Determination of eight Sterols in Rapeseed by Accelerated Solvent Extraction Coupled with Gas Chromatography tandem Mass Spectrometry

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Abstract

For determination of cholesterol, cholestanol, rapeseed sterol, ergosterol, rapeseed sterol, stigmasterol-sitosterol, and stigmasterol in rapeseed, an accelerated solvent extraction followed gas chromatography-tandem mass spectrometry technique was developed. The sterols were isolated after derivatization and identified using accelerated solvent extraction coupled with gas chromatography-mass spectrometry (GC-MS/MS). The results revealed a good linear relationship between the peak area and the corresponding mass concentration of the eight sterols in the range of 2-1000 mg/L, with correlation coefficients all greater than 0.9991, detection limits of 1.12–1.61 mg/kg, and quantification limits of 3.73–5.41 mg/kg, spiked recovery determination ranging from 5 to 20 mg/kg.The average recoveries were 89.5-105.2% at mg/kg supplemental level, and the precision was lower than 5.0%. The developed method was efficient, simple and accurate, and suitable for qualitative and quantitative analysis of eight sterol compounds in rapeseed, providing a method for rapid determination of sterol compounds in rapeseed.

1. Introduction

Sterols are a class of cyclopentane poly-hydro phenanthrene derivatives formed by the condensation of three hexane rings and one cyclopentane, which are extensively present in cells or tissues of living organisms [1]. Sterols are mainly animal sterols, phytosterols, and mycosterols where the main manifestation of cholesterol in animal sterols, is the consequential of β-sitosterol, soy sterol, rapeseed sterol, rapeseed oil sterol in phytosterols, and ergosterol can represent mycosterols. Sterols can exhibit different functional properties:antibacterial [2], anti-aging [3], anti-atherosclerosis [4], antioxidant [5], anti-inflammatory [6], anti-tumor [7] and anti-cardiovascular disease [8], are some of the functions of sterols. Phytosterols, in particular, aid to reduce cholesterol levels. The American Heart Association (AHA) advocated phytosterol consumption to lower cholesterol levels in the US population in 2000, while the Chinese Ministry of Health designated phytosterol foods as new resource foods in 2007. Sterols and their derivatives have become widely employed in nutrition and medicine as a result of study over the last several years.

Rapeseed is one of the four major oilseeds in the world and is widely grown around the world, with China leading the world in rapeseed production. Rapeseed is a raw material rich in sterols, of which cholesterol and ergosterol account for a small percentage, while phytosterols account for a higher percentage [10]. In the human body only the brain and cerebral nerves carry out cholesterol synthesis, while phytosterols and fungal sterols need to be ingested through diet, and the seeds or fruits of rapeseed, a type of oil plant with abundant types of sterol compounds meet the health needs of people for low cholesterol and high phytosterols [11]. A sensitive analytical method for the determination of sterol compounds in rapeseed trace sterols should have an effective extraction and detection technique.

Enzymatic digestion [12], isotope dilution mass spectrometry [13], electrochemical analysis [14], and chromatography [15] are some of the current detection techniques for sterol compounds. High performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [16], gas chromatography-tandem mass spectrometry (GC-MS/MS) [17], and ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [18] are among the chromatographic techniques that have become sterols due to the chromatographic tandem mass spectrometry technique exhibiting the advantages of a perfect[19]. According to the data, most sterols lack a chromophore, which means they can't be detected by UV, visible, or fluorescence spectroscopy. However, sterols can be altered through derivation or oxidation of alcohol substitutes to improve light absorption of sterol structures, which can be applied for GC detection. From the point of view of parameter analysis, the parameter optimization of GC-MS/MS is simpler than that of HPLC-MS/MS, from the point of view of mobile phase, the carrier gas cost consumption of GC-MS/MS is also lower than that of HPLC-MS/MS mobile phase. Furthermore, GC-MS/MS also has the features of high sensitivity, low injection volume and high separation efficiency.

Extraction, purification, and derivatization are important processes in the sample preparation for GC-MS/MS analysis because they are strongly related to the analysis of the components to be measured and have a direct impact on the assay's sensitivity. The complexity of the pretreatment operation is increased by sample derivatization, and extraction techniques can be utilized to optimize the sample pretreatment operation and simplified the experiment. Soxhlet extraction [20,21], supercritical fluid extraction [22], microwave extraction [23], ultrasonic extraction [24], and other extraction techniques are currently used. However, two procedures, Soxhlet extraction and supercritical fluid extraction, have time-consuming and high input costs, microwave extraction might harm plant extracts' unsaturated components, and ultrasonic extraction has significant equipment requirements. Accelerated solvent extraction (ASE) is a technology based on the principle of different solubility of solutes in different solvents, which uses high temperature and high pressure to rapidly extract organic matter, which can effectively shorten extraction time, improve extraction efficiency, and improve extraction safety [25]. This technology has been widely employed in the pre-treatment of agricultural, food, and environmental samples due to its remarkable benefits [26,27].

In this study, total sterols were extracted from rapeseed using petroleum ether as the extraction solvent combined with ASE technique followed by gas chromatography-tandem mass spectrometry (GC-MS/MS) for determination of eight sterols in rapeseed. The developed method is high efficient, simple and accurate to provide convincing supporting scientific evidence for determination of sterols in rapeseed.

2. Experiment

2.1 Instruments and devices

SHIMADZU GCMS-TQ8040 triple quadrupole gas chromatography-mass spectrometry (SHIMADZU technology co., LTD.), ASE 350 accelerated solvent extraction (Thermo mercer technology co., LTD.), AutoEVA30Plus automatic nitrogen blowing concentrator (Reco Group Co., LTD.), IKA T25 high-speed tissue homogenizer (Germany Aika Instrument Equipment Co., LTD.), SK8200H ultrasonic cleaning machine (Shanghai Keguide Ultrasonic Instrument Co., LTD.), VORTEX 2 VORTEX (SI DIGITAL, USA), Mili-q type pure water meter (Merck Millebo Company), Dk-8d Type Thermostatic electric water bath (Shanghai Yiheng Technology Co., LTD.), Gnp-9050 Water-proof Constant temperature Incubator (Shanghai Precision Scientific Instrument Co., LTD.), Model AL104 Electronic analytical balance (Mettler Toledo, Switzerland).

2.2 Standards and reagents

Cholesterol (≥99%, CHER), cholestanol (≥95%, CHAN), brassicasterol (≥95%, BRER), ergosterol (≥95%, ERER), campesterol (≥95%, CAER), stigmasterol (≥95%, STER), β-sitosterol (≥95%, SIER), stigmasterol (≥95%, SIER), stigmastanol(≥95%, STAN), all the above standard substances were purchased from Tianjin Alta Technology Co., LTD. 1-methylimidazole (≥99%), and N-Methyl-N-triMethylsilylheptafluorobutyraMide (≥99%) was purchased from (Shanghai Aladdin Biochemical Technology Co., LTD.), Potassium hydroxide and petroleum ether (30-60 °C) were purchased from Tianjin Kermeo Reagent Co., LTD.), dichloromethane ethanol purchased from (Shanghai Anpu Experimental Technology Co., LTD.) rapeseed samples purchased from Hailar area. dichloromethane ethanol purchased from Shanghai Anpu Experimental Technology Co., LTD.) rapeseed samples purchased from Hailar area.

2.3 Pretreatment method

2.3.1 Fat extraction

Weighed 5.00g (accurate to 0.01g) of rapeseed in a small beaker, added 3-5 g of quartz sand, stirred evenly, then poured into a 22 mL extraction tank, the extraction solvent was petroleum ether 30-60℃, extraction temperature 100℃, extraction pressure 10.0 MPa, static extraction 6 Min, rinsed volume 30%, cycle twice, purge for 100 s, dry the extraction tube, bake to constant weight (the extraction tube needs to be roast to constantly weight in advance), the difference between the two weights is the fat content in the sample.

2.3.2 Total sterol extraction

Quantify and weighed 50 mg (accurate to 0.1 mg) of the oil extract in a centrifuge tube, added 4 mL of potassium hydroxide ethanol solution (2 mol/L) and 0.5 mL of dichloromethane, vortex, and mixed well, then centrifuge for 5 min at 30 °C in a shaker water bath for 18 h at 10,000 r/min. Added 5 mL dichloromethane and 3 mL water, mixed them gently, centrifuged them at 10000 r/min for 5 min, removed the upper water phase, and washed the organic phase with 5 mL water three times until clarified the lower solution. The organic phase obtained was blown with nitrogen in the water bath at 40~50℃ and stopped when the remaining 1 mL was left. And transfer the organic phase to the reaction flask for use.

2.3.3 Derivatization procedure

Under the terms of 40℃ , slowly blow out the solvent in the reaction flask with nitrogen gas, and then 100 μL of silanization reagent (N-Methyl-N-triMethylsilylheptafluorobutyraMide: 1-methylimidazole 950:50) was added. The reaction vials were tightly capped and placed on a thermostat at 75 °C for 20 min. Cool the flask to room temperature, add n-ethane and allow to reflux to 1 mL, standard solution derivatization method is the same as the sample derivatization procedure and supplied to a gas chromatography-tandem mass spectrometer for detection.

2.4 Test conditions

2.4.1 Chromatographic Conditions

Carrier gas control mode: Constant column flow (1.2 mL/min) Chromatographic column :DB-5MS (30 m 0.25 mm 0.25 μm) or equivalent column, Injector temperature :290, Injection method: split injection split ratio 10:1, Flow rate: 1.2 mL/min, Column temperature programmed: the initial temperature was 100 (for 1 min), increased to 220 at 50 ℃/min, and increased to 290 at 5 ℃/min (for 8 min).

2.4.2 Mass spectrometry conditions

Electron bombardment (EI) ion source, Voltage energy:70 eV, Transmission line temperature 300, Ion source temperature 250, Selective ion acquisition mode (SIM).

2.4.3 Preparation of standard sample and preparation of standard curve

2.4.3.1 Reserve solution of the standard curve

To make the stock solutions of -sitosterol and the other seven sterols, accurately weigh 5 mg -sitosterol into a PTFE tube and 1 mg each of cholesterol, cholestanol, rapeseed sterol, ergosterol, campesterol, stigmasterol, and stigmasterol into another PTFE tube, respectively, adding 100 L of silanization reagent, derivatized in an oven at 75 °C

2.4.3.2 Standard curve working solution

The concentration gradients of β -sitosterol were 5.0, 50.0, $5.0×10^2$, $2.5×10^3$, $1.0×10^4$ mg/L by diluting the reserve solution with n-hexane. The concentration gradients of the standard sample solutions of other substances mixed are 2.0, 20.0, $2.0×10^2$, $1.0×10^3$, $2.5×10^3$ mg/L and are now available for use.

3. Results And Discussion

3.1 Analysis of qualitative and quantitative ions of eight sterols

The experimental results show that SIM mode has less interference than SCAN mode and can effectively improve the detection sensitivity of target compounds. Validation of eight sterols in SIM mode. By optimizing the instrument conditions, the qualitative and quantitative ions of the eight sterols were listed in Table 1, and the SIM mode ion flow diagrams for the eight standard solutions were shown in Fig.1.

<Fig 1>

<Table 1>

3.2 Optimization of pretreatment conditions

3.2.1 Selection of extraction solvent

The extraction effects of methylene chloride (DCM) petroleum ether (PE), n-hexane (DCM), tetrahydrofuran (DMF) and ethyl acetate (EAC) as extraction solvent were investigated on eight kinds of sterols. The results are shown in Fig.2 that the extraction rates of 8 species of sterols were 87.9%~105.4% when PE had to be used as the extraction solvent STAN is higher than the other four solvents, which may be because the polarity of PE is closest to the target substance, so the extraction effect is the best. In this experiment, PE was the optimal extraction solvent.

<Fig. 2>

3.2.2 Selection of extraction temperature

The effect of extraction temperature on the extraction efficiency of the eight sterols examined data at 80, 90, 100, 110, 120 ℃. As shown in Fig.3, the influences of temperature on the extraction effect of sterols were as follows: the extraction rate of seven kinds of sterols except SIER was lower than 100℃ when the temperature was lower than 100℃. The extraction rate was the highest, while the extraction rate of SIER was high in the range of 90~100℃, which may be because the melting point of SIER was lower than that of other sterols, so the optimal extraction temperature was low. Based on the above, the extraction temperature for the experiment should be 100℃.

3.2.3 Selection of ASE static extraction time

The effect of static extraction time on the extraction efficiency of the eight sterols was investigated. The samples were pre-treated according to Step 1.3. Static extraction time of ASE was 2, 4, 6, 8, 10 min, the extraction solvent was PE, the extraction temperature was 100, and the extraction pressure was 100 MPa. The results are shown in Fig.4 when the extraction time was 6 min, the maximum extraction efficiency of eight sterols was achieved, and the static extraction time of ASE was 6 min.

<Fig.4>

4. Evaluation Of Analytical Methods

4.1 Linear detection limit Quantitative limit

Under the optimum experimental conditions, vertical coordinates employed peak areas of the eight sterol compounds, mass concentrations of the compounds were used as horizontal coordinates to plot the working curves. The limits of detection (LOD) and limits of quantification (LOQ) of eight sterols were calculated by 3S/N and 10S/N individually. The results were listed in Table 2, indicating that the method had a good linear relationship and sensitivity.

<Table 2>

4.2 Recovery and precision

For the determination of accuracy, recovery experiments were performed using three different concentration levels (5, 10, and 20 mg/kg) of real samples spiked with known amounts of eight sterols standard, the experiment was repeated six times according to the optimized criteria and calculated recovery and precision. The results were shown in Table 3. The average recoveries of sterol compounds were 89.5%-105.2%, and the relative standard deviations (RSDs) were less than 5%. Therefore, the developed method had good precision, accuracy and could meet the detection requirements of sterols in rapeseed.

<Table 3>

4.3 Actual sample determination

The real sample analysis results using the established method were shown in Table 4. the CHAN was not detected in six types rapeseed, the content of SIER was the highest, the content of CHER was the lowest. The highest detected content of SIER in Longyou 10 rapeseed were 4242.28 mg/kg. Sanfeng 66 had the lowest observed SIER concentration of 428.52 mg/kg, with the ratio between the two being 10:1. Longyou 10 rapeseed had the greatest sterol concentration and was a high-quality raw material for the extraction of CAER and BRER, according to the findings.

<Table 4>

5. Conclusion

Accelerated solvent extraction followed by gas chromatography-mass spectrometrywas was used for the analysis of sterol compounds in rapeseed, such as cholesterol, rapeseed sterols, ergosterol, rapeseed sterols, soy sterols, β-sitosterol, and soya sterols. Limits of detection (LODs) and limits of quantification of the established method were less than 1.61 mg/kg and 5.41 mg/kg, respectively. The recoveries were 89.5-105.2 %, and the precision was lower than 5.0 %, indicating that developed method is accurate and reliable. CHAN was not detected in 6 kinds of rapeseed, while SIER was the highest and CHER was the lowest in each sterol. The method has good accuracy and sensitivity, which is applicable for the determination of sterols in oil crops and provides an analytical approach for the expeditious evaluation of oil crops.

Declarations

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Compliance with Ethical Standards

Conflict of Interest Peng Sun declares that he has no conflict of interest. Chao Wang declares that he has no conflict of interest. Yuling Gao declares that she has no conflict of interest.

Author Contribution Peng Sun and Chao Wang conceived and designed this study. Yuling Gao and Chao Wang conducted these experiments. Peng Sun and Chao Wang analyzed the data and prepared the figures and illustrations. Peng Sun wrote the manuscript. All authors read and approved the submission of the manuscript.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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Tables

Table 1 Analysis parameters of eight sterol compounds by mass spectrometry

No.	Componend	Quantitative ion/(*m/z*)	Collision energy/eV	Qualitative ion 1/(*m/z*)	Collision energy/eV	Qualitative ion 2/(*m/z*)	Collision energy/eV
1	CHER	329.00>95.20	21	329.00>109.20	18	329.00>121.20	18
2	CHAN	460.00>215.20	18	355.00>91.10	33	460.00>355.50	12
3	BRER	380.00>69.20	24	365.00>69.20	27	380.00>255.30	9
4	ERER	343.00>95.20	24	363.00>69.10	24	363.00>143.20	24
5	CAER	343.00>83.30	30	343.00>121.10	18	343.00>109.20	18
6	STER	394.00>83.30	27	394.00>159.30	18	484.00>83.20	27
7	SIER	396.00>145.20	36	357.00>121.20	18	357.00>95.10	30
8	STAN	488.00>215.00	21	398.00>215.00	12	383.00>117.00	30

Table 2 The correlation coefficients of standard solutions of eight sterols were linear regression equation, detection limit LODs, quantification limit LOQs

No.	Componend	time/min	Correlation coefficient (r)	Linear equation	LODs/ (mg/kg)	LOQs/ (mg/kg)
1	CHER	18.85	0.9995	$y = 26806.92x - 134.9449$	1.12	3.73
2	CHAN	19.02	0.9996	$y = 20024.71x - 2787.448$	1.24	4.13
3	BRER	19.47	0.9991	$y = 11600.31x - 1781.793$	1.16	3.86
4	ERER	20.15	0.9998	$y = 29687.22x - 4891.185$	1.21	4.05
5	CAER	20.49	0.9993	$y = 13428.22x - 2263.295$	1.46	4.85
6	STER	20.94	0.9992	$y = 7743.004x - 1400.219$	1.35	4.51
7	SIER	22.08	0.9993	$y = 3994.673x - 744.5840$	1.61	5.41
8	STAN	22.33	0.9995	$y = 5919.130x - 1175.498$	1.41	4.66

Table 3 Recovery and precision of eight sterols

No.	Componend	Spiked level 1/ (5 mg/kg)		Spiked level 2/ (10 mg/kg)		Spiked level 3/ (20 mg/kg)	
		Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
1	CHER	96.5	2.6	99.8	3.6	92.5	2.4
2	CHAN	102.6	3.6	104.2	1.7	89.7	4.6
3	BRER	100.5	4.1	98.9	3.8	99.5	4.8
4	ERER	97.6	3.8	89.7	3.4	101.5	2.6
5	CAER	95.2	1.8	102.3	2.6	96.3	2.2
6	STER	99.5	2.2	96.4	4.6	103.4	4.1
7	SIER	102.6	1.7	105.2	3.7	95.7	3.5
8	STAN	90.7	4.5	89.5	2.7	89.8	3.3

Table 4 Determination results of eight sterols compounds in six varieties of rapeseed from farm

No.	Compound	qingza5/ mg/kg	qingza7/ mg/kg	qingza12/ mg/kg	qingza15/ mg/kg	longyou10/ mg/kg	sanfeng66/ mg/kg
1	CHER	5.24	10.47	7.69	6.18	13.11	1.53
2	CHAN	ND	ND	ND	ND	ND	ND
3	BRER	343.87	845.33	604.10	476.18	888.68	102.23
4	ERER	4.51	4.39	4.41	5.61	9.03	2.11
5	CAER	800	1707.66	1007.88	854.25	1710.66	198.91
6	STER	13.10	16.16	15.24	12.92	21.94	3.43
7	SIER	1742.2	3276.26	2202.59	1627.75	4242.28	428.52
8	STAN	7.81	17.34	13.90	9.25	20.99	2.92

Note: Not detected

Figures

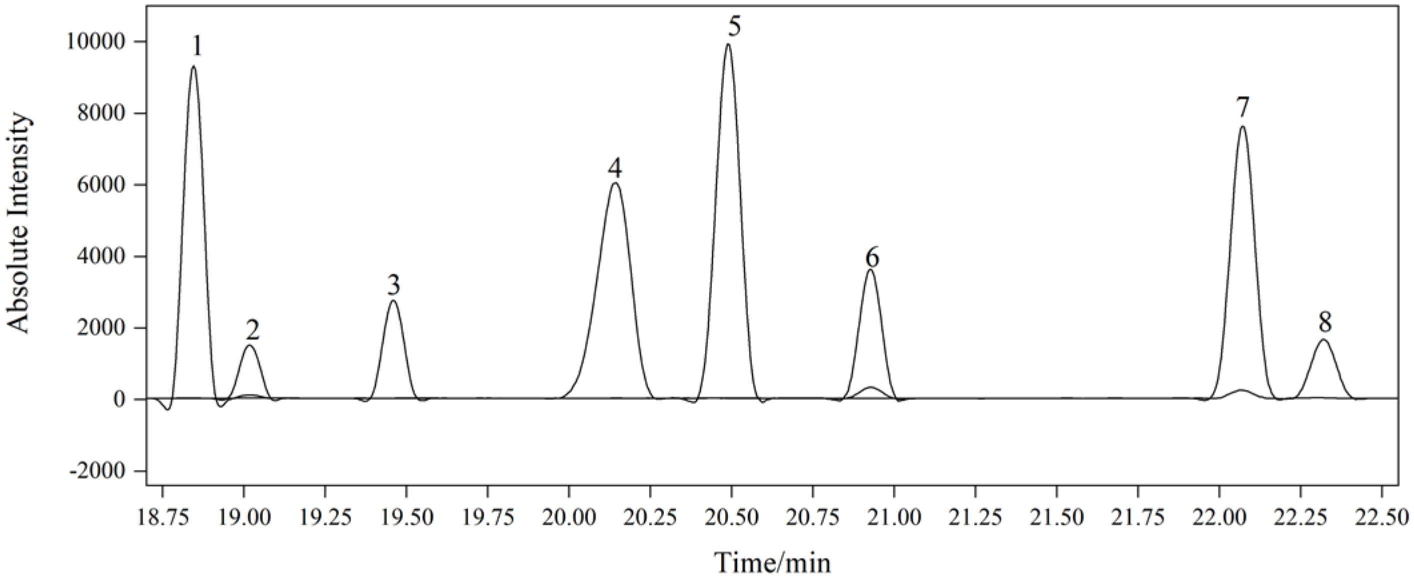

Figure 1

SIM mode ion flow chromatograms of 8 sterols

Note: Compound 1 is CHER; The compound was 2CHAN; Compound 3 is BRER; Compound 4 was ERER; Compound 5 was CAER; Compound 6 was STER; Compound 7 was SIER; Compound 8 is STAN.

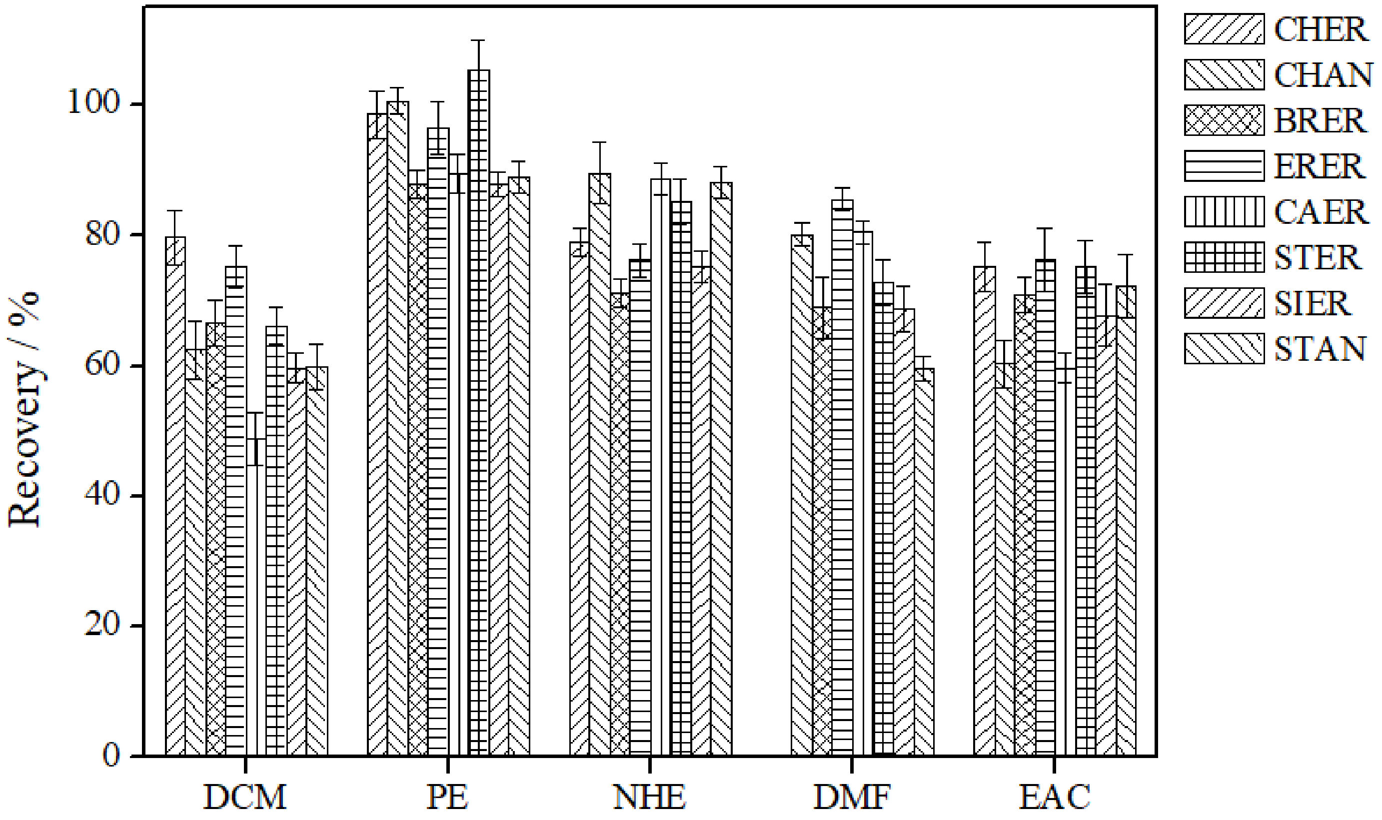

Figure 2

Effects of five extraction solvents on extraction efficiency

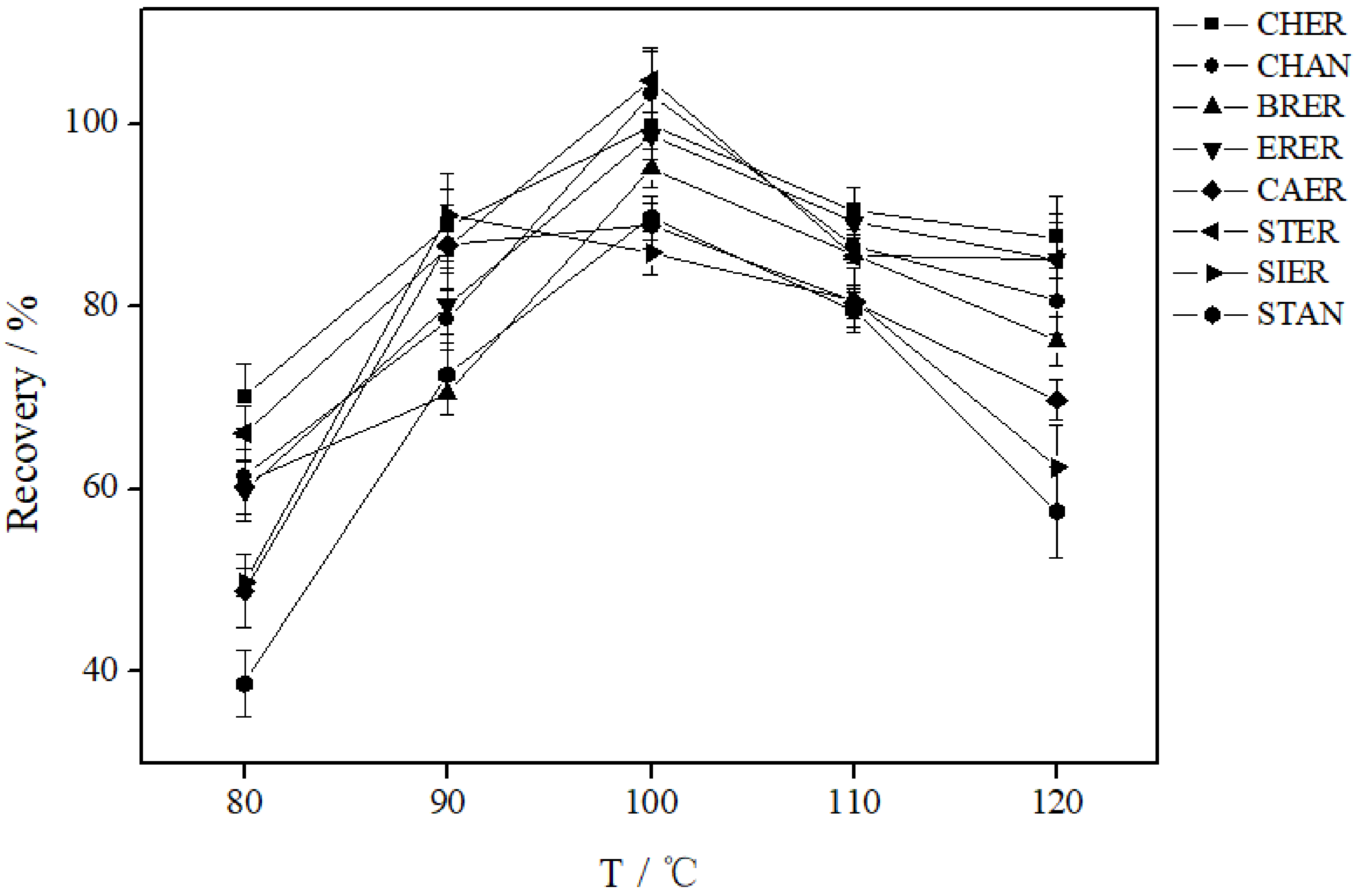

Figure 3

Effect of temperature on extraction efficiency

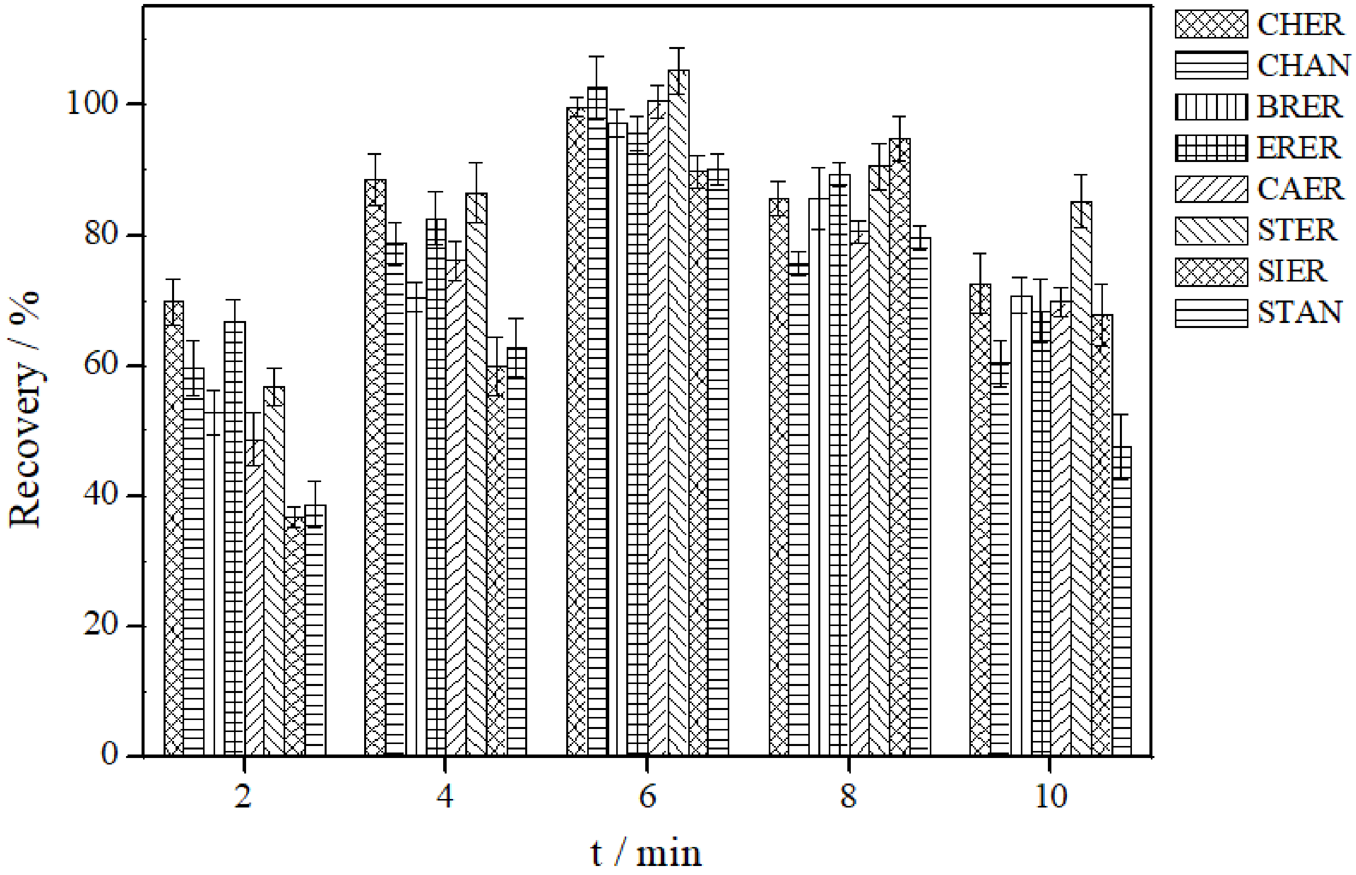

Figure 4

Effect of static extraction time on extraction efficiency