Preventing a Public Health Issue by Quantification of Illegally Added Sexual Enhancers in Natural Dietary Supplements

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Prevención de un problema de salud pública mediante la cuantificación de potenciadores sexuales añadidos ilegalmente en suplementos dietéticos naturales

Prevenció d'un problema de salut pública mitjançant la quantificació de potenciadors sexuals afegits il·legalment en suplements dietètics naturals

RECEIVED: 6 FEBRUARY 2025; ACCEPTED: 27 MARCH 2025 DOI: HTTPS://DOI.ORG/10.55815/432575

SUMMARY.

The lack of regulation for "natural" dietary supplements raises public health concerns as they may contain undisclosed active ingredients, without proper labelling. Natural libido boosters, marketed as dietary supplements for male sexual function improvement, have been linked to serious side effects, prompting health authorities to issue warnings. Liquid chromatography coupled to mass spectrometry (LC-MS) methods are commonly used to detect non-regulated substances in these supplements. In the present work, an LC-MS/MS method was validated to verify that it provides robust and reliable results, with enough sensitivity (LOD ≤ 10 $\mu g \cdot kg^{-1}$ and LOQ ≤ 45 $\mu g \cdot kg^{-1}$), precision (RSD ≤ 15%), and accuracy (80–120% recovery). Then, the concentration of sildenafil, tadalafil, vardenafil, yohimbine and desmethyl carbodenafil was evaluated in several dietary supplements commercially available in Spain. 17 samples were analyzed, and 4 presented contaminations of sildenafil, yohimbine or tadalafil. The outstanding results lead to a robust analytical method that can be used to detect and prevent fraud, ensure food security and prevent public health issues.

Palabra clave*:* Potenciadores sexuales naturales; fraude; inhibidores de la fosfodiesterasa tipo 5 (PDE5); LC-MS; validación de métodos; suplementos dietéticos

RESUMEN

La falta de regulación de los suplementos dietéticos "naturales" plantea preocupaciones de salud pública, ya que pueden contener ingredientes activos no reportados y sin el etiquetado adecuado. Los estimulantes naturales de la libido, comercializados como suplementos dietéticos para mejorar la función sexual masculina, se han relacionado con efectos secundarios graves, lo que llevó a las autoridades sanitarias a emitir advertencias. Los métodos de cromatografía líquida acoplada a espectrometría de masas (LC-MS) se utilizan comúnmente para detectar sustancias no reguladas en estos suplementos. En el presente trabajo, se validó un método LC-MS/MS para verificar que proporciona resultados robustos y fiables, con suficiente sensibilidad (LOD ≤ 10 $\mu g \cdot kg^{-1}$ y LOQ ≤ 45 $\mu g \cdot kg^{-1}$), precisión (RSD ≤ 15%) y exactitud (80-120% de recuperación). Posteriormente, se evaluó la concentración de sildenafilo, tadalafilo, vardenafilo, yohimbina y desmetilcarbodenafilo en varios complementos dietéticos disponibles comercialmente en España. Se analizaron 17 muestras, y 4 presentaron contaminaciones por sildenafil, yohimbina o tadalafil. Los excelentes resultados conducen a un método analítico sólido que puede usarse para detectar y prevenir fraudes, garantizar la seguridad alimentaria y prevenir problemas de salud pública.

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Palabra clave: Potenciadores sexuales naturales; fraude; inhibidores de la fosfodiesterasa tipo 5 (PDE5); LC-MS; validación de métodos; suplementos dietéticos

RESUM

La manca de regulació dels suplements dietètics "naturals" planteja problemes de salut pública, ja que poden contenir ingredients actius no reportats, sense l'etiquetatge adequat. Els potenciadors naturals de la libido, comercialitzats com a suplements dietètics per a la millora de la funció sexual masculina, s'han relacionat amb efectes secundaris greus, fet que ha provocat que les autoritats sanitàries emetissin advertències. Els mètodes de cromatografia líquida acoblada a espectrometria de masses (LC-MS) s'utilitzen habitualment per detectar substàncies no regulades en aquests suplements. En el present treball, es va validar un mètode LC-MS/MS per verificar que proporciona resultats robusts i fiables, amb suficient sensibilitat (LOD ≤ 10 $\mu g \cdot kg^{-1}$ i LOQ ≤ 45 $\mu g \cdot kg^{-1}$), precisió (RSD ≤ 15%) i exactitut (80-120% de recuperació). A continuació, es va avaluar la concentració de sildenafil, tadalafil, vardenafil, iohimbina i desmetilcarbodenafil en diversos suplements dietètics disponibles comercialment a Espanya. Es van analitzar 17 mostres i 4 presentaven contaminacions de sildenafil, iohimbina o tadalafil. Els resultats destacats donen lloc a un mètode analític sòlid que es pot utilitzar per detectar i prevenir el frau, garantir la seguretat alimentària i prevenir problemes de salut pública.

Paraules clau: Potenciadors sexuals naturals; frau; inhibidors de la fosfodiesterasa tipus 5 (PDE5); LC-MS; validació del mètode; suplements dietètics

1. INTRODUCTION

Since many dietary supplements labelled as "natural" are not considered food or pharmaceuticals, there is a lack of regulation to control the manufacturing process and the safety of these kinds of products in the market. However, these products are consumed as supplementary food, and it is crucial to guarantee their security. Otherwise, the uncontrolled and regular consumption of these dietary supplements can lead to the incorporation of certain substances that, without medical control, can be dangerous to human health. For this reason, there are many alerts about medicinal compounds found in natural products, which is an important public health issue. One example is those products considered natural libido boosters. Natural sexual enhancers are products marketed as dietary supplements that are supposed to improve male sexual function. However, many of these products contain active ingredients similar to those found in prescription medications for erectile dysfunction, typically phosphodiesterase type 5 (PDE5) inhibitor family of drugs such as sildenafil, tadalafil, or vardenafil, without being declared on the label[1]tablet, capsule, etc.. These pharmaceuticals are known as counterfeit medicine, which are those compounds deliberately and fraudulently mislabeled concerning identity and/or source[2]. This kind of activity can be dangerous because the added pharmaceuticals can interact with other medications the consumer is taking or can be harmful to their health if used incorrectly. In recent years, several cases of serious side effects, such as headaches, dizziness, fainting, chest pain, and loss of vision or hearing, among others, related to the use of natural sexual enhancers have been reported[3]. In response to this situation, health authorities in several countries, including the United States and the European Union, have issued alerts and recommendations regarding the use of natural sexual enhancers[4]weak quality control system and uncontrolled distribution channels are some of reasons that enhance the informal pharmaceutical market. In recent years, the unfulfilled desire for sex has been a subject that has aroused increasing public interest with respect to improve sexual functions. The use of herbal medicines substantially increased due to escalated prevalence and impact of sexual problems worldwide and estimates predicting the incidence to raise over 320 million by year 2025. The various reasons to use herbal supplements in men may be due to experiencing changes in erectile dysfunction (ED.

Despite these alerts, there are still a large number of natural sexual enhancers on the market that contain medicinal compounds without being declared on the label[5]. This is partly due to the lack of regulation of these products in many countries. In some cases, manufacturers can circumvent regulations by changing the names of the active ingredients or by indicating that the products are "natural" or "herbal", even if they contain synthetic substances. In addition, it was reported in the WHO Global Surveillance and Monitoring System (GSMS) of 2017 that in some cases there is a lack of reporting of inadequate products as a result of the low detection levels required to determine these active principles in complex samples of natural products[6]. Therefore, it is crucial to develop sensitive analytical methods to control the presence of this kind of pharmaceuticals in complex natural samples and thus, ensure the safety of the consumers. In this sense, liquid chromatography coupled to mass spectrometry (LC-MS) is a powerful technique that allows separating, detecting, and quantifying individual components in complex samples.

There are several LC-MS methods described in the literature related to the quantification of PDE5 inhibitor drugs and their analogues[7–10]. However, all these methods include complex sample preparations to extract the active ingredients from the natural sexual enhancer product and purify them before analysis. These processes can involve the partial or total loss of analyte. For this reason, it is necessary to improve the sample preparation, minimizing the number of steps required to obtain an optimal analytes extraction. In the present work, a rapid and simple sample preparation procedure was used and proved to be effective in extracting sildenafil, tadalafil, vardenafil, yohimbine and desmethyl carbodenafil from complex matrices. Moreover, an LC-MS/MS method was developed to

quantify the extracted compounds in dietary supplement samples. Finally, the MRM method was validated to ensure that the results obtained were reliable. The results are compared to established safety thresholds to determine whether the product is safe for consumption. Thus, regulatory authorities can take action to remove dangerous products from the market and protect public health if necessary.

2. MATERIALS AND METHODS

Standards and Reagents

Yohimbine (Yoh) (ref. 146-48-5), and yohimbine-$^{13}C,D_3$ (ref. 1261254-59-4) were supplied by MedChem Express (New Jersey, USA). Desmethyl carbodenafil (DsmC) (ref. 147676-79-7), sildenafil (Sil) (ref. 139755-83-2), tadalafil (Tad) (ref. 171596-29-5), and sildenafil-D_8 (ref. 951385-68-5) were supplied by LGC Standards (UK). Vardenafil (Var) (ref. 224785-90-4) was purchased from Santa Cruz Biotechnology (Texas, USA). Formic acid Optima® for LC/MS (99.5%, ref. A117-50) was purchased from Thermo Fisher Scientific. Methanol for UHPLC (99.9%, ref. 83638.320) and acetonitrile for UHPLC (ref. 83640.320) were supplied by VWR.

Dietary Supplement Samples

For validation purposes, five commercial natural dietary supplement samples were used, covering several formats: tablet, powder, and capsules. Once the method was validated, a total of 17 samples were analyzed. First, samples were ground to obtain a fine powder. Then, 0.2 g of each sample was weighed and mixed with 20 mL of methanol/water (80:20) solution, adjusting the pH to 4.8 with acetic acid. Finally, samples were diluted as required with water and filtered with 0.22 mm nylon filters.

UHPLC-QQQ Analysis

On one hand, the instrumental determination was done using a UHPLC ExionLC™ AD System (AB Sciex, USA) coupled to a Triple Quad™ 6500 System (MS/MS) (AB Sciex, USA) with electrospray ionization (ESI) in positive mode. ESI source parameters: spray voltage, 5500 V; source temperature, 600 °C; nebulizer and auxiliary gas flow (N_2), 60 psi and 60 psi; curtain gas flow (N_2), 35 psi. Mass spectrometry analyses were performed applying a multireaction monitoring (MRM) experiment. Precursor ion, product ion and collision energy were optimized.

On the other hand, instrumental determination was also performed using a UHPLC ExionLC™ AD System (AB Sciex, USA) coupled to a Triple Quad™ 7500 System (MS/MS) (AB Sciex, USA) with ESI in positive mode. ESI source parameters: spray voltage, 3000 V; source temperature, 600 - C; nebulizer and auxiliary gas flow (N_2), 35 psi and 50 psi; curtain gas flow (N_2), 40 psi. Mass spectrometry analyses were performed by applying an MRM experiment. Precursor ion, product ion, collision energy and Q0 were optimized.

In both cases, chromatographic separation was performed using a Luna® Omega Polar C18 (100x2.1 mm id, 1,6 µm and 100 Å particle size) from Phenomenex. Column temperature was set at 40 °C. Mobile phase A was 0.1% formic acid in Milli-Q water and B was 0.1% formic acid in acetonitrile. A linear gradient at a flow rate of 0.3 mL·min^{-1} was used as follows: 0–2 min, from 10 to 40% (B); 2-4 min, from 40 to 45% (B); 4–5 min, from 45 to 50% (B) and maintain for 0.8 min; 5.8–6.5 min, from 50 to 90% (B); then 0.5 min to 90% (B) and back to 10% (B) for 2 min. The injection volume was set at 10 µL.

Finally, the developed method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and uncertainty using the QTrap 6500 system. The robustness of the method was tested by using a QTrap 7500 system.

Yoh, DsmC, Var, Sil and Tad were quantified using isotopic labelled internal standards (IS) (yohimbine-$^{13}C,D_3$ and sildenafil-D_8) and external calibration curve in matrix where the presence of these compounds was not expected. All the standards and samples were spiked with a mix solution of both IS at 2.5 mg·L^{-1} corresponding to 250 mg·kg^{-1} equivalent-in-sample.

Data were processed using SCIEX OS 2.2 software.

3. RESULTS AND DISCUSSION

3.1. Quantification method development

To develop a Multiple Reaction Monitoring (MRM) mass spectrometry method that allows for the unambiguous quantification of Yoh, DsmC, Var, Sil and Tad, using a QTrap 6500 and 7500 instruments, different parameters were evaluated. Key steps involved selecting precursor ions and optimizing declustering potential (DP) for better ionization. A Full Scan mass acquisition method was applied, scanning from m/z 100 Da to 1000 Da, using a 1 mg·L^{-1} solution containing the five analytes under study. Different DP values were evaluated for each compound: 40 V, 60 V, and 80 V. Different DP values were evaluated for each compound: 40 V, 60 V, and 80 V (see Table S1, Supporting Information). The selected precursor ions correspond to the hydrogen adducts $[M+H]^+$ of each standard. Moreover, the DP value that offers a higher signal-to-noise (S/N) ratio varies in function of the compound. Since higher the S/N obtained, better the sensitivity of the method is, several DP values were selected: 80 V for Yoh (S/N 1000), 60 V for DsmC (S/N 750), 80 V for Var (S/N 500), 80 V for Sil (S/N 1050), and 40 V for Tad (S/N 500). The most characteristic fragments were then selected using a Product Ion-Scan method with different collision energies to achieve optimal results. This method was applied on an m/z scan from 100 Da to 1000 Da, using a 1 mg·L^{-1} solution containing the five analytes under study (Yoh, DsmC, Var, Sil and Tad), as well as the internal standards (IS) that will later be undertaken to quantify (sildenafil-D_8 and yohimbine-$^{13}C,D_3$).

The collision energy (CE) and Q0 parameters were evaluated for each compound. It is necessary to mention that in the Qtrap 7500 system, there is a Q0 region where the ions are focused before they enter the Q1 quadrupole avoiding the loss of ions and increasing the sensitivity of the method[11]. The ions are focused

by applying a potential called Q0. This parameter was evaluated by applying the MRM developed method on a 0.02 μg·L^{-1} solution containing the five analytes under study (Yoh, DsmC, Var, Sil and Tad), as well as the internal standards (IS) that will later be undertaken to quantify (sildenafil-D_8 and yohimbine-$^{13}C,D_3$). For each of the compounds, different values of CE were evaluated (see Table 1).

Table 1. *Selection of product ion and collision energy (CE) for MRM method with Triple Quad 6500 and QTrap 7500 systems (Q0 only for 7500 system).*

Compound	Precursor ion	Product ion	CE/V	Q0/V
Yohimbine	355	144	30	10
		212	40	
Desmethyl carbodenafil	439	339	30	40
		311	40	
Vardenafil	489	151	51	30
		312	53	
Sildenafil	475	100	50	10
		283	40	
Tadalafil	390	268	20	-10
		169	40	
Sildenafil-D_8	483	108	55	30
		283	39	
Yohimbine-$^{13}C,D_3$	359	144	40	50
		215	30	

To select the more sensitive transitions of the MRM method, two criteria were followed: that the product ions selected were characteristic of the compound to be determined, and that the collision energy selected allowed high-intensity values. Therefore, the product ions were selected alongside the CE that offered higher intensity for each compound as can be seen in Table 1. Thus, a selective and sensitive MRM method was developed.

Once the compound-dependent parameters of the MRM method were defined, two common parameters were evaluated: ionization spray voltage (SP) and ionization source temperature. It is worth mentioning that in the QTrap 7500 system an OptiFlow Pro ion source is used, whereas, in the Qtrap 6500 system, an IonDrive Turbo V ion source is used. The OptiFlow Pro ion source belongs to the Turbo V ion source family and represents the fourth generation of this technology[12]. It features an orthogonal spray design with a V heater configuration, which focuses heat in the optimal area to achieve high sensitivity through efficient desolvation from the ESI droplets. However, due to the latest changes in the source geometry, probe/electrode configuration, and the inclusion of the E Lens probe all contribute to different optimal source conditions compared to previous generations. In particular, it is important to optimize the ionization spray voltage because usually required lower optimization values compared to the IonDrive Turbo V ion source (typically 5500 V for ESI in positive ionization mode)[13].

The resulting chromatogram can be seen in Figure 1.

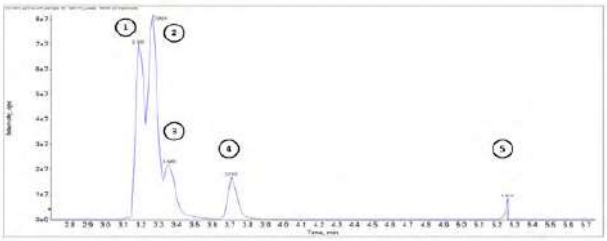

Figure 1. *Chromatographic separation of yohimbine (1), desmethyl carbodenafil (2), vardenafil (3), sildenafil (4) and tadalafil (5).*

3.2. Quantification method validation

3.2.1. Linearity and sensitivity

The linear range refers to the concentration range within quantification results that can be obtained with sufficient precision, accuracy, and reliability[14]. The linear range also considers linearity, which is the ability to obtain a proportional response to the analyte concentration within a certain concentration range. To evaluate this parameter, several standard solutions containing Yoh, DsmC, Var, Sil and Tad at 0.5 μg·L^{-1} (equivalent to 50 μg·kg^{-1} in sample), 1 μg·L^{-1} (equivalent to 100 μg·kg^{-1}), 2.5 μg·L^{-1} (equivalent to 250 μg·kg^{-1}), 5 μg·L^{-1} (equivalent to 500 μg·kg^{-1}), 7.5 μg·L^{-1} (equivalent to 750 μg·kg^{-1}) and 10 μg·L^{-1} (equivalent to 1000 μg·kg^{-1}) were analyzed. All the standard solutions contained the IS sildenafil-D_8, and yohimbine-$^{13}C,D_3$ at 5 μg·L^{-1} (500 μg·kg^{-1}).

Therefore, the influence of the ionization spray voltage was evaluated using the developed MRM method on a 0.02 mg·L^{-1} mix solution of Yoh, DsmC, Var, Sil and Tad. The peak intensity of each compound was recorded when the spray voltage was set at 2000 V, 3000 V, 4000 V, and 5000 V. Results can be seen in Table S2 in the Supporting Information. The maximum S/N is obtained with different values of spray voltage for each compound (Yoh, 4000 V; DsmC, 5000 V; Var, 3000 V; Sil, 2000 V; Tad, 3000 V). Since this parameter must be common for all the analytes in the MRM method, it was decided to set the spray voltage at 3000 V, which is an intermediate value among all the evaluated ones.

Once the ionization spray voltage was selected, the source temperature was evaluated. Once again, to evaluate the influence of this parameter, the MRM method was applied on a mix solution of Yoh, DsmC, Var, Sil and Tad at a concentration of 0.02 mg·L^{-1}, and the peak intensity of the quantifier transition of each compound were recorded when applying 400 °C, 500 °C, 600 °C, and 700 °C. Results can be seen in Table S3 in the Supporting Information. The general trend is for the S/N to increase with temperature, reaching a maximum between 500 °C and 600 °C, depending on the compound. Once again, this parameter is common to all compounds in the MRM method. Therefore, it was necessary to establish a value that favors all compounds. For this reason, the working temperature was set at 600 °C.

The standard solutions were prepared by adding a certain amount of each standard and IS on a blank sample. The potential matrix effect was also considered. Calibration curves were obtained by plotting the [Std]/[IS] vs the A_{Std}/A_{IS} and linearity was evaluated by the correlation coefficient (r) (see Table 3). Moreover, sensitivity was evaluated by calculating the LOD as 3 times the signal-to-noise ratio, and LOQ as 10 times the signal-to-noise ratio (see Table 2). Calibration curves were measured every day before sample analysis, the results presented in Table 2 are one representative example.

As can be seen in Table 2, a linear relationship of the area and the analyte concentration was obtained between 50 $\mu g \cdot kg^{-1}$ and 1000 $\mu g \cdot kg^{-1}$ for each analyte, with a correlation coefficient higher than 0.990 in all the cases. Moreover, the method presents a theoretical detection limit below 10 $\mu g \cdot kg^{-1}$ and a theoretical quantification limit below 45 $\mu g \cdot kg^{-1}$ for all the studied compounds. Since the maximum recommended dose for sildenafil is 100 mg per day and an average tablet weights 600 mg, the maximum expected concentration of sildenafil or their analogues is approximately 200 $g \cdot kg^{-1}$ per tablet[15]especially with the thrive of online commerce. To tackle this threat to public health, new ways to access these products should be identified and detection technologies should be strengthened. The overarching aim of this study was to investigate if herbal supplements sold online claiming to be natural alternatives to Viagra® were amongst these SF medical products and how effective different analytical techniques are in providing information about these products. 3 products which claimed to be herbal supplements for men sexual performance were purchased from an e-commerce platform. Two products were received as unregistered generic sildenafil citrate tablets manufactured in India (and thus different to the products information on the website. Therefore, the developed method presents enough sensitivity.

3.2.2. Precision: Repeatability and Reproducibility

Precision of an analytical method expresses the agreement or degree of dispersion between a series of measurements obtained from multiple samplings and analyses of the same homogeneous sample under prescribed conditions[16]. To assess a wild range of possible concentrations, samples were spiked at three levels: 100 $\mu g \cdot kg^{-1}$, 400 $\mu g \cdot kg^{-1}$, and 750 $\mu g \cdot kg^{-1}$ of Yoh, DsmC, Var, Sil and Tad. The precision acceptance criteria was calculated for each concentration level following the Horwitz equation[18].

$$\%RSD_H = 2^{(1-0.5 \cdot logC)} \quad Equation\ 1$$

where C is the concentration of the added standard. Thus, the acceptance criterium was $RDS_H \leq 23\%$ for 100 $\mu g \cdot kg^{-1}$, $RDS_H \leq 18\%$ for 400 $\mu g \cdot kg^{-1}$, and $RDS_H \leq 17\%$ for 750 $\mu g \cdot kg^{-1}$.

To evaluate the repeatability of the method, six replicate measurements of the Yoh, DsmC, Var, Sil and Tad content at the three levels were performed during the same laboratory session (see Table S4, Supplementary Information). In all cases, the RSD% was lower than the reference values, therefore it can be considered that the method is repeatable.

Furthermore, the method's intermediate precision was evaluated by measuring the analyte contents of the test samples on three different days (see Table S5, Supplementary Information). It is worth mentioning that results from the repeatability test were selected as the first-day measurements. In all cases, the RSD was equal to or lower than 15%. Therefore, the developed method accomplishes the intermediate precision requirements.

In summary, the results obtained show that it is possible to determine the concentration of Yoh, DsmC, Var, Sil and Tad in dietary supplement samples with sufficient precision.

3.2.3. Accuracy

The accuracy is defined as the closeness of the measurement result to the true or accepted reference value. The accuracy of the analytical method was calculated as the percentage of recovery of a spiked sample at a known added amount of analyte, as proposed by Commission Implementing Regulation (EU) 2021/808[19]. Thus, the accuracy of the analytical method was evaluated by spiking a blank sample at three concentration levels: 100 $\mu g \cdot kg^{-1}$, 400 $\mu g \cdot kg^{-1}$, and 750 $\mu g \cdot kg^{-1}$. The recovery results can be seen in Table S6 in the Supplementary Information. The obtained results were well agreed with the nominal analyte concentration values. The recoveries obtained from the prepared samples were comprised of between 80% and 120% (reference value

Table 2. *Study of sensitivity, limit of detection (LOD) and limit of quantification (LOQ) in a linear range from 50 $\mu g \cdot kg^{-1}$ to 1000 $\mu g \cdot kg^{-1}$ of yohimbine, desmethyl carbodenafil, vardenafil, sildenafil, and tadalafil.*

Analyte	Linear range/ µg·kg-1	Slope	Interception	r	R2	LOD/ µg·kg-1	LOQ/ µg·kg-1
Yohimbine	50-1000	$9.73 \cdot 10^{-4}$	$-1.20 \cdot 10^{-2}$	0.9986	0.9972	3	14
Desmethyl carbodenafil	50-1000	$4.70 \cdot 10^{-2}$	-1.46	0.9994	0.9987	4	36
Vardenafil	50-1000	$3.28 \cdot 10^{-3}$	$-1.69 \cdot 10^{-1}$	0.9965	0.9931	6	35
Sildenafil	50-1000	$6.93 \cdot 10^{-4}$	$-2.55 \cdot 10^{-2}$	0.9999	0.9997	8	34
Tadalafil	50-1000	$7.74 \cdot 10^{-3}$	$-2.21 \cdot 10^{-1}$	0.9989	0.9978	10	45

suggested by Commission Implementing Regulation (EU) 2021/808)[19]. The satisfying results demonstrated that the developed analytical method was capable of determining Yoh, DsmC, Var, Sil and Tad in dietary supplement samples with sufficient accuracy.

3.2.4. Influence of the matrix effect

To evaluate the matrix effect, the same concentration of the internal standards (IS) sildenafil-D_8 and yohimbine-$^{13}C,D_3$ was added to different samples spiked with yohimbine, desmethyl carbodenafil, vardenafil, sildenafil, or tadalafil at three levels: 100 $\mu g \cdot kg^{-1}$, 400 $\mu g \cdot kg^{-1}$, and 750 $\mu g \cdot kg^{-1}$. The IS response was compared with the IS response onto a reference sample on two different days. The variation of the chromatographic area was calculated for each matrix (see Table 3).

Based on the results obtained shown in Table 3, it can be affirmed that yohimbine is more affected by the sample matrix than sildenafil since the error values obtained for yohimbine-$^{13}C,D_3$ are higher than those obtained for sildenafil-D_8. Following the SANTE/11312/2021 guidance document, the combined effect matrix of the sample procedure preparation and the ionization enhancement or suppression has to present an error lower than ± 30%[20,21,22]. As can be seen in Table 4, both sildenafil and yohimbine exhibit an error lower than ±30% compared to the reference value, except for yohimbine-$^{13}C,D_3$ in sample 4. These results indicate that sample 4 has the most significant influence on the response of internal standards. However, it has been previously proved that the method allows the quantification of yohimbine, desmethyl carbodenafil, vardenafil, sildenafil, and tadalafil with enough precision and accuracy. Therefore, the observed matrix effect is not an issue for samples quantification.

3.2.5. Uncertainty

The expanded uncertainty is a measure of uncertainty that defines an interval about the measurement result within which the value of the measurand can be confidently asserted to lie and it was evaluated according to the revised Guide to the Expression of Uncertainty in Measurement[23,24]. For the determination of the expanded uncertainty, three components were considered:

- Uncertainty corresponding to the precision of the procedure: RSD_{REC}
- Uncertainty associated with the calculation of the mean recovery value (traceability): $RSD_{REC}/\sqrt{n}$
  Uncertainty associated with the bias (accuracy): $(100 - Rec)/\sqrt{3}$

Thus, the value of the expanded uncertainty (%U) of the analytical method is given by equation 2:

$$\%U_{exp} = K \cdot \sqrt{RSD_{REC}^2 + \left(\frac{RSD_{REC}}{\sqrt{n}}\right)^2 + \left(\frac{|100-Rec|}{\sqrt{3}}\right)^2}$$

Equation 2

where U_{exp} is the expanded uncertainty expressed un %, K is the coverage factor (K = 2 for a confidence interval of 95%), RSD_{REC} is the relative standard deviation of the recoveries expressed in %, Rec is the average recovery obtained in the validation expressed in %, and n is the number of data used in the validation to determine the value of RSD_{REC}.

Regarding the acceptance criteria for the expanded uncertainty, the maximum acceptable value was calculated using equation 3:

$$\%U_{exp}MAX = K \cdot \%RSD_{Horwitz} \quad \textit{Equation 3}$$

where K = 2 to ensure a 95% confidence interval and $\%RSD_{Horwitz}$ was found following equation 1. Thus, the maximum expanded uncertainty of the developed analytical method cannot exceed 33%[25]. Results can be seen in Table S7, in Supplementary Information. The maximum value of extended uncertainty was 29% associated with yohimbine. In all cases, U values are

Table 3. *Evaluation of the matrix effect onto the internal standard sildenafil-D_8 and yohimbine-$^{13}C,D_3$ responses.*

Sample	Spiked level STD ($\mu g \cdot kg^{-1}$)	Peak area (sildenafil-D_8) in matrix ± s /cps	Peak area (sildenafil-D_8) reference ± s /cps	% error	Peak area (yohimbine-$^{13}C,D_3$) in matrix ± s /cps	Peak area (yohimbine-$^{13}C,D_3$) reference ± s /cps	% error
Sample 1 (day 1)	100	$2.61 \cdot 10^5 \pm 6.20 \cdot 10^3$	$2.88 \cdot 10^5 \pm 1.10 \cdot 10^4$	10	$6.34 \cdot 10^6 \pm 3.04 \cdot 10^5$	$8.22 \cdot 10^6 \pm 4.69 \cdot 10^5$	23
	400	$2.45 \cdot 10^5 \pm 8.34 \cdot 10^3$		15	$5.90 \cdot 10^6 \pm 2.03 \cdot 10^5$		28
	750	$2.53 \cdot 10^5 \pm 6.74 \cdot 10^3$		12	$6.25 \cdot 10^6 \pm 2.76 \cdot 10^5$		24
Sample 2 (day 1)	100	$2.89 \cdot 10^5 \pm 4.00 \cdot 10^3$	$2.88 \cdot 10^5 \pm 1.10 \cdot 10^4$	0.2	$9.57 \cdot 10^6 \pm 1.13 \cdot 10^5$	$8.22 \cdot 10^6 \pm 4.69 \cdot 10^5$	16
	400	$2.80 \cdot 10^5 \pm 6.49 \cdot 10^3$		3	$9.81 \cdot 10^6 \pm 2.14 \cdot 10^5$		19
	750	$2.76 \cdot 10^5 \pm 5.15 \cdot 10^3$		4	$9.66 \cdot 10^6 \pm 3.33 \cdot 10^5$		18
Sample 3 (day 2)	100	$2.59 \cdot 10^5 \pm 4.41 \cdot 10^2$	$2.59 \cdot 10^5 \pm 6.91 \cdot 10^3$	0.1	$7.12 \cdot 10^6 \pm 7.58 \cdot 10^4$	$8.32 \cdot 10^6 \pm 4.08 \cdot 10^5$	14
	400	$2.63 \cdot 10^5 \pm 7.15 \cdot 10^2$		2	$7.44 \cdot 10^6 \pm 2.53 \cdot 10^5$		11
	750	$2.57 \cdot 10^5 \pm 4.81 \cdot 10^3$		1	$7.07 \cdot 10^6 \pm 1.03 \cdot 10^5$		15
Sample 4 (day 2)	100	$1.99 \cdot 10^5 \pm 2.76 \cdot 10^3$	$2.59 \cdot 10^5 \pm 6.91 \cdot 10^3$	23	$4.88 \cdot 10^6 \pm 1.00 \cdot 10^5$	$8.32 \cdot 10^6 \pm 4.08 \cdot 10^5$	41
	400	$2.19 \cdot 10^5 \pm 5.49 \cdot 10^3$		16	$5.88 \cdot 10^6 \pm 4.54 \cdot 10^4$		39
	750	$2.19 \cdot 10^5 \pm 1.80 \cdot 10^3$		15	$5.17 \cdot 10^6 \pm 1.80 \cdot 10^5$		38

accomplished with the preestablished criterium of 33%. Therefore, the analytical method presented an acceptable uncertainty.

3.2.6. Robustness

A robustness study evaluates a method's capacity to remain unaffected by small variations in method parameters; it involves deliberately introducing small changes to the method and examining the consequences[25]. To evaluate the robustness of the developed analytical method, it was decided to apply the method using a different LC-MSMS system located in another laboratory. The selected system was an ExionLC system coupled to a QTrap 7500 mass spectrometer. It must be noted that both instruments are high-performance and high-gain instruments from SCIEX, with the QTrap 7500 being the latest triple quadrupole model to be launched on the market in 2020. In this respect, the system offers a higher sensitivity compared to the QTrap 6500 system. For this reason, it was necessary to dilute the standard solutions and samples 100-fold to quantify sildenafil, vardenafil, tadalafil, yohimbine and desmethyl carbodenafil with the QTrap 7500 system. Table 4 shows the concentration results obtained considering the dilution factor 1:100.

As shown in Table 4, the measurements were conducted in two different laboratory sessions, and several replicates were performed by adding the standards to

Table 4. *Evaluation of the robustness of the developed analytical method by using two different LC-QTrap systems*

Analyte	MSMS system	Replicate	[Analyte] / µg·kg^{-1}	[Analyte]$_{average}$ ± s/ µg·kg^{-1}	RSD%	Recovery% (100 µg·kg^{-1})	[Analyte] / µg·kg^{-1}	[Analyte]$_{average}$ ± s/ µg·kg^{-1}	RSD%	Recovery% (400 µg·kg^{-1})	[Analyte] / µg·kg^{-1}	[Analyte]$_{average}$ ± s/ µg·kg^{-1}	RSD%	Recovery% (750 µg·kg^{-1})
Yohimbine	Qtrap 6500	16*	105*	98 ± 16	16	101	404*	413 ± 43	10	101	745*	800 ± 52	7	102
	Qtrap 7500	D1-M1-R1	84				351				743			
		D1-M1-R2	86				368				754			
		D1-M2-R1	85				403				764			
		D1-M2-R2	85				395				749			
		D1-M3-R1	113				455				830			
		D1-M3-R2	118				465				867			
		D2-M1-R1	86				393				833			
		D2-M1-R2	79				375				790			
		D2-M2-R1	113				459				871			
		D2-M2-R2	120				475				856			
Desmethyl carbodenafil	Qtrap 6500	16*	96*	98 ± 16	16	94	367*	389 ± 56	15	93	691*	720 ± 109	15	93
	Qtrap 7500	D1-M1-R1	74				366				646			
		D1-M1-R2	73				360				625			
		D1-M2-R1	103				357				626			
		D1-M2-R2	91				341				617			
		D1-M3-R1	111				450				869			
		D1-M3-R2	120				463				826			
		D2-M1-R1	94				331				645			
		D2-M1-R2	89				325				652			
		D2-M2-R1	108				472				855			
		D2-M2-R2	117				442				870			
Vardenafil	Qtrap 6500	16*	108*	107 ± 9	9	111	422*	412 ± 32	8	105	782*	723 ± 76	10	102
	Qtrap 7500	D1-M1-R1	111				426				714			
		D1-M1-R2	103				413				726			
		D1-M2-R1	90				464				829			
		D1-M2-R2	94				451				801			
		D1-M3-R1	115				381				646			
		D1-M3-R2	111				425				626			
		D2-M1-R1	101				410				804			
		D2-M1-R2	120				400				742			
		D2-M2-R1	119				393				641			
		D2-M2-R2	110				347				639			
Sildenafil	Qtrap 6500	16*	102*	97 ± 6	7	100	395*	374 ± 31	8	96	746*	695 ± 46	7	96
	Qtrap 7500	D1-M1-R1	96				370				663			
		D1-M1-R2	97				362				663			
		D1-M2-R1	95				340				663			
		D1-M2-R2	94				368				628			
		D1-M3-R1	93				372				699			
		D1-M3-R2	94				422				662			
		D2-M1-R1	95				336				770			
		D2-M1-R2	87				337				679			
		D2-M2-R1	106				420				715			
		D2-M2-R2	109				397				759			
Tadalafil	Qtrap 6500	16*	103*	112 ± 9	8	107	405*	366 ± 38	10	97	743*	702 ± 64	9	96
	Qtrap 7500	D1-M1-R1	114				343				655			
		D1-M1-R2	112				346				735			
		D1-M2-R1	99				326				628			
		D1-M2-R2	97				326				626			
		D1-M3-R1	119				429				762			
		D1-M3-R2	115				413				764			
		D2-M1-R1	120				346				637			
		D2-M1-R2	116				331				633			
		D2-M2-R1	120				361				755			
		D2-M2-R2	121				394				781			

** Results from the precision study performed with the LC-MS/MS QTrap 6500 system.*

various matrices. This can be observed in the nomenclature of the replicates "DX-MX-RX," where *D* refers to the measurement day, *M* to the sample matrix, and *R* to the replicate sample. In this way, it was aimed to encompass as many variables as possible to ensure the reliability of the robustness study. Considering all the individual determinations performed in the precision study using the LC-MS/MS QTrap 6500 system (n = 16) and the 10 replicates obtained with the LC-MS/MS QTrap 7500 system (n = 26), the average concentration of each analyte (sildenafil, vardenafil, desmethyl carbodenafil, yohimbine and tadalafil), the standard deviation, and the RSD were calculated. In all the cases, the RSD% was lower than the reference values that were calculated following Horwitz equation (equation 1)[18] . Therefore, it can be considered that the analytical method is robust.

Furthermore, the addition of standards at different concentrations (100 $\mu g \cdot kg^{-1}$, 400 $\mu g \cdot kg^{-1}$, and 750 $\mu g \cdot kg^{-1}$) was also evaluated. As shown in Table 4, in all cases, the recovery is within the range of 93% to 111%. These values fall within the range established by SANTE/11312/2021 guidance, which sets a broader range of 70-120%[20,22]. These results reinforce the robustness and accuracy of the developed method.

Therefore, it can be stated that the LC-MS/MS method developed in this study can be used to determine the concentration of sildenafil, vardenafil, desmethyl carbodenafil, yohimbine and tadalafil with precision, accuracy, robustness, and reliability.

3.3. Samples quantification

The validated LC-MS/MS method was used to analyze 17 samples of natural sexual enhancers commercialized as natural food supplements. Quantification results can be seen in Table 5.

Table 5. *Yohimbine, desmethyl carbodenafil, vardenafil, sildenafil, and tadalafil analysis in natural food supplementary samples.*

Sample name	Brand name	Results
Energisil Vigor Instant 10 caps	Energisil - Pharma OTC SL	< LOQ
Erectab	Labophyto	< LOQ
Viper potenciador masculino	Cobeco	< LOQ
XL Power potenciador erección y afrodisiaco	Labophyto	< LOQ
Penis + erect for men	Cobeco	< LOQ
Pop suplemento para hombres vigorizante	Pops	Sildenafil > 10%
Viazal capsulas potenciadoras	Bio Perine	< LOQ
Sexy hour	DietMed	< LOQ
Yohimbe - food supplement	Solbia	Yohimbine > 1.5%
Gold max Blue - food supplement	R-Vitality	Tadalafil > 1.5%
Male virility supplement	kuh.nekt	Tadalafil > 1.5%
Men's Health - Natural Dietary supplement - Male potency formula	Androsen	< LOQ
Performance Pills For men	Vital Perfect	< LOQ
Supamen - Herbal food supplement, vitamins C, thiamin and niacin	Labo Phyto	< LOQ
Aquilea - Vigor	Aquilea	< LOQ
Aquilea Vigor Él	Aquilea	< LOQ
Love Maculino	Vyacimine	< LOQ

As can be seen in Table 5, 4 out of the 17 samples analyzed showed concentration levels between 1.5% and 10% (w/w) based on the saturated area signals of the chromatographic peaks. The concentration of the positive samples is excessively high for a mass spectrometry technique. In addition, the method had not been validated for such high concentrations. For this reason, no specific concentration value has been reported for those samples but has been expressed with values higher than a level above which we are confident the concentration of the analyte in the sample is found. Moreover, these concentrations are similar to those present in commercial drugs for erectile dysfunction. For example, sildenafil marketed under the brand name Viagra® can be found in doses of 25 mg/capsule, 50 mg/capsule, or 100 mg/capsule. Considering that one capsule weigh approximately 1 g, the concentration of sildenafil in a Viagra® pill would range between 2.5% and 10% approximately. Additionally, different compounds were detected: one sample contains Sildenafil (*Pop suplemento para hombres vigorizante*), one sample contains Yohimbine (*Yohimbe - food supplement*), and two samples contain Tadalafil (*Gold max Blue - food supplement* and *Male virility supplement*). It should be noted that the sample named "Male virility supplement" already had a health alert from the Spanish Food Safety and Nutrition Agency dated 13 September 2022[26]. The rest of the samples that were positive in some of the analytes studied had no alerts at the moment of performing the present study. Finally, among all the samples, those that were purchased in Pharmacies (a total of 4) did not present added sexual enhancers. On the other hand, all positive samples were not found in pharmacies but were only able to be purchased online. The results obtained show the importance of implementing routine controls on dietary supplements, especially for those that are not regulated for any specific normative. Thus, the method developed and validated in the present study can be used as an important tool to prevent potential fraud and related public health issues.

4. CONCLUSIONS

The LC-MS/MS method developed for the simultaneous quantification of yohimbine, desmethyl carbodenafil, vardenafil, sildenafil, and tadalafil demonstrates significant potential for application in the analysis of

complex dietary supplement samples. With a linear range from 50 $\mu g \cdot kg^{-1}$ to 1000 $\mu g \cdot kg^{-1}$ for each analyte and limits of detection and quantification that are well-suited for this context, the method exhibits robust performance in terms of sensitivity and repeatability. The method's precision, both within-day (RSD ≤ 8%) and between-day (RSD ≤ 13%), alongside its recovery rates (92%-108%), highlight its reliability for routine analysis.

Importantly, the successful validation of the method across different LC-QQQ systems further strengthens its applicability in diverse laboratory settings. These results indicate that the method provides a reliable tool for the detection of adulteration in dietary supplements, even when concentrations exceed typical commercial doses. Given the growing concern around the unregulated use of pharmaceutical-grade compounds in over-the-counter supplements, this method offers a valuable means to ensure consumer safety.

This study's findings suggest that routine application of this method could play a pivotal role in identifying adulterated products, thereby preventing public health risks associated with unregulated dietary supplements. Furthermore, the high sensitivity and robustness of the method provide an important step toward the implementation of standardized testing procedures in regulatory and quality control laboratories.

ABBREVIATIONS

Collision Energy, CE; Declustering Potential, DP; Desmethyl Carbondenafil, DsmC;, Electrospray ionization, ESI; Internal standards, IS; Limit of detection, LOD; Limit of quantification, LOQ; Multi-reaction Monitoring, MRM; Sildenafil; Sil; Spray Voltage, SP; Tadanafil, Tad; Vardenafil, Var; Yohimbine: Yoh.

DECLARATIONS

Author Contributions: Conceptualization, Josep Calderón and Margalida Artigues; methodology, Sònia Gotsens, Meritxell Castelló, Eva Muñoz, Josep-Lluis Lliberia, Jordi Díaz, Josep Calderón and Margalida Artigues; validation, Eva Muñoz, Josep-Lluis Lliberia, Jordi Díaz, Josep Calderón and Margalida Artigues; formal analysis, Sònia Gotsens and Meritxell Castelló; investigation, Sònia Gotsens, Meritxell Castelló, Eva Muñoz, Josep-Lluis Lliberia, Jordi Díaz, Josep Calderón and Margalida Artigues; resources, Jordi Díaz and Josep Calderón; data curation, Sònia Gotsens, Meritxell Castelló, Eva Muñoz; writing—original draft preparation, Sònia Gotsens and Margalida Artigues writing—review and editing, Eva Muñoz, Josep-Lluis Lliberia, Jordi Díaz, Josep Calderón and Margalida Artigues; visualization, Sònia Gotsens, Meritxell Castelló, Eva Muñoz, Josep-Lluis Lliberia, Jordi Díaz, Josep Calderón and Margalida Artigues; supervision, Jordi Díaz, Josep Calderón and Margalida Artigues; project administration, Eva Muñoz, Jordi Díaz, Josep Calderón and Margalida Artigues; funding acquisition, Jordi Díaz and Josep Calderón All authors have read and agreed to the published version of the manuscript.

Conflict of Interest: All the authors declare that they have no conflict of interest.

Financial interests: All the authors declare that they have no financial interests.

Non-financial interests: All the authors declare that they have no non-financial interests.

Funding Sources: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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