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A new method research for determination of natural pigment crocin yellow in foods by solid-phase extraction ultrahigh pressure liquid chromatography

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ABSTRACT

Crocin yellow was determined in soft drinks, sausages and sauces by ultra performance liquid chromatography coupled with ultraviolet detector and analyzed within 5 min using a short analytical column ACQUITY UPLC HSS T3 2.1 × 100 mm 1.8 μm) with gradient elution. An innovative pretreatment method based on homemade macroporous resin solid-phase extraction (SPE) column was established. The SPE column packed with macroporous resins could simplify the sample preparation of multi-matrices and be reused by regeneration steps. The recoveries of crocin yellow added to soft drinks, sausages and sauces at three levels ranged from 81.3% to 106.2%, and relative standard deviations (RSDs) were within 8.8%. The limits of quantitation of soft drinks, sausages and sauces were 0.5 mg/kg, 5 mg/kg and 5 mg/kg, respectively.

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1. Introduction

In recent years, the abuse of food additives, especially synthetic food colors, has drawn more and more attention in the world. Synthetic food colors, which are widely used in food industry, cause potential human health concerns; many kinds of synthetic colors are even not permitted to be used in foods on account of the related legal issues [1]. However, the use of food colors is important in food industry because they improve the appearance of foods. Hence the solution is to use natural colors instead, which are much safer and healthier. Crocin yellow is one of the important natural colors [2].

Crocin yellow, which is extracted from *Gardenia jasminoides Ellis*, is widely used as a natural food colorant in Asian countries, while Gardenia extract has been used in Chinese traditional medicine (CTM) for curing a number of ailments [3]. These crocetin derivatives, which are different from most families of carotenoids, are known for their coloring properties owing to their peculiar water-soluble behavior [4]. Numerous studies have dealt with the component structures of yellow pigment extracts isolated from gardenia fruits [4–8], their spectroscopic characterization and radical-scavenging activity [4,9], data concerning the concentration of major components for the determination of optimal time of harvest and extraction process [10,11]. However, reports on the determination of crocin yellow in food products, to our knowledge, are limited in number to date. With the development of food industry and the improvement of people's health consciousness, natural colors will be more and more widely used in food products. Therefore, it is very necessary to establish a simple, fast and accurate method as the industrial standard to detect the addition of natural colors in food products.

A large number of analytical methods for synthetic food colors have been proposed, such as thin-layer chromatography (TLC) [12,13], spectrophotometry [14,15], capillary electrophoresis (CE) [16,17], ion chromatography [18] and high-performance liquid chromatography (HPLC) [19,20]. In HPLC methods, polyamide absorption column was used as the pretreatment method of food samples [21]. The detection methods of synthetic colors are becoming more perfect and standardized. However, the determination methods for natural food colors still need to be improved. We have tested the feasibility of the use of polyamide adsorbent in the sample pretreatment method of natural color detection. Due to the differences in structures and characteristics of natural and artificial colors, polyamide has strong ability to absorb natural color. Strong alkaline ammonia solution is harmful as the common eluent of polyamide adsorbent has destructive effort to most of natural color [22], and the elution rates of some other organic eluents such as methanol and ethanol aqueous solution are low. Therefore, the

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Table 1
Physical characteristics of macroporous resins.

Grade	Surface area (m^2/g)	Ave. pore diam. (Å)	Polarity
X-5	500–600	290–300	Non-polar
AB-8	480–520	130–140	Weak-polar
D-101	480–550	12–15	Non-polar

result was far from ideal. Based on a number of reports stating that macroporous resins could be used to separate and purify natural products by adsorption–desorption process [23–25], we initiatively made solid-phase extraction (SPE) column by using macroporous resins as the stationary phase to pretreat food samples.

Macroporous resin is a new type of nonionic high-molecular-polymer adsorbent mainly using styrene and divinylbenzene as raw materials. This material has the properties of selective adsorption. The adsorption of macroporous resin is caused by Van der Waals' forces or hydrogen bonding between molecules. Simultaneously, the porous structure and various surface functional groups available cause different sized objects to have different adsorption characterstics with the resins. By eluting, different substances are separated. Therefore, the enrichment and filtration of macroporous resins are achieved based on the fact that adsorption performance varies in different substances.

In the present study, the most suitable SPE packing was selected by comparing the recoveries of crocin yellow on different macroporous resins. The studies on the relationship between regenerated times and adsorption–elution efficiency were also conducted.

This paper describes an analytical method for the determination of crocin yellow in drinks, meat products and flavorings in 5 min by ultrahigh pressure liquid chromatography (UHPLC) equipped with UV detector using a short analytical column. The detection method has advantages of short analysis time, high repeatability and accuracy. Compared with commercial SPE columns, the sample pretreatment method of SPE column using macroporous resins as the stationary phase to adsorb crocin yellow is simple with a wide range of applications.

2. Experiment

2.1. Chemicals and reagents

Crocin yellow high-purity extracts were purchased from Tokyo Chemical Industry Co., LTD. The extracts were dissolved in deionized water to give a concentration of 0.5 mg/L. Acetonitrile of HPLC grade was purchased from Fisher Scientific, Fairlawn, NJ (USA). Ethanol and acetic acid were of analytical grade from Beijing Chemical Reagents Co. (Beijing, China). Deionized water was purified by a Milli-Q Water Purification system (Millipore, MA, USA).

Macroporous resins including AB-8, D-101 and X-5 were purchased from Nankai University (Tianjin, China). Their physical properties are listed in Table 1. The resins were pretreated by 4% HCl and NaOH solutions successively to remove the monomers and other agents trapped inside the pores during the synthesis process. Treated resins were soaked in ethanol and subsequently washed by deionized water thoroughly before each use.

2.2. Apparatus and instrumental parameters

All UHPLC measurements for crocin yellow determination were done using a Waters ACQUITY^{TM} 1100 UPLC system equipped with a quaternary solvent delivery system, an autosampler and a UV detector. Separation was achieved on a Waters ACQUITY UPLC HSS T3 (2.1 × 100 mm, 1.8 μm) column. Solvent A was acetonitrile and solvent B was water, which were applied in the gradient elution as follows: 0–3 min, linear gradient from A–B (60:40, v/v) to A–B (80:20, v/v); 3–3.5 min, linear gradient to A–B (98:2, v/v), which was held for 1 min; and 4.5–5 min, linear gradient to A–B (60:40, v/v). The column temperature was set at 30 °C. The flow rate was set at 0.25 mL/min, and the injection volume was 5 μL. The detection wavelength of UV detector was 440 nm.

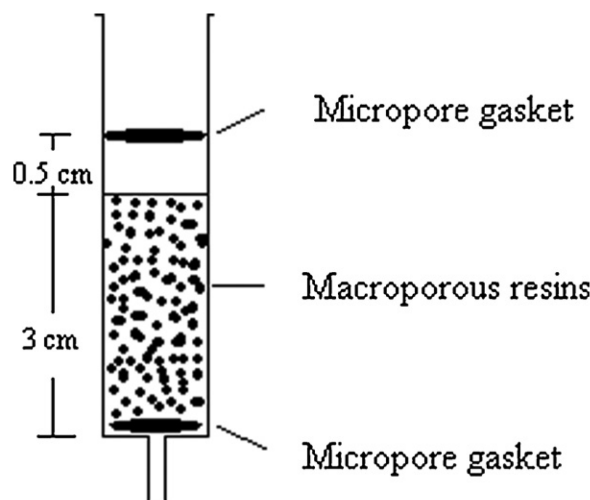

Fig. 1. Diagrammatic drawing of homemade SPE column.

Mass spectrometry was performed on Waters Micromass®-Quattro Premier XE operating in ESI$^+$ modes. The nebulization gas was set to 600 L/h at a temperature of 400 °C, the cone gas was set to 50 L/h, and the source temperature was set to 110 °C. The capillary voltage was set to 3.5 kV, and the cone voltage was set to 40 V. All of the raw data were analyzed by Masslynx V4.1.

2.3. Sample preparation

2.3.1. Preparation of macroporous resin SPE column

Treated hydrated resins (3 cm) were put into the glass column measuring 5 cm in length and 1 cm in diameter. Micropore gaskets were fixed in the column. The void of 0.5 cm was reserved because the resin swells during the elution (Fig. 1).

2.3.2. Optimization of SPE conditions

AB-8, D-101 and X-5 resins were selected to investigate the possibility of their use for the SPE column packing by comparing the recoveries of crocin yellow after adsorption–elution test. According to the Section 2.3.1, different kinds of macroporous resin SPE columns were made. Different loading quantities, pH values, ratios of eluent and velocities of flow were optimized in the process. Accordingly the best macroporous resins and SPE conditions were chosen.

2.3.3. Sample preparation

For drinks, a 5 g amount of sample was weighed accurately in a beaker. If the sample was carbonated, it was degassed by ultrasonication for 5 min. The content was adjusted to approximately pH 4 with 36% acetic acid.

For sausages and sauces, a 0.5 g amount of crushed sample was weighed accurately in a 10 mL centrifuge tube. Five milliliters of acetic acid solution (pH 4) was added to extract the colors. The supernatant was collected and mixed after centrifugation at 12,000 revolutions per minute (rpm). The extraction step was repeated three times.

The sample solution was applied to the column, and then the column was washed with pH 4 acetic acid solutions in order to remove the additives such as inorganic salts, followed by 15 mL of

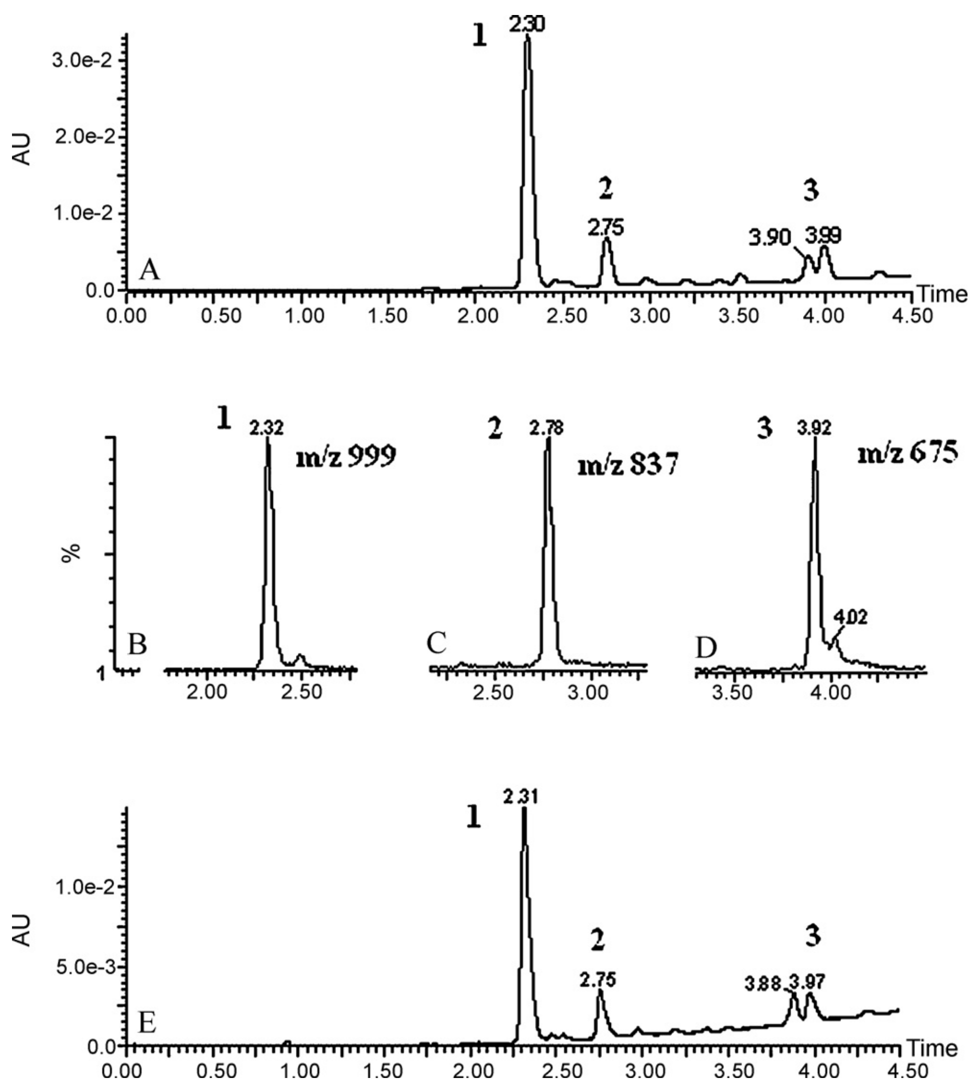

Fig. 2. UHPLC and SIR chromatograms of crocin yellow standard solutions (10 mg/L). (A) was UV chromatogram. (B), (C) and (D) were SIR chromatograms of extracted ion current at m/z 997, 815 and 653. (E) was typical UV chromatogram of samples fortified with crocin yellow at 10 mg/L. Peak 1 was crocin-1 and the retention time was 2.30 min; Peak 2 was crocin-2 and the retention time was 2.75 min; 3.90 min and 3.99 min were the retention times of crocin-3, peaks 3. The component crocin-3 has two forms of optical isomers, so there were two peaks (3.92 and 4.02 min) in the single channel of m/z 675 under SIR mode. The retention times of peaks were postponed for about 0.02 min in the SIR chromatograms because of mass-spectrometer detector after UV.

water to remove the sugar and the residual acetic acid. The column was eluted with 5 mL of 90% ethanol solution, and then the eluate was dried with nitrogen. The residue was dissolved in 2 mL of methanol/water (1:1, v/v). Besides, during the processes, the velocity of flow was kept at about 2–3 s per drop.

2.3.4. Activation and regeneration of SPE column

The used SPE column was washed with 15 mL of water to remove the residual ethanol, and then eluted with 15 mL of 5% hydrochloric acid followed by water until the eluate was neutral, and finally washed with 15 mL of 5% sodium hydroxide again followed by water. The working solution of 5 mg/L was applied to the regenerated SPE column and processed as mentioned above. The eluate was dried and made to volume, and then injected into the instrument. Recoveries were calculated comparing the peak areas of eluate with those of standard solution. Accordingly, the relationship between regenerated times and the recoveries was obtained. The whole process was repeated thrice ($n = 3$).

2.4. Preparation of calibration curve

The maximum UV absorption peak, peak 1, was selected as the quantitation peak (Fig. 2A). Working solution was prepared and diluted to an appropriate concentration range and processed by the SPE steps mentioned above. The eluate was dried and dissolved, and then injected into the instrument. The calibration curve was analyzed in triplicate and constructed from peak areas of the reference standards versus the concentrations.

2.5. Precision and accuracy

Recoveries of fortified samples were calculated comparing the peak areas of the sample eluate with those of standard solution eluate. The blank samples of soft drinks, sausages and sauces were fortified at three additional quantities, and each fortified level was prepared six times. Intra-day and inter-day reproducibility of the method were assessed by performing replicate analyses. Intra-day precision was determined by analyzing the six fortified samples on

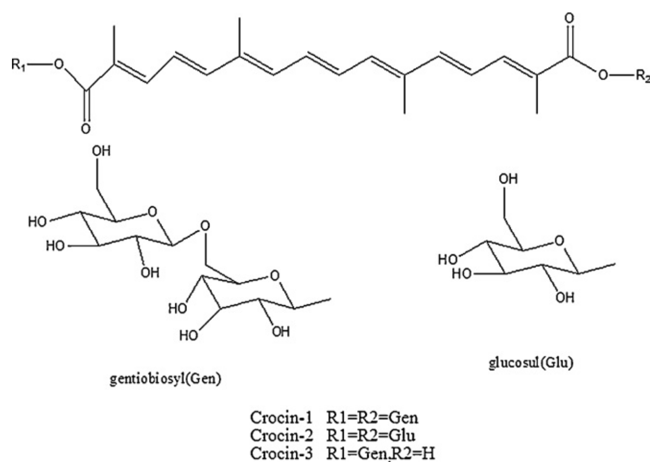

Fig. 3. Structures of crocin yellow compounds.

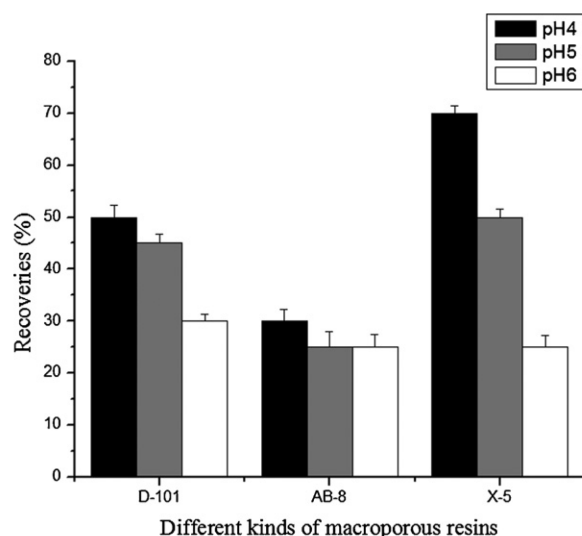

Fig. 4. Effects of different pH values (4, 5, 6) of loading solution on the recoveries of crocin yellow. The error bars represent relative percent differences calculated from working solutions of 1 mg/L, 2 mg/L and 5 mg/L processed by the SPE steps, respectively.

the same day. Inter-day repeatability was determined by analyzing the samples on three days.

3. Results and discussion

3.1. Optimization of separation conditions

According to the maximum absorption data of all the target components on the UV spectra, 440 nm was selected as the detection wavelength, where all kinds of yellow pigments could be detected and had adequate absorption. In order to achieve the best separation of crocin compounds, different types of chromatographic columns were tested to optimize the separation, such as Waters ACQUITY UPLC HSS T3 2.1 × 100 mm, 1.8 μm column, BEH C_{18} UPLC (2.1 × 50 mm, 1.7 μm) column, BEH Shield RP_{18} UPLC (2.1 × 100 mm, 1.7 μm) column and SHISEIDO CAPCELL PAK C_{18} (2.0 × 50 mm, 3 μm) column. As a result Waters ACQUITY UPLC HSS T3 (2.1 × 100 mm, 1.8 μm) was selected. For solvent A, the common reversed-phase solvents such as methanol, acetonitrile and methanol–acetonitrile were compared. Acetonitrile gave the best separation of peaks. For solvent B, water was used. Besides, it was found that the separation was better when the column temperature was kept at 30 °C. The flow rate was set at 0.25 mL/min for satisfactory separation and reasonable analytical time and the gradient elution program was performed to ensure that the each compound was well separated. Fig. 2A showed the typical separation of crocin yellow extract.

According to Chen et al. [2] reports, crocin compounds had three main structures, crocin-1, crocin-2 and crocin-3, as shown in Fig. 3. Under positive SIR (Single Ion Recording) mode, the molecular ion m/z 999 (M+Na$^+$), 837 (M+Na$^+$) and 675 (M+Na$^+$) obtained by adding sodium were chosen as three single channels. The peaks were identified on SIR mode chromatograms (Fig. 2B–D).

3.2. Optimization of SPE condition

Varieties of macroporous resins, different loading quantities, pH values, ratios of eluent and velocities of flow were the main factors influencing recoveries. 1 mg/L, 2 mg/L and 5 mg/L of working solution were processed by the SPE steps and then detected by UHPLC-UV. Recoveries were calculated comparing the peak areas of eluate with those of standard solution and the average was taken. Taking X-5 for example, the effects of different pH values of loading solution on recoveries were compared. Considering that the pH working range of macroporous resins was 4–8 and acidic condition was beneficial to the adsorption of target compounds due to the formation of hydrogen bond between natural colors and the resins, loading solutions of pH 4, 5 and 6 adjusted by acetic acid were compared. As shown in Fig. 4, pH 4 was the best condition. The increase of ethanol to eluent ratio was beneficial to the desorption of target matter from the stationary phase, but also, unfavorable to the solution of crocin yellow. In order to reach a balance point, effects of different ratios of ethanol (50%, 60%, 70%, 80% and 90%) as eluent on recoveries were compared. In Fig. 5, 90% was the best ratio. Besides, it was found that the adsorption–desorption effect was better when the velocity of flow was kept at 2–3 s per drop. According to the highest recoveries of crocin yellow processed by D-101, AB-8 and X-5 resin SPE column, which were 62%, 40% and 82% respectively, the optimum SPE condition was obtained. X-5 macroporous resins used as packing of SPE column had the best adsorption–elution efficiency and the processing steps are described in Section 2.3.3. That means the absorbability and pore size of X-5 macroporous resins were suitable for the enrichment of crocin yellow in food samples.

The used SPE column was regenerated as described in Section 2.3.4, and adsorption–elution recoveries of the regenerated

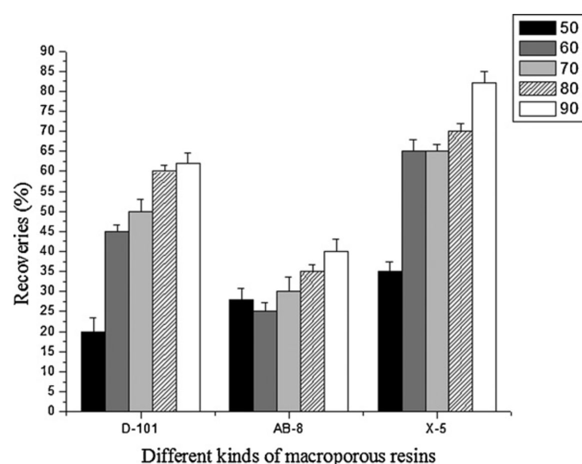

Fig. 5. Effects of different ratios of ethanol (50%, 60%, 70%, 80% and 90%) as eluent on the recoveries of crocin yellow. The error bars represent relative percent differences calculated from working solutions of 1 mg/L, 2 mg/L and 5 mg/L processed by the SPE steps, respectively.

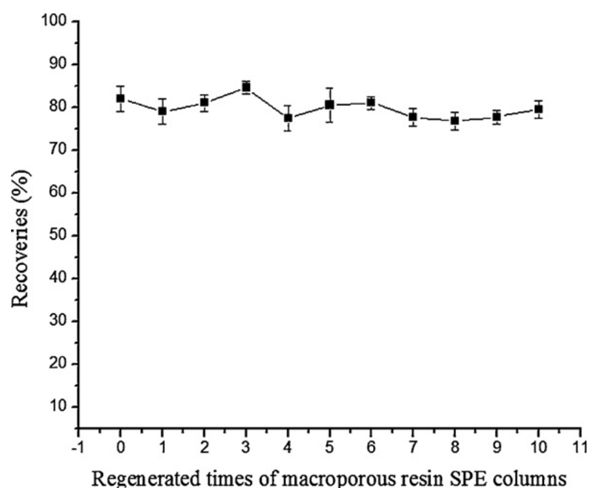

Fig. 6. The relationship between regenerated times and recoveries of crocin yellow on resin SPE column. The regenerated times "0" represented new treated macroporous resins without regeneration. The error bars represent relative percent differences calculated from three parallel solutions of 5 mg/L. The relative standard deviations (RSDs) of the recovery for 11 times were less than 3%.

SPE column were tested. As can be seen from Fig. 6, regenerated resins had the same adsorption–elution properties as those of new treated resins. Therefore, the macroporous resin SPE column can be used repeatedly ten or more times by the regeneration step. Besides, we are also working on how to simplify the approach of regeneration and the modification of macroporous resin to increase performance.

3.3. The advantages of macroporous resin SPE columns

The macroporous resin SPE column is first reported and also a new departure for solid-phase extraction packing. Compared with commercial SPE columns, the greatest advantages of macroporous resin SPE columns are their specific adsorption to natural colors and the fact that they are reusable. We know that commercial SPE column is relatively expensive. Especially for extensive detection, macroporous resin SPE columns have extraordinary advantages over conserving resources. There is no denying that on-off commercial SPE column is a huge waste of resources. Toshiro et al. [26] had established a remarkable CE method involving the pretreatment of commercial SPE cartridge to determinate crocin yellow in candies and noodles. In their reports, the matrices were relatively clean and the extraction solution also needed to be relatively clean and concentrated before the SPE step. In another report [27], the solution loaded into the commercial SPE cartridge was saffron aqueous. By comparison, the matrices used in this study were relatively complex. Because of excellent filtering effect, for sample containing large quantities of sugar (soft drinks), animal fat (sausages) and vegetable oil (sauces), resin SPE column permits direct sampling after simple extraction without the steps of removing the fat and protein. The eluate of crocin yellow was pure and permitted injection into instruments. For this reason resin SPE column could simplify the sample preparation of multi-matrices. On the other hand, in the UHPLC method, three components of crocin yellow were analyzed within only 5 min by using acetonitrile and water as mobile phase. Therefore, compared with the CE detection method described in Ref. [26], UHPLC method has better separation effect, shorter analysis time and simpler system of mobile phase, and is also more suitable for popularization.

Table 2
Results of recoveries for crocin yellow in soda, juice, sausage, sauce samples ($n = 6$).

Matrix	Spiked level (g/kg)	Recovery (%)	RSD (%)	LOQ (mg/kg)
	0.001	92.2	8.17	
Sodas	0.002	98.8	4.07	0.5
	0.005	100.6	5.37	
	0.001	90.6	3.88	
Juice	0.002	83.6	5.27	0.5
	0.005	81.3	1.45	
	0.01	96.4	8.80	
Sausages	0.02	94.8	8.40	5
	0.05	91.4	8.77	
	0.01	104.1	8.67	
Sauces	0.02	106.2	7.25	5
	0.05	96.3	8.80	

3.4. Determination of crocin yellow in foods

The linearity for crocin yellow was checked by analyzing the eluate of standard solution of five different concentrations. Calibration curves were linear ($r^2 = 0.999$) between 0.5 mg/L and 10 mg/L. Typical UHPLC chromatogram of samples is shown in Fig. 2E. Table 2 shows the recoveries of crocin yellow from soft drinks, sausages and sauces fortified at three additional quantities. The intra-day recoveries ($n = 6$) ranged from 81.3% to 106.2%, with relative standard deviations (RSDs) ranging from 1.45% to 8.8%. Both intra-day and inter-day accuracy and precision data showed good RSDs and recovery values. The LOQs (limits of quantification) were based by $S/N \geq 10$.

According to Chinese national standard GB/T 2760–2007 [28], maximum permitted content of crocin yellow in soft drinks, sausages and sauces was 0.3 g/kg, 1.5 g/kg and 1.5 g/kg, respectively. Therefore, the UHPLC analysis method based on pretreatment of homemade SPE can satisfy the requirements of detection of crocin yellow.

4. Conclusions

An analytical method based on innovative pretreatment of homemade SPE was developed for quantitation of crocin yellow in foods by UHPLC with UV detector. Using a short analytical column, these samples were analyzed within 5 min. This method gave reliable and reproducible results with satisfactory detection limits and very short analysis time for the domestic analysis of crocin yellow.

Acknowledgments

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