationary phase is zero ($[BGX]_s=0$). The equilibrium constant of the ion-pairing formation is defined by equation (6).

$$K_{IP} = \frac{[BGX]}{[BG][X]} \qquad (6)$$

In equation (6), [X] is the concentration of the anionic complex. The anionic complex is also assumed to have no interaction with the stationary phase due to its high negative charge. Equation (5) can then be expressed as equation (7).

$$V_{BGX} = \frac{[BG]_m+K_{IP}[BG]_m[X]}{K_{BG}[BG]_m+[BG]_m+K_{IP}[BG]_m[X]} V_0 \qquad (7)$$

In our model, we assumed that the ion-pair would form only in the mobile phase, and $[BG]_s$ would decrease with the formation of BGX because $[BG]_m$ was consumed by the ion-pair formation of BGX. Equation (7) can then be expressed by substituting equations (2) and (6).

$$V_{BGX} = \frac{1+K_{IP}[X]}{K_{BG}+1+K_{IP}[X]} V_0 \qquad (8)$$

Consequently, R_f can be expressed as equation (9).

$$R_f = \frac{1+K_{IP}[X]}{K_{BG}+1+K_{IP}[X]} \qquad (9)$$

When $K_{IP}[X]$ is much smaller than $(K_{BG}+1)$, equation (9) can be expressed as equation (10).

$$R_f = \frac{1+K_{IP}[X]}{K_{BG}+1} \qquad (10)$$

In a low-concentration range, therefore, R_f is directly proportional to the concentration of X.

3. Materials and methods

3.1. Chemicals and reagents

All reagents were of analytical grade and were used without further purification. Boric acid, chromotropic acid disodium salt dehydrate, sodium hydroxide, sodium bromide, sodium chloride, sodium fluoride, sodium nitrate, sodium sulfate, 2-propanol, sodium hypochlorite solution, 2,2,6,6-tetramethyl-1-piperidinyloxy, radical (TEMPO), azomethine H, L(+)-ascorbic acid, ammonium acetate, phosphoric acid, citric acid

monohydrate, and disodium dihydrogen ethylenediaminetetraacetate dehydrate all were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Barbituric acid was bought from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Brilliant Green (BG) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Commercially available eye-drops (ROHTO, Tokyo, Japan) were bought from a local drug store. Ultrapure water was prepared using a Direct-Q UV water purification system (Millipore Co., Ltd., Molsheim, France). The reagent solution for ink-jet printing contained 30 mM BG in a 60% 2-propanol and 40% water solution. Standard solutions of 100 mM boric acid and 100 mM chromotropic acid were prepared by dissolving the appropriate amounts of the reagents in water, respectively. Sample solutions were prepared as follows. First, 1 mL of 100 mM chromotropic acid and an appropriate volume (0-300 µL) of 100 mM boric acid were combined in a beaker. To adjust the pH of the solution, 0.9 mmol of barbituric acid was added to the beaker, and then the pH of the solution was adjusted to 4 by adding 0.5 M of NaOH solution. The solution was transferred to a 10-mL volumetric flask and diluted to 10 mL. The sample solutions contained 10 mM chromotropic acid, 90 mM barbiturate, and 0.3 to 3 mM borate at pH 4. A 40-µL aliquot of a sample solution was introduced into the PAD to develop the BG color band. The leading ends of the BG bar and the solvent were marked after 50 min in order to calculate the R_f value.

3.2. Fabrication of the PAD

Sheets of filter paper (200×200 mm, Chromatography Paper 1CHR, Whatman, GE Healthcare) were initially oxidized by TEMPO according to a procedure reported in the literature.[19] To oxidize the paper, a solution was prepared by dissolving 0.164 g of TEMPO and 1.08 g of sodium bromide in 1.05 L of water, which was then poured into a 19×25×4 cm container. Then, three sheets of paper were immersed in the solution, and oxidization was initiated by adding 27 mL of 12%(w/v) sodium hypochlorite solution, and the pH was adjusted to 10 via the addition of 0.1 M HCl. The oxidation reaction was carried out for 20 min by maintaining the pH at 10 via monitoring and adjustment using 0.5 M NaOH. The oxidized paper sheets were washed with water three times and dried at room temperature.

The chromatographic PAD was designed using Microsoft Office Power Point 2013, as shown in Fig. 1. The PADs were printed on oxidized paper sheets using a wax printer (ColorQube 8580N, Xerox, CT, USA), which was followed by heating at 120 °C for 2 min in a drying machine (ONW-300S, AS ONE Corp., Osaka, Japan). The reagent solution of the BG was then printed at the connection between the sample zone and the flow channel 8 times using an ink-jet printer (PIXUS, iP2700, Canon, Tokyo, Japan), according to a method reported by the Henry group and also by the Citterio group.[8,20] After drying, both faces of the paper sheet were laminated using a laminator (NEL-101A4, Nakabayashi, Tokyo, Japan). The sample zone was punched (5.5 mm, CARL, Tokyo, Japan), and the backside of the punched sample zone was covered with clear tape. To prevent bending of the filter paper, the PADs were fixed on the lab bench using double-sided tape.

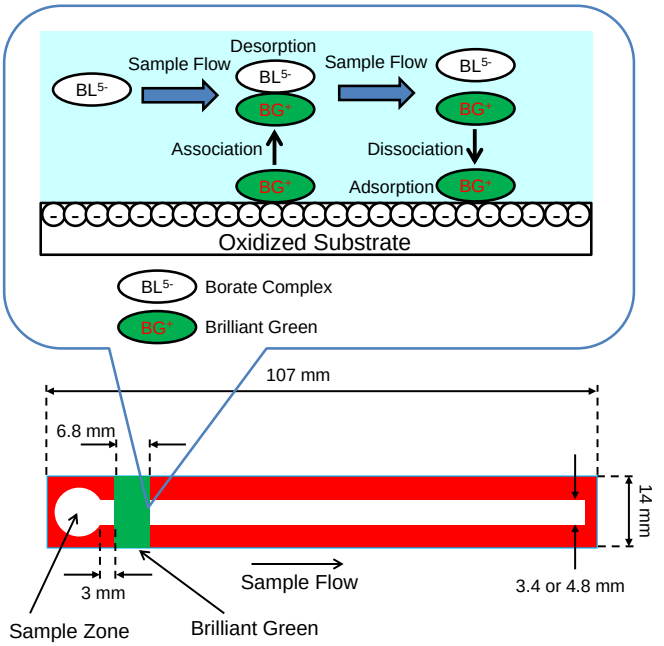

Fig. 1 Design and retention model of the chromatographic PAD.

4. Results and Discussion

4.1. Retention behavior of the BG.

As described in the theory section, the R_f value of BG depends on the concentration of the anionic species that forms an ion-pair with the BG. Thus, the principle of the chromatographic PAD was validated in this study using the complex between the boric acid and the chromotropic acid as an analyte, because the anionic complexes between 1,8-dihydroxynaphthalene derivatives (including chromotropic acid) and boric acid form an ion-pair with the cationic BG.[21] Figure 2 shows the results for samples with and without boric acid (3 mM) in a buffer solution containing 10 mM chromotropic acid and 90 mM barbiturate buffer (pH 4.0). The distance for the elongation of the BG slightly increased when the sample contained a complex of boric acid and chromotropic acid, as seen in Fig. 2. However, the difference in the distance was too small to allow a discrimination of lower concentrations. Therefore, the distance for the blank sample (without boric acid) was shortened in order to improve the sensitivity.

The long distance of the blank sample was attributed to a weak interaction of the BG with the paper substrate. Therefore, we oxidized the paper substrate in order to strengthen its interaction with the BG. The oxidation reaction converted the hydroxyl groups of the filter paper to carboxyl groups, which dissociated to produce anionic functional groups. Thus, the electrostatic interaction of the BG with the paper substrate was enhanced, which resulted in a shorter distance for the blank sample. We oxidized paper samples at reaction times of 10, 20, and 30 min, and found that a reaction time of 30 min

deteriorated the paper and resulted in a pulping effect that causes the cellulose fibrillation and the long cellulose fibers to short fragments.[22] Therefore, the oxidation time was maintained at 20 min for the remaining experiments.

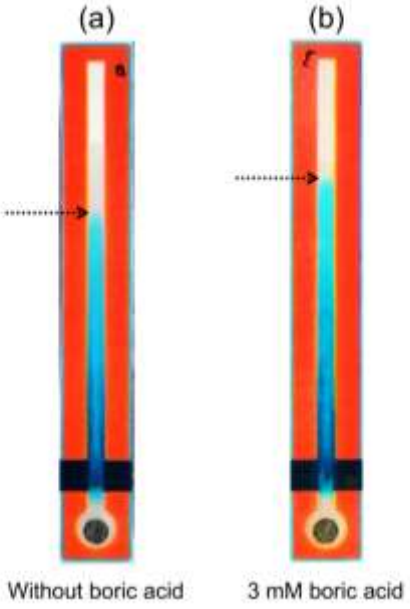

Fig. 2 Signals of the chromatographic PAD fabricated with untreated filter paper. Channel width, 4.8 mm; samples, 90 mM barbiturate buffer and 10 mM chromotropic acid (pH 4.0) with and without 3 mM boric acid; sample volume, 40-µL.

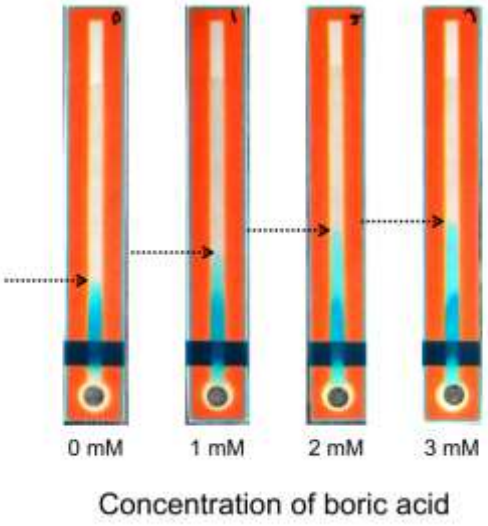

Fig. 3 Signals of the chromatographic PAD fabricated with oxidized filter paper. Channel width, 4.8 mm; samples: 90 mM barbiturate buffer and 10 mM chromotropic acid (pH 4.0) containing 0, 1, 2, and 3 mM boric acid; Sample volume, 40-µL.

Figure 3 shows retention of the BG on the oxidized paper substrate when samples of boric acid were introduced in various concentrations, 0, 1, 2, and 3 mM, into the sample zone. The length of the colored bar for the blank sample was significantly shortened via enhancement of the interaction between the BG and the oxidized paper substrate, compared with that shown in Fig. 2. According to eq. 10, the linearity of the calibration curve ranged from 0 to 3 mM ($y = 85.5x + 0.286$, $R^2 = 0.999$) with limits of detection (LOD) of 0.3 mM, which showed an increased length that was 3-fold that of the standard deviation for a blank sample. The relative standard deviations for the R_f values were within less than 4.2% for n=3. The LOD is not low enough to determine boron in drinking water since the permissible concentration of boron in drinking water is defined as 2.4 mg L^{-1} in the WHO guidelines, which corresponds to 0.2 mM.[23] Therefore, the PAD is only useful for rough estimation for judging whether the concentration of boric acid is under the safety level.

4.2. Curve fitting

Using equation (9), we fitted the experimental data to a theoretical curve representing the relationship between the concentration of boric acid and the R_f value. The relationship was linear up to 3 mM according to equation (10), but the curve deviated from linearity above 3 mM, as expected from equation (9). We estimated K_{BG} from the intercept of the y-axis (R_f=0.28), as indicated in equation (10), resulting in K_{BG}=2.57. Using this value, 580 M^{-1} for K_{IP} was obtained by fitting the experimental data to equation (9). Figure 4 shows a fitting curve using equation (9) that is in good agreement with the experimental data. It is noteworthy that K_{BG} varied depending on the oxidation of the paper substrate whereas K_{IP} was independent of the paper substrate. For example, another batch of oxidized paper substrate gave the values of K_{BG}=3.12 and K_{IP}=580 M^{-1}. According to our model, K_{BG} is determined by the interaction between the paper substrate and the solution, while K_{IP} represents equilibrium in the solution (mobile phase). Therefore, a constant K_{IP} value also supported the validity of the present model for a chromatographic PAD.

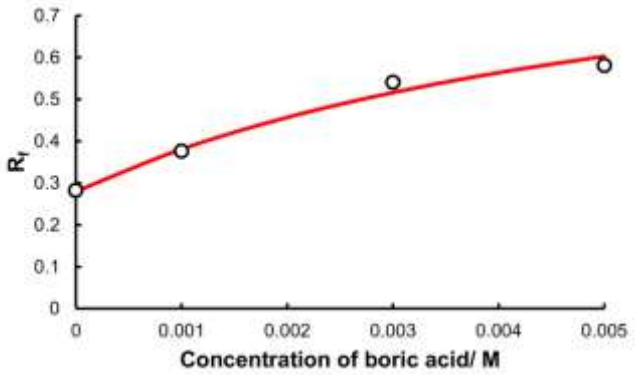

Fig. 4. Dependence of the R_f value on the concentration of boric acid. Circles indicate the experimental data. The solid line is drawn to fit the data according to equation 9 using the following constants: K_{BG}=2.57, K_{IP}= 580 (M^{-1}). The channel width is 3.4 mm.

In the present study, the conditions for the samples were optimized to obtain the maximum slope of the calibration curve in terms of pH and the concentration of chromotropic acid (Supplementary Information, Fig.S1). The maximum slope was

obtained at pH 4, whereas the concentration of chromotropic acid was optimal at 10 mM. Under these conditions, boric acid exists as $B(OH)_3$, $B(OH)_2(chr)^{3-}$, and $B(chr)_2^{5-}$ whereas chr^{2-} represents a divalent anionic chromotropate ion. The reported constants for the acid dissociation of chromotropic acid and the complex formation between boric acid and chromotropic acid are listed in Table 1.[24,25] Using these values, the fractions of the chemical species for boron were calculated as given in Table 2. More than 95% of boron exists as a 1:2 complex, $B(chr)_2^{5-}$, with a pentavalent negative charge, although the fractions of the chemical species slightly depended on the concentration of boric acid under the present experimental conditions (pH 4, 10 mM chromotropic acid). Therefore, during curve fitting, it was understandable that the concentration of the complex approximated that of boric acid.

Table 1 Acid dissociation constant and complex formation constants

Definition	Value	Ref
$K_{a1} = \frac{[H^+][Hchr^{3-}]}{[H_2chr^{2-}]}$	pK_{a1} = 5.35	[23]
$\beta_1 = \frac{[H^+][B(OH)_2chr^{3-}]}{[B(OH)_3][H_2chr^{2-}]}$	$\log\beta_1$=-1.57	[24]
$\beta_2 = \frac{[H^+][B(chr)_2{}^{5-}]}{[B(OH)_3][H_2chr^{2-}]^2}$	$\log\beta_2$=2.35	[24]

Table 2 Fractions of chemical species.

Species	Fraction		
	Concentration of boric acid/ mM		
	1	3	5
$B(OH)_3$	0.0096	0.0059	0.0183
$B(OH)_2(chr)^{3-}$	0.0174	0.0137	0.0238
$B(chr)_2^{5-}$	0.9729	0.9804	0.9578

4.3. Interference and application

Interference from other anions was examined using solutions containing 3 mM of boric acid and 20 mM of each of the anions listed in Table 3. As shown in Table 3, no significant interference was observed for Cl^-, NO_3^- and SO_4^{2-} while F^- caused a negative error. The interference of F^- can likely be attributed to the complex formation with boron during the production of partially fluorinated complexes. Similar results were obtained in the study on the solvent extraction-spectrophotometric determination of boron with 2,4-dinitro-1,8-naphthalenediol and BG.[20] Therefore, the borate complex forms the ion-pair with BG more selectively than small inorganic ions as demonstrated in ref. 20 although some large anions may lead to interferences.

To demonstrate one such practical application, we measured the boric acid contained in commercial eye-drops. The concentration of boric acid in eye-drops may be an important factor which influences lens center thickness and postlens tear film thickness for users of hydrogel contact lens.[26] The result was validated by comparing a standard azomethine-H method for the measurement of boric acid. The chromatographic PADs produced a reading of 160±20 mM, which was in good agreement with the value obtained via the azomethine-H method at 150±0 mM. This result indicates that the proposed method could be applied to the determination of boric acid in eye-drops although the reproducibility is much poorer than the azomethine-H method.

Table 3 Recovery of 3 mM boric acid in the presence of coexisting ions

Concentration of ion	Recovery, %
None	100
20 mM Cl^-	111±8
20 mM NO_3^-	96±8
20 mM SO_4^{2-}	103±4
20 mM F^-	25±2
2 mM F^-	92±4

The concentration of boric acid, 3 mM. n=3

Conversely, the PADs are more advantageous than the azomethine-H method in terms of the throughput and portability. The analysis time of the PADs was less than 90 min including the sample preparation whereas the azomethine-H method required more than 2 h. The throughput of the PADs is also better than the azomethine-H method because many samples can be measured in parallel. Furthermore, the PADs permit the measurements without any large instruments.

5. Conclusions

Here, we used chromatographic principles to propose a novel detection scheme for PADs and demonstrated a practical measurement of boric acid by coupling an ion pair formation with a complex of chromotropic acid and measuring the reaction with BG dye. The complex in a sample solution weakened the interaction of the BG with the paper, which resulted in an elongation of the colored bar flowing in the channel of the chromatographic PAD. Oxidation of the paper substrate strengthened the interaction of the BG in the stationary phase and decreased the length of the bar for a blank sample. Thus, the length of the bar exhibited an apparent dependence on the concentration of the borate complex. The calculated results based on the proposed model were in good agreement with the experimental results, which indicated the validity of the method. Furthermore, we successfully demonstrated the determination of boric acid in commercial eye-drops wherein the obtained results were comparable to a standard spectrophotometric method. This example of a chromatographic PAD could further be extended to different modes of chromatography including ion exchange, chelate

formation, and inclusion-complex formation. Other modifications of the paper substrate are also potentially applicable to a chromatographic PAD. The operation of the proposed detection scheme is simple and easy, so it may be advantageous for use under ill-equipped conditions.

Conflicts of interest

There are no conflicts to declare.

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