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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1633–1641
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A rapid and simple procedure for the determination of ephedrine alkaloids in dietary supplements by gas chromatography–mass spectrometry

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Received 2 January 2006; received in revised form 15 February 2006; accepted 16 February 2006
Available online 31 March 2006

Abstract

A simple method for the determination of ephedrine alkaloids: ephedrine (EF), pseudoephedrine (PE), norpseudoephedrine (NPE), norephedrine (NE) and methylpseudoephedrine (MPE) in dietary supplements by gas chromatography–mass spectrometry is described. After the addition of 3,4-methylenedioxypropylamphetamine as internal standard, a liquid–liquid extraction procedure in alkaline conditions with chloroform/isopropanol (9:1, v/v) was applied to the samples prior to analysis. Chromatography was performed on a fused capillary column and analytes, derivatized with pentafluoropropionic anhydride, were determined in the selected-ion-monitoring (SIM) mode. The method was validated in the range 0.3–10 μg/mg for EP, 0.06–2.5 μg/mg for PE and NPE and 0.04–1 μg/mg NE and MPE. Mean recovery ranged between 65.7 and 81.3% for the different analytes in dietary supplements. The quantification limits were 0.3 μg/mg for EP, 0.06 μg/mg for PE, 0.04 μg/mg for NPE, NE and MPE. The method was applied to analysis of various dietary supplements containing Ma-huang (Ephedra Sinica) and Sida Cordifolia plant extracts promoted for aiding weight control and boosting sports performance and energy.
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Keywords: Ephedrine alkaloids; Ma-Huang; Sida Cordifolia; Dietary supplements

1. Introduction

Ephedra Sinica, or *Ma Huang*, is an evergreen shrub native to central Asia [1]. It is contained in various herbal preparations (e.g. Chinese traditional medicine preparations) and has been utilized for respiratory, antitussive, central nervous system stimulant, antipyretic and anti-inflammatory purposes [2]. In particular, the stimulant effects of *Ma Huang* are linked to presence in aerial parts of the plant of six alkaloids: ephedrine (EP), pseudoephedrine (PE), methylephedrine (ME), methylpseudoephedrine (MPE), norephedrine (NE) and norpseudoephedrine (NPE). The total alkaloids content of *Ma Huang* is approximately 1 and 2% with EP being the most abundant alkaloid [3] and EP and PE constituting more than 80% of the alkaloid content of the dried herb [4,5].

EP and PE are also major alkaloids of *Sida Cordifolia*, or malva branca (white mallow), a plant found in several parts of the Brazil [6]. The plant is used in folk medicine for the treatment of stomatitis, blenorrhea, asthmatic bronchitis and nasal congestion [6–8].

In recent years, many dietary supplements containing *Ma Huang* or/and *Sida Cordifolia* alone or in combination with other botanical ingredients (guaranà, kola nut and Willow bark) have received increasing attention for their use in aiding weight control and boosting sports performance and energy [3,9]. Whereas, in US, Food and Drug Administration issued a final rule prohibiting the sale of dietary supplements containing ephedrine alkaloids because such supplements present an unreasonable risk of illness or injury due to adverse health effects (including heart attack and stroke), these products are freely sold in esoteric and nature stores (also called “smart shops”) along Europe and internet web sites for their “supposed” nutritional and health benefits [10].

Different methods have been reported for the determination of ephedrine alkaloids in dietary supplements by liquid chromatography [9,11–13], liquid chromatography coupled to mass spectrometry [14,15], capillary electrophoresis [9,16,17] and gas chromatography–mass spectrometry [18–20].

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doi:10.1016/j.jpba.2006.02.043

It has to be said that methodologies involving mass spectrometry as detector are preferred to identify with a high grade of certainty substances contained in products of unknown origin. Whereas, a standard gas chromatograph–mass spectrometer is an apparatus generally found in analytical laboratories and easy to use, the same is not with liquid chromatographs coupled to mass spectrometry or tandem mass spectrometry.

Previous assays involving mass spectrometry require lengthy extraction procedures—solid phase extractions or more than three different steps [14,15,19] and/or require large amounts of extraction solvents (range: 20–450 ml) [18–20] and bulk material [20], finally appearing complex and time-consuming.

We here present a relatively easy and rapid method, based on gas chromatography–mass spectrometry coupled with simplified sample preparation (two extraction steps and 3 ml extraction solvent), for the determination of ephedrine alkaloids in dietary supplement rendering the assay suitable for high throughput laboratories. Furthermore, the assay has been validated to meet the acceptance criteria for bioanalytical method validation [21,22].

2. Experimental

2.1. Instrumentation

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out on a 6890 Series Plus gas chromatograph equipped with an Agilent 7683 autosampler and coupled to a 5973 N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation, Palo Alto, CA, USA).

2.2. Chemicals and materials

EP, PE, NE, NPE, MPE and 3,4-methylenedioxypropylamphetamine (used as internal standard, IS) were supplied by Salars (Como, Italy). Pentafluoropropionic anhydride (PFPA) was obtained from Sigma–Aldrich (Milano, Italy). Ultrapure water and all other reagents of analytical grade were purchased from Carlo Erba (Milano, Italy). Eighteen different dietary supplements containing "herbal" capsules (weight range of capsules: 900–1000 mg), whose label reported the presence of "Mahuang" (Ephedra Sinica) or Sida Cordifolia or ephedra extract were purchased in autumn 2003 from esoteric and nature stores in Italy. The blank products used in the validation studies (products similar in the composition to those previously mentioned but without any presence of ephedrine alkaloids, reported as "drug-free food products") were purchased from the same nature stores and analyzed to assess the absence of any substance before spiking them with ephedrine alkaloids standard solutions.

2.3. Preparation of standard solutions

Working solutions containing EP, PE, NE, NPE and MPE at 10 mg/ml concentration were prepared in methanol and stored at −20 °C until analysis. The internal standard (IS) working solution was used at a concentration of 1 mg/ml.

Calibration standards containing 100 μg IS working solution and six different microgram amounts of EP (30–1000 μg), PE and NPE (6–250 μg) and NE and MPE (4–100 μg) were prepared for each analytical batch by preparing tubes with suitable amounts of methanol working solutions, which were evaporated under nitrogen before adding 100 mg of pre-checked drug-free food products. Several aliquots of quality control samples (low, medium and high control) at 0.6, 4 and 8.5 μg/mg for EP; 0.12, 1 and 2 μg/mg for PE and NPE; 0.08, 0.4 and 0.85 μg/mg for NE and MPE concentration were prepared in different blank products to be used for calculation of validation parameters. Calibration and quality control samples were treated and processed as unknown samples.

2.4. Sample preparation and extraction

All the samples were blended and homogenized in a standard mixer (Heidolf Reax Top, WWR International Srl, Milano, Italy). An amount of 100 mg product, added to 100 μl of IS working solution, was dissolved in 2 ml 0.1 M phosphate buffer (KH_2PO_4, pH 10.0). After centrifugation at 3500 rpm for 10 min, the alkaline solution was extracted with two different aliquots of 1.5 ml chloroform/isopropanol (9:1, v/v). The organic phases, transferred to another tube, were evaporated to dryness under a stream of nitrogen. The dried residue was derivatized in capped test tubes with 50 μl of PFPA at 80 °C for 20 min. At the end of derivatization process, the solution was evaporated under nitrogen flow and, after ambient temperature cooling, the residue was dissolved in 50 μl ethyl acetate. For GC–MS analysis, a 1 μl amount was injected.

2.5. GC–MS conditions

Analytes separation was achieved on a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent Technologies). The oven temperature was programmed at 120 °C for 2 min, increased to 290 °C at 10 °C/min. Split injection mode (15:1) was used. Helium (purity 99%), with a flow rate of 1 ml/min was used as carrier gas. The injection port, ion source, quadrupole and interface temperatures were: 260, 230, 150 and 280 °C, respectively.

The electron-impact (EI) mass spectra of the analytes were recorded by total ion monitoring mode (scan range 40–550 *m/z*) to determine retention times and characteristic mass fragments (Fig. 1). For quantitative analysis, the chosen characteristic mass fragments were monitored in selected-ion-monitoring (SIM) mode: *m/z* 119, 160 and $\underline{204}$ for EP-diPFPA, *m/z* 160, 204 and $\underline{294}$ for PE-diPFPA, *m/z* 119, $\underline{190}$ and 280 for NE-PFPA, *m/z* 119, 190 and $\underline{280}$ for NPE-PFPA, *m/z* 72, $\underline{134}$ and 162 for MPE-PFPA and *m/z* 86, 105 and $\underline{135}$ for IS. The underlined ions were selected for the quantification measurement.

2.6. Validation procedures

Prior to application to real samples, the method was tested in a 4-day validation protocol [22]. Selectivity, recovery, matrix effect, linearity, precision, accuracy, freeze–thaw cycles and

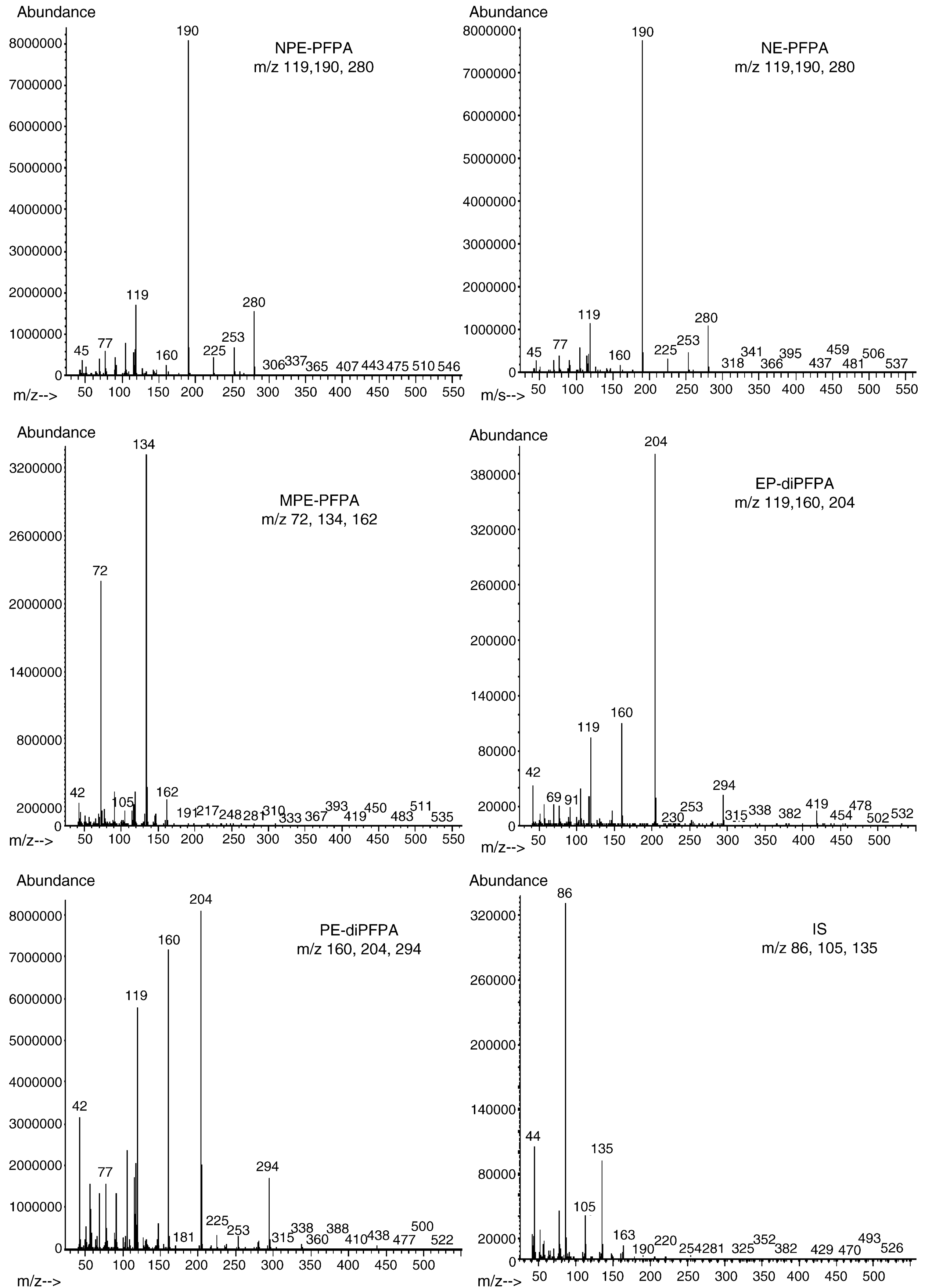

Fig. 1. Mass spectra of EP-diPFPA, PE-diPFPA, NE-PFPA, NPE-PFPA, MPE-PFPA and IS.

mid-term stability and limits of detection (LOD) and quantification (LOQ), were assayed.

The drug-free dietary supplements were extracted and analyzed for assessment of potential interferences due to substances other than analytes under investigation. The apparent responses at the retention times of ephedrine alkaloids and IS were compared to the response of analytes at the LOQ and IS at its lowest quantifiable concentration. The potential for carryover was investigated by injecting extracted drug-free products, with added IS, immediately after analysis of the highest concentration point of the calibration curve on each of the 4 days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analytes under investigation.

Analytical recoveries were calculated by comparing the peak areas obtained when quality control samples were analyzed by adding the analytical reference standards and the IS in the extract of drug-free food products prior to and after the extraction procedure. The recoveries were assessed at three concentration levels, using four replicates at each level.

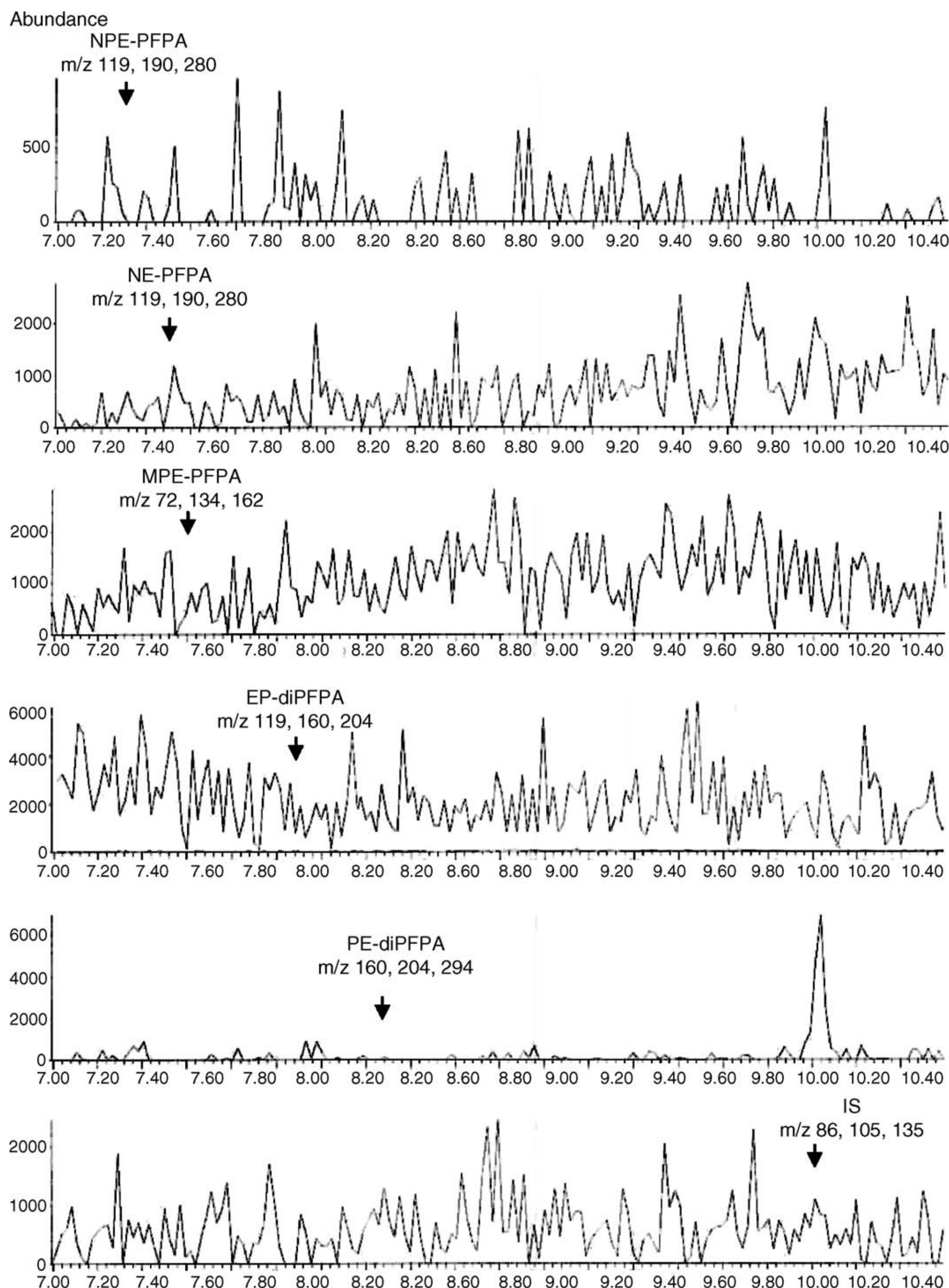

Fig. 2. Representative SIM chromatogram of an extract of pre-checked drug-free food products.

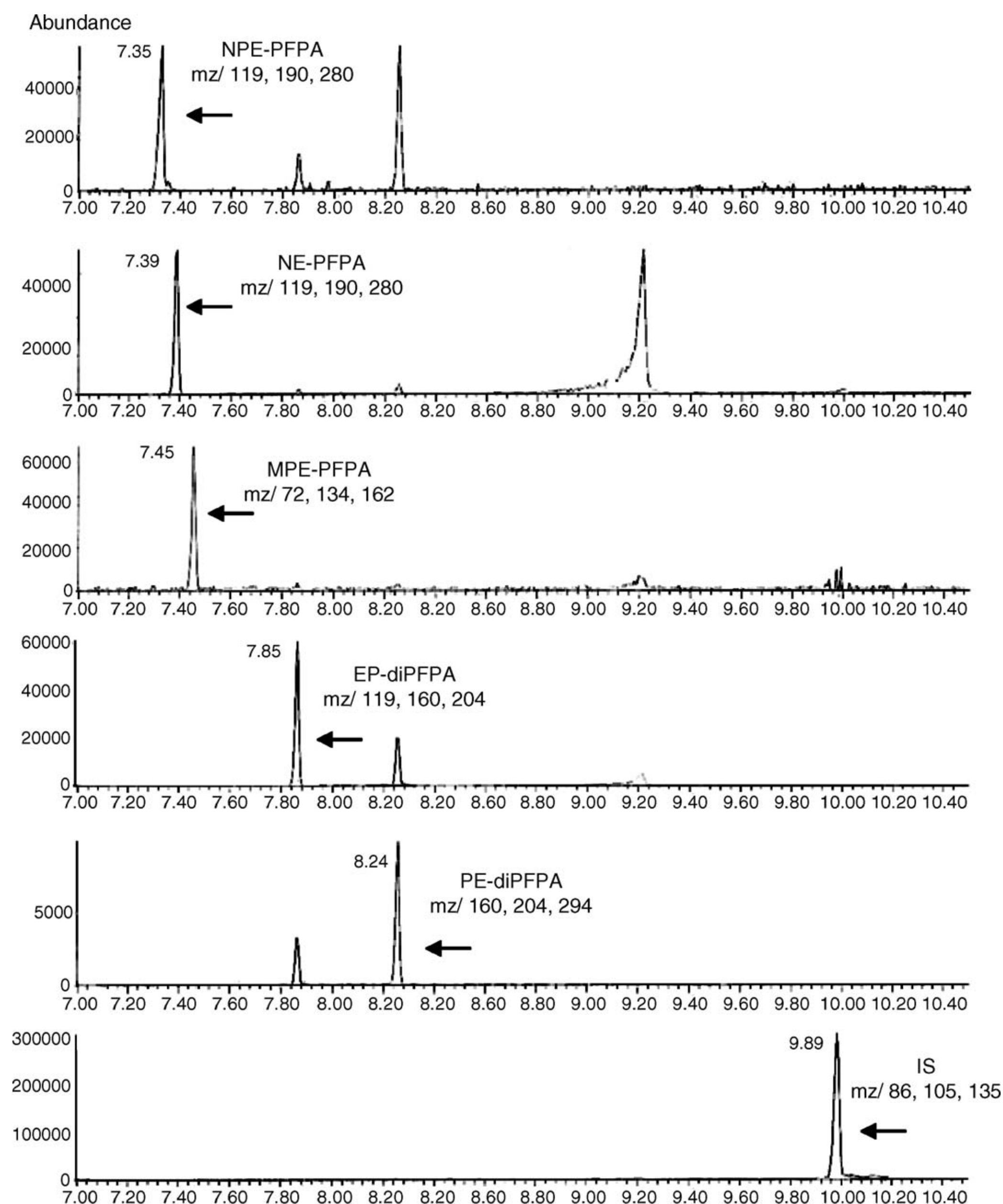

Fig. 3. Representative SIM chromatogram of an extract of pre-checked drug-free food products spiked with 0.5 μg/mg EP, 0.1 μg/mg PE and NPE, 0.05 μg/mg NE and MPE and 1 μg/mg IS.

Table 1
Method calibration data

Analyte	Correlation coefficient ($n = 3$) (r^2)	Analytical recovery % (mean ± S.D., $n = 4$)[a]									LOD ($n = 10$) (μg/mg)	LOQ ($n = 10$) (μg/mg)
		Low control			Medium control			High control				
		0.06	0.12	0.6	0.4	1	4	0.85	2	8.5		
EP	0.993 ± 0.002	–	–	70.2 ± 3.5	–	–	72.8 ± 0.9	–	–	70.6 ± 3.5	0.1	0.3
PE	0.995 ± 0.003	–	68.4 ± 2.1	–	–	70.4 ± 2.6	–	–	70.9 ± 2.1	–	0.02	0.06
NPE	0.998 ± 0.003	–	65.7 ± 3.5	–	–	68.3 ± 2.8	–	–	67.5 ± 3.5	–	0.02	0.06
NE	0.995 ± 0.004	73.6 ± 0.7	–	–	70.6 ± 0.3	–	–	72.3 ± 0.7	–	–	0.012	0.04
MPE	0.996 ± 0.001	79.9 ± 1.4	–	–	81.3 + 2.5	–	–	80.9 ± 1.4	–	–	0.012	0.04

[a] S.D.: standard deviation.

For an evaluation of matrix effects, the peak areas of extracted drug-free products samples spiked with standards at a mean concentration level (0.5 μg/mg) after the extraction procedure, were compared to the peak areas of pure diluted substances.

Calibration curves ($n = 3$) were tested over the quantification limit: 10 μg/mg for E, over the quantification limit: 2.5 μg/mg for PE and NPE and over the quantification limit: 1 μg/mg for NE and MPE. Peak area ratios between compounds and IS were used for calculations. A weighted (1/concentration) least-squares regression analysis was used (Statistical Package for the Social Sciences, SPSS, Version 9.0.2 for Microsoft Windows, Microsoft Italia, Milano, Italy). Ten replicates of drug-free product samples were used for calculating the limit of quantification. Standard deviation (S.D.) of the mean noise level over the retention time window of each analyte was used to determine the detection limit (LOD = 3 × S.D.) and quantification limit (LOQ = 10 × S.D.). Once calculated, LOQ was tested for accuracy and precision to meet the established international criteria [21,22].

A total of five replicates at each of three quality control concentrations were added to drug-free products samples were extracted, as reported above, and were analyzed for the determination of intra-assay precision and accuracy. The inter-assay precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Inter-assay precision was expressed as the relative S.D. (R.S.D.) of concentrations calculated for quality control samples. Inter-assay accuracy was expressed as the relative error of the calculated concentrations.

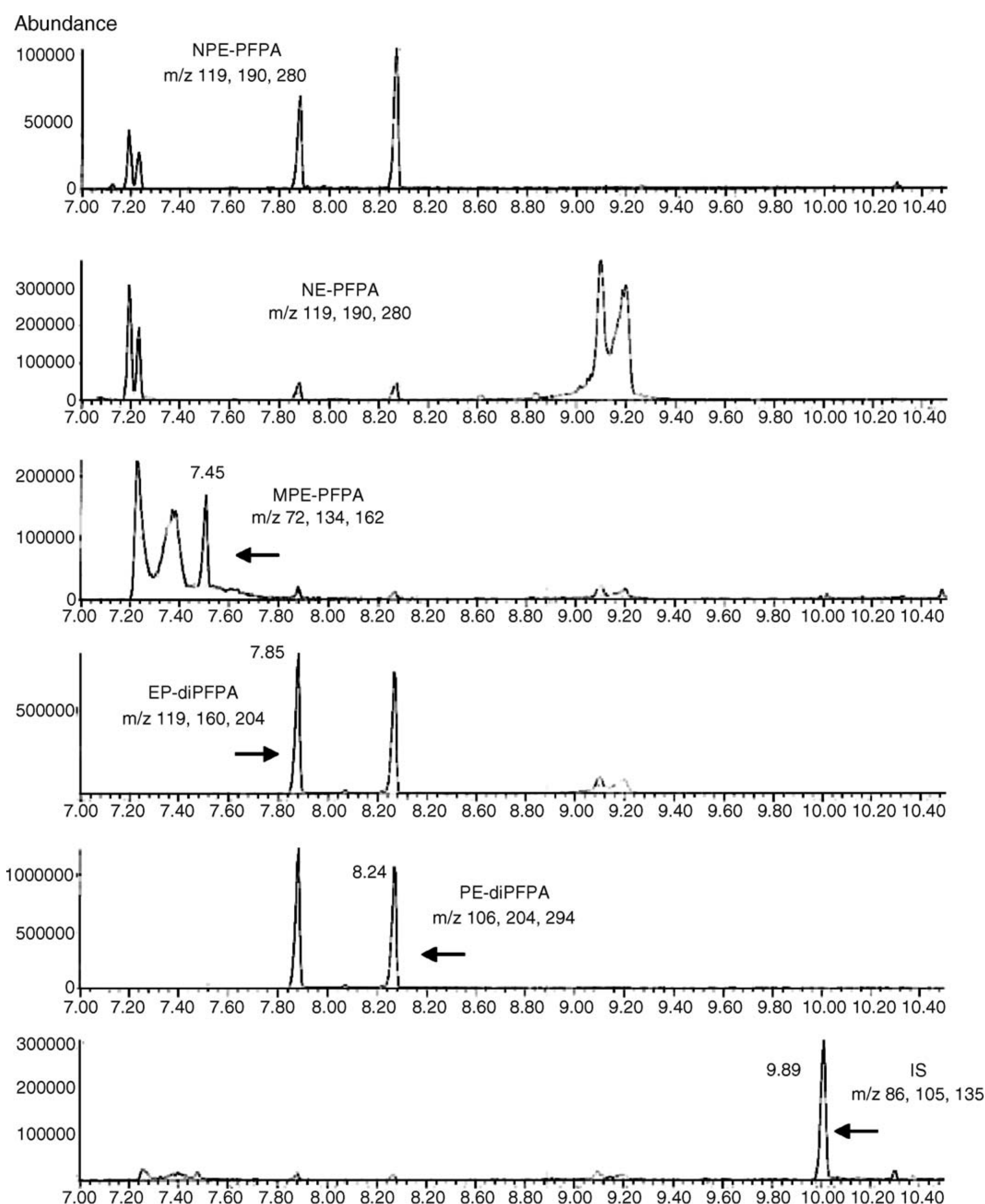

Fig. 4. Representative SIM chromatogram of a sample extract containing 6.25 μg/mg EP, 1.33 μg/mg PE and 0.13 μg/mg MPE.

Table 2
Intra-assay ($n = 5$) and inter-assay ($n = 15$) precision and accuracy obtained from analytes under investigation

Analyte	Intra-assay precision (R.S.D.)									Intra-assay accuracy (error%)								
	Low control			Medium control			High control			Low control			Medium control			High control		
	0.06	0.12	0.6	0.4	1	4	0.85	2	8.5	0.08	0.12	0.6	0.4	1	4	0.85	2	8.5
EP	–	–	5.3	–	–	11.7	–	–	12.5	–	–	2.7	–	–	8.3	–	–	10.1
PE	–	6.3	–	–	11.1	–	–	6.3	–	–	13.5	–	–	10.7	–	–	5.4	–
NPE	–	8.2	–	–	4.5	–	–	10.5	–	–	6.4	–	–	10.1	–	–	7.1	–
NE	11.2	–	–	7.3	–	–	8.1	–	–	5.8	–	–	9.2	–	–	4.6	–	–
MPE	2.3	–	–	10.1	–	–	7.5	–	–	6.5	–	–	10.8	–	–	3.6	–	–

Analyte	Inter-assay precision (R.S.D.)									Inter-assay accuracy (error%)								
	Low control			Medium control			High control			Low control			Medium control			High control		
	0.06	0.12	0.6	0.4	1	4	0.85	2	8.5	0.08	0.12	0.6	0.4	1	4	0.85	2	8.5
EP	–	–	1.2	–	–	5.5	–	–	6.4	–	–	10.8	–	–	12.5	–	–	7.5
PE	–	6.6	–	–	9.6	–	–	3.1	–	–	10.2	–	–	2.3	–	–	5.3	–
NPE	–	10.2	–	–	4.7	–	–	2.8	–	–	8.4	–	–	7.5	–	–	3.6	–
NE	11.7	–	–	9.5	–	–	10.2	–	–	10.4	–	–	13.5	–	–	12.1	–	–
MPE	8.1	–	–	7.3	–	–	9.9	–	–	8.3	–	–	7.3	–	–	11.3	–	–

The effect of three freeze–thaw cycles (storage at −20 °C) on ephedrine alkaloids stability in different was evaluated on quality control samples in triplicate. The stability was expressed as a percentage of the initial concentration of the analytes spiked in blank dietary supplements and quantified just after preparation. Mid-term stability test was performed for samples stored at ambient temperature. Three replicates of three different herbal capsules were included in each analytical batch during a 3 months period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes within the products.

3. Results and discussion

3.1. GC–MS

Representative chromatograms obtained following the extraction of pre-checked drug-free dietary supplement and the same product spiked with 0.5 μg/mg EP, 0.1 μg/mg PE and NPE, 0.05 μg/mg NE and MPE and 1 μg/mg IS are shown in Figs. 2 and 3, respectively. Chromatograms of sample extract containing 6.25 μg/mg EP, 1.33 μg/mg PE and 0.13 μg/mg MPE are presented in Fig. 4. When analytes concentrations in samples resulted higher than those in the calibration curve range, a smaller amount of samples (usually 1/10 amount) was re-extracted and analyzed following standard procedure. Samples following the one exceeding the linear range in the chromatographic run were re-injected to check eventual contamination by carryover. Nonetheless, nor in this case any carryover was observed, nor when drug-free dietary supplement samples were injected after the highest point of the calibration curve. A chromatographic run was completed in 10 min, and afterwards initial conditions were restored in 3 min. No additional peaks due to substances in ephedrine alkaloids-free dietary supplements that could have interfered with the detection of compounds of interest were observed. With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted drug-free products samples versus those for pure diluted standards showed less than 10% analytical signal suppression.

3.2. Validation results

Tables 1 and 2 summarize the method validation data. Linear calibration curves were obtained for the compounds of interest with a correlation coefficient (r^2) higher than 0.99 in all cases. The analytical recoveries (mean ± S.D.) obtained after liquid–liquid extraction at different concentration levels showed that there were no relevant differences in recoveries between different products. Limits of detection and quantification were considered adequate for the purposes of the present study and coefficients of variation for precision and accuracy at LOQ were always better than 20%. The results obtained for intra-assay and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria [21,22]. With reference to the freeze–thaw stability assays for quality control samples, no relevant degradation was observed after any of the three freeze–thaw cycles, with differences from the initial concentration less that 10%. Similar results (differences to the initial concentration always lower than 5%) were obtained in case of mid-term stability test.

3.3. Analysis of samples

The concentration of ephedra alkaloids in the different dietary supplements products are shown in Table 3 as mean and standard deviation (S.D.) of three different replicates. All the analyzed products contained EP (concentration range: 4.2–78.6 μg/mg) and PE (concentration range: 0.09–1.42 μg/mg). Only three “Ma-huang” herbal capsules contained also MPE (concentration range: 0.13–0.58 μg/mg). Concerning the other two alkaloids

Table 3
Ephedra alkaloids content (μg/mg ± S.D., $n = 3$) in dietary supplements under the study

Products	Label ingredients	EP	PE	NE	NPE	MPE
A	Ma-huang	43.0 ± 5.4	0.8 ± 0.1	ND	ND	ND
B	Ma-huang	78.6 ± 2.2	1.2 ± 0.2	ND	ND	ND
C	Ephedra extract	9.6 ± 0.7	0.4 ± 0.1	ND	ND	ND
D	Ma-huang	15.5 ± 1.0	0.7 ± 0.2	ND	ND	ND
E	Ma-huang	7.6 ± 1.4	0.6 ± 0.05	ND	ND	ND
F	Ma-huang	12.5 ± 0.8	1.3 ± 0.1	ND	ND	0.1 ± 0.01
G	Ma-huang	10.2 ± 0.7	0.6 ± 0.1	ND	ND	0.5 ± 0.1
H	Ma-huang	25.5 ± 2.6	0.3 ± 0.1	ND	ND	ND
I	Ephedra extract, Sida Cordifolia	13.3 ± 1.1	1.4 ± 0.1	ND	ND	ND
J	Sida Cordifolia	9.5 ± 0.8	0.1 ± 0.03	ND	ND	ND
K	Sida Cordifolia	9.7 ± 0.8	0.1 ± 0.04	ND	ND	ND
L	Ephedra extract	21.1 ± 1.7	0.2 ± 0.1	ND	ND	ND
M	Sida Cordifolia	7.4 ± 0.5	0.7 ± 0.04	ND	ND	ND
N	Sida Cordifolia	9.6 ± 0.7	0.5 ± 0.6	ND	ND	ND
O	Ma-huang	9.2 ± 1.0	0.1 ± 0.04	ND	ND	ND
P	Ma-huang	14.1 ± 1.7	0.1 ± 0.01	ND	ND	0.6 ± 0.1
Q	Ma-huang	4.2 ± 0.3	0.1 ± 0.02	ND	ND	ND
R	Sida Cordifolia	6.8 ± 0.4	0.1 ± 0.03	ND	ND	ND

(NE and NPE), they were not found in any of the "Ma-Huang" or "Ephedra Extract" samples. This fact is clearly explainable in case of NPE: indeed this substance (norpseudoephedrine or cathine) is included in the list of narcotic and psychotropic drugs under international control by the United Nations [23], in the Schedule IV of US substance control act and in the tables of illicit drugs of abuse whose sale is prohibited in Italy and in many other European countries. Therefore, dietary supplements freely sold in nature shops cannot contain this substance. The absence of NE in the analyzed products is in agreement with what was reported by Betz et al. [19]. In that case, NE was found only in 2 out of 18 dietary supplements. The authors attributed this finding to the different ephedrine alkaloids concentration among different plant species [5,24–29] varieties [30] and part [28–31].

Looking at the results obtained, it appears that in some products (e.g A, B, H and L) the total amount and proportion of ephedrine to the other alkaloids was not within the range of plant patterns, and the possibility that pure natural substance could be added to such products cannot be excluded. Furthermore, due to the high ephedrine content in these particular products, ingestion (outside any medical supervision) of more than one capsule can lead to a daily intake of this alkaloid higher than the usual oral adult dose (e.g. not exceeding 150 mg ephedrine sulfate per day) for nasal decongestion or bronchodilation with possible adverse secondary health effects [32].

4. Conclusion

GC–MS method reported in this article allows the determination of EP, PE, NPE, NE and MPE in different dietary supplements freely sold in nature and "smart" shops. The main characteristics of the assay are the rapid and simple sample extraction and preparation and total analysis time. Owing to the minimum handling and time required, this procedure can be useful when large stocks of samples from different origin have to be processed.

Acknowledgement

This study was supported by Dipartimento Nazionale per le politiche antidroga della Presidenza del Consiglio dei Ministri.

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