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Article

A GC–EI-MS-MS Method for Simultaneous Determination of Seven Adulterants in Slimming Functional Foods

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A gas chromatography–electron impact–tandem mass spectrometric method was established for the simultaneous determination of seven adulterants, including fenfluramine (FEN), norpseudoephedrine (NPE), pseudoephedrine (PSE), ephedrine (EPH), amfepramone (AMF), sibutramine (SIB) and strychnine (STR) in slimming functional foods. The target chemicals were extracted with 2% formic acid solution and then cleaned-up with solid-phase extraction using a strong cation exchange cartridge from tablet, liquid, mixed plant powder and capsule formulations. Chromatographic separation was accomplished on a VF-5MS column within 23 min. Leucomalachite green was employed as an internal standard. The recoveries of seven target chemicals in two formulations ranged from 80.1 to 106%. Limits of detection of the method were from 7.5 to 375 μg/kg with relative standard deviations of 1.6 to 13.9%. The linearity of the method ranged from 90 to 1500 ng/mL for NPE, 150 to 1500 ng/mL for STR, 10 to 500 ng/mL for AMF, 5.0 to 500 ng/mL for PSE and EPH and 3.0 to 500 ng/mL for FEN and SIB. This method was applied to the determination of six brands of slimming functional foods. SIB was detected in five of the samples with the contents in the range of 10.3 – 8.55 × 10^5 μg/kg.

Introduction

Functional food is a kind of food that is claimed to have certain health-promoting or disease-preventing properties beyond the basic function of foods (1). Some producers of these foods intentionally violate the food safety laws of China and illegally add or abuse one or more pharmaceutical drugs in the functional food. Those illegal products could be hazardous to human health and could even threaten consumers' lives. A gas chromatography–tandem mass spectrometry (GC–MS-MS) method was established in this study for the determination of seven chemicals: fenfluramine (FEN), norpseudoephedrine (NPE), pseudoephedrine (PSE), ephedrine (EPH), amfepramone (AMF), sibutramine (SIB), and strychnine (STR). The chemical structures of the seven analytes are shown in Figure 1. NPE, PSE and EPH are ephedrine analogues commonly used as substances of cold medicine to relieve nasal congestion. Negative reports have revealed that the existence of EPH in functional foods can threaten consumers' health, and the legitimacy of its existence in functional foods was consequently denied by the US Food and Drug Administration (FDA) (2). FEN has been used in diet pills with the curing dosage limited to 80 mg per day. Because of FEN's adverse effects on the cardiovascular system, China suspended the production, sale and use of this substance in both raw material and product forms in 2009 (3). AMF is an appetite suppressant. Overdosing and long-term taking of AMF may cause drug dependence, and it was classified as a psychotropic drug in 1996 by the Ministry of Health in China. STR is a medicine that can improve the tension of skeletal muscle with a narrow safe dosage range. The minimal lethal dose of STR is only 30 mg for adults and 5 mg for children; only 20% of the administered dose can be excreted, and the remaining accumulates in the body, so it is not for daily use and is seldom used clinically these days. SIB was used for curing obesity in European countries (EU), the United States and China, until 2010. It works by generating a sense of satiety to suppress the appetite (4), so it was once used as an anti-obesity drug. Sibutramine Cardiovascular and Diabetes Outcome Study (SCOUT) was designed to evaluate the efficacy/safety ratio of SIB in a high-risk population (5). Because the final results of SCOUT showed that (6) the benefit of SIB as a weight-loss medicine did not outweigh the cardiovascular risks, it was withdrawn from the domestic market by authorities in the EU, the United States, and China in 2010. To inspect the adulterants in functional foods, it is necessary and urgent to establish simultaneous determination methods for these chemicals. Therefore, a GC–electron impact (EI)–MS-MS method was established as an alternative method.

For the chromatographic separation of chemicals of this sort, liquid chromatography (LC) has been used more frequently than GC (7). Yoe-Ray *et al.* (8) reported a GC–MS method for detecting six synthetic anorexics, including FEN, in Chinese traditional medicines with a limit of detection (LOD) of 0.4 μg/mL for FEN. Barroso *et al.* (9) developed a GC–MS method after solid-phase microextraction (SPME) to determine STR in human blood, with an LOD of 6.83 ng/mL. Sporkert and Pragst (10) developed a method for detecting several chemicals, including AMF, in human hair. They used headspace SPE (HS-SPE) to extract the target chemicals from hair samples. The LOD was lower than 100 μg/kg for AMF. The advantage of HS-SPE is its high purification ability for complex matrix samples such as biological materials. Furthermore, it does not involve the use of organic solvents, and is easily coupled with a GC system. However, the recoveries of this pretreatment method were not satisfactory. Liu *et al.* (11) developed a high-performance liquid chromatography–diode array detection

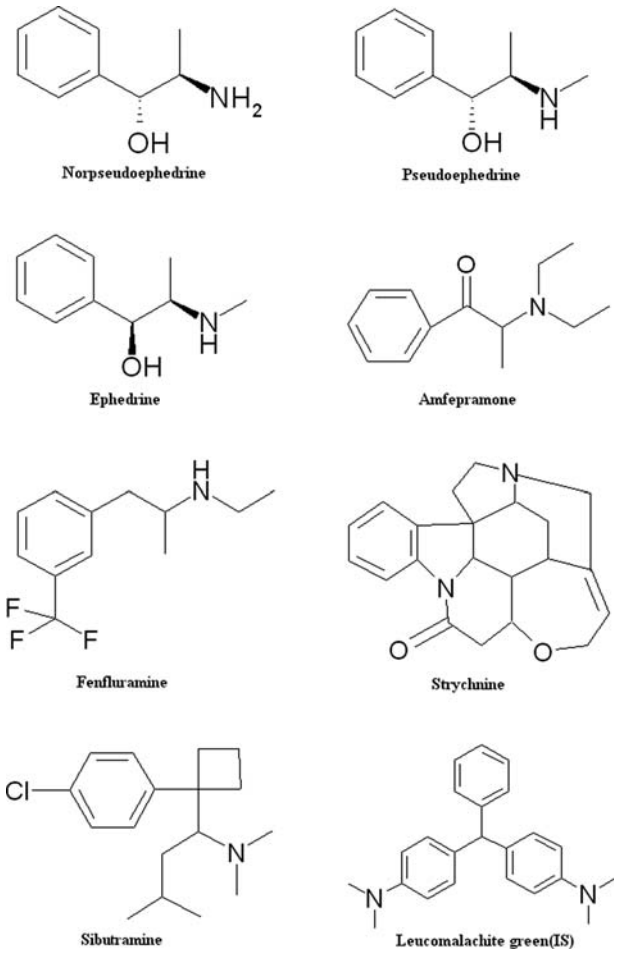

Figure 1. Chemical structure of the target chemicals and IS.

(HPLC–DAD) and GC–MS screening method for 266 therapeutic substances, including EPH, FEN and STR, which might be used as adulterants in Chinese traditional medicines. The purification capability of the method was limited and the LODs were not satisfactory. For example, the LOD for FEN detected by GC–MS was 18 μg/mL. Jamie *et al.* (12) introduced a quick and sensitive method for detecting SIB in dietary supplements using portable ion mobility spectrometers with an LOD of 2 ng/μL. This method provided a good option for the supervision of the functional food products market.

Compared with the reported methods, the method established in this study is suitable for routine qualitative and quantitative detection of the seven chemicals in four common formulations of slimming functional foods.

Experiment

Reagents and materials

FEN, PSE, EPH, AMF, SIB and STR standards were purchased from the National Institute for Control of Pharmaceutical and Biological Products of China. NPE was purchased from China Chifeng Arker Pharmaceutical Technology Co. Leucomalachite green (LMG) was purchased from Dr. Ehrenstorfer GmbH (Germany). HPLC-grade methanol was purchased from Fisher (Loughborough, UK). Analytical grade formic acid was purchased from Merck (Darmstadt, Germany). Ammonia of analytical grade (25–28%) was obtained domestically. The concentration of ammonia changes during storage, so its concentration was titrimetrically determined before use. Purified water was from a Millipore water purification system (resistivity, 18.2 MΩ.cm, Millipore; Milford, MA). The SPE column was an LC-SCX (Supelclean, 500 mg/3 mL, Sigma–Aldrich, St. Louis, MO).

For each analyte (FEN, NPE, PSE, EPH, AMF, SIB, STR and LMG), the 1.0 mg/mL stock solution was prepared in methanol and stored at −30°C. Standard solutions (10.0 μg/mL) were prepared by pipetting 100 μL of each chemical into a series of 10-mL volumetric flasks and diluting to the scale with methanol. One milliliter of each of the secondary solutions except NPE and STR was added to a 10-mL volumetric flask and diluted with methanol to prepare the 1.00 μg/mL mixed standard solution of the five chemicals. LMG (internal standard, IS) was diluted to 1.0 μg/mL with methanol before use. The 1.0 μg/mL mixed solution of five chemicals and the 10.0 μg/mL solutions of NPE and STR were used to prepare the mixed standard solution series. The concentrations of NPE and STR were 50, 150, 250, 500, 1,000 and 1,500 ng/mL, respectively, and the concentrations of the other analytes were 5.00, 20.0, 150, 250, 380 and 500 ng/mL, respectively. The IS concentration was 60 ng/mL in the mixed standard solution series.

Instruments

Chromatographic separation was performed on a Varian 450 GC system (Varian, Palo Alto, CA) equipped with a split/splitless injector (cp-1177) and an autosampler (cp-8400). The capillary column was a VF-5ms (30 m × 0.25 mm i.d., DF = 0.25 μm, Varian). Detection was performed using a Varian 320 tandem mass detector (Varian) with an EI source. The instrument control, data acquisition and data processing were performed with a Varian MS workstation (system control, version 6.9.1). The GC oven temperature was initially 100°C, held for 1 min, and then increased to 140°C at 10°C/min, held for 5 min, and finally increased to 300°C at 30°C/min, held for 10 min. The injector temperature was 260°C; the injection volume was 1 μL. The sample solutions were injected in the instantaneous splitless mode (splitless at 0 min, splitter opened at 0.75 min with the split ratio 1:100, shifted to 1:20 at 3.0 min and held to the end of the analysis program); the carrier gas was helium and its flow rate was 1.0 mL/min. The mass conditions were as follows: the transferline temperature was 250°C, the source temperature was 230°C, the filament current was 50 μA, the discharge was 1,500 volts and the electron energy was 70 eV. The retention times of the seven compounds and the IS are shown in Table I.

Sample preparation

Samples of six brands of slimming functional foods were purchased from local drug stores, in four formulations: tablet, plant powder, capsule and oral liquid. These functional foods claim that their primary raw materials are natural ingredients; for instance, lotus leaf, hawthorn, fleeceflower root and green tea. The solid samples, including the tablets, powders and the

Table I
MRM Monitor Pairs and Parameter for Each Chemical

Segment	Chemicals	Retention time (min)	Q1 (amu)	Q3 (amu)	CE (V)	Dwell time (s)
1	FEN	5.389	159.0	83.0	25	0.1
				109.0*	20	0.1
	NPE	7.905	56.9	41.9	10	0.2
				56.4*	5	0.2
	PSE/EPH	8.164	71.1	42.2	10	0.2
				56.1*	15	0.2
2	AMF	11.101	100.1	71.9*	15	0.15
				99.6	15	0.15
	SIB	13.862	114.0	56.2	25	0.15
				70.8*	25	0.15
3	LMG (IS)	18.593	330.0	208.5	25	0.1
				253.0*	20	0.1
	STR	22.113	334.0	121.1	15	0.25
				306.3*	10	0.25

*Represents the quantitative ions.

contents of the capsules, were pulverized. To each 0.2 g of sample, 5.0 mL of formic acid solution (2%, v/v) was added. After being thoroughly mixed on a vortex mixer, the samples were ultrasonically extracted for 20 min, and then centrifuged at 8,000 rpm for 5 min (CR3i, Thermo). Finally, the supernatant was filtered through a 0.45-μm filter membrane and 4.0 mL of the filtered sample solutions underwent an SPE clean-up procedure. Before loading sample solutions, the LC-SCX cartridge was conditioned with 3 mL formic acid–methanol (0.1%, v/v) and then formic acid solution (2%, v/v). After the sample solution was loaded, the column was washed with 2 mL of formic acid solution (2%, v/v), 2 mL of methanol–water (30%, v/v), and 1 mL of methanol. Finally, the column was eluted with 6 mL ammoniated methanol (2%, v/v). The eluant was collected and 30 μL IS solution (1.0 μg/mL) was added. The SPE process was conducted without vacuum and the drop speed was not greater than 1 mL/min while loading sample and eluting. The collected solution was blown to dryness under nitrogen and the residue was redissolved in 0.50 mL of methanol to produce the test solution.

Internal standard

LMG, which only appears in animal-origin products, was chosen as the IS in this study. It can be easily gasified and the retention time was appropriate in the range of the retention time of the target analytes. Because LMG shows different natures from the seven target compounds in the SCX-SPE course, it was only used to quantify and not used to evaluate the sample preparation procedure. Additionally, the IS was added after the SPE extraction and before the concentration of the collected solution (13–15).

Qualitative and quantitative methods

The qualitative analysis was accomplished by utilization of the abundance ratios of the qualitative ion pairs together with the retention time of chromatographic peaks of the target chemicals. The IS calibration curves were used for quantification. The calibration curves were established by use of the linear regression of concentration of each standard and the peak area ratio of each standard versus the IS.

Results and Discussion

Optimization of the mass parameters

The multiple reactions monitoring (MRM) mode was applied for detection in this study. The reference standards were tested to decide the proper monitored ion pairs and to optimize collision energies (CE). First, in full scan mode, standard solution of each analyte (10.0 μg/mL) was successively injected into the GC–MS system and the spectra were collected. Second, for each chemical, the fragments with relatively large molecular weight and high abundance were chosen as parent ions. The first quadrupole (Q1) was set in selective ion monitoring (SIM) mode to detect the chosen parent ions of a certain chemical. The third quadrupole (Q3) was set in scan mode and the spectra were collected. The CE was changed by a step length of 5 V in the range of 5–30 V to reveal the product ion spectra generated by different CEs for each parent ion. The product ions with a relatively stable signal and a high signal-to-noise ratio were chosen as the monitoring product ions. The chosen parent ion and product ions composed the monitoring ion pairs for each analyte (Table I). Because PSE and EPH are isomers, they generated the same spectra in EI and the mass collision chamber. As a result, the monitoring ion pairs for PSE and EPH are exactly identical. Furthermore, it was difficult for the capillary column used in this study to separate these isomers. It was concluded that PSE and EPH could not be distinguished by this method. The scan process was divided into three segments to solve the contradiction of dwell time and scan time, according to the sensitivity and retention time of the target chemicals.

Optimization of the SPE procedure

Selection of the SPE cartridge

In this study, we established an SPE procedure to fulfill the requirements for analysis of samples with a complex matrix.

According to the pKa of some of the analytes (FEN 9.6, NPE 8.9, PSE 9.9, Ephedrine 9.6 and STR 9.5) and their chemical structures, all of the analytes are weak organic bases and can be extracted with a weak acid solution. To purify the sample solution and isolate the weak alkaline compounds, the SCX cartridge was chosen. For the components adsorbed in the solid phase after the sample solution was loaded, methanol can elute the neutral and weak acid chemicals; ammoniated methanol can elute the weak alkaline chemicals; the strong alkaline chemicals were kept in the cartridge.

The retention properties of the chemicals of interest on the SCX cartridge can be understood by the eluting curves of each chemical. The washing and eluting parameters, including the solvent category, concentration and volume, are determined based on the curves.

Elution curves

One hundred microliters of the mixed standard solution (1.00 μg/mL), diluted with 2% formic acid solution to 4.00 mL, was taken as a simulation sample solution. Ammoniated methanol solutions of different concentrations, i.e., 0.5, 2 and 5% (v/v), were used as the elute solvents for establishing the eluting curves. First, the SPE column was conditioned. Then,

the sample solution was loaded and washed with 3 mL formic acid solution (2%, v/v) and 3 mL methanol. Finally, the column was eluted with 5–8 mL of ammoniated methanol, depending on the percentage of the ammonia in methanol. When washing with methanol, each milliliter of eluent was separately collected and the target analytes in the eluent were quantitatively detected with GC–EI–MS-MS. The percentages of each chemical detected in every milliliter formed a basis for establishing the elution curves. All of these solutions of ammoniated methanol could almost completely elute the target compounds. A tea sample was purified with the SPE procedure and these ammoniated methanol solutions were separately used as elutes. The 2% and 0.5% ammoniated methanol showed lower noise than the 5% ammoniated methanol; less ammoniated methanol volume was used for 2% than for 0.5%. As a result, 2% ammoniated methanol was selected as the elute solvent. Figure 2 shows the eluting curves for the seven target chemicals eluted with 2% ammoniated methanol. The total eluting efficiencies were 93.4% for FEN, 97.8% for NPE, 93.8% for PSE and EPH, 97.5% for AMF, 94.1% for SIB and 95.1% for STR.

Optimization of the SPE procedure

Approximately 30% SIB was eluted by the second milliliter of methanol. If this methanol were discarded, there would be a loss to the amount of the SIB, but this methanol could wash interfering substances. We eluted the SPE cartridge with 2 mL methanol–water between the acid and methanol elution to increase the resolution of SIB and the interfering substances, especially for the colored compounds. Methanol–water solutions of two concentrations, 30% and 50% (v/v), were tested. The colored sample with a complex matrix (mixed-plant powder) that was spiked with 100 μL of SIB standard solution (1.0 μg/mL) was cleaned up with the SPE procedure. The collected eluent was analyzed with GC–EI–MS-MS. Both of the methanol solutions improved the separation of the colored components with SIB. By comparison of the quantitative results, the recoveries of SIB were approximately 95% for 30% methanol–water and approximately 65% for 50% methanol–water. As a result, 30% methanol–water solution was selected. The total eluting efficiencies of the seven chemicals were not influenced by the addition of this 2 mL 30% methanol–water washing step.

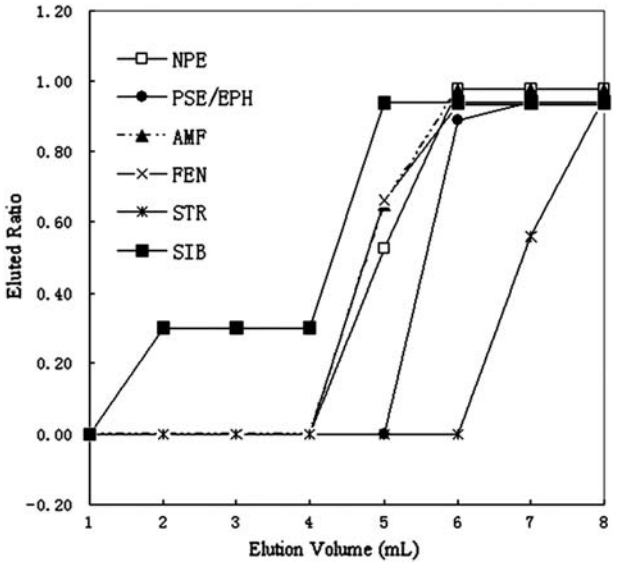

Figure 2. The elution curves for the seven target chemicals eluted with 2% ammoniated methanol.

Method validation

Linearity

The mixed standard series of 50.0, 150, 500, 1,000 and 1,500 ng/mL for NPE and STR, and 5.00, 20.0, 150, 250, 380 and 500 ng/mL for the other compounds were prepared in methanol. The same amount of IS solution was added to make each tube contain 60 ng/mL of IS. Finally, the standard solutions were injected twice. The samples were quantified with the IS curves. These relationships were fitted to a linear regression to calculate the slope, intercept and correlation coefficient for each calibration curve. Linear coefficients of greater than 0.999 were obtained in the linear ranges of these chemicals (Table II).

Limits of detection

The LODs were calculated as three times the signal-to-noise ratio (S/N = 3) and the limits of quantification (LOQs) were calculated as 10 times the S/N ratio (S/N = 10). The LODs and LOQs of this method are listed in Table II. As shown in Table II, the LODs and LOQs of this method were in the range of 7.5–375 and 25–1,250 μg/kg, respectively.

Recoveries

Recovery tests were performed by applying the standard addition method to two formulation samples: tablet and mixed plant powder. Each sample (0.2 g) was spiked with three levels of mixed standards; the addition amounts were 0.15 μg for NPE, 0.25 μg for STR, 0.05 μg for others for low level, 0.30 μg for NPE, 0.50 μg for STR, 0.10 μg for others for intermediary level and 0.60 μg for NPE, 1.0 μg for STR, 0.20 μg for others for high level. Each sample was also spiked with 30 μL of IS solution (1.0 μg/mL) after clean-up with the SPE course. The average recoveries obtained from two formulation samples of each analyte ranged from 80.1 to 106% (Table III).

Precision

The precision of the method was expressed in relative standard deviations (RSDs) obtained from each chemical at different concentration levels. The RSDs of the method ranged from 1.6 to 13.9%.

Table II
Linear range, LODs and LOQs of the Method

Chemicals	Linear range (μg/mL)	LOD (μg/kg)	LOQ (μg/kg)
FEN	0.010–0.50	7.5	25
NPE	0.30–1.5	225	750
PSE/EPH	0.017–0.50	12.5	42
AMF	0.033–0.50	25	85
SIB	0.010–0.50	7.5	25
STR	0.50–2.5	375	1,250

Table III
Recoveries (R) of the Analytes in Two Different Formulations (n = 3)

Formulation	Added (μg kg)	FEN		NPE		PSE/EPH		AMF		SIB		STR	
		Found	R (%)	Found	R (%)	Found	R (%)	Found	R (%)	Found	R (%)	Found	R (%)
Tablet	0	ND	—	ND	—	ND	—	ND	—	13.1	—	ND	—
	250 (750, 1,250)	226	90.4	770	103	236	94.4	262	105	220	82.6	1,118	89.4
	500 (1,500, 2,500)	470	94.1	1,677	112	483	96.6	484	96.9	417	80.8	2,354	94.2
	1,000 (3,000, 5,000)	9,100	91.0	3,175	106	975	97.5	964	96.4	976	96.3	5,011	101
Capsule (tea)	0	ND		ND	—	ND	—	ND	—	10.2	—	ND	—
	250 (750, 1,250)	223	89.3	619	82.5	209	83.8	269	107	237	90.9	605	80.7
	500 (1,500, 2,500)	998	99.8	1,288	85.9	429	85.9	423	84.7	450	88.0	1,275	85.0
	1,000 (3,000, 5,000)	854	85.4	2,859	95.3	1,045	83.6	852	85.2	1,021	101	2,580	86.0

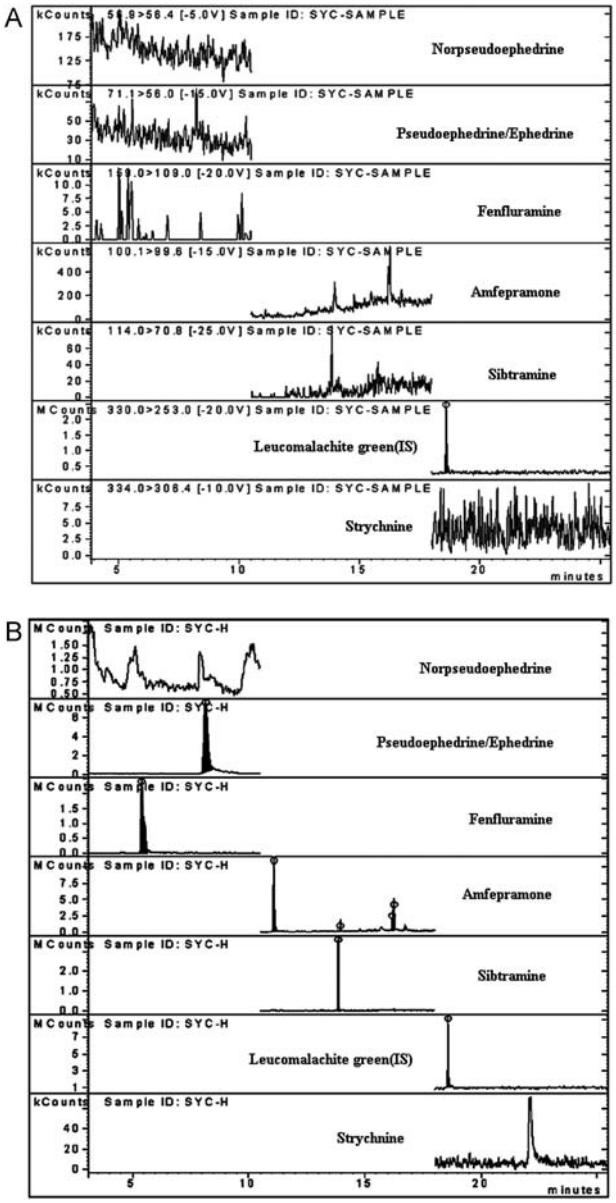

Figure 3. The chromatograms of a powder (brand code: SYC) (A); spiked sample (B).

Method application

The method was used to analyze the seven target chemicals in six brands of slimming functional foods purchased in local drugstores (one brand of powder formulations/tea, three brands of tablets, one brand of oral liquid and one brand of capsule). SIB was detected in all of the samples, except the liquid sample, with a content range of $10.3–8.55 \times 10^5$ μg/kg. Other target chemicals were not detected in all of the samples. The chromatograms of a powder and the sample that was spiked with 3.0 mg/kg of NPE, 5.0 mg/kg of STR and 1.0 mg/kg of the other five chemicals are shown in Figure 3.

Conclusions

A simple and fast GC–EI–MS-MS method was presented in this paper for the simultaneous determination of seven adulterating chemicals, namely, pseudoephedrine, norpseudoephedrine, ephedrine, fenfluramine, amfepramone, sibutramine and strychnine, in different forms of slimming functional foods. The method was fully validated and successfully applied to the analysis of the target chemicals in six brands of functional foods. Sibutramine was detected in all except one of the samples. This method was demonstrated to be effective and sensitive, and may provide a useful tool to control the quality of slimming functional foods.

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