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Short communication

A rapid and simple procedure for the determination of synephrine in dietary supplements by gas chromatography-mass spectrometry

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Abstract

A simple and rapid procedure based on gas chromatography-mass spectrometry (GC-MS) is described for determination of synephrine, active principle of *Citrus aurantium* plant, in solid and liquid dietary supplements. After the addition of 3,4-methylenedioxypropylamphetamine as internal standard (I.S.), 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 synephrine and I.S., derivatized with pentafluoropropionic anhydride, were determined in the selected-ion-monitoring (SIM) mode. The method was validated in the range 0.1–50 μg/mg or μg/mL synephrine. Mean recovery ranged between 89.3% and 90.5% in both solid and liquid dietary supplements. The quantification limit was 0.1 μg/mg or μg/ml. The method was applied to analysis of various dietary supplements promoted for aiding weight control containing, among other constituents such as ephedrine alkaloids and methylxanthines, *Citrus aurantium*. Amount of synephrine present in such products ranged from 3.1 μg/mg solid product to 480.2 μg/mL liquid product.

Keywords: *Citrus aurantium*; Synephrine; dietary supplements

1. Introduction

Citrus aurantium var. *amara.* (also called Seville orange, sour orange or bitter orange) is a plant belonging to the Rutaceae family [1,2]. The ripe fruit is eaten only in Iran [3] and in Mexico where the fresh fruits are sometimes eaten with salt and chili paste [4], since the fruit is too sour to be popular for eating. Indeed, the most common use of *Citrus aurantium* is medicinal rather than culinary. Historically, the dried, entire unripe fruit has been used in Asian herbal medicine primarily to treat digestive problems [2].

Recently, the fruits extracts have been used for the treatment of obesity [5]. In fact, weight loss dietary supplements containing *Citrus aurantium* extract have rapidly replaced products containing ephedrine alkaloids [6], which have been banned by the United States Food and Drug Administration (FDA) in April 2004 because of an association with serious adverse health effects [7,8]. The active constituents of *Citrus aurantium* fruits are flavonoids and adrenergic amines (synephrine, octopamine and tyramine), [9]. Structurally related to endogenous neurotransmitters (epinephrine and norepinephrine) and ephedrine [2]. Synephrine, the most abundant active component, produces effects on human metabolism which could be useful for reducing fat mass in obese human since it stimulates lipolysis, rises metabolic rate and oxidation of fat through increased thermogenesis [10,11]. However, it is also well known that due to adrenergic stimulation effect, mainly synephrine but also the other amines found in *Citrus aurantium* can produce adverse effects the cardiovascular system (e.g. hypertension, tachyarrhytmias, etc) [12,13].

The sale of dietary supplements containing synephrine is prohibited in Canada because of risk of adverse health effects [14] and for the same reason Food and Drug Administration discourages the use of these products as substitutes of dietary supplements containing ephedrine alkaloids, recently banned in United States [15]. Conversely, these products are freely sold in esoteric and nature stores (also called “smart shops”) within

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Europe, including Italy, and internet web sites for their "supposed" nutritional and health benefits.

Several methods have been reported for the determination of synephrine in herbal products, fruits/peels of various *citrus* species and dietary supplements by capillary electrophoresis with electrochemical detection [16], capillary gas chromatography with flame ionization detector [17] and by liquid chromatography with ultraviolet [18,19], photodiode array [20] detectors. Such methods require large amounts of initial bulk material and volumes of extraction solvents [18] thus appearing complex, time [17,19,20] and material consuming. None of the presented assays include mass spectrometry as detection method.

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. Indeed, recently a liquid chromatography-tandem mass spectrometry method for evaluation of synephrine [21] has been reported. Nonetheless, 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.

The present paper describes a new simple and rapid procedure for the determination of synephrine in dietary supplements by gas chromatography-mass spectrometry.

2. Experimental section

2.1. Gas chromatography

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

Synephrine and pentafluoropropionic anhydride (PFPA) were purchased from Sigma–Aldrich (Milan, Italy); 3,4-methylenedioxypropylamphetamine (used as internal standard, I.S.) was supplied by Salars (Como, Italy). Three different dietary supplements containing "herbal" capsules (weight range of capsules: 540–950 mg) and one liquid dietary supplement (aqueous syrup soft drink, 20 mL), whose labels reported the presence of *citrus aurantium* or synephrine 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 synephrine, reported as "drug-free food products") were purchased from the same nature stores or at local supermarkets and analyzed to assess the absence of the substance under investigation before spiking them with synephrine standard solutions.

2.3. Sample preparation and extraction

All the samples were blended and homogenized in a standard mixer. An amount of 100 mg powdered solid sample and 1 mL liquid sample, added to 100 μL and of I.S. working solution (1mg/mL for solid samples, 10 μg/mL for liquid samples), were dissolved in 2mL 0.1 M phosphate buffer (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 layer was evaporated to dryness at 40 °C under a nitrogen stream. 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.

2.4. GC-MS conditions

Analyte 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, Palo Alto, CA, USA). 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 analyte and I.S. were recorded by total ion monitoring mode (scan range 40–550 *m/z*) to determine retention times and characteristic mass fragments. The chosen characteristic mass fragments were monitored in selected-ion-monitoring (SIM) mode: *m/z* 119, 190 and 428 for synephrine-triPFPA and *m/z* 86, 105 and 135 for I.S. The underlined ions were selected for the quantification measurement.

2.5. Validation procedures

Prior to application to real samples, the method was tested in a 4-day validation protocol. Selectivity, recovery, matrix effect, linearity, precision, accuracy, freeze-thaw cycles, mid-term stability and limits of detection (LOD) and quantification (LOQ), were assayed as previously reported [22]. Following the indication of products labels and the weights or volumes of dietary supplements under investigation, calibration was performed in the range of μg/mg for solid samples and in the range of μg/mL for liquid samples.

Calibration standards containing 0.1, 0.5, 1.0, 5.0 10 and 50 μg/mg or μg/mL synephrine and 1 μg/mg or μg/mL I.S. were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 100 mg solid samples and 1 mL liquid samples. Peak area ratios between compound and I.S. were used for calibration curves calculations. A weighted (1/concentration) least-squares regression analysis was used (SPSS, version 9.0.2 for Windows). Five replicates of

blank products samples were used for calculating the limits of detection and quantification. Standard deviation (S.D.) of the mean noise level over the retention time window of each analyte was used to determine detection limit (LOD = 3 S.D.) and the quantification limit (LOQ = 10 S.D.).

Quality control (QC) synephrine samples at 30 μg/mg or μg/mL (high control), 3 μg/mg or μg/mL (medium control), 0.3 μg/mg or μg/mL (low control) and samples at LOQ were prepared in drug-free food products, aliquoted and stored at −20 °C to be used for calculation of validation parameters as previously described [22]. Blank products containing 100 and 500 μg synephrine per 100 mg or 1 mL products were prepared as over-curve samples, to be tested for accuracy and precision once diluted 10 times.

3. Results and discussion

Representative SIM chromatograms obtained following the extraction of liquid and powdered solid drug-free dietary supplements (A, left and right, respectively), drug-free liquid and

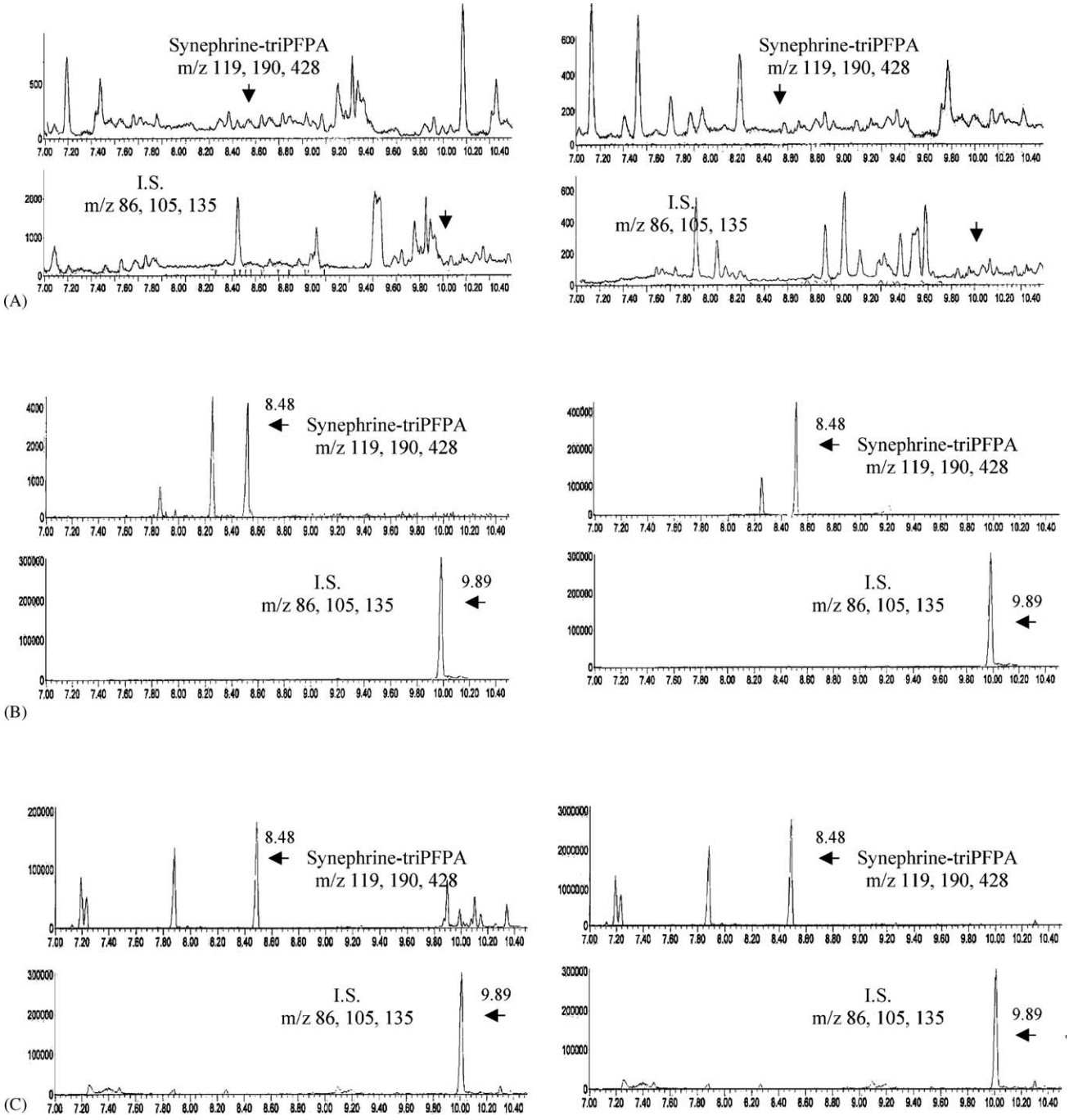

Fig. 1. (A) SIM chromatogram of extracts of: 1 mL liquid (left) and 100 mg solid (right) drug-free dietary supplements. (B) SIM chromatogram of extracts of drug-free liquid and powdered solid dietary supplements spiked with 1 μg/mL and 1 μg/mg synephrine and 1 μg/mg or μg/mL I.S., respectively. (C) SIM chromatograms of extracts of two different dietary supplements containing 48.0 μg/mL (left) and 7.1 μg/mg (right) synephrine.

Table 1
Intra ($n = 5$) and inter-assay ($n = 15$) precision and accuracy

	Concentration μg/mg or μg/Ml	Intra-assay		*Inter − assay*	
		Precision (R.S.D.%)	Accuracy (Error %)	Precision (R.S.D.%)	Accuracy (Error %)
Sinephrine	0.3	8.5	6.7	7.9	8.3
	3	5.8	7.1	9.7	7.7
	30	7.6	6.3	7.4	4.1

powdered solid dietary supplements spiked with 1 μg/mL and 1 μg/mg synephrine and 1 μg/mg or μg/mL I.S. (B, left and right, respectively), are shown in Fig. 1A and B.

Retention time for synephrine was 8.48 min and that of I.S. was 9.89 min, respectively. Peaks present at retention times different from the above-reported, both in Fig. 1 B and C, were not identified and attributed to endogenous substances present in the synephrine-free dietary supplements or in products under investigation.

When analyte concentration in dietary supplements resulted higher than those in calibration curve range, samples were re-processed once diluted 10 times (over-curve samples). 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 synephrine-free dietary supplements that could have interfered with the detection of compound of interest were observed. With respect to the matrix effect, the comparison betweens of synephrine spiked in extracted drug-free products samples versus those for pure diluted standard showed less than 10% analytical signal suppression.

The method exhibited good linearity along the calibration range studied. Mean calibration curves ($n = 3$) presented the following parameters: slope 0.122, intercept −0.0384, determination coefficient (r^2) 0.998. Absolute analytical recoveries (mean ± S.D.) ranged between 89.3% ± 2.1 in solid samples and 90.5% ± 1.8 in liquid samples and relative analytical recovery. The inter and intra-assay precision and accuracy data are presented in Table 1. Over-curve samples, tested for accuracy and precision after diluting 10 times, gave values always better than 10% relative standard deviation (RSD) and error %. LOD was 0.03 μg/mg and 0.03 μg/mL and LOQ was 0.1 μg/mg or μg/mL. 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 [23]. 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.

Extract of dietary supplements containing 48.0 μg/mL (corresponding to the liquid sample with 480 μg/mL synephrine, once diluted 10 time to enter the calibration curve) and 7.1 μg/mg synephrine, are shown in Fig. 1C (left and right, respectively).

The concentration of synephrine in the different dietary supplements examined are shown in Table 2 as mean and standard deviation (S.D.) of three different replicates. The products greatly varied in the amount of contained synephrine with the liquid dietary supplement (product C), showing the highest amount of the drug. It has to be taken into consideration that Due to the high synephrine content in products such as B and C, ingestion (outside any medical supervision) of more than one capsule or more than one liquid preparation can lead to a daily intake of this substance higher than the 30 mg/day established in the Official Italian Gazette [24] as a maximum daily consumption corresponding to an amount of 800 mg *Citrus aurantium* with a 4% active principle. In case of ingestion of higher doses, adverse effects can include hypertension, increase of hearth rate, tremors, headaches [12,13]. Furthermore, the examined products not only contained synephrine, but also other substances, such as methylxanthines and ephedrine alkaloids (ephedrine, pseudoephedrine, caffeine, theobromine and thephylline in case of sample A), methylxanthines (caffeine, teobromine and theophilline in case of sample C) or methylxanthines and taurine (in case of samples B and D), identified with already published methodologies [22,25]. It has to be mentioned that all these other components show stimulant effects on central nervous system with ephedrine alkaloids presenting adverse

Table 2
Synephrine content (mean ± S.D. $n = 3$) in dietary supplements under the study

Products	Label ingredients (weight of a single capsule or mL liquid supplements)	Synephrine (μg/mg)
A	Synephrine, guaranà, Ma Huang (950 mg)	3.1 ± 0.9
B	Synephrine, caffeine, taurine (540 mg)	60.3 ± 0.8
C	*Citrus aurantium*, caffeine (20 mL)	480.2 ± 8.3*
D	*Citrus aurantium*, taurine, caffeine, guaranà extract (700 mg)	7.1 ± 0.8

* Liquid dietary supplement, μg/mL.

health effects including heart attack and stroke, methylxanthines toxic manifestations such as tremors, tachycardia up to seizures and finally excess taurine cardiomyopathy and hepatic toxicity [26–28]. Hence, the concomitant presence of these substances in dietary supplements containing synephrine likely increase the risk of secondary effects and can constitute a serious concern for health safety.

4. Conclusions

This GC-MS method reported allows the determination of synephrine in different dietary supplements. The main characteristics of the assay are the rapid and simple extraction and sample preparation procedures, little volumes of extraction solvents and total analysis time. Owing to the minimum handling, time required, high assay sensitivity and unequivocal detection this procedure can be useful when large stocks of food samples from different origin and unknown amount of synephrine or *Citrus aurantium* extract have to be processed.

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