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A high throughput chemiluminescence method based on molecularly imprinted sol-gel films for determination of sibutramine

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ABSTRACT

A novel and high throughput chemiluminescence (CL) method for determination of sibutramine was originally developed based on potassium permanganate-hydrochloric acid-(tween-80) CL system. Molecularly imprinted sol-gel film (MIF) was synthesized and used as selective material to improve the selectivity of CL. In the proposed procedure, sibutramine molecule was adsorbed into the MIF, and then potassium permanganate, hydrochloric acid and tween-80 were added in wells of a 96-well plate, Based on which, sibutramine was oxidized and CL was produced and detected by a photomultiplier tube (PMT). The CL intensity was correlated linear with the concentration of sibutramine over the range of 5.0×10^{-8}-1.0×10^{-6} g·mL^{-1} and the detection limit was 1.8×10^{-8} g·mL^{-1}. The relative standard deviation (RSD) was 3.1% for determination of 1.0×10^{-7} g·mL^{-1} sibutramine (n = 11). The fabricated molecularly imprinted sol-gel film possessed good selectivity and could be regenerated. This method was successfully applied to the determination of sibutramine in weight-reducing tonic.

Keywords: Sibutramine; chemiluminescence; molecularly imprinted film; high throughput

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Introduction

Sibutramine, a synthetic anorecxic, makes people lose weight by controlling the nerve centre to decrease appetite. However, excessive use of it can lead to serious consequences, such as cardiac arrhythmias, paresthesia, mental/mood changes including excitement, restlessness, confusion, depression, rare thoughts of suicide and some other injury [**1**]. On December 22, 2008, the food and drug administration an alert to consumers naming 27 different products marketed as "dietary supplements" for weight loss, which illegally contain undisclosed amounts of sibutramine [**2**]. Nevertheless, sibutramine has still been added in some weight- reducing tonic illegally by the manufacturers to earn commercial profits. Hence, it is of great significance to monitor the content of sibutramine in diet food. Some methods have been reported for determination of sibutramine including gas chromatography (GC) [**3**], HPLC [**4, 5**], LC-MS [**6**] and spectrophotometry [**7**]. However, they often suffer from a variety of limitations: GC, HPLC and MS call for expensive instruments and long time to analyze, and spectrophotometry has low sensitivity. Chemiluminescence (CL) method [**8**] is known to be a powerful analytical technique with high sensitivity, fast response time, wide dynamic range and simple instrumentation, and it has been successfully applied to the determination of fenfluramine [**9**] and phenolphthalein [**10**]. In general, they still exhibit low selectivity and display low throughput of sample. In this work, a novel CL method for monitoring sibutramine level in diet food has been developed.

Molecularly imprinted polymers (MIP) have many advantages such as predetermination, recognition and wide practicability, which has been widely used in many areas including chromatography stationary phase for separation purpose [**11**], solid-phase extraction [**12, 13**], artificial antibody and enzyme [**14**], biosensors [**15**], fluorescence sensor [**16**] and electrochemical sensor [**17**]. Application of the sol-gel method to construct thin MIP film is favorable since it's easier to control the thickness, porosity, and surface area, while the selectivity and diffusion are comparable and even better than acrylic polymer-based films [**18, 19**].

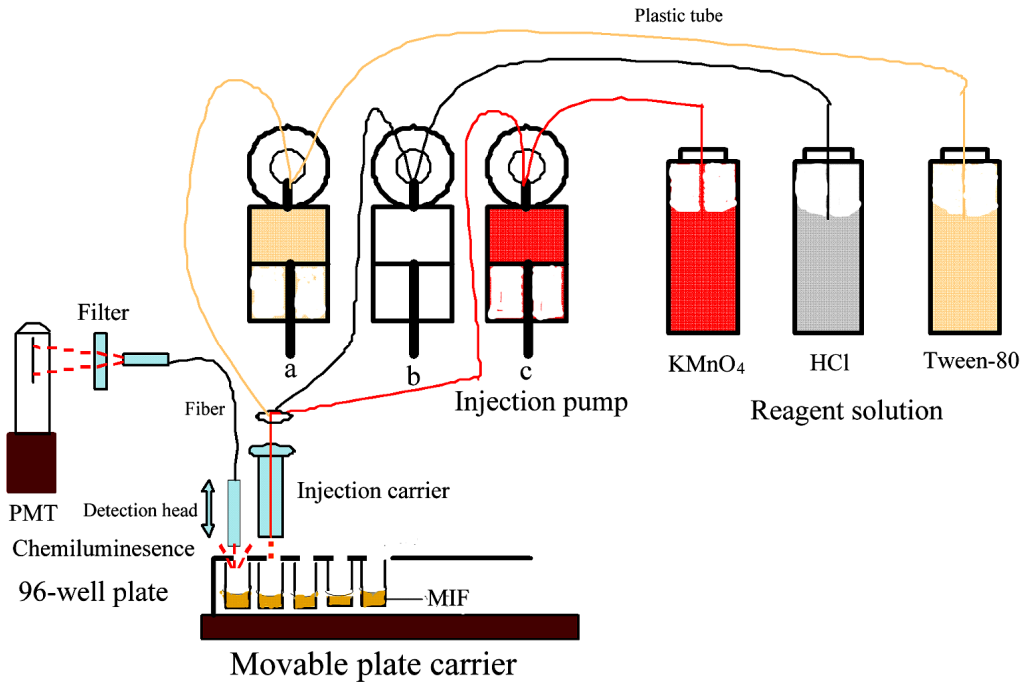

Fig. 1. Schematic diagram of CL system for the determination of sibutramine

In this study, a novel and high throughput method with high selectivity was established successfully for the determination of sibutramine in diet food sample based on potassium permanganate ($KMnO_4$)-hydrochloric acid (HCl)-(tween-80) CL system. The scheme was shown in **Fig. 1**. Using this method, a complete analysis can be performed in a 96-well plate format, giving high throughput detection. Compared with the traditional CL methods, this novel method requires much smaller volume of solutions, offers greater selectivity, and the 96-well plate was so small that easy to be taken. Also the rapid and high throughput detection has made it a powerful analytical tool to monitor the sibutramine level in diet food.

Experimental

Reagents

Tetraethyl orthosilicate (99%, TEOS), and potassium permanganate were purchased from Tian Jin Da Mao Chemical Reagent Factory (Tian Jin, China). Hydrochloric acid (HCl) and ethanol were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Phenyltrimethoxysilane (99%, PTMOS) and methyl trimethoxysilane (98%, MTMOS) were purchased from Alfa Aesar. All chemicals were of analytical reagent grade or above.

Double distilled water used throughout was obtained by SYZ-550 quartz sub-boil high-purified water distiller. The standard solution of sibutramine (1.0×10^{-4} g·mL^{-1}) was prepared by dissolving 0.0100 g sibutramine in 100 mL water. The 5.00×10^{-3} mol·L^{-1} stock solution of potassium permanganate was prepared by dissolving 0.0790 g potassium permanganate in 100 mL water. Solution (5%) of Tween-80 was prepared by dissolving 4.0 g Tween-80 in water and diluting in 80 mL water.

Apparatus

TriStar LB 941 Multimode Reader (Berthold Technologies GnbH & Co. KG, Bad Wildbad, Germany) was used for CL *determination*. The detection cell was a 96-well plate (Berthold white plate). CL was produced and simultaneously transmitted with an optical fiber and collected with a photomultiplier tube (PMT). The signal was recorded using a computer. UV absorption spectra were measured on a UV-3101 spectrophotometer (SHIMADZU, Japan).

Preparation of the sibutramine-imprinted polymer film

The sibutramine-imprinted polymer film (MIF) was prepared according to the literature [**17**]. 3.0 mL of TEOS, 3.0 mL of ethanol, 0.37 mL of PTMOS, 0.3 mL of MTMOS, 1.0 mL of 0.1 mol·L^{-1} hydrochloric acid, and 0.7 mL H_2O were mixed thoroughly for 2 h to yield the initial sol. Then, 2.0 mL of the sol was mixed with 2.6 mg sibutramine to prepare the imprinted sol. A 60 μL of the imprinted sol was then transferred into each well of 96-well plate with a transferpettor, followed by placing the 96-well plate in a covered airer overnight. The final 96-well plate was rather stable for more than 3 months when kept at 4 ℃ in a refrigerator. The binding measurement of the sibutramine-imprinted polymer film (MIF) was studied using 7 films of above, and the other films used as CL recognizers. Non-imprinted blank films (NIF) without the template molecule were prepared and treated in the same manner.

Binding experiments

Before binding experiments, the sibutramine molecules in the MIF were removed by washing with the mixture of methanol-acetic acid (9:1, v/v) until the absorbance of sibutramine at 224 nm was no longer detected in the elution. The polymer was dried to a constant weight at 60 ℃ under vacuum. Then a MIF was mixed with 10.0 mL sibutramine solution with various concentrations in a 50 mL conical flask and oscillated for 6 h at room temperature. After centrifuging at 3000 rpm for 10 min, the concentration of free sibutramine in the supernatant was detected by UV spectrophotometry at 224 nm. The amount of sibutramine bound to the polymer was calculated by subtracting the concentration of free sibutramine from the initial sibutramine concentration. The data obtained was used for the Scatchard analysis.

Procedure for the determination of sibutramine

Investigations of CL behaviors were performed using the system schemata shown in **Fig. 1**. Flow tubes (a, b and c) were connected with potassium permanganate, hydrochloric acid and tween-80. CL behaviors were studied in the well of 96-well plate. For a new sibutramine-MIF, the merged stream of acidic potassium permanganate and tween-80 was injected into the well of 96-well plate with pumps to react with the sibutramine adsorbed on the MIF until a stable CL was recorded, leaving specific cavities of sibutramine on the MIF. Then, water was injected on the MIF to clean the polymer. Sibutramine molecule in the sample solution was selectively adsorbed in the cavities on the polymer. The mixture of potassium permanganate, tween-80, and hydrochloric acid were injected on the MIF to produce CL. The concentration of sibutramine was quantified via the relative CL intensity, which was obtained by subtracting the blank CL intensity from that of the sibutramine standard solution or sample.

The determination process was summarized as four steps.

a) Recognition and adsorption of sibutramine: In this step, sibutramine solution was injected into the well by transferpettor. Sibutramine molecule in the sample solution was selectively adsorbed in MIF.
b) Removing the impurity: In this step, water was injected on the MIF to remove the impurity adsorbed in the MIF until a stable CL was recorded.
c) Chemiluminescence detection: In this step, potassium permanganate, tween-80, and hydrochloric acid were injected into the well by the pumps to react with sibutramine adsorbed in the MIF to produce CL.

d) Cleaning the MIF: In this step, water was injected on the MIF by transferpettor to remove the reaction products in the MIF for next determination.

Results and discussion

Binding analysis of MIF and NIF

The binding characteristics of MIF and NIF were investigated by equilibrium binding experiments according to the literature [**20**]. Varying the concentration of sibutramine from 1.0 to 30.0 μg·mL^{-1} in the presence of one film, the equilibrium binding experiments were carried out. The results were shown in **Fig. 2**. As it can be seen from **Fig. 2**, not merely MIF but also NIF could adsorb sibutramine in aqueous solvent. However, the adsorption capability of MIF was remarkablely better than the NIF.

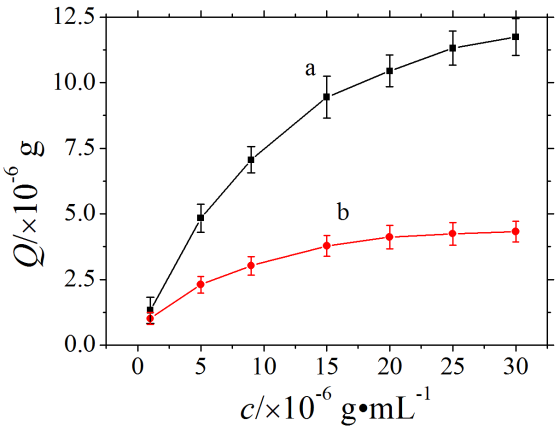

Fig. 2. Binding isotherms for sibutramine-imprinted films and the blank polymer; Q is the amount of sibutramine absorbed in 60 μL sol-gel; T = 25 °C; V = 10.0 mL; binding time: 6 h; a) MIF; b) NIF.

The data of the MIF obtained was used for the Scatchard analysis to estimate the binding parameters of the MIF. Scatchard equation was given as $Q/c = (Q_{max}-Q)/K_d$, in which K_d is equilibrium dissociation constant, Q_{max} is apparent maximum amount of binding sites, and c is concentration of sibutramine in adsorbed solution. As shown in **Fig. 3**, the Scatchard plot was linear within the whole sibutramine concentration range which indicated the binding sites in MIF were uniform. However, it is observed that one section within the plot can be regarded as straight lines, which indicated there was one class of binding sites in MIF. The equilibrium association constant $K_d = 1.2 \times 10^{-2}$ g·L^{-1} and the maximum number $Q_{max} = 2.8 \times 10^{-3}$ g·L^{-1}.

Kinetic characteristics of the CL reactions

Kinetic characteristic of the $KMnO_4$–HCl-(tween-80) CL reaction were examined. The CL intensity–time curve was shown in **Fig. 4**. When 20 μL potassium permanganate solution (3.0×10^{-4} mol·L^{-1}), 20 μL hydrochloric acid (1.0 mol·L^{-1}) and 20 μL tween-80 (5 %) were injected into the well, the mixture solution reacted with sibutramine molecule absorbed on the MIF, CL signal was produced and recorded over 0–100 s. The maximum CL intensity was obtained within 0.3–1 s. After 65 s approximately, the CL signal (curve a) declined to baseline. Thus, in order to save the analysis time, 0.5 s was selected as the time point of CL detection. In addition, CL signal was not detected by using distilled water instead of the sibutramine solution under the same condition (curve b).

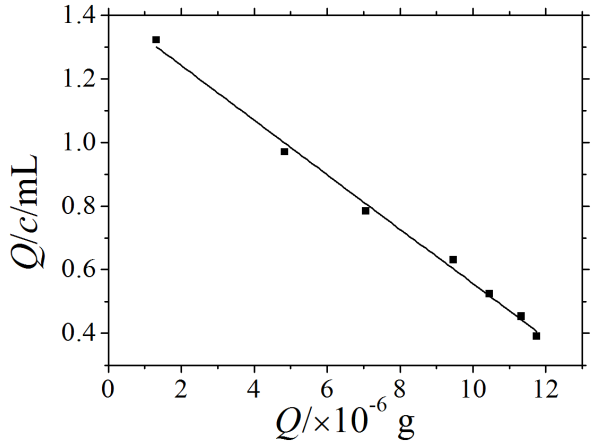

Fig. 3. Scatchard plot to estimate the binding characteristic of sibutramine imprinted films.

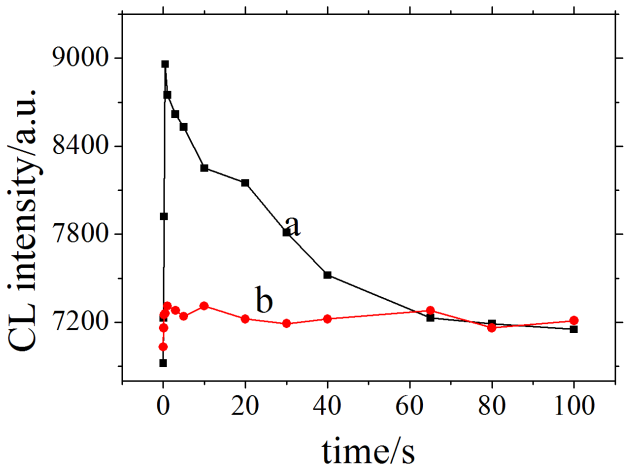

Fig. 4. Chemiluminescence kinetic curve of the system, a) 20 μL potassium permanganate (3.0×10^{-4} mol·L^{-1}) + 20 μL hydrochloric acid (1.0 mol·L^{-1}) + 20 μL tween-80 (5 %) + 20 μL sibutramine (1.0×10^{-7} g·mL^{-1}); b) 20 μL potassium permanganate (3.0×10^{-4} mol·L^{-1}) + 20 μL hydrochloric acid (1.0 mol·L^{-1}) + 20 μL tween-80 (5 %) + 20 μL distilled water.

Optimization of experimental conditions

A series of experiments were performed to optimize analytical conditions using a 1.0×10^{-7} g·mL^{-1} sibutramine solution. The optimum conditions included separate conditions (adsorption time, washing time, response time, cleaning time), concentration of hydrochloric acid solution, concentration of potassium permanganate solution, and dosage of tween-80 solution.

Adsorption time

The adsorption time is the time of MIF was immersed in standard solution or sample solution. This determines the amount of sibutramine adsorbed in the MIF, thereby influencing the sensitivity of the detection and the linear range of the method. When 20 μL potassium permanganate solution (3.0×10^{-4} mol·L^{-1}), 20 μL hydrochloric acid (1.0 mol·L^{-1}) and 20 μL tween-80 (5 %) was injected into the well, the relation between the CL intensity and the adsorption time within the time range of 1-15 min was examined using 1.0×10^{-7} g·mL^{-1} sibutramine solution. The CL intensity increased with the increase of adsorption time up to 9 min. Above 9 min, the CL intensity remained constant. Considering analytical efficiency, 10 min was finally selected as adsorption time. It should be mentioned that for the analysis of a sample with lower sibutramine content, the sensitivity of the detection could be improved by increasing the adsorption time.

Washing time

Because the other substances remain on the surface of the MIF, it is necessary to wash the MIF modified 96-well plate. The washing times should be enough to remove impurities completely and not cause loss of sibutramine adsorbed in MIF. Because Ascorbic acid [**21**] also reacted with potassium permanganate and tween-80 to produce CL in acidic medium, it was selected as interference indicator to optimize the washing times. The effect of washing times on CL intensity was examined in the range of 1-8 time. The experimental results (**Fig. 5**) showed that when the washing time was ≥ 6 time, the CL intensity showed no obvious change with that of the same concentration (1.0×10^{-7} g·mL^{-1}) of sibutramine standard solution in the absence of Ascorbic acid, indicating the interference indicator, Ascorbic acid, was effectively removed. Hence, 6 times was the optimum washing time.

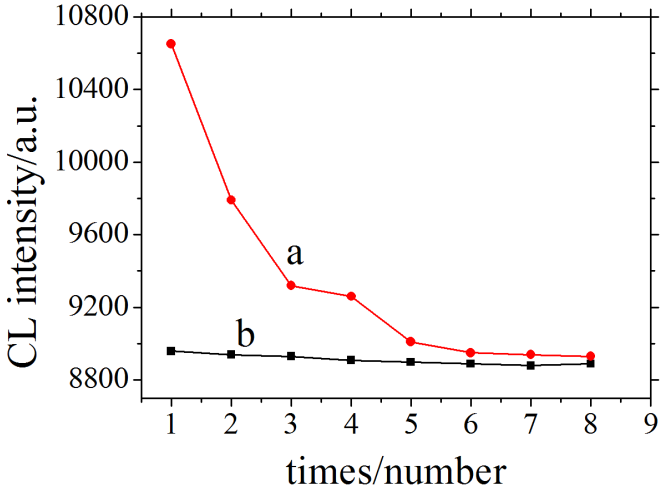

Fig. 5. Effect of washing times on CL intensity, a) sibutramine (1.0×10^{-7} g·mL^{-1}) + Ascorbic acid (1.0×10^{-5} g·mL^{-1}); b) sibutramine (1.0×10^{-7} g·mL^{-1}).

CL reaction conditions

The effects of reagents concentrations including the concentrations of potassium permanganate, and hydrochloric acid and the dosage of the tween-80 on the CL reaction were also examined. The results of the experiments showed that the optimum concentrations of potassium permanganate and hydrochloric acid were chose to be 3.0×10^{-4} and 1.0 mol·L^{-1} respectively, and the volume of tween-80 solution was 20 μL for further determination, respectively.

Analytical performance

Under the optimum conditions the analytical performance was studied. The relative CL intensity (I) was linear to the concentrations (c) of sibutramine from 5.0×10^{-8} to 1.0×10^{-6} g·mL^{-1}. The regression equation is $I = 631.62 + 1.93 \times 10^{9}\ c$ (g·mL^{-1}) ($r = 0.9904$). The detection limit is 1.87×10^{-8} g·mL^{-1}. The relative standard deviation (RSD) for 11 replicate determinations of 1.0×10^{-7} g·mL^{-1} sibutramine is 3.1 %.

Selectivity against interferences

To examine the selectivity of the method, the interference from foreign species to the determination of 1.0×10^{-7} g·mL^{-1} sibutramine were investigated using the molecular imprinting chemiluminescence (MI-CL) method and the traditional CL method, respectively. The tolerable ratios of foreign species in samples were compared when relative error was less than ±5 %. The results were showed in **Table 1**. As it can be seen from table 1, the MI-CL method exhibited an excellent selectivity for the determination of sibutramine and the tolerable ratios for all foreign species were improved greatly.

Table 1. Tolerable ratio of interfering species to sibutramine.

Species	With MIF	Without MIF	Species	With MIF	Without MIF
Cyclodextrin	1000	50	Sucrose	800	30
Tetracycline	500	10	Fructose	800	20
Chloramphenicol	800	30	Starch	1000	50
Fenfluramine	500	10	Zn^{2+}	500	5
Ascorbic acid	500	1	Fe^{3+}	300	2
L-Lysine	300	2	Sn^{2+}	200	1
L-Histidine	100	1	Ca^{2+}	500	2
Mg^{2+}	300	1	Cu^{2+}	100	1

To evaluate the selectivity of MI-CL method, the tolerable ratios of foreign species using the proposed method were compared with that of the references methods [**7, 20, 22**]. The results showed that MIP can enhance the selectivity of analytical methods [**20, 22**]. In the reference [**7**] of determination of sibutramine, foreign species had more serious interference than this proposed MI-CL method.

Application

The practical usage of the MI-CL method was assessed by the determination of sibutramine in two kinds of diet capsules under optimum conditions. The powder of capsule was dissolved in double distilled water and diluted to 100 mL, then filtered. The filtrate was first analyzed with the proposed MI-CL method, and then analyzed with

the high-performance liquid chromatography (HPLC) [23] method. The results were shown in **Table 2**. Comparison between the MI-CL method and reliable HPLC was also carried out. It can be seen that the results measured by the MI-CL method showed a good agreement with those measured by chromatogram method. At the same time, the recovery of added sibutramine and the *t*-test assumed that there is no significant difference between the proposed method and HPLC at confidence level of 95 %. These results showed that the sensor has good accuracy to the determination of sibutramine in weight-reducing tonic.

Table 2. Determination of sibutramine in weight-reducing tonic.

Samples No.	Found[a] (mg·g^{-1})	Added (10·mL^{-1})	Recovered[a] (10·mL^{-1})	Recovery (%)	R.S.D. (%)	HPLC Determination[a] (mg·g^{-1})
		10.0	9.8	98.0	2.6	
1	0	40.0	41.2	103.0	3.1	0
		80.0	77.3	96.6	2.9	
	4.91	10.0	9.7	97.0	2.3	
2	5.03	40.0	38.7	96.8	2.7	5.13
	4.99	80.0	83.4	104.3	2.5	

a. Average of six measurements

Compared with other similar systems

The results of the MI-CL method were compared with the other reported similar systems in terms of determination limit, apparent maximum amount of the MIP, and the functional monomer. The results were shown in **Table 3**. It can be seen that the MI-CL method had higher sensitivity and capability than the other reported similar systems. The materials we used were different with these papers, which expand the synthetic method of MIP.

Table 3. The results of comparing with some similar systems.

Terms	Results	References
Sensitivity (Determination limit)	2.0×10·mL^{-1}	[21]
	1.0×10·mL^{-1}	[24]
	3.4×10·mL^{-1}	[25]
Capability (Apparent maximum amount)	1.07×10·g^{-1}	[25]
	5.46×10^{-4}mol·g^{-1}	[26]
	3.7×10·g^{-1}	[27]
Materials development (Functional monomer)	Methacrylic acid	[20]
	Acrylamide	[9]
	Methacrylic acid	[27]

Conclusion

This work reported a novel method for determination of sibutramine with an effective combination of chemiluminescence and molecular imprinting technique (MIT). The MI-CL method exhibited high selectivity to sibutramine due to the specific binding function of the MIF to sibutramine. Meanwhile, the high sensitivity for the sensor was obtained owing to the preconcentration of the sibutramine on the MIF and the sensitive CL detection method. Moreover, the sensor can give a high throughput of 96 samples per 60 min, which makes it capable of detecting a large number of samples. The proposed method was successfully applied to the determination of sibutramine in weight-reducing tonic.

Acknowledgements

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