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Analytical Methods

Chemiluminescence screening assay for diethylstilbestrol in meat

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ABSTRACT

A simple, rapid, and sensitive flow injection method with chemiluminescence detection was developed for the screening of meat samples containing diethylstilbestrol, based on the enhancement by diethylstilbestrol of the cerium(IV)-rhodamine 6G chemiluminescence system in sulfuric acid medium. Under the optimal conditions, the chemiluminescence intensity was linear for the diethylstilbestrol concentration in four types of meat (chicken, beef, mutton, and pork) matrix, with the linear ranges of CL detection more than three orders of magnitude and the detection limits (3σ) in the range 0.75–1.12 pg/mL. The relative standard deviations for intra-day and inter-day precision were less than 3.0%. The proposed method was found to be highly reliable for screening purpose and successfully applied to the screening of diethylstilbestrol residue in four types of meat samples, with the good quantitative recoveries for the different concentration levels varied from 93.1% to 104.5%. The mechanism of this chemiluminescence reaction has also been proposed.

1. Introduction

Diethylstilbestrol (DES) is a synthetic estrogen that was used to prevent spontaneous abortion clinically. The long-term consequence of DES exposure to mothers has been increased risk of breast cancer, while DES-exposed daughters have shown increased risk of cervicovaginal cancer. Other abnormalities in DES daughters and sons have also been reported which include immune system disorders, psychosexual effects, and reproductive abnormalities (Giusti, Iwamoto, & Hatch, 1995). Moreover, DES has also been employed as a growth-promoting agent to accelerate weight gain and improve feeding efficiency in cattle. Use of growth-promoting drugs for fattening livestock has been banned in the European Union since 1986 to protect consumers from possible harmful effects due to the intake of estrogen residues (Council of the European Communities (Eds.) Council Directive 86/469/EEC, 1986). The Commission Decision 2002/657/EC is in force in the EU since 1 september 2002 (postponed 2 years: 2004) and gives the rules for the analytical methods to be used and specific common criteria for the interpretation of analytical results of official control laboratories (Council of the European Communities (Eds.) Council Directive, 2002). However, the research on food toxicology showed that the illegal application of DES as a growth promoter has been widely reported throughout the world and became a main hazard to human health (Pfaffl, Reck, Dreher, & Meyer, 2003; Santarius, 2004), thus prompting continuous ministerial surveillance to control its abuse. Therefore, it is very important to develop a sensitive and rapid method for the determination of DES residue in food-safety area.

The selected analytical method should be of low detection limit, since DES in biological matrices was usually found at the concentrations in the low nanograms per gram range. To date, various analytical methods for the biological determination of DES residue have been described in the literature. The molecular imprinted polymer–solid-phase extraction–high performance liquid chromatography (MIP–SPE–HPLC) method showed higher selectivity and good recoveries higher than 87.5% (RSD 11.6%), with the detection limit of 60 ng/mL in fish samples (Jiang, Zhao, Jiang, Zhang, & Liu, 2008). Detection and quantitation of DES were performed on a HPLC system coupled to a triple quadrupole mass spectrometer. Validation of this method established a decision limit between 0.2 and 0.9 μg/L and a detection capability between 0.3 and 1.0 μg/L (Rubies, Cabrera, & Centrich, 2007). The pressurized capillary electrochromatography (pCEC) with UV-detection was used to determine estrogens in fish muscle samples (Liu et al., 2005). The DES residue was analyzed by electron-ionization–gas chromatography–mass spectrometry (EI–GC–MS) and solid-phase microextraction (SPME) GC-MS method with the detection limit of 0.3 μg/kg in meat (Seo, Kim, Chung, & Hong, 2005) and 0.004–0.474 μg/L in blood serum (Yang, Luan, & Lan, 2006), respectively. Sheep urine and chicken muscle samples were tested for residues of DES using enzyme-linked immunosorbent assay (ELISA) (Sawaya, Lone, Husain, Dashti, & Al-Zenki, 1998). Although most of the methods cited above have the advantage of sensitive and specific, they suffered from instrument-expensive, approach-complicated and time-consuming.

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The chemiluminescence (CL) technique provides methods for trace analysis that are attractive because of their high sensitivity and fast kinetics. This technique, combined with flow injection (FI) systems, provides good accuracy and precision. FI–CL method has extensively been applied in the different fields of analytical chemistry, including in food, pharmaceutical formulation, in environmental matrices or in biological matrices (Calokerinos & Palilis, 2001, chap. 14; Gámiz-Gracia, García-Campaña, Soto-Chinchilla, Huertas-Pérez, & González-Casado, 2005; Haghighi & Dadashvand, 2006; Huertas-Pérez, Gámiz-Gracia, García-Campaña, González-Casado, & Martínez-Vidal, 2005; Rose, Moffett, & Waite, 2008; Satienperakul, Cardwell, Kolev, Lenehan, & Barnett, 2005; Song, Yue, & Wang, 2006; Townshend, Pulgarín, & Pardo, 2003; Wang, Wang, & Yang, 2007). Two papers have been published on the analysis of DES based on the CL enhancement effect on tetrasulfonated cobalt phthalocyanine (CoTSPc)-luminol-H_2O_2 and HCHO–$KMnO_4$ reaction using FI–CL method, which applied to the determination of DES in tablets and tap water with the detection limits of 6.42×10^{-8} mol/L (Wang, Ye, Jiang, Chen, & Huang, 2004) and in tablets and injections with the detection limits of 1.08×10^{-9} g/mL (Liao, Wu, & Xie, 2005). A electrochemiluminescence (ECL) method for the determination of DES with a nano-cluster-structured manganese dioxide modified glassy carbon electrode was applied to detecting DES tablet. The ECL intensity was linear with the concentration of DES over the range from 3.5×10^{-11} to 6.5×10^{-9} mol/L, and the detection limit was 2.0×10^{-11} mol/L (Li, Zhang, Guo, & Li, 2007). However, there are no reports of the determination of DES in biological matrices by FI–CL method.

Our preliminary experiments showed that weak CL emission could be produced when cerium(IV) reacted with rhodamine 6G in sulfuric acid medium, and the CL intensity was strongly enhanced by DES. The enhancement in the CL emission is proportional to the concentration of DES, which can be determined by measuring the increase in the CL intensity. This phenomenon allowed us to develop a sensitive CL method for the screening of DES residue in meat samples (chicken, beef, mutton, and pork) by using a simple FI system. Finally, the CL mechanism for cerium(IV)-rhodamine 6G-DES has been proposed.

2. Experimental

2.1. Reagents

All chemicals were of analytical grade and were used without further purification. Ultrapure water was used throughout. $Ce(SO_4)_2 \cdot 4H_2O$ was obtained from Shanghai Yaolong Metal Company (Shanghai, China) and prepared in sulfuric acid solution daily. Rhodamine 6G was obtained from Merck (Darmstadt, Germany). Diethylstilbestrol was obtained from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A stock solution of 0.001 mol/L rhodamine 6G was prepared by dissolving 0.024 g rhodamine 6G in 50 mL ultrapure water. A stock solution of 0.1 μg/mL diethylstilbestrol was prepared with methanol and standard solutions by diluting with ultrapure water. The solutions of diethylstilbestrol remained stable during two months if stored in refrigerator at 4 °C.

2.2. Apparatus

The CL detection was conducted on a flow injection CL system (Remax, China) consisting of a model IFFM-M peristaltic pump, a mixing tee and a model IFFS-A CL detector equipped with a glass coil (13 cm × 1 mm i.d.), which was used as a reaction coil and detection cell, and a photomultiplier. The data from the CL detector was acquired by an IBM compatible personal computer. The CL and fluorescent spectra were measured by using a model RF-5301 fluorimeter (Shimadzu, Japan). The UV–visible spectra were conducted on a model 8453E UV–visible spectroscopy system (Agilent Technologies, USA).

2.3. Screening procedure

A schematic diagram of the FI–CL system is shown in Fig. 1. The solutions of 5.0×10^{-3} mol/L cerium(IV) containing 1.5 mol/L H_2SO_4 and 7.0×10^{-5} mol/L rhodamine 6G were pumped continuously at a rate of 2.5 mL/min into the flow cell. The sample solution was introduced using a 100-μL loop valve injector. The light output from the flow cell was detected by the photomultiplier (at the negative voltage of −800 V). The full CL intensity vs time curve was recorded. The concentration of diethylstilbestrol was determined by measuring the enhanced CL intensity according to $\Delta I = I_S - I_0$, where I_s and I_0 are the CL signals in the presence and absence of diethylstilbestrol, respectively.

2.4. Sample preparation

Meat samples including chicken, beef, mutton, and pork were obtained from local market and stored frozen at −20 °C or below until analysis. Meat (ca. 200 g) was cut into small pieces and homogenized in a household mixer. Meat samples were pretreated according to the literature (Han, Yu, Ge, & Yu, 2004). A 3 g aliquot of sample was extracted with 10 mL of methanol in an ultrasonic bath for 30 min and centrifuged at 3500 rpm for 10 min. The upper layer was collected, and the residue was extracted and centrifuged as above again. The combined methanol extract was evaporated to dryness in a water bath at 78 °C. The concentrate was dissolved by 15 mL of 1.0 mol/L NaOH and purified by washing with 6 mL of chloroform twice. The pH of NaOH layer was then adjusted to 10.3. The adjusted NaOH solution was repeatedly extracted by 8 mL of chloroform thrice, and the extracts (24 mL) were evaporated to dryness at 45 °C. The residue was dissolved and volumed to 25 mL with ultrapure water. Sample solutions were determined by the proposed method directly after dilution, respectively.

3. Results and discussion

3.1. Optimization of the FI–CL system

The effects of various oxidants, including H_2O_2, $K_3Fe(CN)_6$ in sodium hydroxide medium and $KMnO_4$, cerium(IV) in sulfuric acid medium on CL intensity were studied, as shown in Table 1. The CL was obtained when $KMnO_4$ or cerium(IV) was used as an oxidant in sulfuric acid medium. However, the CL emission produced by $KMnO_4$ oxidation was very weak. Therefore, cerium(IV) was selected as an oxidant in the coming work.

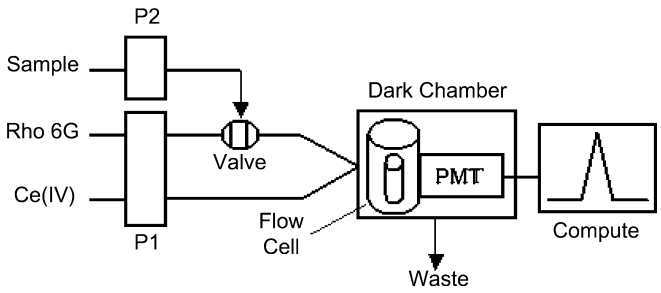

Fig. 1. Schematic diagram of the FI–CL system used for the determination of diethylstilbestrol. P1, P2: peristaltic pump; PMT: photomultiplier tube.

Table 1
Effects of different oxidants on the CL signal of diethylstilbestrol[a]

Oxidant	Concentration (mol/L)	Medium	ΔI
H_2O_2	5.0×10^{-2}	0.1 mol/L NaOH	ND
$K_3Fe(CN)_6$	1.0×10^{-3}	0.5 mol/L NaOH	ND
$KMnO_4$	1.0×10^{-4}	1.0 mol/L H_2SO_4	12.3 ± 0.31
Ce(IV)	3.0×10^{-3}	1.0 mol/L H_2SO_4	155.6 ± 2.18

ND: Not detected.
ΔI: relative CL intensity, mean ± SD, $n = 5$.
[a] The sample concentration is 0.15 ng/mL. CL conditions: Rho 6G, 5.0×10^{-6} mol/L; flow rate, 2.5 mL/min.

A series of experiments were conducted to establish the optimum reaction conditions for the cerium(IV)-rhodamine 6G-DES CL system. The optimized parameters included chemical variables, such as the concentrations of the reagents used for the CL reactions, and physical variables. The DES concentration used for the optimization experiments was 0.15 ng/mL.

3.1.1. Effect of sulfuric acid concentration

Cerium(IV) is not readily soluble in water, but becomes stable when dissolved in sulfuric acid. The effect of the concentration of sulfuric acid used for preparing cerium(IV) solution was studied in the range 0.5–1.9 mol/L. It was found that CL intensity increased with increasing sulfuric acid concentration up to 1.5 mol/L, above which the CL intensity increased slightly. The reason that high concentrations of sulfuric acid were needed for maximal enhanced CL intensity may be due to that higher fluid viscosity caused by higher concentration of sulfuric acid decreased the collision of excited-state molecular, resulting in the stronger CL intensity. However, stronger sulfuric acid badly eroded the tubes of flow injection. As a compromise, 1.5 mol/L sulfuric acid was then chosen to prepare and dilute the cerium(IV) solution.

3.1.2. Effect of cerium(IV) concentration

The effect of cerium(IV) concentration upon the CL intensity was examined in the range 1.0–8.0 $\times 10^{-3}$ mol/L in 1.5 mol/L sulfuric acid medium. It was found that the CL emission increased until 6.0 $\times 10^{-3}$ mol/L cerium(IV) and then decreased with increasing cerium(IV) concentration. Lower concentrations of oxidant caused lower CL emission because of incomplete oxidation reaction, whereas higher concentrations produced absorption of radiation by the deep yellow color from the superfluous cerium(IV) solution. However, the decrease in cerium(IV) concentration from 6.0 $\times 10^{-3}$ to 5.0 $\times 10^{-3}$ mol/L could spare the consumption of reagents with only a slight loss of CL intensities. Therefore, 5.0 $\times 10^{-3}$ mol/L cerium(IV) was recommended for the further work.

3.1.3. Effect of rhodamine concentration

The effect of the concentration of rhodamine 6G on the CL intensity was examined over the range 3.0×10^{-6}–1.0×10^{-4} mol/L. The results show that 7.0×10^{-5} mol/L rhodamine 6G provides maximum CL intensity. Lower concentrations of rhodamine 6G gave lower CL emission because of fewer energy receptors, and higher concentrations produced decreased CL emission due to that some excited-state rhodamine 6G molecules return to the ground state by a non-radiative internal transfer process, which would decrease the chemiluminescent quantum yield because of the increased collisional energy transfer between molecules caused by higher rhodamine 6G concentration.

3.1.4. Effect of flow rate

The flow rates of solutions are very important to the CL reaction and should be regulated. At the flow rates that are too slow or too high, CL is not emitted in the flow cell and hence the emitter can not be detected. Under the above selected conditions, the effect of flow rate on the CL intensity of DES was studied over the range 0.5–3.8 mL/min in each stream. It was found that CL intensity increased with increasing flow rate up to 2.5 mL/min, and that a approximate plateau of CL intensity was reached in the range of 2.5–3.0 mL/min and then CL intensity decreased with increasing flow rate. Therefore, a flow rate of 2.5 mL/min was used because of greater precision of CL signal and lower consumption of reagents.

3.1.5. Effect of the tube length between T-piece and flow cell

The tube length between T-piece and flow cell is critical when working with CL detection in flowing streams. The effect of tube length in the 2.5–18.0 cm range was investigated, with the maximum CL intensity obtained at a tube length of 12.0 cm. For this reason, 12.0 cm was then selected as the optimum tube length for the determination.

3.2. Sensitivity, repeatability and reproducibility studies

Under the optimum conditions described above, the calibration curves were prepared over the range 1.0 pg/mL–10.0 ng/mL, and at least 10 DES samples covering the whole range were used for four types of meat (chicken, beef, mutton, and pork) matrices, respectively. The meat matrix was selected from the meat samples free from DES used in the spiking experiments. Each point of the calibration graph corresponded to the mean value from five independent peak measurements. The linearity between the logarithm of DES concentration (C) and the logarithm of CL intensity (ΔI) was good, as shown by the data in Table 2 that the regression coefficients (r) were greater than 0.9993 for four curves. For all tested matrices, the linear ranges of CL detection were more than three orders of magnitude. Table 2 indicates that the detection limits for DES defined as three times the standard deviation (3σ) for the reagent blank signal were 0.95 pg/mL for chicken, 0.75 pg/mL for beef, 0.98 pg/mL for mutton, and 1.12 pg/mL for pork, and the number of measurements performed for the calculation of σ was seven, respectively. Repeatability and reproducibility studies were carried out. The intra-day precision was tested with seven repeated injections of DES solution at the concentration level of 5.0 pg/mL. The inter-day precision of the proposed method was studied by analyzing 8.0 pg/mL DES solution, injected six times every day, on five consecutive days. The relative standard deviations (RSD) were all below 3.0%. The recoveries obtained for repeatability and reproducibility studies were in the range of 95.5–102.8%. These values are in accordance with the required accuracy in trace analysis.

3.3. Selectivity study

Possible interferences from cations, anions, sugars, amino acids, and organic acids in biological samples and some common organic solvents were investigated by considering their effects on the CL intensities of the cerium(IV)-rhodamine 6G-DES system. The tolerance limits for various foreign species in the determination of 8.0 pg/mL DES by the described procedure were studied. When a relative error is within ±5.0%, it is used as the criterion. The tolerable ratios of foreign species with respect to 8.0 pg/mL DES were determined as 1600 for K^+, Cl^-, 1200 for Na^+, ethanol, 1000 for glucose, acetone, Ca^{2+}, $H_2PO_4^-$, 800 for methanol, acetic acid, 600 for CO_3^{2-}, 400 for ascorbic acid, 200 for citric acid, tartaric acid, 110 for Mg^{2+}, Zn^{2+}, and 20 for glycine. In the selected foreign species, methanol, ethanol and acetone are common solvents, and K^+, Cl^-,

Table 2
Linear range, regression equation, and detection limit of diethylstilbestrol in four types of meat matrices

Matrix	Linear range	Regression equation[a]	Detection limit (pg/mL)	RSD
Chicken	2.7 pg/mL–3.0 ng/mL	$\log(\Delta I) = 9.9538 + 1.0061 \log C$	0.95	2.8
Beef	2.0 pg/mL–3.2 ng/mL	$\log(\Delta I) = 8.7742 + 1.0013 \log C$	0.75	3.0
Mutton	3.5 pg/mL–8.0 ng/mL	$\log(\Delta I) = 9.0389 + 1.0129 \log C$	0.98	2.0
Pork	2.7 pg/mL–4.5 ng/mL	$\log(\Delta I) = 9.9169 + 1.0005 \log C$	1.12	2.5

[a] ΔI: relative CL intensity; C: concentration of diethylstilbestrol; regression coefficients: $r \geqslant 0.9993$; $n \geqslant 10$.

Na^+, Ca^{2+}, $H_2PO_4^-$, CO_3^{2-}, Mg^{2+}, and Zn^{2+} often exist in water. The rest glucose, acetic acid, citric acid, tartaric acid and glycine are the ingredients in meat. Therefore, the proposed method shows higher selectivity to DES and less interference. After the sample pretreatment, protein was eliminated, which made it suitable for the screening of DES in meat samples.

3.4. Reliability of the screening assay for DES

DES is often used as a growth-promoter drug for fattening livestock and become a food residue problem. The method was applied to the determination of DES in meat samples. The meat samples including chicken, beef, mutton, and pork were analyzed, and only chicken 1 sample gave a positive response (see Table 3). The standard addition calibration was applied to minimize the matrix interference. The regression equation in chicken 1 matrix was $\log(\Delta I) = 10.0238 + 0.9987 \log C$ ($r = 0.9991$, $n = 12$), and the DES concentration was 22.1 pg/mL. To confirm the validity of this method, known amounts of DES were spiked into meat samples, and then the tested solution of spiked meat samples was prepared as in Section 2.4. The good quantitative recoveries for the different concentration levels varied from 93.1% to 104.5%, with the relative standard deviations were below 3.0%. In conclusion, the present method is applicable for the screening of DES residue and can be used to monitor the occurrence at trace level of DES in meat samples found in the market.

3.5. CL mechanism

The CL spectra of the cerium(IV)-rhodamine 6G reaction in the absence and presence of DES were measured. Both CL spectra were almost identical with a maximum wavelength at about 556 nm, which was in agreement with that of the fluorescent spectrum of rhodamine 6G. Thus, the luminophor could be ascribed to rhodamine 6G. The UV–visible absorption spectra of the cerium(IV)-rhodamine 6G-DES CL reaction were recorded, as shown in Fig. 2. The absorption spectrum of the mixture of cerium(IV), rhodamine 6G and DES shows that the absorption of rhodamine 6G at 526 nm disappears, suggesting that rhodamine 6G was oxidized to form a new compound. Similarly, the 240 nm peak of DES shifts to 253 nm, at the same time the 320 nm peak of cerium(IV) disappears, which meant that DES was oxidized into its corresponding DES quinone (Metzler & McLachlan, 1978). Therefore, it was evident that rhodamine 6G and DES were oxidized by cerium(IV) to the oxidized product of rhodamine 6G and DES quinone, respectively. On the other hand, the fluorescent spectrum taken from the mixture of cerium(IV), rhodamine 6G and DES exhibits the characteristic emission of cerium(III) at 350 nm, indicating that cerium(IV) was reduced to cerium(III) (Huang & Chen, 2002).

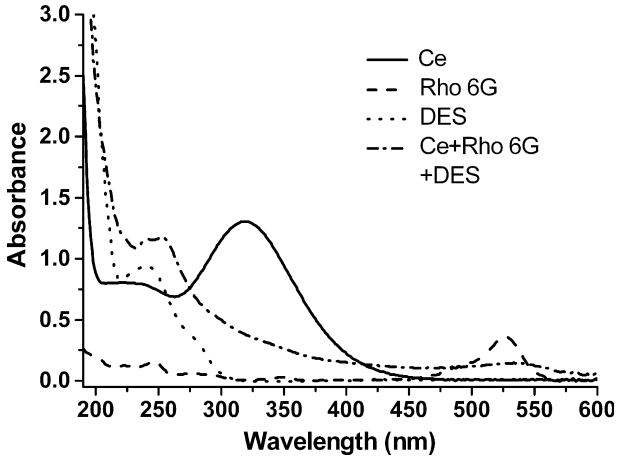

Fig. 2. Absorption spectra of the cerium(IV)-rhodamine 6G-diethylstilbestrol reaction. Reference solution: water; Ce(IV): 3.0×10^{-4} mol/L; Rho 6G: 4.0×10^{-6} mol/L; DES: 30 mg/L.

Based on the above discussion, the mechanism of the cerium(IV)-rhodamine 6G-DES CL reaction can be explained as shown in Scheme 1. The reaction of cerium(IV) with rhodamine 6G and DES in sulfuric acid medium forms the excited-state cerium(III). The reaction rate between cerium(IV) and DES may be faster than that of cerium(IV) with rhodamine 6G. Thus, the presence of DES can accelerate the generation of the excited-state cerium(III), and then energy is transferred from cerium(III)* to rhodamine 6G to form the excited-state rhodamine 6G, which emits its characteristic radiation at 556 nm.

Table 3
Results for the determination of diethylstilbestrol in meat samples by FI–CL method ($n = 5$)

Sample	Screening response	Found (pg/mL)	Added (pg/mL)	Recovery (%)	RSD (%)
Chicken 1	Yes	22.1	20.0	102.6	2.8
Chicken 2	No	–	30.0	93.1	2.4
Beef 1	No	–	30.0	103.7	3.0
Beef 2	No	–	40.0	101.4	2.5
Mutton 1	No	–	30.0	93.7	2.7
Mutton 2	No	–	40.0	96.5	1.9
Pork 1	No	–	30.0	103.3	2.0
Pork 2	No	–	40.0	104.5	2.5

$$\mathrm{Ce(IV) + Rho\ 6G \xrightarrow[H_2SO_4]{slow} Ce(III)^* + Rho\ 6G_{ox}}$$

$$\mathrm{Ce(IV) + DES \xrightarrow[H_2SO_4]{fast} Ce(III)^* + DES\ quinone}$$

$$\mathrm{Ce(III)^* + Rho\ 6G \longrightarrow Ce(III) + Rho\ 6G^*}$$

$$\mathrm{Rho\ 6G^* \longrightarrow Rho\ 6G + h\nu}$$

where Rho $6G_{OX}$ is the oxidized form of Rho 6G; hν is the CL emission.

Scheme 1.

4. Conclusions

In this paper, a new cerium(IV)-rhodamine 6G-DES CL reaction combined with FI system was used to screen DES in meat samples. For the first time, the DES residue in biological matrices was screened by FI–CL method. Compared to the reported FI–CL methods for the analysis of DES, the present method shows lower detection limit, higher selectivity, and less interference. It offers the advantages of simplicity, rapidity, low cost, and high sensitivity and has a potential application in the screening of DES residue in food-safety area. Further research will be developed in this sense.

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