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The Development and Validation of a Targeted LC-HRAM-MS/MS Methodology to Separate and Quantify *p*-Synephrine and *m*-Synephrine in Dietary Supplements and Herbal Preparations

Celine Vanhee [1,*], Sophia Barhdadi [1], Angélique Kamugisha [1], Tanika Van Mulders [2], Kevin Vanbrusselen [1], Marie Willocx [1] and Eric Deconinck [1]

[1] Service Medicines and Health Products, Scientific Direction of Chemical and Physical Health Risks, Sciensano, J. Wytsmanstraat 14, B-1050 Brussels, Belgium
[2] Platform Chromatography and Mass Spectrometry, Scientific Direction of Chemical and Physical Health Risks, Sciensano, J. Wytsmanstraat 14, B-1050 Brussels, Belgium
* Correspondence: celine.vanhee@sciensano.be

Abstract: Dietary supplements containing *Citrus aurantium* or *p*-synephrine remain very popular in Europe and the United States of America (USA). They are primarily sold as weight loss enhancers, although their efficacy and the safety are still under scrutiny. To this end, several countries have set maximum threshold levels of *p*-synephrine that are permitted in dietary supplements. Moreover, there have also been reports of possible chemical adulteration of these supplements with the synthetic positional isomer, *m*-synephrine, known to be used as a medicinal product. Therefore, it is pivotal for regulatory agencies to be able to discriminate between the two positional isomers and also quantify the amount of each when encountered in dietary supplements. Here, we present the development and the validation of a simple and fast "dilute and shoot" procedure, employing liquid chromatographic (LC) separation in combination with high-resolution accurate mass (HRAM) tandem mass spectroscopy (LC-HRAM-MS/MS) to separate these two isomers and subsequently quantify them. The quantification methodology has been validated using the "total error approach", applying accuracy profiles, and is consequently compliant with ISO17025. Moreover, ten real-life samples, either purchased online or encountered by Belgian regulatory agencies, were analyzed using the described procedure. Startlingly, only one sample out of ten was compliant with Belgian legislation in terms of labeling, the presence of a batch number, expiration date and dosage (with a tolerated error of ±20%). Moreover, three samples also contained banned substances such as yohimbine and sibutramine.

Keywords: tandem mass spectrometry; liquid chromatography; plant preparations; isomerism; food adulteration

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1. Introduction

According to the world health organization (WHO), at least 2.8 million people die each year as a result of obesity-related health ailments, including hypertension, diabetes, and cardiovascular diseases [1]. Consequently, there is growing demand for medication and/or dietary food supplements used to attain and maintain normal body weight. Indeed, the global weight loss supplement market was valued at USD 38 billion in 2022, and is projected to pass USD 50 billion in 2030 [2,3]. These supplements are thought to act either by reducing hunger and therefore food intake, reducing the absorption of nutrients, or by stimulating the body to burn fat. Herbal dietary supplements containing green tea extract, green coffee bean extract, *Garcina cambogia* extract, and the extract of *Citrus aurantium* or bitter orange are frequently used for their thermogenic properties and subsequent weight loss and energy level promotion. The effects of bitter orange extracts are attributed to

the presence of biogenic phenethylamines, of which synephrine is the most abundant, in the unripe fruit of *Citrus aurantium* [4–7]. This alkaloid has some structural similarities with other bioactive compounds, including adrenaline and ephedrine (see Figure 1). The latter is also a naturally occurring alkaloid, which can be found in some plant species from the *Ephedra* genus, and to whom thermogenic properties have been attributed [8,9]. However, in 2004, the United States of America (USA) Food and Drug Administration (FDA) and later the European Commission (EC) banned the use of ephedrine alkaloids in dietary supplements due to severe adverse effects, including high blood pressure, rapid or irregular heartbeat, stroke, and addiction [10,11]. Since then, *Citrus aurantium* extracts and synephrine-containing dietary supplements have been marketed as a safer alternative to *Ephedra* extracts [12], as synephrine is thought to exhibit only a small effect on α-1, α-2, β-1, and β-2 adrenergic receptors compared to ephedrine [13]. The agonistic effect of ephedrine alkaloids on these receptors is the main reason that these molecules were banned from usage in dietary supplements. Moreover, several studies have demonstrated that *p*-synephrine promotes the activation of β-3-adrenergic receptors that modulate the mechanism of action of thermogenesis and, consequently, stimulate weight loss [9].

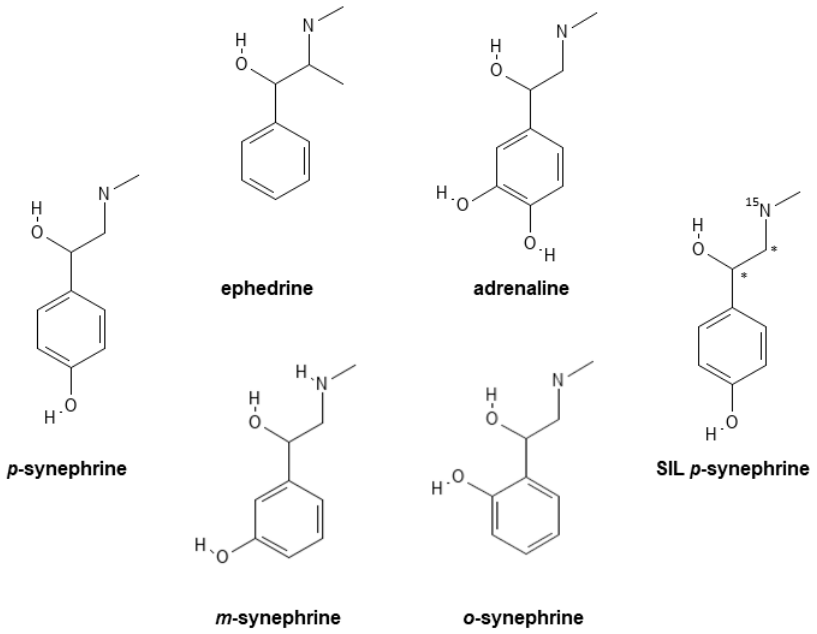


Figure 1. Chemical structures of *p*-synephrine, ephedrine, adrenaline, the stable isotope-labeled internal standard (SIL) used for quantification purposes, and the positional isomers *m*-synephrine and o-synephrine. These isomeric forms differ in their positioning of the hydroxyl group on the phenyl ring. The asterisks present on the SIL structure represent the ^{13}C labeled carbons.

Even today, synephrine remains quite popular; the number of dietary supplements that claim on their label to contain "synephrine" on the market in 2023 was more than 6900, according to the National Institute of Health's (NIH) dietary supplement label database, corresponding to 4.2% of the total labels present in the database [14]. Synephrine exists in three different positional isomeric forms depending on the positioning of the hydroxyl group on the phenyl ring (see Figure 1). Based on previous studies, a general consensus has arisen that only para-synephrine (*p*-synephrine) can be found in bitter orange fruits [15–17]. Phenylephrine or meta-synephrine (*m*-synephrine) and ortho-synephrine (*o*-synephrine) are derived via chemical synthesis, and there exists no proof of their natural occurrence. Moreover, *m*-synephrine, the most potent α-adrenergic agonist of the tested synephrines, is labeled in the USA and in the European Union (EU) as a medicinal product. Due to its effect on the α-, β-1, and β-2 adrenergic receptors, *m*-synephrine is used by the pharmaceutical industry as a vasopressor to increase blood pressure, and as a nasal decongestant [13].

o-synephrine, on the other hand, is thought to have no pharmacological effect in humans, and has until today not been found in dietary supplements [18].

Although some studies performed in animals and humans report that there are little to no cardiovascular adverse effects resulting from *p*-synephrine intake [13], its safety and efficacy after prolonged exposure have not yet been thoroughly studied [18–21]. However, some studies or case reports documented the occurrence of clinical adverse effects of *p*-synephrine alone and in combination with caffeine [20,21]. Because of a lack of consistent data on the safe daily intake of *p*-synephrine, different countries apply different national laws and threshold values for food supplements containing either *p*-synephrine alone or in combination with caffeine (see Table 1). In Belgium, similarly to France, the recommended daily intake of *p*-synephrine from dietary supplements should not exceed 20 mg [22,23]. Moreover, analyses should demonstrate the absence of detectable amounts of *m*-synephrine, as there have been reports of the occurrence of this isomer in commercially available *Citrus* extracts [24]. This phenomenon is likely the result of chemical adulteration [25]. Therefore, it is pivotal that regulatory agencies discriminate between the two positional isomers, *p*-synephrine and *m*-synephrine, and are able to quantify both these molecules to see if the encountered products comply with national legislation. Although such methodologies have been generated in the past, they either made use of an additional derivatization step, or were not fully validated according to ISO17025 [15–17,24,25]. The latter might be of importance if for a certain product further legal steps are required. In this paper, we describe the generation and the validation of a simple and fast dilute and shoot procedure, employing a liquid chromatographic (LC) separation in combination with high-resolution accurate mass (HRAM) mass spectrometry (LC-HRAM-MS) for screening and quantification purposes. The quantification methodology has been validated using the "total error approach" and applying accuracy profiles, and consequently is compliant with ISO17025. Moreover, ten real-life samples, either purchased online or encountered by Belgian regulatory agencies, were analyzed using this novel procedure.

Table 1. Overview of the maximum intake levels suggested or set by the different national health authorities for *p*-synephrine.

	USA [a]	Canada [b]	Australia	UK	Germany [c]	Italy [d]	France [e] and Belgium [f]	Finland and Sweden	The Netherlands
Maximum intake levels suggested or set by national health authorities for *p*-synephrine	No official maximum intake levels are set [17]	Up to 50 mg/day of *p*-synephrine and up to 40 mg *p*-synephrine combined with maximum 320 mg of caffeine per day [26]	30 mg/day [27]	*p*-synephrine is deemed a prescription-only drug [28]	6.7 mg/day [27]	30 mg/day [26]	20 mg/day [22,23]	*Citrus aurantium* is not to be used as food [29]	27 mg/day [30]

[a]. Although no official maximum levels are set for *p*-synephrine, FDA letters were sent to a company selling highly concentrated *p*-synephrine extracts. [b]. The following information must be present on labels: contraindications for children, and pregnant and lactating women; not for use alongside blood pressure-increasing or -lowering drugs, thyroid drugs, sympathomimetics or monoamine oxidase inhibitors. [c]. Germany requires the presence of a safety warning on the label, stating that these products are not suitable for people with existing hypertension, who are overweight, or who suffer from other cardiovascular illnesses. [d]. Italy demands the following warnings: "During pregnancy and while breastfeeding, the use of this product is forbidden, while in children under 12 years of age, use of this product is discouraged. If you have cardiovascular problems, consult a doctor before use". [e]. The following information should be clearly made available to consumers: The consumption of *p*-synephrine is strongly discouraged for individuals with heightened risks of adverse effects (people under treatment for high blood pressure, heart disease, or depression, in particular), pregnant or breastfeeding women, children and adolescents. [f]. The following information should be present on the label: "Do not use if you are pregnant or breastfeeding. Not suitable for people being treated for high blood pressure. Should not be used by children under the age of 12. Intake should be stopped if there are signs of restlessness or nervousness".

2. Materials and Methods

Reagents and reference standards: MS-grade methanol and water were purchased from Thermo Fisher Scientific (Waltham, MS, USA). MS-grade formic acid and MS-grade ammonium acetate originated from Biosolve (Valkenswaard, The Netherlands).

Standards and stock solutions: Reference standards of *p*-synephrine (purity = 97%) and *m*-synephrine (purity = 100%) were acquired from Dr. Ehrenstorfer™ (Augsburg, Germany) and Fagron (Waregem, Belgium), respectively. The labeled internal reference standard of *p*-synephrine, synephrine-13C2,15N (purity = 99.6%) was bought from Toronto Research Chemicals (Ontario, Canada). The initial standard stock solutions of 1 mg/mL of either reference standard were prepared in methanol prior to a dilution to a working stock solution of 5 µg/mL in methanol/water (50:50). Both stock solutions were stored at −20 °C and kept for 3 months. In order to determine the limit of detection of the different positional isomers, serial dilutions were made in 50:50 methanol/water from the standard stock solutions. For the generation of the calibration curves for quantitative analysis, standard stock solutions were diluted into nine different concentrations in 50:50 methanol/water, ranging from 20 to 1500 ng/mL. For validation of the screening and quantification method, the reference standards were diluted in the chosen matrices (see "Validation of the synephrine separation methodology").

Sample set: A total of ten dietary food supplements that contained *p*-synephrine were analyzed. Four samples were obtained in 2022 upon inspection by federal government authorities, and were positive for "synephrine", according to our general screening methodology, as described in [31]. The general screening methodology is not able to make the distinction between the two positional isomers. The remaining six supplements were recently purchased from online websites that delivered to Belgium. All samples were stored at room temperature (15 to 25 °C) and protected from light.

Instrumental conditions: High-resolution accurate mass (HRAM) tandem MS (MS/MS) analyses were carried out on Thermo Scientific™ Vanquish™ ultra-high performance liquid chromatography (UHPLC) system coupled to a Q Exactive™ Focus orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Different types of column chemistries, including high-strength silica (HSS) columns, C8 and C18 reverse-phase columns, HILIC columns, and two different pentafluorphenyl (PFP) columns, with different stationary phases compatible with further upstream MS, were tested.

The selected chromatographic separation made use of an Acquity UPLC HSS PFP column (150 mm × 2.1 µm, 1.8 µm particle size) (Waters, Milford, MA, USA). The LC methodology was as follows: isocratic elution for 1.5 min at 50% mobile phase A (10 mM ammonium acetate pH 7.6 in water) at a constant flow rate of 0.3 mL/minute and a column temperature of 40 °C, followed by a steep increase to 80% B (10 mM ammonium acetate pH 7.6 in methanol) in 0.5 min, which was kept for 4 min. Next, a washing step of 2 min with 95% B was included, as well as a final re-equilibration step of 2 min (total run time 10 min). A high-resolution, accurate-mass analysis was performed on a Q-Exactive Focus Orbitrap MS (Thermo Fisher Scientific, Waltham, MS, USA) equipped with a heated electrospray ionization (HESI) source operating in positive ion mode. The optimized tune method was set as follows: the flow rate of sheath gas (nitrogen, purity ≥ 99.99%) and auxiliary gas (nitrogen, purity ≥ 99.99%) was set at 51 and 14 arbitrary units, respectively; the temperature of the capillary was set at 269 °C; the voltage of spray was 3.5 kV; and the S-lens RF level was set at 50 V. The MS data were acquired within a mass range of *m/z* from 50 to 250 at a resolving power of 70,000 (at *m/z* 200). The MS/MS data, with a resolving power of 35,000, were obtained with the use of a parallel reaction monitoring (PRM) scanning mode. The collision energy of the targeted precursor ion, which triggered collision-induced dissociation (CID), was adjusted to 30. The monitored precursor *m/z* values (inclusion list) corresponded to the *m*/*z* of *p*-synephrine (with predominant *m/z* = 150.091 [(M+H)-H_2O]), *m*-synephrine (with predominant *m/z* = 168.102 [M+H]), and the internal standard synephrine-13C2,15N (with predominant *m/z* = 153.095). All data

were acquired using Thermo Xcalibur 4.0 software and processed using Tracefinder 5.1 software (Thermo Fisher Scientific, Bremen, Germany).

Validation of the synephrine separation methodology: Evidently, according to the International Council for Harmonisation (ICH)'s guidelines, the developed methodology should be able to distinguish the two positional isomers, *p*-synephrine and *m*-synephrine, from each other and from the matrix ingredients [32]. To ensure the selectivity of the screening method, the validation strategy followed was the same as described in "Analytical quality control and method validation procedures for pesticide residues analysis in food and feed" and "Method validation and quality control procedures for pesticide residues analysis in food and feed" [33]. According to both documents, for a qualitative screening method, it is required that detection of an analyte is established at a given concentration level, the so-called screening detection limit (SDL). This has to be demonstrated for a certain concentration level, i.e., the screening detection limit (SDL) at which the respective isomers can be correctly identified in 95% of the samples. The SDL of the components was experimentally determined by serial dilutions in four different matrices, and corresponded to the lowest concentration at which the signal to noise (S/N) ratio reached a value equal to or exceeding 3.3, and where the fragment ions were still present in the MS/MS spectrum. These matrices were devoid of any synephrine, and corresponded to typical matrices that are encountered in products branded as dietary supplements for weight loss. Matrix 1 contained a mixture of extracts from green tea (*Camellia sinensis*), guarana (*Paullinia cupana*), Yerba maté (*Ilex paraguarensis*), and cherry stem (*Prunis cerasus*); matrix 2 consisted of an extract of *Amorphophallus konjac* root, matrix 3 was coffee powder, and matrix 4 contained *Opuntia ficus indica* fibers.

Validation of the quantification methodology:

The limit of quantification or LOQ was determined as the lowest level of concentration at which accuracy and precision could be demonstrated [32,33]. Furthermore, the LOQ had to have an S/N of at least $\geq$10, and was also taken as the lowest point of the calibration curve.

Linearity of the response and matrix effect: Additionally, for the validation of the quantification methodology, the linearity of the PRM response was assessed for concentrations ranging from 20–1500 ng/mL (at least nine intermediate concentrations were analyzed) by applying a least squares regression analysis; this also applied to the four different matrices. Adequate linearity was achieved when the regression coefficient (r) was $\geq$0.98. Non-linearity was tested with a Mandel's fitting test, evaluating whether a quadratic regression model fits better than a linear regression model [34]. Subsequently, matrix effects were evaluated by performing a *t*-test on the slope of the calibration curves of *p*-synephrine and *m*-synephrine with and without a matrix. All injections were performed in triplicate. Moreover, the potential matrix effects were also evaluated by comparing the recovery of *p*-synephrine and *m*-synephrine in four different matrices at five different concentration levels (20 ng/mL, 125 ng/mL, 250 ng/mL, 1000 ng/mL and 1.5 µg/mL); the acceptance limits were set to 100% $\pm$ 20% [33].

Trueness, accuracy, precision, and uncertainty: Validation was performed via the "total error approach" according to the ISO 17025 guideline [35–37]. This approach estimates the "total error" by combining the systemic error (trueness) and the random error (intermediate precision) to find the difference between the observed result and the true value. Through this approach, it is possible to calculate trueness, accuracy, and precision, and estimate the total error and uncertainty of the developed method. Indeed, calculation of the β-expectation tolerance limits, calculated at each concentration level, can be used as a predictive tool that guarantees that 95% of future results produced using the analytical method will fall inside the predefined acceptance limits [$-\lambda$; λ]. The predefined acceptance limits [$-\lambda$; λ] used to construct the accuracy profiles were set to $\pm$ 20% due to the inherent complexity of the matrix.

Briefly, spiked samples were daily made in triplicate at five concentrations (20 ng/mL, 125 ng/mL, 250 ng/mL, 1000 ng/mL and 1.5 µg/mL) and analyzed for four consecutive

days. The corresponding concentrations were back-calculated using the calibration lines generated on the same day. These calculated concentrations were then used to determine the linearity of the results, trueness, precision (repeatability and intermediated precision), and accuracy by means of a validated excel sheet that has previously been used successfully by our research group [38–41].

Sample preparation: Dietary supplements were found in the form of capsules, tablets or powders. At least five capsules were weighed and opened, and their content was mixed and homogenized prior to analysis. One sample (sample 5) consisted of tablets, and five tablets were ground using a mortar and pestle. About 50 mg of dry finely ground and powdered sample was weighed into a volumetric flask, resuspended in 5 mL of methanol, sonicated for 10 min, and passed through a 0.2 µm polytetrafluoroethylene (PTFE) filter. Then, 1 mL of this filtrate was mixed with 1 mL water and left for 5 min at room temperature, and subsequently again passed through a 0.2 µm PTFE filter prior to spiking with SIL *p*-synephrine and subsequent injection into the LC-HRAM-MS systems. For quantification purposes, serial dilutions were made with 50:50 methanol–water until a concentration within the interval of the calibration line was obtained. All quantifications were performed in triplicate.

3. Results and Discussion

3.1. Development and Validation of the Identification Methodology

The separation of the two positional isomers was performed on a Thermo Scientific™ Vanquish™ ultra-high performance liquid chromatography (UHPLC) system equipped with a Q-Exactive focus mass spectrometer. The applied chromatographic separation methodology made use of a pentafluorophenyl (PFP) stationary phase, as this has been proven successful in the separation of these isomers in the past [16,17]. Chromatographic separation of *p*-synephrine and *m*-synephrine (see Table 2) was carried out using an Acquity UPLC HSS PFP column (150 mm × 2.1 µm, 1.8 µm particle size). The total run time, including washing and equilibration, was 10 min, resulting in the total consumption of 3 mL solvents per run; this is currently the fastest methodology described. For the detection of target analytes, high-resolution acute mass tandem mass spectrometry (HRAM MS/MS) was chosen due to its highly selective power. Moreover, the MS settings also resulted in a difference of precursor ions for these isomers (see Table 2 and Figure 2), adding an additional discrimination parameter compared to previous methodologies. The monitored precursor *m/z* values (inclusion list) were the *m/z* of *p*-synephrine (with predominant *m/z* = 150.091 $[(M+H)\text{-}H_2O]^+$), *m*-synephrine (with predominant *m/z* = 168.102 $[M+H]^+$) and the internal standard *p*-synephrine-13C2,15N (with predominant *m/z* = 153.095 $[(M+H)\text{-}H_2O]^+$). In accordance with what has been encountered in the past [24], a difference in intensity of the fragment ions could be observed (see Table 2). For *p*-synephrine, the fragment with *m/z* 107.050 (100%) was the most intense, followed by *m/z* of 91.055 (60%) and *m/z* of 135.069 (20%). For *m*-synephrine, the fragment with *m/z* 91.055 (100%) was the most intense, followed by 107.050 (55%) and 135.069 (45%). A default setting with a relative error of 20% for relative intensities between 40–90% and a relative error of 30% for relative intensities between 10–40% was utilised.

The LC-HRAM MS/MS separation method was subsequently also validated by assessing the sensitivity, specificity and selectivity. This was achieved by checking if it was possible to correctly identify the components in the spiked blank matrix and the four spiked matrices. Moreover, we also verified the absence of the different components in blank matrix and the four different matrices. As can be seen in the Supplementary Data, no peaks could be detected in the indicated *m/z* in matrix alone, while peaks were present in the spiked matrix. Moreover, the obtained intensities of the different fragment ions are in agreement with the tolerated error for their relative intensities. This resulted in a preferred maximum limit of detection (LOD) of 5 ng/mL for *p*-synephrine and 10 ng/mL for *m*-synephrine, calculated as $S/N \geq 3.3$ (see Supplementary Data). Compared to previously published LC-MS/MS methodologies, our LODs are three to six times higher [16,17]. How-

ever, the developed methodology is advantageous, as it makes use of HRAM technology and includes an additional discrimination parameter to separate these positional isomers, thereby increasing the specificity and selectivity of the procedure.

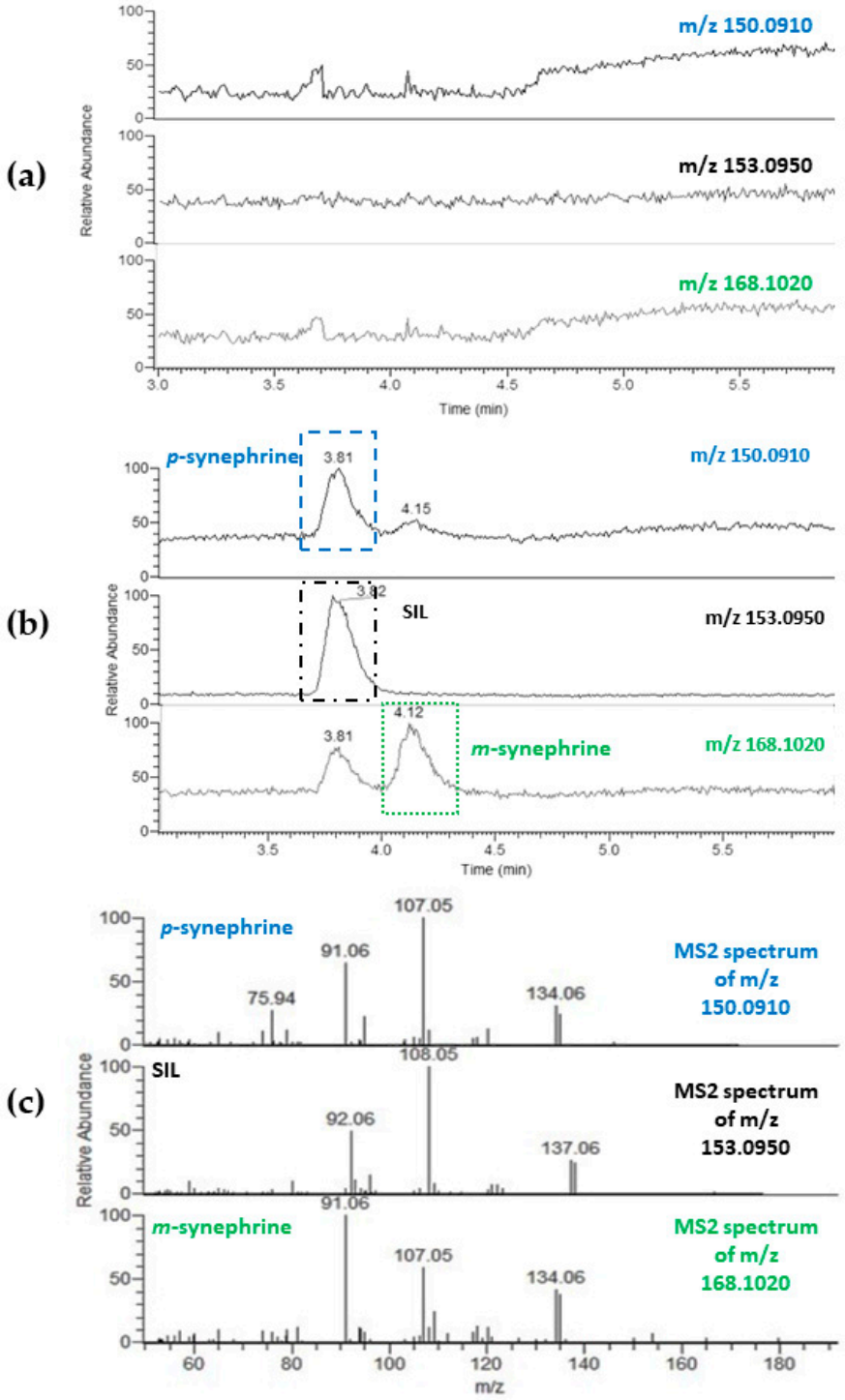


Figure 2. The targeted LC-MS/MS chromatograms obtained for blank matrix (**a**), the matrix spiked with 20 ng/mL of both synephrines and 40 ng/mL of the SIL-internal standard (**b**), and the obtained MS/MS spectra for the different compounds (**c**). The boxes indicate which peak is used for quantification purposes (*p*-synephrine: *m/z* 150.0910 at 3.8 ± 0.2 min; *m*-synephrine *m/z* 168.1020 at 4.2 ± 0.2 min and SIL: *m/z* 153.0950 at 3.8 ± 0.2 min).

Table 2. Summary of the LC-MS/MS characteristics and the performance characteristics for the three different compounds. Abbreviations: SDL = screening detection limit; SIL = stable isotope-labeled.

	Retention Time (minutes)	Precursor Ion (*m*/*z*)	Fragment Ions (*m*/*z*) and Their Relative Intensities	Screening Detection Level (SDL)	Limit of Quantification (LOQ)	Linear Range
p-synephrine	3.8	150.091 $[M+H-H_2O]^+$	91.055 (60%) 107.050 (100%) 135.069 (20%)	10 ng/mL (2 µg/g powder)	20 ng/mL (4 µg/g powder)	20–1.5 µg/mL
m-synephrine	4.2	168.102 $[M+H]^+$	91.055 (100%) 107.050 (55%) 135.069 (45%)			
SIL *p*-synephrine	3.8	153.095 $[M+H-H_2O]^+$	92.059 (50%) 108.054 (100%) 137.065 (30%)			

Moreover, as our analysis does not allow any false positive or false negatives, we set our screening detection limit (SDL) at 10 ng/mL for both components. This allows the detection of at least 2 µg of synephrine per gram product. These values are at least ten times lower than the lowest value encountered in the 59 analysed commercial dietary supplements labelled to contain synephrine and available in 2019 in the U.S.A. [23]. This value is also 60 times lower than the lowest value reported in unripe *Citrus aurantium* fruit [15–17,24], demonstrating that our methodology is sensitive enough for its envisioned purpose.

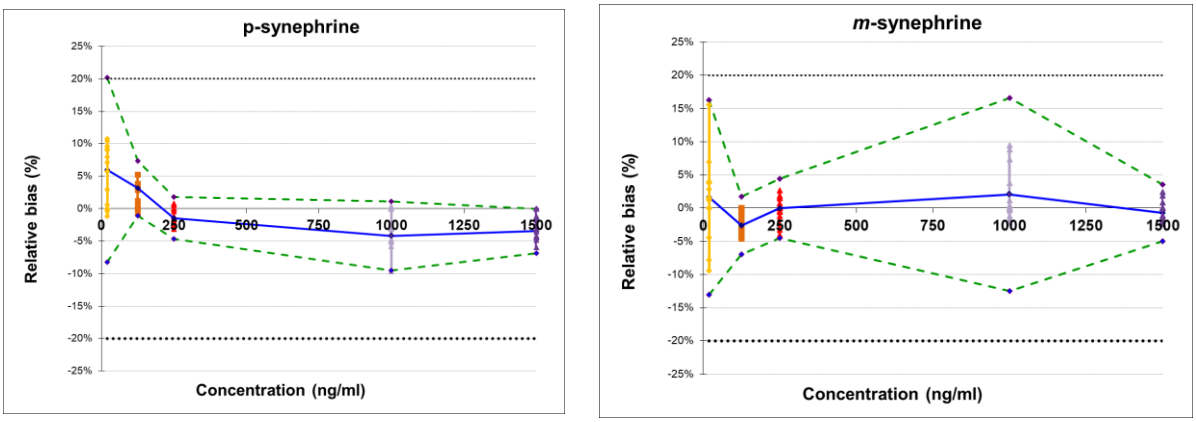
3.2. Validation of the Quantification Methodology

The developed LC-HRAM-MS/MS methodology has been shown to be selective and specific (Section 3.1), and could be used for quantification purposes. In order to determine the proper quantification approach, the linearity of the calibration line and possible matrix effects were assessed. The latter should be minimized due to the use of a stable isotope-labelled (SIL) internal standard of *p*-synephrine. Such a standard is more expensive than that of its structural close relative terbutaline [16]. However, with the current methodology, the use of terbutaline was not possible, as large differences in retention time (>2 min) were obtained, which resulted in a lower method accuracy due to matrix effects.

Calibration curves were obtained for concentrations ranging from 20–1500 ng/mL (at least nine intermediate concentrations were analyzed) by applying a least squares regression analysis to the blank matrix and spiked matrices (n = 4). Adequate linearity was achieved when the regression coefficient (r) was $\geq$ 0.98. Non-linearity was tested with a Mandel's fitting test. All calculated F-values were lower than the critical F-values, thus demonstrating that the linear model is adequate. Subsequently, matrix effects were evaluated by performing a *t*-test on the slope of the calibration curves of *p*-synephrine and *m*-synephrine with and without a matrix, and no significant differences were observed. Moreover, the potential matrix effects were also evaluated by comparing the recovery of *p*-synephrine and *m*-synephrine in four different matrices at five different concentration levels (20 ng/mL, 125 ng/mL, 250 ng/mL, 1000 ng/mL and 1.5 µg/mL). The recoveries ranged from 98–112.6%, confirming that there were indeed no significant matrix effects (see Table 3 [33]). Consequently, the present method was validated according to ISO-17025 applying accuracy profiles which are based upon the "total error" approach (see Materials and Methods). The results are given in Table 4 and Figure 3. Matrix 1, consisting of a herbal mixture of four different plant species, was chosen as a mock matrix for the validation, as this matrix resulted in the highest noise compared to the other matrices.

Table 3. Summary of the obtained recoveries for different concentrations in different matrices. Matrix 1: mixture of extracts from green tea (*Camellia sinensis*), guarana (*Paullinia cupana*), Yerba maté (*Ilex paraguarensis*), and cherry stem (*Prunis cerasus*); Matrix 2: extract of *Amorphophallus konjac* root; Matrix 3: coffee powder; Matrix 4: *Opuntia ficus indica* fibers. The numbers in bold indicate the lowest and highest values obtained for the positional isomers.

	Concentration (ng/mL)	Recovery *p*-Synephrine (%)	Recovery *m*-Synephrine (%)
Matrix 1	20	112.6	115.8
	125	102.4	99.6
	250	104.4	98.8
	1000	99.5	94.4
	1500	105.8	103.5
Matrix 2	20	102.2	101.7
	125	100.7	104.2
	250	103.2	97.8
	1000	98.5	92.7
	1500	100.0	99.6
Matrix 3	20	103.1	104.1
	125	99.7	98.3
	250	102.7	103.4
	1000	98.4	97.7
	1500	100.1	101.6
Matrix 4	20	102.7	99.7
	125	**98.0**	100.2
	250	102.3	104.4
	1000	97.6	96.0
	1500	98.4	**95.9**



(**a**) (**b**)

Figure 3. Accuracy profiles of *p*-synephrine (**a**) and *m*-synephrine (**b**) with the β-expectation interval (black dotted line), relative bias (solid blue line), 95% β-expectation tolerance limits (dashed green line); back-calculated concentrations of reference concentrations (20 ng/mL (yellow), 125 ng/mL (orange), 250 ng/mL (red), 1000 ng/mL (lila) and 1.5 µg/mL (purple)).

A linear relationship is mandatory between the theoretical and measured concentration. The linearity of the obtained method is acceptable when R^2 values are above 0.999 and the *p*-values of the lack-of-fit (LOF) test are above 0.05. As can be seen in Table 4, the R^2 values surpassed the requirements. Moreover, the trueness (a measure for the systematic error of the method) and the precision of the analytical method were calculated. The precision was investigated at two levels: (i) the repeatability, which gives the precision under the same operating conditions over a short time interval; and (ii) the intermediate

precision, which expresses the intra-laboratory variations, as assessed on different days. The precision is a measure for the relative errors of the method, and is expressed using relative standard deviations (RSD). Finally, the accuracy takes into account the total error of the test results, and is represented by the β-expectation tolerance limits. Taking into account the complex matrix, the acceptance limits were set at [−20%; 20%]. As shown in Table 4 and Figure 3, the β-expectation tolerance limits did not exceed the acceptance limits, which means that 95% of the future measurement of unknown samples will be included within the tolerance limits.

Table 4. Summary of the validation data of the quantification methodology, including trueness, precision, accuracy and relative expanded uncertainty. [a] R^2 of the linear relationship between the theoretical and measured concentration. Values in bold indicate the lowest and highest values obtained for the β-expectation tolerance limits.

		Concentration (ng/mL)	*p*-Synephrine	*m*-Synephrine
Linearity [a] expressed as R^2		/	0.99994	0.99966
Trueness	Relative bias (%)	20	2.20	6.67
		125	1.86	1.07
		250	0.93	2.02
		1000	2.40	1.75
		1500	1.15	1.30
Precision	Intermediate precision (%)	20	4.73	6.67
		125	1.91	1.66
		250	1.28	2.04
		1000	2.40	4.58
		1500	1.52	1.73
	Repeatability (%)	20	5.98	1.61
		125	3.13	−2.63
		250	−1.45	−0.03
		1000	−4.20	2.06
		1500	−3.46	−0.75
Accuracy	β-expectation tolerance limits (%)	20	[−8.23; **20.20**]	[−**13.06**; 16.28]
		125	[−1.11; 7.37]	[−7.00; 1.75]
		250	[−4.68; 1.78]	[−4.48; 4.43]
		1000	[−**9.49**; 1.09]	[−12.49; **16.62**]
		1500	[−6.85; −0.06]	[−5.04; 3.53]
Uncertainty	Relative expanded uncertainty (%)	20	10.41	13.75
		125	3.96	3.59
		250	2.75	4.17
		1000	4.96	10.14
		1500	3.16	3.71

In addition to the accuracy, the relative expanded uncertainty was also determined (see Table 4). This uncertainty is calculated as the ratio of the expanded uncertainty at a 95% confidence level forthe respective concentration levels. A maximum expanded uncertainty value of 13.75% was measured for *m*-synephrine, which is deemed a scientifically reasonable uncertainty margin.

From Table 4, it can be concluded that the lowest limit of quantification (LLOQ) corresponded to 20 ng/mL, provided that the LLOQ has an S/N $\geq$ 10 and the upper limit of quantification (ULOQ) corresponds to 1.5 µg/mL. Nevertheless, the developed methodology allows for the quantification of at least 4 µg of synephrine per gram of product, a value which is at least five times lower than the lowest value encountered in the 59 previously analyzed commercial dietary supplements [17].

Compared to the previously published LC-MS/MS methodologies, our LLOQ is three to four times higher than what is reported. However, the previous published methodologies

defined their LOQ as the lowest concentration with an S/N $\geq$ 10, and did not apply any accuracy requirements for their LOQ [15–17].

3.3. Analysis of the Samples

Prior to the analysis of the ten dietary food supplements, the labels of the products were screened for the presence of a batch number and an expiration date, which are mandatory in the EU [18,42,43], the presence of the specific warning information (see Table 1), and the amount of synephrine or *Citrus* extract claimed to be present in the sample. As can be seen in Table 5, only one sample had all the required information on its label.

All samples were analyzed using the developed methodology. As can be seen in Table 5, all samples contained *p*-synephrine, and were devoid of any detectable amount of *m*-synephrine. Additionally, the amount of *p*-synephrine was quantified, and none of the samples purchased online contained quantities of *p*-synephrine that exceeded 10 mg, taking into account the tolerated error of the methodology (±20%). Moreover, none of these samples purchased online (sample 1–sample 6) surpassed the recommended daily intake of 20 mg. However, deviations in label accuracy were found for half of the samples wherein the label accuracy could be determined (n = 4). The amount encountered in the sample and the amount claimed on the label ranged from 34% to 101%, with only two out of four samples containing 100 ± 20% of what was mentioned. The remaining two contained 52% and 34% of the labelled amount. Although no conclusions can be made about the overall label accuracy of *p*-synephrine-containing products in Belgium, due to the limited amount of samples analysed, label accuracy issues have been described for *p*-synephrine samples available in the USA [17]. In their study, Pawar et al. demonstrated that from their 59 analysed samples, only 13 samples were correctly labelled for their *p*-synephrine content. Unfortunately, such findings are not scarce, as several reports have demonstrated poor labelling accuracy regarding the amount of the desired component in dietary food supplements [44–48]. Additionally, these four samples, which were intercepted by federal regulatory agencies, were analyzed as they had been shown to contain synephrine upon standard routine screening for suspected illegal substances (see Supplementary Data). Of these four samples, two were slimming coffees, while the other two samples claimed to contain herbal extracts. Only for sample 10 was the presence of *Citrus* extracts mentioned on the label. Samples 7 and 8 contained, respectively, 5.4 ± 0.7 and 7.7 ± 1.3 mg of *p*-synephrine per gram of coffee powder. Assuming that one coffee cup can be generated from 2 g of powder, one cup would contain, respectively, a maximum of 12.2 and 18 mg of *p*-synephrine. No maximum daily servings were mentioned for these samples; consequently, the amount of *p*-synephrine consumed may exceed the daily recommended amount, provided that in the case of sample 7, more than two cups, and in the case of sample 8, more than three cups are consumed. Startlingly, sample 9 and sample 10 contained quantities of *p*-synephrine that exceeded the daily recommended amount by far. Sample 10 contained 68.1 ± 4.3 mg of *p*-synephrine, while sample 9 contained 124.7 ± 15.7 mg of *p*-synephrine. Moreover, there were also no maximum servings mentioned on the label, nor was there any readable warning on the label.

Table 5. Information provided on the label and actual content present in dietary supplements that were either purchased online or that were seized by federal controlling agencies in 2022.

Product Code	Mandatory Warning Present on the Label	Lot n° and Expiration Date (m/y)	Maximum Servings per Day as Mentioned on the Package	Labeled *p*-Synephrine-Containing Ingredients and Amount of *p*-Synephrine (mg/capsule) [c]	Type of Synephrine Detected and Quantity Found (mg/Capsule or mg/g Powder) [d]		Maximum Daily Intake [e] (mg)	Amount of Caffeine Present (per Capsule or per Gram Powder)	Illicit APIs Identified
Sample 1 [a]	yes	Yes January 2025	1 capsule	162 mg *Citrus aurantium extract*, containing *p*-synephrine	10	*p*-synephrine	10.1 ± 0.2 mg	10.1	-	-
Sample 2 [a]	yes	-	2 capsules	*Citrus aurantium extract*, 30% synephrine [e]	n.a.	*p*-synephrine	11.4 ± 0.9 mg	22.8	253 ± 6.8 mg	Yohimbine (4.9 mg/capsule)
Sample 3 [a]	not present	Yes December 2024	2 capsules	400 mg *Citrus sinensis*	n.a.	*p*-synephrine	0.2 ± 0.04 mg	0.4	-	-
Sample 4 [a]	incomplete	Yes December 2024	1 capsule	125 mg *Citrus aurantium* extract (8% synephrine)	10	*p*-synephrine	8.4 ± 1.0 mg	8.4	-	-
Sample 5 [a]	incomplete	- November 2024	1 tablet	*Citrus aurantium* extract (8% synephrine)	10	*p*-synephrine	3.4 ± 0.2 mg	3.4	-	-
Sample 6 [a]	incomplete	Yes April 2025	3 capsules	100 mg *Citrus aurantium* (10% synephrine)	10	*p*-synephrine	5.2 ± 0.8 mg	15.6	-	-
Sample 7 [b]	not present	-	-	*Citrus aurantium* [f]	-	*p*-synephrine	5.4 ± 0.7 mg	-	2.4 ± 0.1 mg	Sibutramine (0.7 mg/g) and bisacodyl (≈0.05 mg/g)
Sample 8 [b]	not present	-	-	-	-	*p*-synephrine	7.7 ± 1.3 mg	-	10.8 ± 0.2 mg	Sibutramine (1.9 mg/g) and a trace of benzylsibutramine
Sample 9 [b]	not present	Yes November 2023		Herbal extracts mentioned but no *Citrus* sp. [g]	-	*p*-synephrine	124.7 ± 15.7 mg	-	79.6 ± 9.1 mg	-

Table 5. *Cont.*

Product Code	Mandatory Warning Present on the Label	Lot n° and Expiration Date (m/y)	Maximum Servings per Day as Mentioned on the Package	Labeled *p*-Synephrine-Containing Ingredients and Amount of *p*-Synephrine (mg/capsule) [c]	Type of Synephrine Detected and Quantity Found (mg/Capsule or mg/g Powder) [d]		Maximum Daily Intake [e] (mg)	Amount of Caffeine Present (per Capsule or per Gram Powder)	Illicit APIs Identified
Sample 10 [b]	not present or not readable	Yes April 2024	-	*Citrus aurantium* extract and *Citrus sinensis* extract	-	*p*-synephrine	68.1 ± 4.3 mg	-	36.1 ± 1.7 mg	-

[a]. Samples were purchased online either on April 2022 (sample 1 and sample 2) or on May 2023 (sample 3–sample 6). [b]. Samples seized in 2022 by federal controlling agencies. Samples 7 and 8 were coffee powders, while samples 9 and 10 were in the form of capsules containing a beige-brown colored powder. [c]. A full description of what is labeled can be found in the Supplementary Data. [d]. The uncertainty of the measurement is expressed as a confidence interval using the standard deviation of the generated quantification results. [e]. Maximum daily serving intake was calculated by multiplying the quantity of an ingredient found in an individual serving size by the maximum servings per day recommended on the label. [f]. Product is labeled to contain coffee, cactus extract, *Garcinia cambogia*, and white kidney bean extract. [g]. Product is labeled to contain green tea extract, *Aloe vera* extract, and lemongrass extract.

In addition to the presence of *p*-synephrine and *m*-synephrine, the samples were also screened for the occurrence of caffeine or illicit active ingredients using our routine methodology, employed to analyze suspected illegal medicinal products [31]. Next, the amount of caffeine or illicit active ingredient was quantified by means of previously validated quantification methodologies [49,50]. The obtained quantification data are displayed in Table 5. Sample 2 contained pharmaceutical quantities of yohimbine (9.42 ± 1.5 mg/capsule). Yohimbine is an alkaloid derived from the bark of *Pausinystalia johimbe*, and was used two decades ago as a prescription medicine, with dosages ranging from 5 mg to 10 mg. However, its usage could result in significant adverse effects including headaches, hypertension, and panic attacks [44,49]. Therefore, the usage of yohimbine in dietary supplements has been banned in Europe [51]. Additionally, sample 2 also contained ± 253 mg of caffeine per capsule, which taking into account the mentioned maximal daily serving, will result in a caffeine intake of more than 500 mg per day, thus exceeding the safe caffeine limit set by EU and the USA by at least 100 mg [52,53].

Both samples 7 and 8, consisting of coffee powder, were positive for sibutramine and contained, respectively, 0.7 ± 0.03 mg/g and 1.9 ± 0.3 mg/g of this molecule. Sibutramine, initially used as a prescription drug with dosages forms ranging from 5 mg to 15 mg, was banned in the USA and EU due to safety concerns, as it has been linked to undesirable cardiovascular side effects [54,55]. Assuming that one coffee cup can be generated from 2 g of powder, this would result in a respective intake of 1.4 mg and 3.8 mg sibutramine per cup. These are considerable quantities that might result in adverse effects, particularly as no maximum daily servings were mentioned for these samples. Moreover, sample 7 also contained a small amount of the synthetic laxative bisacodyl (≈0.05 mg/g), and sample 8 contained a small trace of a sibutramine homolog, benzylsibutramine (see Supplementary Data).

4. Conclusions

Demand for herbal dietary weight loss supplements has risen in the last decade as a means to attain and maintain normal body weight. Supplements containing extracts of *Citrus aurantium* or its most abundant biogenic phenethylamine, synephrine, are some of the most popular. Synephrine exists in three different positional isomeric forms depending on the positioning of the hydroxyl group on the phenyl ring, and it is assumed that only *p*-synephrine can be found naturally in these Citrus extracts. Nevertheless, there have been reports of chemical adulteration of Citrus extracts with *m*-synephrine. Consequently, it is pivotal for regulatory agencies to be able to discriminate between these two isomers, and also quantify the correct amount of *p*-synephrine. Moreover, several countries have established a maximum threshold level of *p*-synephrine that is allowed in dietary supplements. Here, we present the development and validation, according to ISO17025, of a targeted LC-HRAM-MS/MS methodology to distinguish and quantify both isomers. The validation method according to ISO17025 is of importance for regulatory agencies, if for a certain product further legal steps are advisable. Previously described methodologies have not been validated as compliant with this norm [16,17]. Furthermore, the developed methodology also bears several other advantages, as it is currently, to the best of our knowledge, the fasted described separation methodology for these two isomers, and utilizes only 3 mL of solvent per analysis. Moreover, the additional parameter of retention time (a precursor to ion selection) was applied to discriminate between the two studied positional isomers, increasing the specificity and selectivity of this procedure compared to previous methodologies. Next, we also demonstrated the applicability of the procedure by analyzing ten real-life samples, and showed the complementary power of this methodology alongside more general screening methodologies. Our results indicate that all the samples contained *p*-synephrine, and were devoid of any detectable amount of *m*-synephrine. Startingly, only two samples were accurate in their labelling for *p*-synephrine. From these two samples, one sample contained the proper European mandatory warnings written on the label, as well as the national information requirements, while the other sample contained incomplete warnings. Moreover, three samples also contained banned substances (yohimbine and

sibutramine). Taken together, our results illustrate the applicability of the methodology to real-life samples. Moreover, the fact that only one sample from the sample set was compliant with Belgian national legislation suggests that more quality controls on these supplements are pertinent; due to the limited amount of samples analyzed, no general conclusion can be made on the overall quality of *p*-synephrine-containing products available on the Belgian market.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10080444/s1, Figure S1: The MS spectra obtained for *p*-synephrine and *m*-synephrine; Figure S2: The targeted LC-MS/MS chromatograms obtained in matrix for different concentrations of *p*-synephrine; Figure S3: The targeted LC-MS/MS chromatograms obtained in matrix for different concentrations of *m*-synephrine; Figure S4: Overview of identification of *p*-synephrine, caffeine, and the different illicit substances in sample 2; Figure S5: Overview of identification of *p*-synephrine, caffeine, and the different illicit substances in sample 9; Figure S6: Overview of identification of *p*-synephrine, caffeine, and the different illicit substances in sample 10.

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