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A Rapid and Sensitive Method for Semicarbazide Screening in Foodstuffs by HPLC with Fluorescence Detection

Guoliang Li · Chenhong Tang · Ying Wang · Jing Yang · Hongliang Wu · Guang Chen · Xiaojian Kong · Weiheng Kong · Shucheng Liu · Jinmao You

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Abstract Semicarbazide (SEM) has been proven to extensively exist in foodstuffs due to anthropogenic factor in food processing and possesses various toxic effects on human health. Although many methods have been developed, they often require long analytical time, complex laboratory equipment, trained personnel, difficultly prepared antibodies, or relatively expensive equipment. The present study developed a new method for SEM determination by HPLC with fluorescence detection (FLD). The fluorescence reagent, 2-(11H-benzo[a]carbazol-11-yl) ethyl chloroformate (BCEC), was first used for SEM labeling. The fluorescent labeling conditions were optimized systematically. SEM can be labeled in only 10 min at 40 °C. The labeled SEM was analyzed on an eclipse XDB-C8 column in 8 min. The new method offered the low LOD of 0.4 μg/kg at a signal-to-noise ratio of 3 and also exhibited excellent reproducibility, precision, and accuracy. When applied to analyze several foodstuffs, it showed good applicability. The developed method has been proven to be simple, inexpensive, selective, sensitive, accurate, and reliable for SEM analysis in foodstuffs. Furthermore, this developed method should have a powerful potential in the analysis of SEM from many other food samples.

Keywords Semicarbazide · HPLC · Fluorescence labeling · Foodstuffs

Introduction

Semicarbazide (SEM) belonging to the hydrazine family of chemicals is determined in food as a marker to detect the illegal use of the banned antibiotic nitrofurazone (Jiang et al. 2012; Jin et al. 2011; McCracken et al. 2013). It was recently found that SEM is also present in glass jars and bottles closed with metal lids sealed with plastic gaskets that are foamed using the azodicarbonamide (ADC) as blowing agent (Fig. 1) (Mulder et al. 2007). ADC is also widely used as a flour additive in some countries such as China, Canada, USA, and Brazil (Ye et al. 2011). Recent studies proved that semicarbazide can be formed in processed foods prepared with ADC-containing flour (Becalski et al. 2004; Noonan et al. 2008). The thiol groups of flour proteins are readily converted to disulfide bridges by ADC which is, in turn, reduced to biurea (Fig. 1). This reaction improves the physical properties of the flour and is commonly used in the cereal industry to improve the quality of flours, particularly those poor in gluten. In Singapore, the use of ADC can result in up to 15 years imprisonment and a fine of $450,000 (Ye et al. 2011). However, ADC is not listed as a permitted food additive in many countries such as Australia and Europe. Another possible source for SEM's formation is hypochlorite treatment, when used for disinfection and bleaching reasons. Due

Chenhong Tang contributed equally to this work.

G. Li · S. Liu (✉)
Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety, College of Food Science and Technology, Guangdong Ocean University,
524000 Zhanjiang, People's Republic of China
e-mail: Lsc771017@163.com

G. Li · Y. Wang · J. Yang · G. Chen · X. Kong · W. Kong · J. You
Key Laboratory of Life-Organic Analysis of Shandong Province, Qufu Normal University, 27365 Qufu, People's Republic of China

C. Tang
Center for Bioenergetics, Biodesign Institute, and Department of Chemistry & Biochemistry, Arizona State University, Tempe, AZ 85287, USA

H. Wu · J. You (✉)
Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences,
810008 Xining, People's Republic of China
e-mail: jmyou6304@163.com

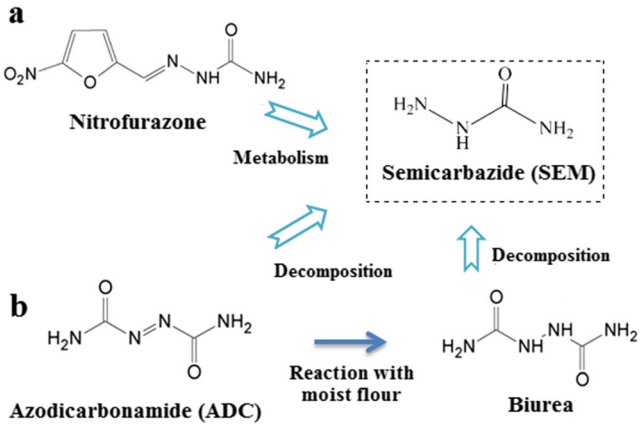

Fig. 1 The formation of semicarbazide (**a** nitrofurazone abuse, **b** flour-improving agent and blowing agent of gasket seals in food jars)

to the widespread existence of SEM in foodstuffs, there is a sudden concern about SEM safety in recent years. Available experimental data showed SEM possessed carcinogenic potential and can induce genotoxic effects and other toxic effects in the cardiovascular (i.e., aorta) and skeletal systems (Becalski et al. 2004; Noonan et al. 2008; Vass et al. 2008).

In view of possible exposure from various foods, the efficient screening of trace SEM becomes urgent. Currently, many methods are established for detecting SEM in complicated food samples including enzyme-linked immunosorbent assay (ELISA) (Cháfer-Pericás et al. 2010; Cooper et al. 2007; Vass et al. 2008), immunochromatographic assay (Tang et al. 2011) and liquid chromatography tandem mass spectrometry (LC–MS/MS) (Bogialli and Di Corcia 2009; Cháfer-Pericás et al. 2010). These methods are accurate and sensitive, but they require complex laboratory equipment, trained personnel, difficultly prepared antibodies, or relatively expensive equipment (e.g., HPLC–MS/MS). For example, HPLC–MS is the main method for SEM screening owing to its superior detection limit and selectivity, but SEM possesses a low mass located in the range of MS background noise; furthermore, being a very polar compound, its retention time on a reversed-phase column would be very low, and the signal is significantly affected by the MS background. In order to overcome these problems, derivatization is required to increase the mass of SEM and improve the retention time on the reversed-phase column. This derivatization procedure will take for 16 h on a rotary shaker at 37 °C (Rezaee et al. 2010). Furthermore, for HPLC–MS analysis, the stable isotope-labeled internal standards ($^{13}C^{15}N_2$-SEM or d_4-SEM derivatives) are often required (de la Calle and Anklam 2005).

As the mature and reliable coupled detection techniques in routine use, ultraviolet or fluorescence detection (FLD) is relatively cheap and convenient. HPLC with FLD is more selective and sensitive (Yu et al. 2010; Kabashima et al. 2008; Di Stefano et al. 2014), which is much preferable to the analysis of trace SEM in foodstuffs. SEM does not absorb in the UV range of the spectrum, it is not fluorescence-active. Thus, fluorescent labeling becomes a necessary procedure before the detection. To the best of our knowledge, the method based on fluorescent labeling combining with HPLC–FLD for SEM determination in foodstuffs remains poorly investigated. In this study, 2-(11H-benzo[a]carbazol-11-yl) ethyl chloroformate (BCEC) was first used as fluorescent labeling reagent for SEM screening, which possesses strong fluorescence and excellent stability. Here, a cheap and efficient method for SEM determination in foodstuffs using BCEC as labeling reagent by HPLC–FLD has been developed. Due to the specific structure of SEM, the labeling reaction between BCEC and SEM was systematically optimized by response surface methodology, ensuring the sufficient labeling. This method can achieve a short analysis time, high sensitivity, and specificity. The established method was employed to determine the concentration of SEM in commercial food products and gained satisfactory suitability and reproducibility.

Materials and Methods

Reagents

SEM was purchased from Sigma-Aldrich (Sigma-Aldrich Company, USA). High purity water purified with a Milli-Q water purification system (Millipore, Molsheim, France) was used throughout the experiment. HPLC grade acetonitrile (CH_3CN, ACN) was purchased from Yucheng Chemical Reagents Corp. BCEC was synthesized by our laboratory (You et al. 2007). Other reagents used were of analytical reagent grade (Shanghai Chemical Reagents Corp., Shanghai, China).

BCEC solution (1×10^{-3} mol/L) was prepared by dissolving 3.23 mg BCEC in 10 mL of anhydrous acetonitrile prepared by distilling the dried HPLC grade acetonitrile with P_2O_5. The SEM solution (5.0×10^{-3} mol/L) was prepared in water and diluted to the work solutions with different concentrations by water. When not in use, all reagent solutions were stored at 4 °C.

Instrumentation

Experiments were performed using an Agilent 1100 Series high-performance liquid chromatography (Agilent Technologies, Palo Alto, CA, USA). The HPLC system consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (FLD). The ultrasound-assisted extraction of SEM was performed using an ultrasonic cleaner (SB-5200DTD, 40 kHz, Xinzhi Biotech Co., Ningbo, China).

Sample Extraction

The SEM extraction was carried out according to previous publications with minor modifications (Becalski et al. 2004; Noonan et al. 2005; Ye et al. 2011). A variety of commercial products were purchased from local food stores. The products including bread, instant noodles, pork, shrimp, prawn, mutton, chicken, and fish were dried under a stream of nitrogen and ground in a blender. A subsample (1 g) was homogenized with 5 mL of hydrochloric acid (0.2 mol/L, pH 3.5) in a centrifuge tube. The homogenate was extracted for 20 min by ultrasound and then centrifuged for 10 min at 4500 rpm. Three milliliter aliquots of the supernatants were blown to dryness under a stream of nitrogen, and the residues were dissolved (assisted by ultrasonication) in 0.5 mL water/ACN solution (1:1; *v*/*v*), filtered through a 0.45-μm filter, and stored in a refrigerator for the next analysis.

Fluorescence Labeling of SEM

The fluorescence labeling proceeded under the optimum conditions. A 30 μL of SEM standard solutions (or 100–150 μL sample extracted solutions) was added into a vial (2.0 mL) and then successively added 50 μL of BCEC-Cl solution. The vial was then sealed and the mixture was heated at 40 °C for 10 min in a thermostatic water-bath, and then 10–15 μL acetic acid (36 %, w/w) was added to stop the reaction. The labeled sample was directly injected into the HPLC system. The derivatization scheme of BCEC with SEM is shown in Fig. 2.

HPLC Conditions

HPLC separation of SEM derivative was carried out by an Eclipse XDB-C_8 column with binary gradient elution. Eluent A was 5 % aqueous acetonitrile and B was acetonitrile (100 %). During conditioning of the column prior to injection, the mobile phase composition was 50 % (A) and 50 % (B). The gradient elution program was as follows: 50–65 % (B) from 0 to 5 min, 65–100 % (B) from 5 to 6 min, and 100 % (B) from 6 to 8 min. The flow rate was constant at 1.0 mL/min, and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set to λ_{ex}= 279 and λ_{em}=380 nm, respectively.

Method Validation

The method was validated in order to concur with the FDA guidelines on the validation of analytical methods. Evaluated parameters included limit of detection (LOD), linearity of calibration curve, accuracy, precision, reproducibility, and recovery. Calibration curves were constructed by plotting peak area (*Y*) versus concentration (*X*) in the range of 0.75–750 μg/kg for SEM. LOD was calculated at the signal-to-noise (S/N) ratio of 3. The method repeatability was investigated by six injections of 10 μL standard solution. Intra-day and inter-day precisions were expressed as the percentage relative standard deviation (R.S.D.%). The accuracy of the analytical method was determined by spiking with a known amount of standards (low level, middle level, and high level) into real samples.

Results and Discussion

Optimization of Fluorescence Labeling

The conditions for fluorescence labeling can significantly affect the labeling efficiency and the subsequent method sensitivity. Thus, the conditions for SEM labeling were systematically investigated. Compared with single-factor experiments, response surface methodology (RSM) is more efficient, requires fewer data and reagent depletion, and provides interaction effects on the response besides factor effects (Bezerra et al. 2008). In this study, pH of buffer solution, labeling time, and labeling temperature were the main parameters affecting the fluorescence labeling yield and were chosen for further optimization by employing a three-level, three-variable Box-Behnken design (BBD) from RSM. According to BBD design, a total of 17 runs were given in Table 1. The peak area was used to express the labeling yield. The design expert software was applied to analyze the experimental data. The analysis of variance (ANOVA) for the experimental results indicated that all the linear parameters and quadratic parameters were significant at the level of $p<0.05$. The interaction effect between pH of buffer solution and labeling temperature (X_1X_2) was significant at the level of $p<0.05$, while the interaction between pH of buffer solution and labeling time (X_1X_3), labeling temperature, and labeling time

Fig. 2 The fluorescent labeling scheme for BCEC with semicarbazide (SEM) under the optimum conditions

Table 1 The conditions used and experimental data for the peak area of SEM obtained from Box–Behnken design (n=3)

No.	Parameters			Response
	pH	Temp. (°C)	Time (min)	(Peak area)
1	7.5	20	5	1240
2	7.5	40	10	1978
3	7.5	60	5	1171
4	7.5	20	15	1421
5	5	40	15	1301
6	7.5	40	10	1900
7	10	40	5	177
8	7.5	40	10	1966
9	10	60	10	146
10	7.5	60	15	1376
11	7.5	40	10	1985
12	5	60	10	732
13	10	40	15	656
14	5	40	5	1221
15	10	20	10	193
16	7.5	40	10	1950
17	5	20	10	1347
18-Opt[a]	7.5	42	10	1990
19-Verif[b]	7.5	42	10	2029

[a] The optimized conditions by the model and the predicted peak area

[b] The results for verified experiments under the optimized conditions (n=3) with observed responses

(X_2X_3) were insignificant. The value of R^2 (0.992) revealed that the experimental data were in good agreement with the predicted values. F value for the lack of fit was insignificant (p>0.05), meaning that this model was sufficiently accurate for predicting the relevant responses. The final estimative response model equation (based on the actual value of 17 runs) was given as follows:

$$Y = 1984 - 428.6\,X_1 - 97\,X_2 + 118.1X_3 - 921.4X_1^2 - 458.1\,X_2^2 - 223.8\,X_3^2 + 142X_1X_2 + 99.8\,X_1X_3 + 6X_2X_3$$

Response surfaces were plotted to study the effects of parameters and their interactions on extraction yield. Three-dimensional response surface plots are presented in Fig. 3. These types of plots show effects of two factors on the response at a time. Figure 3a is the response surface showing the effect of pH and temperature on the response at the fixed value of labeling time. There is an increase in the labeling yield with an increase in pH. When the pH value got up to about 7, the labeling yield achieved the maximum value. Beyond this level, the labeling yield slightly decreased, which suggested that a greater yield could be achieved when the moderate pH was selected. Figure 3b showed the effect of buffer pH and labeling time on labeling yield. The peak increased with the increased time and reached up to a maximum value, further increase did not produce obvious effect. Figure 3c depicts the interaction effect of labeling time and labeling temperature on labeling yield. It can be seen that by increasing the labeling temperature, the labeling yield increased as well, reached a maximum value, and the further increase of temperature led to its decrease. This phenomenon indicated that the high temperature could induce decomposition of SEM derivative.

The optimum conditions given by the model were as follows: buffer pH of 7, labeling temperature of 40 °C, and labeling time of 10 min. In order to validate the adequacy of the model equation, a verification experiment was carried out under the optimal conditions mentioned above. Under the optimal conditions, the model predicted a maximum response of 1990. A mean value of 2029 (n=3) was obtained from actual experiments and demonstrated the validation of the model equation. The good correlation between these results undoubtedly confirmed that the model was adequate for reflecting the predicted optimization.

HPLC Separation of SEM Derivative

The primary challenge in analyzing the present single-reactor derivatization products is the separation of the products of interest from the large excess of labeling reagents. First of all, the chromatographic conditions were optimized. Five LC columns with different stationary phases were trialed (i.e., Hypersil C18, Hypersil BDS C8, Hypersil BDS C18, Eclipse XDB-C8, and Spherisorb C18), with a variety of mobile phases (ultrapure water, MeOH, and ACN). Results indicated Hypersil BDS C8, Hypersil C18, Hypersil BDS C18, and Spherisorb C18 columns gave poor separation and peak shape compared to the Eclipse XDB-C8 column. Therefore, Eclipse XDB-C8 column was chosen for further optimization. Acetonitrile was selected as organic solvent due to its better elution power. The proportion of acetonitrile in the mobile phase was investigated. The results showed that the retention time of the SEM–BCEC derivative gradually reduced with increasing acetonitrile percentage. An excessively high proportion of acetonitrile was not beneficial to the complete separation of SEM derivative in samples because of the co-elution of other components. Considering the separation efficiency and accuracy, the optimum eluent A was 5 % of aqueous acetonitrile and eluent B was acetonitrile (100 %). Fluorescence excitation and emission spectra were obtained by wavelength scanning spectrometer in the range of 200–700 nm. Results indicated the maximum fluorescence excitation and emission wavelengths were λ_{ex}=279 and λ_{em}=380 nm, respectively.

As an amidogen-reactive reagent, BCEC used in this research can also react with other coexisted amidogen-

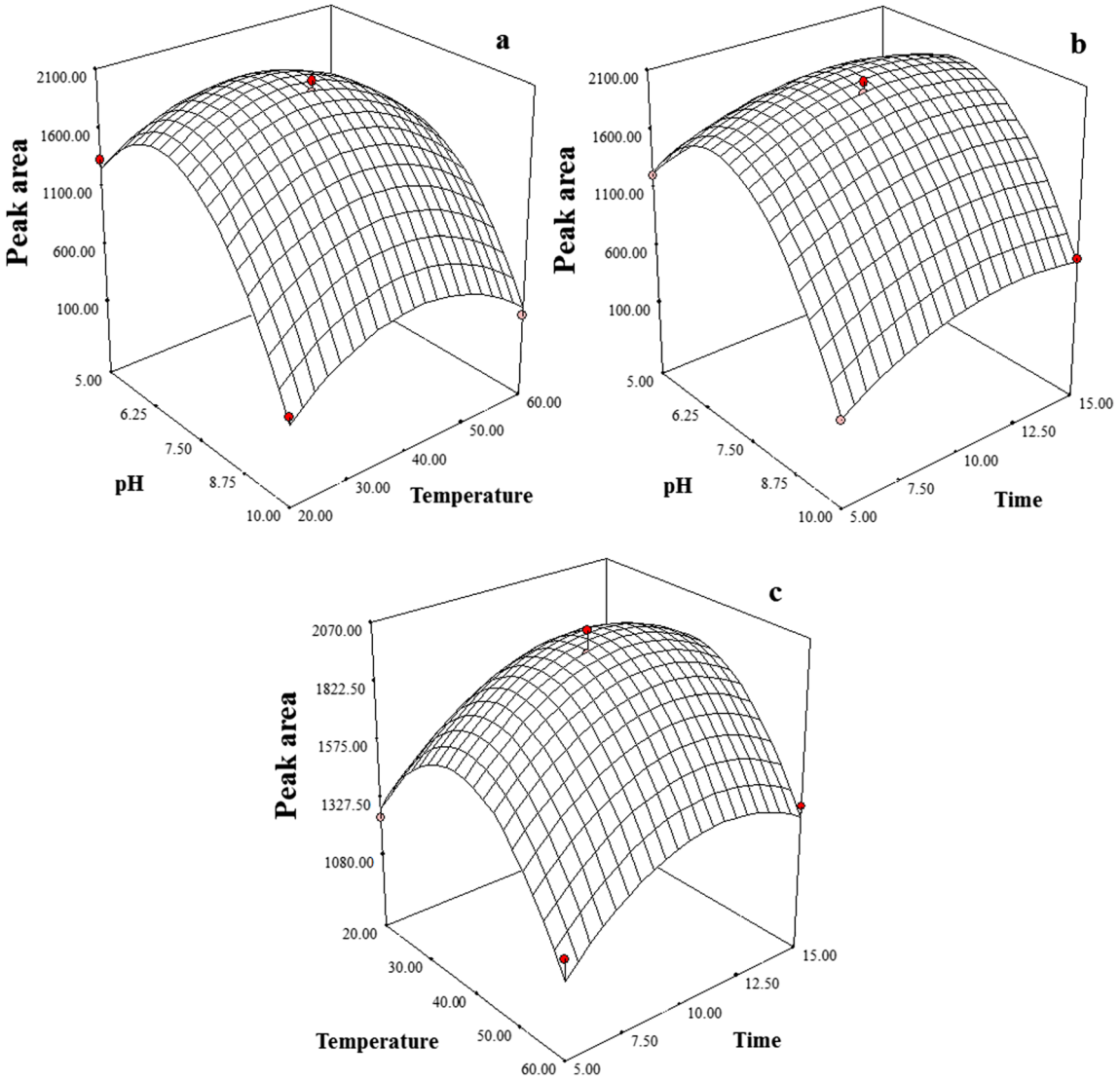

Fig. 3 The 3D response surface plots of fluorescent labeling efficiency for SEM affected by buffer solution pH, labeling time, and labeling temperature

containing compounds, like amino acids or polypeptide. After systematic experiments, it was found that although amino acids or polypeptide could react with BCEC, their derivatives are easily separated by gradient elution because of their different structures.

HPLC Method Validation

The linear regression equation for SEM was calculated as $Y=153.8X-0.79$($R^2=0.9997$), in which X is SEM concentration and Y is peak area. The detection limit for SEM was determined by diluting SEM standard derivatization solution and found to be 0.4 μg/kg at a signal-to-noise ratio of 3, which indicated that the proposed HPLC method is ultrasensitive to quantify SEM in foodstuffs. RSD values of retention time and peak area were less than 0.06 and 2.5 %, respectively, which satisfied the criteria of quantitative analysis. The intra- and inter-day precisions (expressed in terms of % R.S.D.) were found to be in the range of 1.32–3.83 % and 4.21–5.93 %, respectively, which demonstrated the good precision of the proposed method. The accuracy of the method was examined by adding known amount of standard substance to sample solution. The percentage of recovery obtained by comparing the results from the original samples and the fortified samples are reported in Table 2. Recoveries for food samples were in the range of 91.0–100.4 %, indicating a good accuracy.

Sample Analysis

In order to evaluate the feasibility of the proposed method, it was applied for the determination of SEM in the food matrices (bread, instant noodles, pork, shrimp, prawn, mutton, chicken, and fish). The contents of SEM in all food samples are summarized in Table 2. The representative chromatograms

Table 2 Analytical recovery and contents of SEM in selected samples (n=3)

Samples		SEM		Recovery	RSD (%)
	Original (μg/kg)	Added (μg/kg)	Found (μg/kg)		
Bread	35.5	10	45.21	97.1	2.91
		50	82.65	94.3	3.04
		100	128.70	93.2	3.50
Instant noodle	45.8	10	55.84	100.4	3.80
		50	92.15	92.7	3.05
		100	138.90	93.1	3.14
Pork	ND	2	1.82	91.0	3.15
		6	5.57	92.9	2.68
		10	9.14	91.4	3.37
Shrimp	ND	2	1.83	91.7	2.72
		6	5.76	96.0	2.32
		10	9.83	98.3	2.15
Prawn	ND	2	1.81	90.4	4.00
		6	5.37	89.5	2.10
		10	9.13	91.3	2.72
Mutton	ND	2	1.88	94.1	3.30
		6	5.50	91.7	4.20
		10	9.57	95.7	3.63
Chicken	ND	2	1.92	95.4	3.14
		6	5.68	94.7	2.80
		10	9.69	96.9	3.24
Fish	ND	2	1.85	92.5	2.76
		6	5.72	95.3	3.47
		10	9.77	97.7	3.23

ND not detected

for SEM standard, blank, bread sample, and instant noodle sample are shown in Fig. 4a–d. It was found that SEM was found in bread and instant noodle samples, and the contents were 35.5 and 45.8 μg/kg, respectively. These results indicated that ADC may be used as a flour-improving agent in bread and instant noodle products, which degraded to form SEM. The meat samples were all tested negative for SEM according to the prohibition of nitrofurazone established by China Food and Drug Administration (CFAD).

Comparison of the Proposed Method with Some Reported Methods

To evaluate the proposed method further, a thorough comparison of the present method with several recently reported methods is presented in Table 3, and these methods were based on HPLC–MS, surface-enhanced Raman spectroscopy, HPLC–FLD, and Biochip array sensing technique. Compared with these methods, the present method has many advantages

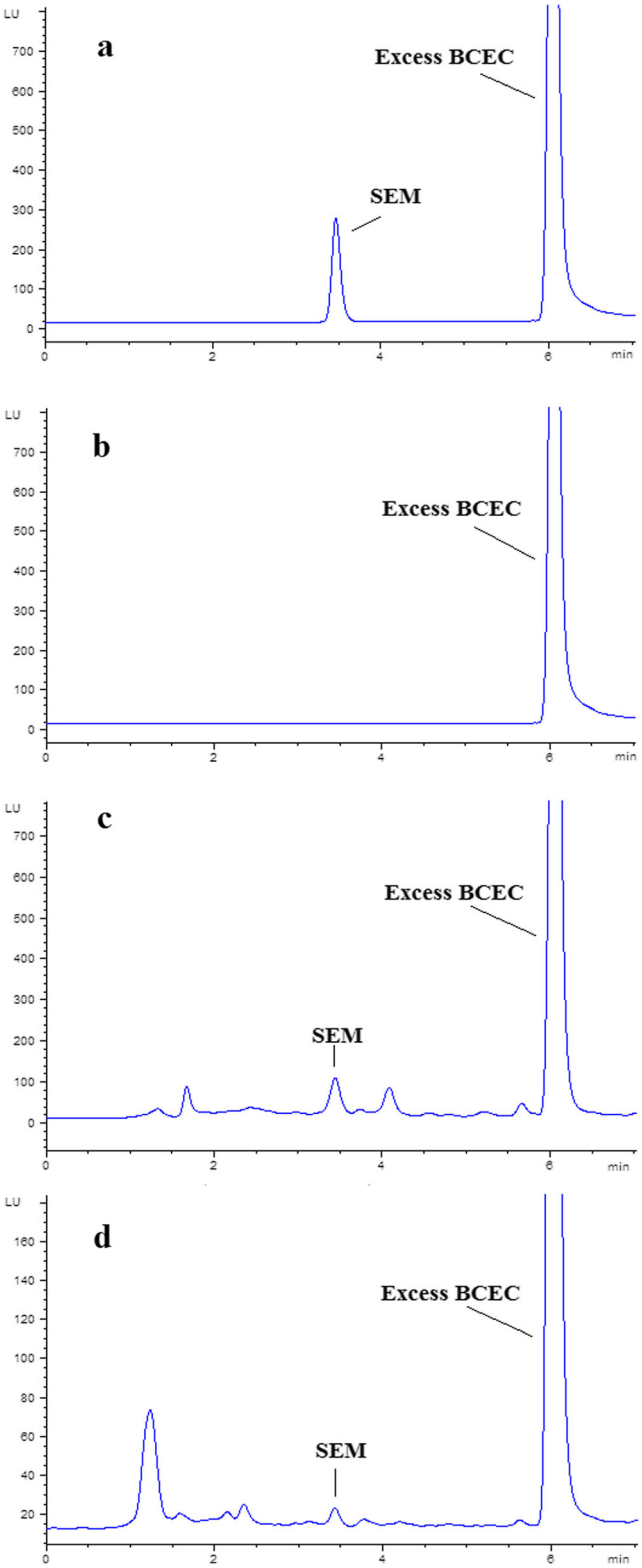

Fig. 4 The representative chromatograms for **a** SEM standard, **b** blank, **c** bread sample, and **d** instant noodle sample

such as low cost, high sensitivity, good selectivity, simple sample preparation, and so on. For example, the new method provides the LOD of 0.4 μg/kg without complex enrichment,

Table 3 Comparison of the proposed method with some reported methods

Method	Matrix	Derivatization conditions	LOD	Reference
HPLC–MS	Animal tissue	Derivatization time, 16 h; temperature, 37 °C	3 μg/kg	(Li et al. 2014)
HPLC–MS	Animal tissue, plants, eggs	Derivatization time, 16 h; temperature, 37 °C	10 μg/kg	(Chen et al. 2014)
HPLC–MS	PVC gaskets	Derivatization time: *overnight*; temperature, 37 °C	Not mentioned	(Chen et al. 2012)
UHPLC–MS	Seafood	Derivatization time: *overnight*; temperature, 37 °C	0.6 μg/kg	(Valera-Tarifa et al. 2013)
Surface-enhanced Raman spectroscopy	Flour	No derivatization	10 μg/mL	(Xie et al. 2013)
Chemiluminescence-based biochip array sensing technique	Honey	No derivatization	0.9 μg/kg	(O'Mahony et al. 2011)
HPLC–FLD	Pork muscle	Derivatization time, 2 h; temperature, 60 °C	0.21 μg/kg	(Sheng et al. 2013)
HPLC–FLD	Several foodstuffs	Derivatization time, 10 min; temperature, 40 °C	0.4 μg/kg	The present work

which is lower than most of methods in Table 3. Furthermore, the mostly used method for SEM determination is HPLC–MS, but a tedious derivatization procedure (16 h) using 2-nitrobenzaldehyde (2-NBA) is often required to overcome the implications of ion suppression and low retention in reversed HPLC column (Table 3). The isotopically labeled internal standard is also necessary in HPLC–MS analysis of SEM, which is relatively expensive and not readily available. Sheng et al. developed a HPLC–FLD method for nitrofuran metabolites determination using 2-hydroxy-1-naphthaldehyde as labeling reagent (Sheng et al. 2013). This method offered a similar LOD of 0.21 μg/kg, but it required a long labeling time of 2 h. The method proposed in this study only needs the short derivatization time of 10 min at the mild temperature of 40 °C. Moreover, the labeling reagent of BCEC possesses a large conjugated-system not only ensuring the fluorescent properties but also increasing the retention capability of the labeled SEM, which significantly facilitates the separation in a reversed column. The labeled SEM was detected at the specific excitation and emission wavelengths, eliminating the interference of many other inclusions. Moreover, compared to HPLC–MS, surface-enhanced Raman spectroscopy and Biochip array sensing technique in Table 3, HPLC–FLD is more convenient, cheaper, and easily available in common analytical laboratories. It is worthy to note that it is the first trial of using fluorescence labeling (BCEC as labeling reagent) followed by HPLC–FLD for SEM screening in foodstuffs. This method provides a potential choice for SEM monitoring.

Conclusion

A new pre-column fluorescence labeling method using BCEC as the labeling reagent has been developed for trace SEM determination by HPLC–FLD. The labeling conditions were optimized by RSM, ensuring sufficient and rapid labeling. This method has been proven to be simple, inexpensive, selective, sensitive, accurate, and reliable for SEM analysis in foodstuffs. Furthermore, this developed method should have a powerful potential in the analysis of SEM from many other food samples.

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Conflict of Interest Guoliang Li declares that he has no conflict of interest. Chenhong Tang declares that he has no conflict of interest. Ying Wang declares that she has no conflict of interest. Jing Yang declares that she has no conflict of interest. Hongliang Wu declares that he has no conflict of interest. Guang Chen declares that he has no conflict of interest. Weiheng Kong declares that he has no conflict of interest. Xiaojian Kong declares that he has no conflict of interest. Shucheng Liu declares that he has no conflict of interest. Jinmao You declares that he has no conflict of interest.

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