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Development and validation of a high-performance liquid chromatography–mass spectrometry assay for methylxanthines and taurine in dietary supplements

Emilia Marchei*, Manuela Pellegrini, Roberta Pacifici, Ilaria Palmi, Simona Pichini

Drug Research and Evaluation Department, Istituto Superiore di Sanitá, V.le Regina Elena 299, 00161 Rome, Italy

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Abstract

A procedure based on liquid chromatography–mass spectrometry (LC–MS) is described for determination of caffeine, theobromine, theophylline, taurine in different dietary supplements. After addition of tryptophan as internal standard, both solid and liquid specimens were extracted with 4 ml of hexane/isopropanol (9:1).

Chromatography was performed on a C_{18} reversed-phase column using water/methanol/acetic acid (75:20:5, v/v/v) as a mobile phase. Analytes were determined in LC–MS single ion monitoring mode with atmospheric pressure ionization-electrospray (ESI) interface. The method was validated in the range 0.1–500 and 0.06–500 μg/ml or μg/g for taurine and caffeine, respectively; 0.06–100 μg/ml or μg/g for theobromine and theophylline. Mean recoveries ranged between 70.1 and 94.4% for different analytes. The quantification limits were 0.1 μg/ml or μg/g for taurine and 0.06 μg/ml or μg/g for methylxanthines either in liquid samples or in solid samples. The method was applied to the analysis of various dietary supplements containing methylxanthines and taurine. Energetic drinks contained amounts of taurine in the range of hundreds to thousands μg/ml and ten times lower amounts of caffeine. Conversely, herbal powders, tablets and capsules mainly contained mg amounts of caffeine per gram of product with the other two methylxanthines in the range of ten to hundred μg/g.

Keywords: Methylxanthines; Taurine; Dietary supplements; Liquid chromatography–mass spectrometry

1. Introduction

The methylxanthines caffeine, theobromine and theophylline are present in most commonly consumed beverages (coffee, tea, cola drinks) and in several food products. These alkaloids show physiological stimulatory effects on various body systems, including of central nervous, gastrointestinal, cardiovascular, renal and respiratory system [1,2]. In recent years, caffeine itself and plant extracts containing methylxanthines (e.g. *Paullina cupana* known as guaraná, *Cola nitida*, *Ilex paraguaiensis*, *Yerba mate*) have received increasing attention as components of the so-called "energy" dietary supplements, supplemental products used for weight loss and energy drinks [3–5]. These food products are commonly sold in esoteric and nature stores and Internet web sites for their "supposed" nutritional and health benefits and for the high and fixed content of caffeine. Indeed, caffeine-rich sources have been claimed to improve endurance when included in athlete's diet and to increase the basal metabolic rate [6]. Nonetheless, intake of excessive amount of caffeine and methylxanthines in general can give rise to toxic manifestations such as tremors, tachycardia up to seizures and even death. Furthermore, combined exposure to different methylxanthines may potentiate the toxic effects of either drug [7–9].

Eventual undesired side effects due to consumption of dietary supplements rich in methylxanthines could be increased since certain dietary supplements contain not only caffeine (or methylxanthines), but also taurine. Taurine (2-aminoethanesulphonic acid) is a conditionally essential amino acid, which is not utilized in protein synthesis, but rather is found free or in simple peptides [10]. Taurine is able

* Corresponding author. Tel.: +39 06 49903654; fax: +39 06 49902016.
E-mail address: emarchei@iss.it (E. Marchei).

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to act as a neurotransmitter, antioxidant and modulator of cellular calcium levels [10–12]. Nonetheless, excess taurine has also been associated with cardiomyopathy and hepatic toxicity [13,14]. Since the last 2 years, different types of dietary supplements (beverages, herbal preparations, capsules, tablets, etc.) containing methylxanthines together with taurine and other components gradually spread into the Italian natural foods stores. In this context, a control on methylxanthines and taurine content was decided by governmental legal authorities and the development of easier to use, sensitive and specific method for simultaneous determination of caffeine, theobromine theophylline and taurine in different solid and liquid dietary supplements turned out to be necessary.

Liquid chromatography has been applied as method of choice for simultaneous determination of methylxanthines in food products. Most of these procedures were based on amperometric [15], UV spectrophotometric [16,17] and mass spectrometric detection [18,19]. On the other hand, methods for measuring taurine in biological samples included amino acids analysis [20,21], gas chromatography [22], electrophoresis [23,24], thin-layer chromatography [25] and high-performance liquid chromatography [26–33]. In general, these procedures involve pre-column or post-column derivatization and the entire procedure appears to be complex and time-consuming.

In this study, we report a simple liquid chromatography–mass spectrometry assay for determination simultaneous of caffeine, theobromine, theophylline and taurine in different dietary supplements. Differently from the aforementioned methodologies, pre-analytical extraction phase is simple and easy to perform, no sample derivatization is required and mass spectrometry detection confers certainty to the results obtained. The assay has been extensively validated in order to meet the accepted criteria for bioanalytical method validation [34,35].

2. Experimental

2.1. Chemicals and reagents

Caffeine, theobromine, theophylline, taurine and tryptophan as internal standard (I.S.) were supplied by Sigma-Aldrich (Sigma Chemical, St. Louis, MO, USA). Methanol was HPLC grade (Lab-Scan Analytical Science, Dublin, Ireland). Ultrapure water and all reagents of analytical grade were obtained from Carlo Erba (Milan, Italy).

A diverse range of dietary supplements were included: guaraná powder, “Cola Nitida” powder, “Pausinystalia Yohimbe” and “Yerba Mate” herbal tablets, three different energy carbonated drinks and 14 different “Ephedra Sinica” herbal capsules with a label indicating the presence of guaraná and *C. nitida* powder and in some cases taurine in combination with ephedrine. These products, sold as coffee substituted and energy supplements, were purchased from esoteric and natural 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 methylxanthines and taurine reported in the product label) were “energy supplement” powder, tablets, capsules and soft drinks purchased from the same nature stores or at local supermarkets and analyzed to assess the absence of any substance under investigation before spiking them with methylxanthines and taurine standard solutions.

2.2. Instrumentation

LC–MS analyses were performed using an Agilent 1100 series HPLC system consisting of a G 1312A binary pump, a G1322A degasser, and an ALS G1329A autosampler (Agilent Technologies, Palo Alto, CA) interfaced to an Agilent 1100 series G1946D mass spectrometer equipped with an atmospheric pressure ionization-electrospray (ESI) interface. Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation, Palo Alto, CA, USA).

2.3. Standard solutions

Standard stock solutions (1 mg/ml) of caffeine, theobromine, theophylline, taurine and I.S. were prepared in hot deionized water and stored at +4 °C. The internal standard (I.S.) working solution was used at a concentration of 500 μg/mL.

Calibration standards containing 100 μg I.S. working solution and different μg amounts of taurine (0.1–500 μg), caffeine (0.06–500 μg) and theobromine and theophylline (0.06–100 μg) per 1 ml beverages and 1 g powders were prepared for each analytical batch by adding suitable amounts of standard solutions to 1 ml of blank carbonated beverages and 1 g blank “energy supplement” powder, tablets and capsules. Calibration samples were treated and processed as unknown samples. Several aliquots of quality control samples at 0.3, 25 and 45 and 450 (this last only for taurine and caffeine) μg/ml or μg/g concentration of analytes under investigation were prepared in different blank products to be used for calculation of validation parameters. Blank products containing 500 and 1000 μg analytes under the investigation per ml or per g products were prepared as over-curve samples, to be tested for accuracy and precision once diluted 5 and 10 times, respectively.

2.4. Sample preparation and extraction

Commercial bulk powders and capsules were used as received. Tablets were pulverized and homogenized in a standard mixer. Beverages were degassed by sonication for 5 min prior to extraction.

In case of solid samples, 1 g amount was dissolved in 10 ml hot water. A 500 μl amount of this solution, added with 100 μl I.S., was diluted up to 1 ml by adding LC–MS mobile phase and filtered through 0.45 μm filter (Millipore, Cork, Ireland).

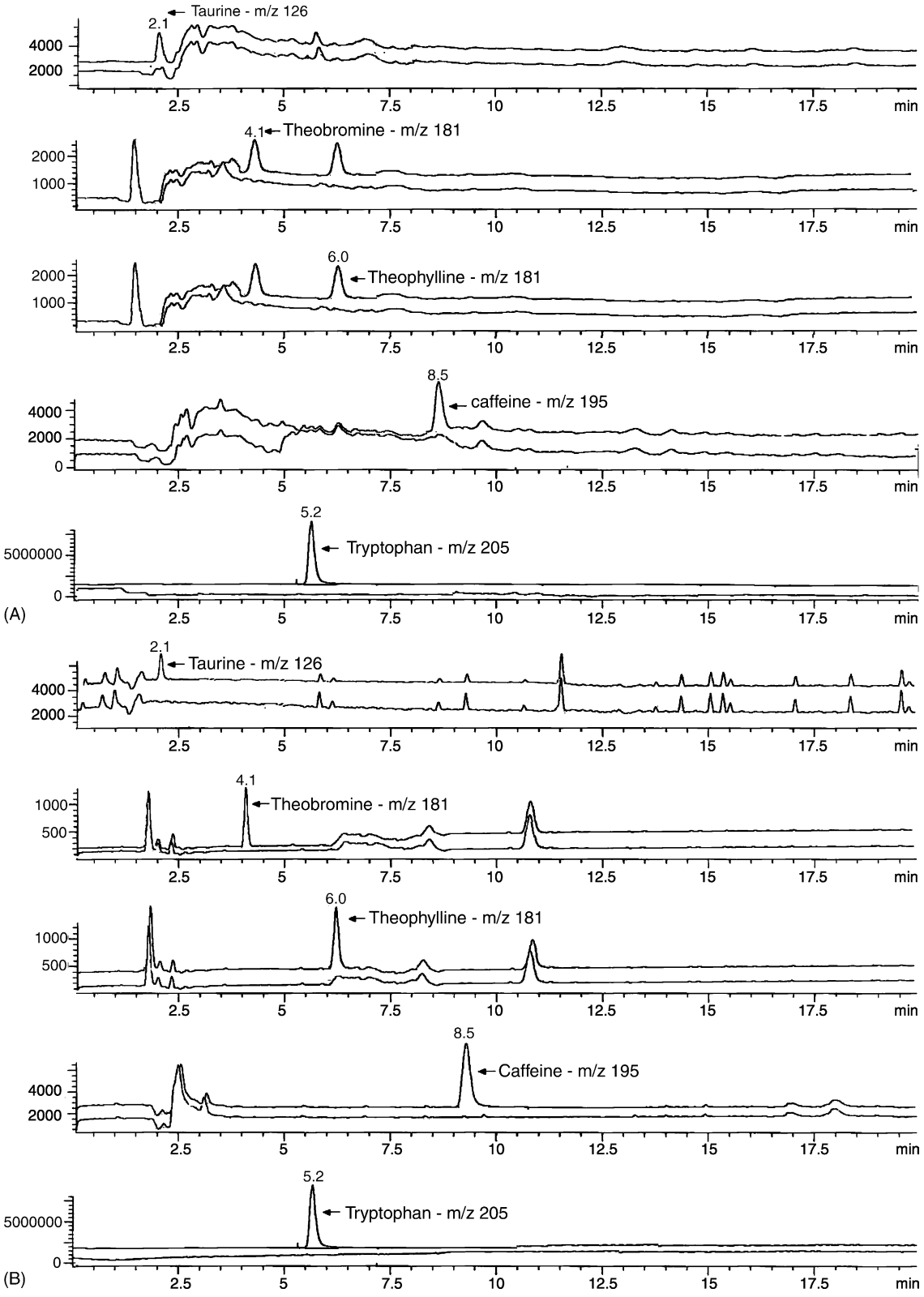

Fig. 1. SIM chromatogram of an extract of pre-checked drug-free food products: (A) carbonated beverage and (B) powder overlaid with blank products spiked with 0.1 μg taurine and 0.06 μg methylxanthines.

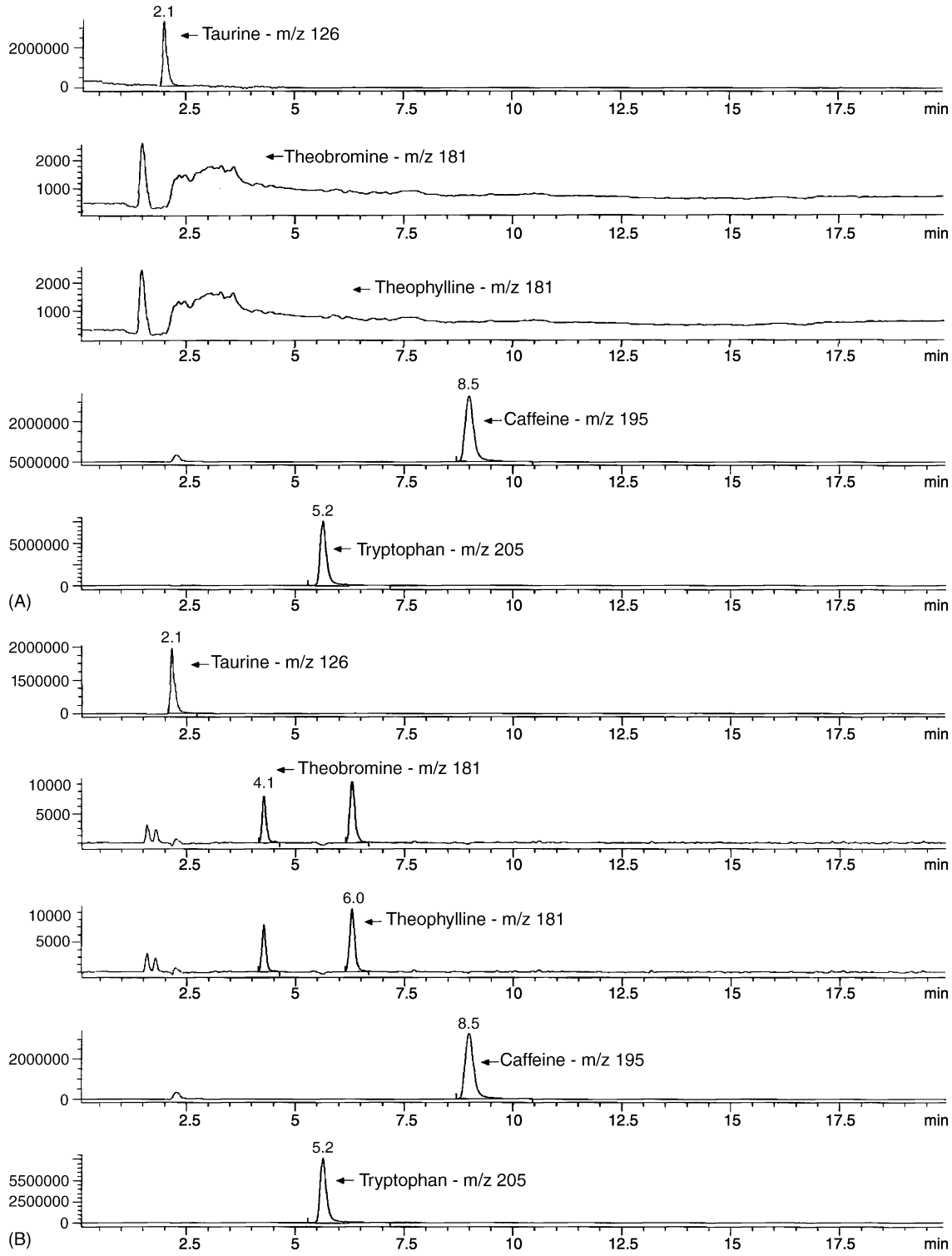

Fig. 2. SIM chromatogram of an extract of two different commercial carbonated beverages: (A) 1 ml of drink containing 300 μg/ml caffeine and 390 μg/ml taurine; (B) 1 ml of drink containing 230 μg/ml caffeine, 1.2 μg/ml theobromine and theophylline, 370 μg/ml taurine.

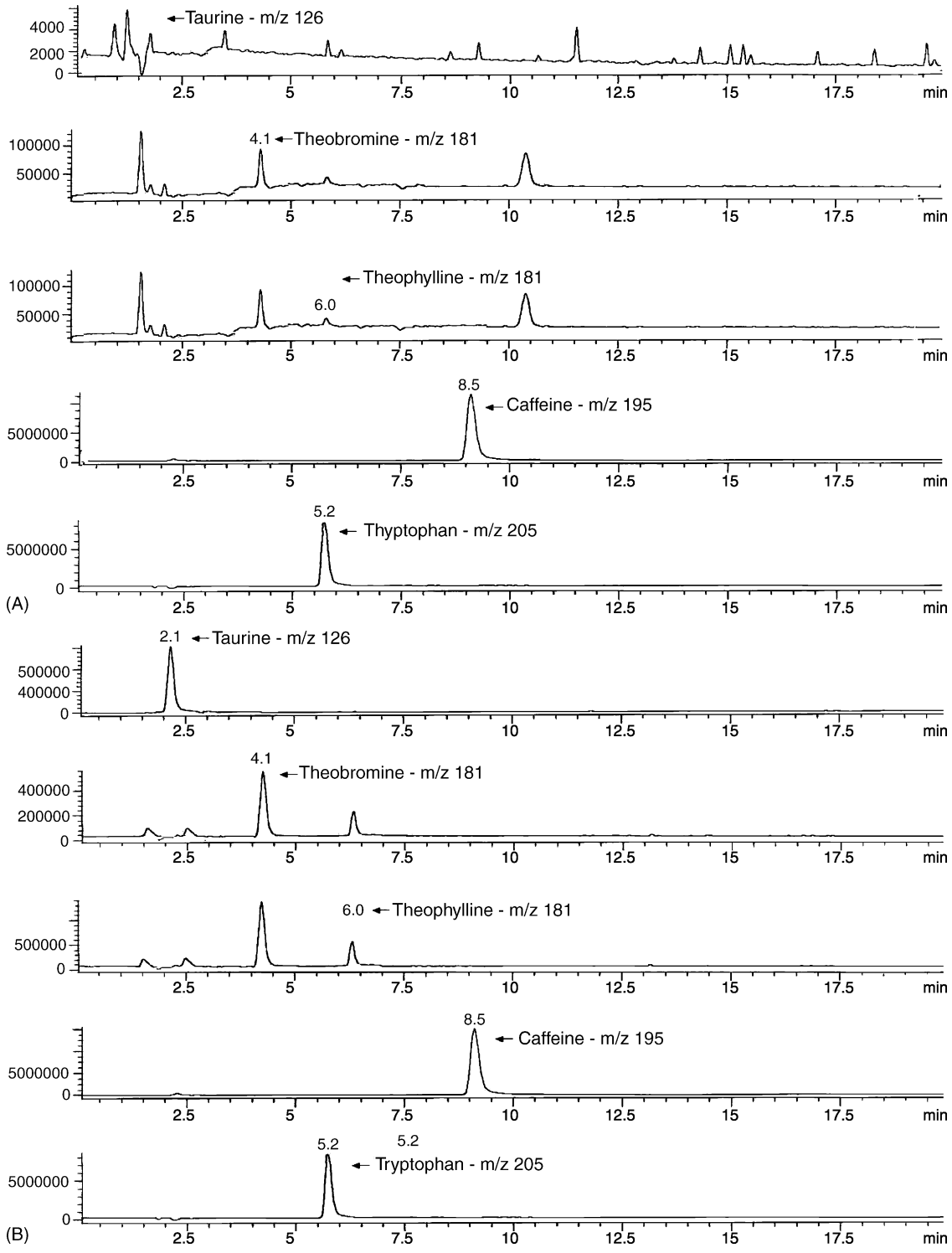

Fig. 3. SIM chromatogram of an extract of: (A) guaranà powder containing 395.7 μg/g caffeine, 66.0 μg/g theobromine and 3.7 μg/g theophylline; (B) herbal capsules containing 467.2 μg/g caffeine, 54.3 μg/g theobromine, 32.7 μg/g theophylline and 129.5 μg/g taurine.

In case of liquid samples, a 100–400 μl amount (depending on methylxanthines and taurine concentration declared in the drink label), added with 100 μl of I.S, was also diluted up to 1 ml by adding LC–MS mobile phase and filtered through 0.45 μm filter. These aqueous-mobile phase layers were extracted with two different aliquots of 2 ml chloroform/isopropanol (90:10, v/v). The organic phases, transferred to another tube, were evaporated to dryness under a stream of nitrogen. The dried residue was reconstituted with 1 ml of mobile phase. A 20 μl amount was injected into chromatographic column.

2.5. LC–MS conditions

Chromatographic separation was achieved using a Phenomenex Luna C18 (150 mm × 4.6 mm, 3 μm) column (Waters, Rome, Italy) with water/methanol/acetic acid (75:20:5, v/v/v) as eluent at a flow of 0.7 ml/min. All chromatographic solvents were degassed with helium before use.

Mass spectrometer was operated in positive ESI mode with selected ion monitoring (SIM) acquisition. The following ESI–MS conditions were applied: drying gas (nitrogen) heated at 350 °C at a flow rate of 11 ml/min; nebulizer gas (nitrogen) at a pressure of 35 psi; capillary voltage at 3500 V, fragmentor voltage (applied to the exit end of the capillary) at 100 V, dwell time at 41 ms and mass peak width at 0.12 min. Qualifying ions were *m/z* 218 (sodium adduct), 195 and 138 for caffeine, *m/z* 203 (sodium adduct), 181 and 124 for theobromine, *m/z* 203 (sodium adduct), 181 and 124 for theophylline, *m/z* 148 (sodium adduct), 126 and 108 for taurine and *m/z* 205, 188 and 146 for tryptophan as I.S. The $[M + H]^+$ ions at *m/z* 195 for caffeine, *m/z* 181 for theophylline and theobromine, *m/z* 126 for taurine and *m/z* 205 for tryptophan were selected for quantification.

2.6. Validation procedures

Prior to application to real samples, the method was tested in a 3-day validation protocol [34,35]. Selectivity, recovery, matrix effect, linearity, precision, accuracy and limit of quantification were assayed.

The blank "energy supplements" products and drinks were extracted and analyzed for assessment of potential interferences due to endogenous substances. The apparent responses at the retention times of the analytes under investigation and I.S. were compared to the response of analytes at the LOQ and I.S. at its lowest quantifiable concentration. The potential for carryover was investigated by injecting extracted blank "energy supplement" products, with added I.S., immediately after analysis of the highest concentration point of the calibration curve on each of the 3 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 calibration samples were analyzed by adding the analytical reference standards and the I.S. in the extract of blank products prior to and after the extraction procedure. The recoveries were assessed at different concentration levels (0.3, 25, 45 and 450 – this last for caffeine and taurine, only – μg/ml or μg/g for beverages and powders, respectively), using four replicates at each level.

For an evaluation of matrix effects, the peak areas of extracted blank products samples spiked with standards at a mean concentration levels (25 and 45 μg/ml or μg/g) after the extraction procedure, were compared to the peak areas of pure diluted substances.

Calibration curves were tested in triplicate over the quantification limit – 500 μg/ml or μg/g for caffeine and taurine and over the quantification limit – 100 μg/ml or μg/g for theobromine and theophylline. Peak area ratios between compounds and I.S. were used for calculations. A weighted (1/concentration) least-squares regression analysis was used (SPSS, version 9.0.2 for Windows). Back-calculated values and deviations from nominal values (expressed as the relative error of the calculated concentrations) were calculated for each of the points of the calibration curves to confirm linearity of the calibration model. Five replicates of blank

Table 1
Method calibration data

Analyte	Correlation coefficient (r^2)	Analytical recovery (%) (mean ± S.D., $n = 4$)[a]				LOD ($n = 5$) (μg/ml or μg/g)	LOQ ($n = 5$) (μg/ml or μg/g)
		0.3 (μg/ml or μg/g)	25 (μg/ml or μg/g)	45 (μg/ml or μg/g)	450 (μg/ml or μg/g)		
Beverages							
Caffeine	0.999 ± 0.003	90.29 ± 7.8	93.89 ± 1.1	90.69 ± 2.3	90.29 ± 1.5	0.02	0.06
Theobromine	0.9989 ± 0.002	75.39 ± 8.5	72.19 ± 3.2	71.99 ± 9.5	–	0.02	0.06
Theophyline	0.9969 ± 0.001	72.19 ± 2.2	74.69 ± 2.6	70.19 ± 5.7	–	0.02	0.06
Taurine	0.9999 ± 0.001	77.99 ± 1.2	78.49 ± 7.4	79.99 ± 1.0	79.59 ± 3.5	0.03	0.1
	Powder						
Caffeine	0.9989 ± 0.002	90.29 ± 6.8	94.49 ± 4.6	94.29 ± 5.7	93.89 ± 5.8	0.02	0.06
Theobromine	0.9969 ± 0.004	89.49 ± 3.7	75.79 ± 2.4	73.39 ± 5.5	–	0.02	0.06
Theophyline	0.9999 ± 0.001	80.29 ± 3.8	75.49 ± 4.1	73.79 ± 3.6	–	0.02	0.06
Taurine	0.9959 ± 0.003	78.39 ± 2.2	76.79 ± 5.6	77.29 ± 1.3	77.99 ± 3.8	0.02	0.1

[a] S.D., standard deviation.

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.).

A total of five replicates at each of quality control concentrations added to blank products and over-curve samples (opportunely diluted) extracted as reported above 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.

The effect of three freeze–thaw cycles (storage at −20 °C) on the methylxanthines and taurine stability 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 products and quantified just after preparation. Mid-term stability test was performed for samples stored at ambient temperature. Three replicates of different products (one drink, one type of tablets and one type of capsules) were included in each analytical batch during a 3-month 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. LC–MS

Representative chromatograms of an extract of prechecked drug-free food products (A: carbonated beverage; B: powder) overlaid with blank products spiked with 0.1 μg taurine and 0.06 μg methylxanthines are shown in Fig. 1. Chromatograms of a carbonated beverages, guaranà powder and herbal capsule containing methylxanthines and taurine are presented in Figs. 2 and 3. When analytes concentrations in dietary supplements resulted higher than those in the calibration curve range, samples were re-injected opportunely diluted.

The influence of organic modifier concentration on retention time was studied during the method development; a 20% (v/v) amount of methanol allowed complete separation of the compounds under investigation, without any interference from other substances eventually present in dietary supplements. A volatile acid, such as acetic acid, was used to improve the ionization process.

No interfering peaks were detected at the retention time of compounds of interest. Drug-free food product samples injected after the highest point of the calibration curve did not present any traces of carryover. With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted drug-free food products samples versus those for

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

Analyte	Intra-assay precision (R.S.D.)				Intra-assay accuracy (error%)				Inter-assay Precision (R.S.D.)				Inter-assay accuracy (error%)			
	0.3 (μg/ml or μg/g)	25 (μg/ml or μg/g)	45 (μg/ml or μg/g)	450 (μg/ml or μg/g)	0.3 (μg/ml or μg/g)	25 (μg/ml or μg/g)	45 (μg/ml or μg/g)	450 (μg/ml or μg/g)	0.3 (μg/ml or μg/g)	25 (μg/ml or μg/g)	45 (μg/ml or μg/g)	450 (μg/ml or μg/g)	0.3 (μg/ml or μg/g)	25 (μg/ml or μg/g)	45 (μg/ml or μg/g)	450 (μg/ml or μg/g)
Beverages																
Caffeine	4.3	7.7	11.5	10.2	10.3	8.4	10.0	9.6	6.8	7.6	7.9	8.3	9.3	9.5	9.7	8.9
Theobromine	6.7	5.4	9.1	–	6.9	7.4	7.5	–	6.9	8.6	6.7	–	6.9	10.4	6.6	–
Theophyline	8.5	7.4	5.0	–	3.8	7.4	2.5	–	5.1	8.1	9.0	–	5.1	8.5	6.3	–
Taurine	5.6	3.8	6.6	7.1	5.4	6.9	4.7	6.7	3.4	3.7	10.5	5.8	7.9	8.7	4.8	7.4
Powder																
Caffeine	6.1	5.9	6.3	5.9	7.6	9.6	5.5	6.9	5.9	6.3	5.6	6.5	9.9	9.1	10.7	11.5
Theobromine	5.5	3.9	7.1	–	8.5	7.1	9.8	–	5.1	4.2	6.0	–	8.3	8.4	8.10	–
Theophyline	5.9	3.5	7.6	–	10.1	9.7	10.4	–	5.3	4.3	6.3	–	6.4	8.7	11.2	–
Taurine	5.7	4.4	6.9	7.8	8.4	8.6	8.2	8.9	5.7	5.2	6.1	5.9	9.1	8.3	9.8	10.6

Table 3
Methylxanthines and taurine content (mean ± S.D., $n = 3$) in dietary supplements under the study

Products	Caffeine	Theobromine	Theophylline	Taurine
Drink (μg/ml)				
A	300.39 ± 9.3	N.D.[a]	N.D.	39009 ± 120.9
B	230.19 ± 20.5	12.09 ± 0.1	12.09 ± 0.1	3709 ± 10.2
C	318.29 ± 4.3	N.D.	N.D.	40589 ± 25.53
Drink (μg/ml)				
Powder (μg/g)				
Guaranà	79509 ± 600	66.09 ± 1.0	3.79 ± 0.1	N.D.
Cola nitida	48.89 ± 3.8	56.69 ± 7.7	48.39 ± 3.0	N.D.
Tablet (μg/g)				
Pausinystalia Yohimbe	438.79 ± 61.9	N.D.	N.D.	N.D.
Yerba Mate	1114.09 ± 156.5	64.89 ± 2.3	49.99 ± 1.7	N.D.
Capsule (μg/g)				
A	1444.39 ± 309.2	127.99 ± 8.2	42.79 ± 2.1	N.D.
B	2763.09 ± 147.4	71.69 ± 2.7	12.79 ± 0.2	1488.59 ± 55.9
C	1423.79 ± 44.7	142.69 ± 1.6	19.19 ± 0.2	N.D.
D	2408.09 ± 125.4	64.99 ± 2.1	42.39 ± 1.5	418.99 ± 13.1
E	1868.79 ± 131.8	54.39 ± 2.5	32.79 ± 2.2	129.59 ± 16.3
F	984.79 ± 89.1	111.39 ± 6.1	14.79 ± 0.6	N.D.
G	3098.09 ± 257.6	97.49 ± 6.3	54.79 ± 3.1	N.D.
H	2620.39 ± 107.4	64.49 ± 0.8	39.89 ± 0.8	N.D.
I	2242.79 ± 101.5	110.29 ± 12.7	47.19 ± 1.0	N.D.
J	2328.59 ± 173.3	192.99 ± 6.5	58.39 ± 3.5	N.D.
K	1635.39 ± 27.1	71.69 ± 1.6	24.09 ± 0.2	N.D.
L	1856.09 ± 111.9	60.79 ± 2.8	22.99 ± 1.2	N.D.
M	1510.09 ± 15.1	33.39 ± 18.5	22.49 ± 0.6	N.D.
N	14939 ± 250.6	108.89 ± 15.7	24.99 ± 2.4	N.D.

[a] Not detected.

pure diluted standards showed less than 10% analytical signal suppression due to co-eluting endogenous substances.

3.2. Validation results

Tables 1 and 2 summarizes the method validation data. Linear calibration curves were obtained for the compounds of interest with correlation coefficients (r^2) higher than 0.99 in all cases. The relative error of the back-calculated values for each of the points of the calibration curves ranged between a maximum of 9.2% for lowest to a maximum of 7% for the highest concentration values in case of all the analytes (data not shown). The recoveries (mean ± S.D.) obtained after liquid–liquid extraction at different concentration levels showed that there were no relevant differences between various food products. Limits quantification of and the limits of detection were considered adequate for the purposes of the present study. The results obtained for intra-assay and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria both [34,35]. In addition, over-curve samples, containing 500 and 1000 μg analytes under the investigation per ml or per g products tested for accuracy and precision once diluted 5 and 10 times, gave values always better than 10% R.S.D. and error%. 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 lower 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 methylxanthine and taurine in the different in dietary supplements products are shown in Table 3 as mean and standard deviation (S.D.) of three different replicates. Energetic drinks contained amounts of taurine in the range of hundreds to thousands μg/ml and ten times lower amounts of caffeine. Conversely, herbal powders, tablets and capsules mainly contained mg amounts of caffeine per gram of product with the other two methylxanthines in the range of tens to hundreds μg/g. Three different capsules of “Ephedra Sinica” also contained taurine in the range of hundreds to thousands μg/g.

The values of substances found in beverages were in agreement with the amounts reported in the labels. Conversely, nor powders or tablets and capsules presented a label with indication on quantitative amounts of methylxanthines and taurine in order to compare with the obtained results.

4. Conclusion

The LC–MS method reported allows the determination of caffeine, theobromine, theophylline and taurine

concentration in different food products. The main characteristics of the assay are the rapid and simple extraction and sample preparation procedures and total analysis time. Owing to the minimum handling and time required, this procedure can be useful when large series of food samples from different origin have to be processed.

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