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Development and validation of a GC/IT-MS method for simultaneous quantitation of *para* and *meta*-synephrine in biological samples

Luciana Grazziotin Rossato*, Paula Guedes de Pinho, Renata Silva, Helena Carmo, Félix Carvalho, Maria de Lourdes Bastos, Vera Marisa Costa, Fernando Remião*

REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal

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ABSTRACT

After the FDA's ban of ephedrine-containing supplements, *Citrus aurantium* appeared as an alternative to ephedra in herbal weight loss products. Synephrine, the most abundant active component of *C. aurantium*, exists in three different structural or positional isomeric forms (*ortho*—*o*-, *meta*—*m*-, and *para*—*p*-). Dietary supplements contain *m*- and *p*-synephrine, both α-adrenergic agonists,while the m-isoform is the most potent at α_1-adrenoreceptors. In spite of the pharmacokinetic and toxicological interest in the study of these compounds, adequate methods for their quantification in biological samples are yet to be developed. Thus, in the present study, a sensitive gas chromatography–ion trap mass spectrometry (GC/IT-MS) method was developed and validated for the simultaneous quantitation of *m*- and *p*-synephrine in a cellular matrix after solid phase extraction (SPE). The validation of the method was performed through the evaluation of the following parameters: selectivity, linearity, specificity, precision, accuracy, limit of detection, limit of quantification, and recovery. The method's applicability was studied in two different biological matrices by evaluating *p*- and *m*-synephrine uptake in Caco-2 cells and also in freshly isolated cardiomyocytes from adult rat. The developed GC/IT-MS method was shown to be selective, accurate, precise, and valid for simultaneous determination of *p*- and *m*-synephrine in biological samples.

1. Introduction

Synephrine is an alkaloid, present in *Citrus aurantium*, which has been frequently included in dietary supplements, mainly after the ban of ephedrine in weight loss products, by the Food and Drug Administration (FDA) [1,2].

Synephrine is an adrenergic agonist, and can exist in three different positional isomeric forms (*ortho*—*o*-, *meta*—*m*-, or *para*—*p*-) (Fig. 1) with nonequivalent pharmacological properties. *o*-Synephrine is not used as a pharmaceutical agent and its presence in nature has not been documented [3]. Furthermore, it is well known that *p*- and *m*-synephrine, also named phenylephrine, are trace endogenous amines widely distributed among bacteria, invertebrates, and vertebrates, including humans [4]. Although there are some studies that report the presence of *m*-synephrine in *C. aurantium* [5–7], the majority of works report that only *p*-synephrine can be found in plants [4,8,9]. Nevertheless, when considering weight loss products, the presence of *m*-synephrine is also reported, probably due to synthetic addition [10,11].

The use of synephrine or synephrine-containing extracts in dietary supplements is attributed to β_3 receptors stimulation, which could help to reduce fat mass through lipolysis stimulation, increase of metabolic rate, and promotion of fat oxidation with increase of thermogenesis [12]. However, synephrine is also an unspecific adrenergic agonist and several adverse effects, mainly cardiovascular effects, have been associated to the use of synephrine-containing products [13–16]. Importantly, the synephrine's pharmacokinetic and toxicokinetic data are still very scarce, which is probably due to the lack of methodologies adapted common use laboratory equipment [17]. Indeed, until now, only liquid chromatography–mass spectrometry (LC/MS) [18,19] and radioactivity methodologies [20] have been applied in synephrine's pharmacokinetic studies.

Thus, the main aim of the present study was to develop and validate a sensitive gas chromatography–ion trap mass spectrom-

Abbreviations: *p*-synephrine, *para*-synephrine; *m*-synephrine, *meta*-synephrine; FDA, Food and Drug Administration; LC/MS, liquid chromatography–mass spectrometry; GC/IT-MS, gas chromatography–ion trap mass spectrometry; DMEM, Dulbecco's modified eagle's medium with 4500 mg/L glucose; NEAA, nonessential amino acids (GlutMAX™); FBS, fetal bovine serum; IS, internal standard; TFAA, trifluoracetic anhydride; EMEA, European Medicines Agency; DMSO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid); SPE, solid phase extraction; LOQ, limit of quantification; LOD, limit of detection; LDH, lactate dehydrogenase; CV, coefficient variation.

* Corresponding authors. Tel.: +351 222078979.
E-mail addresses: luciana@gratto.com.br (L.G. Rossato), remiao@ff.up.pt (F. Remião).

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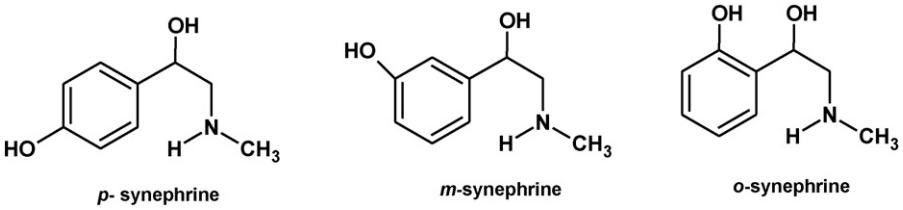

Fig. 1. Chemical structures of synephrine positional isomers.

etry (GC/IT-MS) method for simultaneous determination of *p*- and *m*-synephrine. The present methodology was further applied to evaluate the synephrine's uptake in an immortalized cell line of heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2 cells) and in freshly isolated cardiomyocytes from adult rat.

The Caco-2 cell line is one of the most widely used human cell culture models and has been largely accepted as a reliable *in vitro* model for the rapid screening of intestinal drug absorption and excretion in humans [21–23]. In addition, since cardiovascular events have been associated with synephrine-containing products, the *m*-synephrine uptake in freshly isolated cardiomyocytes was evaluated since this positional isomer has been reported to be the most potent adrenergic agonist of synephrine [24]. This cellular model was already used to evaluate the uptake of catecholamines and its related compounds in previous studies [25–27].

The present method could be helpful to evaluate the physiologic levels of synephrine as a biogenic amine and its pharmacokinetic profile.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were of analytical grade. Trifluoroacetic anhydride (99%) (TFAA), (±) *p*-synephrine (≥98%), (−) *m*-synephrine hydrochloride, 4-hydroxy-3-methoxybenzylamine hydrochloride, collagenase (type IA), and *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid) (HEPES) were obtained from Sigma–Aldrich (St. Louis, MO). Collagenase type II was obtained from Worthington (Lakewood, NJ). Dimethyl sulfoxide (DMSO) and perchloric acid ($HClO_4$) were purchased to Merck (Darmstadt, Germany).

2.2. Biological matrices

Dulbecco's modified eagle's medium with 4500 mg/L glucose (DMEM) and GlutMAX™, nonessential amino acids (NEAA), fetal bovine serum (FBS), trypsin (0.25%)–EDTA (1 mM), antibiotic (10,000 U/mL penicillin, 10,000 μg/mL streptomycin), fungizone (anphotericin B 250 μg/mL) and human transferrin (4 mg/mL) were obtained from Gibcco Laboratories (Lenexa, KS). AccuGENE® (1× PBS buffer) was obtained from Lonza Laboratories (Verviers, Belgium).

Caco-2 cells were cultured at 37 °C in a 5% CO_2 atmosphere using DMEM medium supplemented with 10% FBS, 1% antibiotic, 1% fungizone, 1% NEAA, and transferrin 6 μg/mL.

The validation of the method was performed using Caco-2 cells as matrix. Aliquots of 1 mL Caco-2 cell suspensions (2.5×10^5 cells/mL) were centrifuged ($18 \times g$ for 2 min) and cellular matrix was obtained after precipitation of the produced pellet with 5% HClO4 After a centrifugation ($16{,}000 \times g$, 10 min, 4 °C), the supernatant was used as the matrix where *p*- and *m*-synephrine were added.

Adult male Sprague–Dawley rats (Charles River Laboratories, Barcelona, Spain) weighing 250–350 g were used for cardiomyocyte isolation. Calcium-tolerant cardiomyocytes were isolated by Langendorff retro perfusion of adult rat heart, as previously described [25,26]. At the beginning of the experiments, cell viability was always greater than 60%, as evaluated by the lactate dehydrogenase (LDH) leakage assay and by the microscopic evaluation of the cardiomyocyte's morphology. Incubations were performed in a water bath at 37 °C, using a density of 2.5×10^5 cells/mL in modified Krebs–Henseleit buffer supplemented with 1 mM $CaCl_2$ (pH 7.4) and saturated, every hour, with an airstream of carbogen.

2.3. Calibrators and quality control samples

Stock solutions of *m*-synephrine and *p*-synephrine (150 μg/mL), as well as all intermediate solutions, were prepared in 5% $HClO_4$. Synephrine standards were prepared by adding intermediate solutions to the acidic supernatant of Caco-2 cells, in order to achieve the final concentrations of 10, 100, 500, 1000, 5000, and 10,000 ng/mL. Samples with intermediary concentrations (250, 2500, and 7000 ng/mL) were prepared in order to evaluate accuracy. Internal standard (IS) stock solution of 4-hydroxy-3-methoxy-benzylamine (1 mg/mL) was prepared in methanol. Ten microliters of IS solution (250 μg/mL) was added to each sample.

2.4. Sample preparation for GC/IT-MS analysis

2.4.1. Solid phase extraction (SPE)

All samples (blank, standards, and cellular matrices) were extracted using Waters OASIS SPE columns. The totality of sample was passed through the column, after which 2 mL of HCL 0.1N and 2 mL methanol were applied. Finally, the compounds of interest (both synephrine positional isomers and the IS) were eluted to a glass tube using 2 mL of a methanol solution with 5% NH_4OH. The obtained solution was dried under nitrogen flow. To eliminate residual water, all tubes were left open in the excicator overnight.

2.4.2. Derivatization procedure

Dried residues were submitted to derivatization with 50 μL ethyl acetate and 50 μL TFAA. The incubation was performed at 80 °C for 20 min. After cooling to room temperature, the solution was dried under nitrogen flow. The obtained residue was dissolved in 100 μL of ethyl acetate and injected into the GC/IT-MS system.

2.5. Analytical instrument settings

GC/IT-MS analysis was performed with a Varian CP-3800 GC (USA) equipped with a VARIAN Saturn 4000 ion trap mass detector (USA) and a Saturn GC/MS workstation software version 6.8. The GC was equipped with a VF-5ms (30 m × 0.25 mm × 0.25 μm) from VARIAN. The carrier gas was Helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min in split mode 1:10. One microliter of the derivatized standard or of the cellular extract was injected. The injector port was heated to 250 °C. The initial column temperature of 100 °C was held for 1 min, followed by temperature ramp of

15 °C/min to 300 °C, with a 10 min post-run hold. Total separation run time was 9 min. The ion trap detector was set as follows: the transfer line, manifold, and trap temperatures were 280, 50, and 180 °C, respectively. All mass spectra were acquired in the electron impact mode. Ionization was maintained off during the first 3:50 min, to avoid solvent overloading. The mass range was 50–600 *m*/*z*, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 *m*/*z*. The analysis was performed in full scan mode. The selected qualifier ions were: IS *m*/*z* 69, *m*/*z* 232, and *m*/*z* 345; *p*- and *m*-synephrine *m*/*z* 69, *m*/*z* 140, and *m*/*z* 455.

2.6. Method validation

2.6.1. Evaluation of selectivity

Blank samples (5% $HClO_4$) and cellular matrix control samples were extracted by SPE, derivatized, and analysed by GC/IT-MS to detect possible interferences from endogenous components or experimental procedures. Chromatographic selectivity was evaluated by the presence or absence of co-eluting peaks at the retention times of the analytes or IS. First of all, IS was also tested alone, under the same conditions, in order to evaluate possible interferences of other compounds.

2.6.2. Linearity

Linearity, as indicated by the evaluation of regression curves and squared correlation coefficients (r^2), was performed in 3 days using 6 different concentration levels of the analytes. All standards of *m*-synephrine and *p*-synephrine were extracted in the presence of IS by SPE, before derivatization. The linear range of the method was investigated by performing calibration curves in the concentration range of 10 ng/mL to 10 μg/mL (10, 100, 500, 1000, 5000, and 10,000 ng/mL) and the minimal accepted r^2 for the two synephrine positional isomers was of 0.99.

2.6.3. Limits of detection and quantification

The limit of detection and quantification (LOD and LOQ, respectively) were determined from calibration curves data, following European Medicines Agency (EMEA) criteria [28]. The LOQ was defined as the lowest concentration of the calibration curve and it was estimated after 5 injections based on a signal-to-noise ratio of 10. A signal-to-noise ratio of 3 was considered acceptable for estimating the LOD [28].

2.6.4. Precision and accuracy

Intra-day precision was determined by preparing and analyzing by GC/IT-MS, on the same day, 5 replicates of 3 different concentrations (100, 1000, and 10,000 ng/mL) of *m*- and *p*-synephrine. Inter-day precision was evaluated by repeating the intra-day precision study in 3 different days. Precision was assessed by calculating the mean, standard deviation, and coefficient of variation (CV%) of those values.

Accuracy was determined by evaluating a low, a medium, and a high concentration of *m*- and *p*-synephrine (250, 2500, and 7000 ng/mL) and through the calculation of the percentual deviation between the calculated value and the nominal value.

2.6.5. Extraction recovery

Recovery for synephrine positional isomers through SPE was assessed by adding the IS to samples of *m*- and *p*-synephrine at low, medium, and high concentrations (100, 1000, and 10,000 ng/mL). IS was added to one set of samples before the extraction procedure and to another set after extraction, but before solvent evaporation. Each concentration of both sets was analysed in 3 replicates. The percentage of recovery was calculated by comparing the peak area ratios of the analyte to IS for these two set conditions.

2.6.6. Specificity

Solutions containing structurally related substances, namely biogenic amines or substances that could be co-ingested with synephrine as dietary supplements impurities or adulterants were evaluated in the specificity study. The possibility of interference by dopamine, adrenaline, octopamine, ephedrine, caffeine, and by the metabolites *m*-hydroxymandelic acid and *p*-hydroxymandelic acid were analysed by evaluating the presence or absence of interfering peaks at the retention times of the analytes. Solutions containing 1000 ng/mL of each substance were prepared and analysed.

2.6.7. Sample storage/stability

Short-term stability was evaluated using aliquots of low, medium, and high concentrations of *m*- and *p*-synephrine (100, 1000, and 10,000 ng/mL) prepared in 5% $HClO_4$. One set of samples was kept at room temperature, another set was kept at 4 °C and the last set was stored at −20 °C. After 3 days, all samples were analysed by GC/IT-MS in 3 replicates.

2.7. Proof of applicability

2.7.1. Study of synephrine uptake by Caco-2 cells

The Caco-2 cell model was used to predict the potential intestinal absorption of synephrine. The Caco-2 cells were subcultured at 90% confluence with trypsine (0.25%)–EDTA (1 mM) and seeded into 24-well plates at a density of 60,000 cells/cm^2 to obtain confluent monolayers at the day of the experiment. On the day of the experiment, cells were washed with PBS buffer (pH 7.4), and then incubated at 37 °C for 3 h with *p*- or *m*-synephrine (500 μM and 1 mM each). Each group had 3 replicates and was tested in 3 different and independent experiments. DMSO (1%) was required to prepare *p*-synephrine solutions. Thus, one group of cells was exposed only to DMSO (1% final concentration).

After 3 h incubation, the medium was aspirated and cells were lysed with 5% $HClO_4$ and centrifuged (16,000 × *g*, 10 min, 4 °C). The IS was added to the obtained supernatants and samples were prepared as referred in Section 2.4 to be analysed by GC/IT-MS.

2.7.2. Study of synephrine uptake by isolated cardiomyocytes

The validated method was also applied to another cellular matrix, calcium-tolerant freshly isolated cardiomyocytes from adult rat, to study synephrine uptake by these cells. After a preincubation period of 30 min, the cell suspensions were exposed to *m*-synephrine (1 mM). After 3 After 3 h incubation, aliquots of cell suspensions were centrifuged (18 × *g* for 2 min), the supernatant discarded, and cells were lysed with 5% $HClO_4$, and centrifuged (16,000 × *g*, 10 min, 4 °C). The IS was added to supernatant and samples were analysed by GC/IT-MS.

2.7.3. Viability evaluation and protein determination

The protocol used for the LDH leakage assay in Caco-2 cells was performed as already described [29].

In the cardiomyocyte suspensions, the percentage of rod-shaped cells and the LDH leakage assay were determined as previously described [30–32]. In order to confirm the sampling homogeneity, the protein levels were determined by the method described by Lowry [33].

2.8. Statistical analysis

Results are presented as means ± the standard deviation (SD) (n = 9 per condition out of 3 independent experiments). Nonpara-

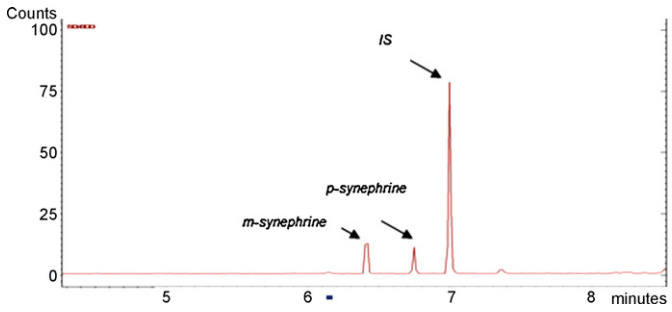

Fig. 2. Representative separations of *m*-synephrine (500 ng/mL), *p*-synephrine (500 ng/mL), and IS (4-hydroxy-3-methoxy-benzylamine) in Caco-2 cells by GC/IT-MS (full scan).

metric tests were used. Kruskal–Wallis test (one-way ANOVA on ranks) was used to compare means of different treatment groups, followed by the Student–Newman–Keuls *post hoc* test, if a significant *p*-value had been obtained. Statistical significance was accepted at *p*-values less than 0.05.

3. Results

3.1. Method validation

3.1.1. SPE extraction, GC/IT-MS separation, and selectivity

The SPE was able to purify and pre-concentrate the analytes. Fig. 2 shows a full scan of a chromatogram of a Caco-2 cells extract, with the retention times of 6.18 and 6.52 min for *m*- and *p*-synephrine, respectively. The IS retention time was 7.0 min and the total time of the analysis was 9 min. It was possible to obtain a good resolution of the peaks and no interference peaks were observed at the retention times of the analytes and of the IS.

The *m*/*z* ions of the two synephrine positional isomers analysed were the same, with the ions *m*/*z* 69, *m*/*z* 140, and *m*/*z* 455. The mass spectrum of the IS was *m*/*z* 69, *m*/*z* 232, and *m*/*z* 345.

3.1.2. Linearity, LOD, and LOQ

The method was considered linear at the concentration range between 10 ng/mL and 10 μg/mL. Linear regression analysis was determined with correlation coefficients (r^2) greater than 0.99 for the calibration curves of both synephrine positional isomers standards. The results for linearity are presented in Table 1.

The LOQ and LOD were estimated following EMEA criteria [28] and were 10 and 3 ng/mL, respectively.

3.1.3. Precision, accuracy, and recovery

Precision, accuracy, and recovery results are summarized in Table 2. Results of intra- and inter-day precision showed CV% values not exceeding 15%, which mean that the method is precise [34].

As the calculated values of accuracy were always within 15% of the nominal value, the method could be considered accurate [34].

Recoveries varied between 79.1% and 95.1% for *p*-synephrine and between 81.1% and 96.8% for *m*-synephrine.

3.1.4. Specificity

Biogenic amines (dopamine and adrenaline), synephrine biotransformation products (*m*-hydroxymandelic acid and *p*-hydroxymandelic acid) and caffeine, which is the stimulant mostly associated with synephrine in weight loss products, do not react with the derivatization reagent used and could not be analysed by this method. Octopamine, a biogenic amine also present in *C. aurantium* fruits, ephedrine, and amphetamine, exhibited retention times of 6.8, 5.7, and 4.7 min respectively, thus, do not interfering with synephrine and IS.

3.1.5. Stability

Short-term stability of synephrine solutions in 5% $HClO_4$, when stored at room temperature, 4 or −20 °C, was evaluated. The best synephrine storage condition, for at least 3 days, was to keep the samples at 4 °C. The other conditions presented chromatograms with smaller analyte' peaks when compared with fresh solutions of the same concentration. Additional peaks from possible degradation products were not observed in none of the conditions evaluated.

3.2. Determination of intracellular synephrine content in Caco-2 cells

Both positional isomers *p*- and *m*-synephrine (1 mM and 500 μM) and DMSO (1%) were not cytotoxic after 3 h incubation (data not shown).

In 3 independent experiments and after a 3 h incubation with 1 mM of each synephrine isomer, the intracellular contents of *m*- and *p*-synephrine were 25.55 ± 5.6 and 26.1 ± 10.5 μM, respec-

Table 1
Linear regression analysis from *m*- and *p*-synephrine in a Caco-2 cells matrix performed in 3 different days using 6 levels of concentration.

Analyte	Day	Equation	Range (ng/mL)	r^2
p-synephrine	Day 1	$y = 0.0005x - 0.0185$	10–10,000	0.999
	Day 2	$y = 0.0004x$	10–10,000	0.998
	Day 3	$y = 0.0004x + 0.0308$	10–10,000	0.999
m-synephrine	Day 1	$y = 0.0004x$	10–10,000	0.999
	Day 2	$y = 0.0003x + 0.014$	10–10,000	0.998
	Day 3	$y = 0.0004x + 0.0243$	10–10,000	0.999

Table 2
Precision, accuracy and recovery from *m* and *p*-synephrine in Caco-2 cells by GC/IT-MS.

Analyte	Concentration (ng/mL)	Intra-day (*n* = 5)	Inter-day (*n* = 5)	Recovery (*n* = 3)	Accuracy (*n* = 3)	
		Precision (%)	Precision (%)	(%)	Concentration (ng/mL)	Accuracy (%)
m-synephrine	100	2.9	10.1	81.1	250	98.2
	1000	7.3	2.1	90.1	2500	110.9
	10,000	4.5	7.2	96.8	7000	115.1
p-synephrine	100	7.5	4.6	79.1	250	95.0
	1000	7.2	10.2	90.6	2500	115.8
	10,000	2.8	8.6	95.7	7000	103.9

tively. When Caco-2 cells were exposed to smaller concentrations, the intracellular content detected significantly decreased ($p < 0.05$). When cells were incubated with 500 μM *m*-synephrine for 3 h, the intracellular content was 12.0 ± 4.18 μM, while with 500 μM *p*-synephrine the intracellular concentration was 11.6 ± 4.3 μM. In *m*-synephrine groups, a residual quantity of *p*-synephrine was detected. The *p*-synephrine content in *m*-synephrine groups was 312 ± 0.20 nM (*m*-synephrine 500 μM) and 240 ± 0.03 nM (*m*-synephrine 1 mM). *m*-Synephrine was not detected in either of the *p*-synephrine groups.

No interfering peaks in the chromatogram were found in control group or vehicle group (data not shown).

3.3. Determination of intracellular synephrine content in cardiomyocytes

No significant differences in viability were observed between treatments in LDH leakage assay after 3 h incubation with *m*-synephrine (data no shown).

In 3 independent experiments, the cardiomyocyte suspensions was exposed to 1 mM of *m*-synephrine and the detected intracellular concentration was about 4.72 ± 0.8 μM.

4. Discussion

The proposed GC/IT-MS method was successfully developed and validated (Fig. 2, Tables 1 and 2). It was linear in a large range of concentration with low LOD and LOQ (3 and 10 ng/mL, respectively). Curiously, the recovery results presented better results for the higher concentrations used when compared with the medium and low levels (Table 2). Moreover, the present method is able to quantify both isomers showing no interferences from cellular matrix. The biogenic amines generally present in biological samples, namely adrenaline, dopamine, and octopamine, or possible adulterants or compounds present in weight loss products, such as caffeine, ephedrine, and amphetamine [4,35] did not interfered with synephrine's analysis.

The literature reports on the chemical analysis of synephrine commonly refer the use of liquid chromatography coupled to ultraviolet detection (HPLC/UV) and most of them are applied to herbal products, fruits, or leaves of *Citrus* sp. [3,9,36]. Furthermore, UV detection presents several interferences, since dietary supplements and vegetable biological samples are very complex matrices. These limitations can be overcome by the use of mass detection. There are two reports using LC/MS and LC/MS/MS analysis to detect and quantify synephrine in biological samples, namely urine, aqueous humor, serum, and plasma [18,19]. However, the proposed GC/IT-MS method presents better recovery results. Furthermore, SPE presents some advantages when compared with liquid extraction, which is non-selective, time-consuming, requires the utilization of huge amounts of organic solvents, and it is an approach that may allow the formation of emulsions. These limitations are important especially when applied to complex matrices such as biological samples.

There is a notorious lack of studies with synephrine in biological samples other than vegetables, urine, and plasma matrices. Regarding synephrine analysis, GC/IT-MS represents a good potential to be exploited since there are only two methods described using this approach and none of those using cellular matrices [8,37]. The GC methods previously published are applied to dietary supplements and they show linearity range, LOD, and LOQ to concentrations higher than those required for biological analysis [8,37]. Furthermore, in the work of Andrade et al., samples must be subjected to a maceration process of 60–90 min and the derivatization procedure with cyclohexanone lasts 1 h [8]; thus it is a time-consuming method. To the best of our knowledge, this is the first time that a sensitive method to quantify simultaneously both synephrine isomers by GC/IT-MS, in a cellular matrix, is described.

Considering the high polarity and low volatility of synephrine, a derivatization step is required for GC/MS analysis [8]. The reaction with TFAA was successfully employed in the present work, minimizing matrix interferences. The validated GC conditions have resulted in well resolved peaks, in which the synephrine positional isomers and the IS have different retention times (Fig. 2). Furthermore, the complete runs last less than 9 min, which also represents an advantage for this methodology. Different peaks other than the synephrine or IS were not identified and were attributed to endogenous substances.

Although there is no consensus about the presence of *m*-synephrine in *Citrus* sp., it is important that analytical protocols are able to identify simultaneously *p*- and *m*-synephrine considering that it is well known that dietary supplements contain both synephrines [5,6,10,11].

The method developed in the present study can help to understand synephrine behaviour in *in vitro* or *in vivo* models and to predict and clarify its pharmacokinetic profile. *In vitro* studies with synephrine are nearly nonexistent. Although *in vitro* studies have extrapolation limitations, they provide important insights and new mechanistic data, namely when considering toxicology and pharmacokinetics. Moreover, as the present method is able to analyse both synephrine positional isomers, it could also help to clarify whether *m*-synephrine is actually present in nature. Considering that the lack of sensitive methods for the detection of trace amines as synephrine, in biological samples, has limited their investigation [38], the present methodology can be applied even to elucidate synephrine's physiological role.

Through the present study, it was possible to observe that the uptake of *p*- or *m*-synephrine by Caco-2 cells, after 3 h, was around 2.5%. The detected intracellular concentrations were about 26 and 12 μM for both positional isomers when exposed to concentrations of 1 and 0.5 mM, respectively. To the best of our knowledge, it was the first time that a study concerning synephrine Caco-2 uptake is described. We showed that, although *m*-synephrine is more potent when concerning the activation of adrenergic receptors [24], when it comes to the uptake transport, both synephrine positional isomers have the same ability to enter Caco-2 cells, as highlighted by the similar intracellular concentrations detected for *m*- and *p*-synephrine.

In the Caco-2 cells study, a residual quantity of *p*-synephrine was detected in cells exposed to *m*-synephrine. This intriguing result could not be explained by the occurrence of impurities in the *m*-synephrine added since the same was not observed in standard curves, which were constructed from the same origin. In fact, only chiral inversion of synephrine has already been reported *in vivo* [39] or when samples are extracted with high temperatures and for long periods of refluxing [40]. Our samples were not subjected to drastic chemical conditions, thus this positional conversion may be related to the high enzymatic competency of Caco-2 cells [41], although it was never described before.

Cardiomyocytes were chosen as an alternative model to show the applicability of the method to matrices other than Caco-2 cells because of the important cardiovascular adverse effects related to synephrine. In fact, these cardiotoxic effects determined the prohibition of dietary supplements containing synephrine in Canada [13–16]. Through the present methodology, it was possible to detect and quantify *m*-synephrine in freshly isolated Ca^{2+} tolerant cardiomyocytes from the adult rat.

5. Conclusion

The proposed GC/IT-MS method can be regarded as selective, accurate, precise, and valid for simultaneous determination of *m*- and *p*-synephrine with a total running time of 9 min. Through this method it was possible to evaluate, for the first time, the uptake of both isomers in Caco-2 cells and of *m*-synephrine in freshly isolated cardiomyocytes.

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