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Development and Validation of Gas Chromatography–Mass Spectrometry Method for Quantification of Sibutramine in Dietary Supplements

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Abstract: The use of dietary supplements (DSs) has dramatically increased in recent decades. However, around 20% of these products are reported to contain pharmacologically active undeclared compounds, most of which could expose consumers to serious side effects. According to recent data, some of the most commonly detected undeclared compounds are also considered doping and are prohibited by the World Anti-Doping Agency (WADA). One of the most frequently detected undeclared substances in DSs used for promoting weight loss is sibutramine. In 2011, all medicines containing sibutramine were urgently withdrawn from Europe and US markets because of serious side effects. In the present study, in order to detect and quantify sibutramine in DSs, a rapid, sensitive, and reliable gas chromatography with mass spectrometry (GC-MS) method was developed. The method was validated according to the ICH guidelines and demonstrated good linearity, accuracy, precision, and robustness. The limits of detection and quantification were 0.181 µg/mL and 0.5488 µg/mL, respectively. The method was applied to analyze 50 DSs promoting weight loss, fat burning, and performance enhancement. Sibutramine was detected in six of them in a range of 16.59–14,854.94 µg/per capsule. The high concentrations of sibutramine detected in some samples raise concerns about the potential health risks associated with the use of adulterated DSs. The proposed GC-MS method could be used successfully in the quality control of DSs or in different research programs, contributing to safety and the prevention of associated side effects.

Keywords: sibutramine; GC-MS; dietary supplements; food supplements; unintentional doping; obesity

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1. Introduction

Dietary supplements (DSs) are considered concentrated sources of a wide range of nutrients, such as vitamins, minerals, and other bioactive compounds [1,2]. In the past few decades, the use of DSs has progressively risen [3–5]. Some of the main factors responsible for the growth of the DS market are the rapid introduction of new DSs into the market, liberal legislation, and consumers' belief that these products are safe, as well as the association of DSs with a healthy lifestyle. In the United States, more than 50% of adults report using DSs, and in some studies, almost 40% have taken DSs in the past 30 days [6,7]. However, there are some important challenges in the use, definition, regulation, and safety of DSs [8]. For example, there is no uniform international definition of DSs [5,8]. Furthermore, regulations vary from country to country, and in most countries, manufacturers of DSs are not obliged to comply with good manufacturing practice (GMP) or good laboratory practice (GLP), and in general, the regulations on DSs globally are much less stringent than those

for medicines. For example, in the US and the European Union, there are no mandatory regulations for pre-market quality control of a DS. Manufactures of DSs are not required to comply with the same regulations as manufactures of pharmaceuticals [9].

Nowadays, one of the most popular categories of DSs is those alleged to promote weight management [10]. Most of these products have natural origins and are claimed to possess thermogenic and lipotropic activity (example: green tea extracts, green coffee extracts, etc.) or satiety properties (example: choline and glucomannan) [11]. Dietary supplements intended to promote weight loss are used by many consumers at different ages and with different medical conditions. Such DSs are not only used to support obesity/overweight management, but are also included in many lifestyle programs for people who exercise regularly for better and faster body-shaping results [11]. Although consumers consider DSs as safe and natural healthy products, recent studies have reported that almost 20% of all DSs available worldwide could be adulterated with pharmaceuticals or other undeclared substances, because of the lack of obligatory analytical control [12–17].

Recently, the safety of DSs used for weight management has come under particular question [11,18]. Many researchers have reported the detection of different undeclared compounds with serious side effects [15,19]. Nowadays, sibutramine is one of the most frequently detected unlabeled compounds in weight loss DSs [16,20–22].

Sibutramine (Figure 1) or 1-(1-(4-Chlorophenyl) cyclobutyl)-3-methyl butyl-N, N-dimethylamine is a serotonin and norepinephrine reuptake inhibitor that increases neuropeptide release in the arcuate nucleus and prevents the reduction in basal energy expenditure [23].

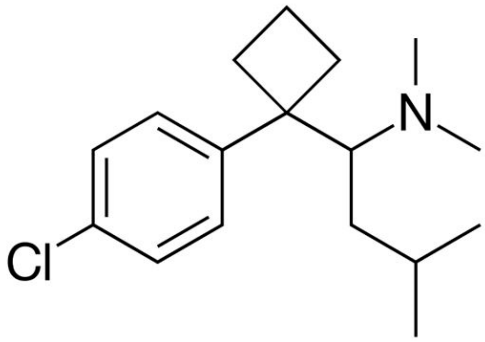

Figure 1. Chemical structure of sibutramine.

Sibutramine was approved in 1997 as a medicine that suppresses appetite and reduces body weight. It was a promising pharmaceutical for obesity/overweight management. However, in 2010, sibutramine was banned and is no longer used due to an increase in cardiovascular events, such as arrhythmias, tachycardia, hypertension, and myocardial infarction [23–26].

The Cardiovascular Outcomes Trial including 9804 people confirmed that individuals with pre-existing cardiovascular disease or diabetes mellitus had a significantly increased risk of myocardial infarction and non-fatal stroke after long-term treatment with sibutramine 10–15 mg daily [25,27]. A case of non-ischemic cardiomyopathy has also been reported [28]. Other side effects of sibutramine intake included headache, dry mouth, and constipation [29]. Although sibutramine has been withdrawn from the market as a medicine, the problem of sibutramine-adulterated DSs remains. Even more, these products are mostly labeled as natural and safe [16].

Recently, several cases of sibutramine overdose from adulterated DSs were reported [30,31]. If obese/overweight adults with cardiovascular diseases have unintentional sibutramine intake, it may cause an increase in blood pressure and other cardiovascular side effects. Also, sibutramine can lead to tachycardia through its pharmacological effects on the body. By blocking the reuptake of neurotransmitters, sibutramine increases their levels in the synaptic clefts, thereby enhancing their effects on various receptors. Increased levels of

norepinephrine, in particular, play a significant role in sibutramine-induced tachycardia. By inhibiting the reuptake of norepinephrine, sibutramine prolongs its effect on β1 adrenergic receptors in the heart. This prolonged stimulation results in an increased heart rate, leading to tachycardia. These effects can be associated with an elevated risk of cardiovascular events such as heart attacks, strokes, and arrhythmias [24,25,32–34]. In addition, increased levels of norepinephrine following sibutramine intake can cause sleep disturbances, anxiety, and restlessness. Dizziness and vertigo are also associated with sibutramine intake [35,36].

Furthermore, sibutramine intake was associated with different drug interactions. The undeclared sibutramine in DSs could lead to not only well-known cardiovascular side effects but also serious drug interactions. In such cases, it is highly likely that consumers are not able to judge that the source of these health issues could be the consumed DSs. Sibutramine affects the serotonin levels in the brain, and combining it with other medications that increase serotonin levels, such as selective serotonin reuptake inhibitors (SSRIs), serotonin–norepinephrine reuptake inhibitors (SNRIs), and monoamine oxidase inhibitors (MAOIs), could cause serotonin syndrome [37,38]. In recent years, depression and anxiety have been increasing among the global population [39]. Furthermore, a coloration between depression and obesity has been observed. Obesity can lead to physiological changes in the body, including inflammation and hormonal imbalance, lack of social interactions, and low self-esteem, which may contribute to the development of depression [40–43]. Considering these factors, there is a significant risk of drug interactions when using DSs containing sibutramine.

Combining sibutramine with stimulants such as amphetamines can further enhance the effects and potentially increase the risk of cardiovascular problems [44]. When taken together, sibutramine and caffeine may have additive stimulant effects.

Sibutramine is metabolized in the liver by certain cytochrome P450 enzymes, mostly by CYP3A4 and CYP2B6. The primary metabolite formed by the CYP3A4 enzyme is N-desmethylsibutramine, commonly referred to as M1. This metabolite is pharmacologically active. M1 exhibits similar properties to sibutramine, including the inhibition of serotonin, norepinephrine, and dopamine reuptake [32,45]. Drugs that induce or inhibit these enzymes can potentially affect the metabolism and clearance of sibutramine. Examples of medications that can interact with sibutramine through this mechanism include ketoconazole, erythromycin, and rifampicin [46].

It is essential to screen and determine sibutramine in dietary supplements to ensure consumer safety. There are few reported methods for the detection of sibutramine [16,20,22,47,48].

The aim of this study is the development and validation of a simple, sensitive, and reliable method for the screening of sibutramine in DSs.

2. Materials and Methods

2.1. Chemicals and Reagents

Standard of sibutramine hydrochloride monohydrate, HPLC $\geq$ 98%, was purchased from Sigma-Aldrich, Steinheim, Germany. Methanol with analytical-grade purity was purchased from Merck KGaA, Darmstadt, Germany.

2.2. Gas Chromatography–Mass Spectrometry/Selected Ion Monitoring Mode (GC-MS/SIM) Analysis

2.2.1. Standard Preparation

Stock solutions at a concentration of 1 mg/mL of sibutramine were prepared by diluting in methanol, then vortexed and stored at 4 °C until use.

2.2.2. Sample Preparation

For the purposes of analysis, 50 DSs for weight loss were purchased from drug stores and the internet. One capsule or tablet of each sample was ground and homogenized and then dissolved in 10 mL of methanol. The dissolution was carried out with an ultra-

sonic bath (Bandelin, Berlin, Germany) for 30 min and then filtrated through a 0.45 µm syringe filter.

2.2.3. GC-MS Conditions

GC-MS analyses were carried out using Bruker Scion 436-GC SQ MS, Bremen, Germany. A Bruker BR-5ms fused silica capillary column (0.25 µm film thickness and 15 m × 0.25 mm i.d.) was used. The injector was splitless. The oven temperature was initially maintained at 60 °C for 1 min, then increased to 160 °C at 40 °C/min, and after that increased to 225 °C at 10 °C/min and held for 2 min. The flow rate of the carrier gas, helium, was 1 mL/min. The injection volume was 1 µL. The method was carried out in 12 min. The transfer line temperature was maintained at 300 °C. An electron impact ionization (EI) source was applied, and electron energy was 70 eV. The monitored ions 114, 72, and 58 m/z were used for the quantitative analyses.

2.3. Method Validation

The method was validated according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) with the following validation parameters: linearity range, accuracy, precision, limits of detection (LOD) and quantification (LOQ), and robustness [49].

2.3.1. Linearity

The calibration curve was constructed by using nine concentration levels in the range from 0.3 to 30 µg/mL, each with six replicates. To construct a calibration curve, working standard solutions were prepared by diluting the stock solution with methanol. The regression line was calculated with the following equation: $y = ax \pm b$, where x is the concentration and y is the peak area of each measurement, b is the y-intercept, and a is the slope of the regression line. The coefficient of determination (R^2) was presented.

2.3.2. Limit of Detection and Limit of Quantification

The limit of detection and the limit of quantification were evaluated based on the standard deviation on the response (σ) and the slope of the calibration curve (S) with the following equations: LOD = 3.3 σ/S and LOQ = 10 σ/S.

2.3.3. Accuracy and Precision

To evaluate accuracy and precision, a stock solution of sibutramine was prepared and diluted in three known concentrations with methanol. The concentration levels used were as follows (high—20 µg/mL, medium—10 µg/mL, low—1 µg/mL). Furthermore, known concentrations from freshly prepared stock solution (high—20 µg/mL, medium—10 µg/mL, low—1 µg/mL) were spiked into two blank samples (dietary supplements). To evaluate the accuracy and precision, working solutions were analyzed in six replicates. Inter-day precision was performed within 6 days.

2.3.4. Robustness

In order to assess the robustness of the developed method, changes were made to the flow rate and initial oven temperature. The working standard solution of 10 µg/mL used for robustness was prepared by diluting the freshly prepared stock solution.

3. Results and Discussion

The GC-MS parameters were optimized to achieve the best instrument performance in terms of sensitivity. The sibutramine standard was introduced into the detector in full scan mode in the range of 50–500 m/z, using standard equipment parameters to evaluate the characteristic ions, and the most abundant ion was selected. To achieve effective analyte separation and acceptable peak shape, various chromatographic conditions were tested. Parameters include injector temperature, which was changed in the range of 200–250 °C,

initial column temperature (±10 °C), and change in gas flow rate (1.0 ± 0.2 mL/min). A list of conditions is provided in Section 2.2.3.

MS identifications were established of the compound and retention time. The selected characteristic mass fragments were tracked in selected ion monitoring: 114, 72, and 58 m/z for the quantification measurement. The fragmentation of sibutramine by electron ionization at 70 eV with a single quadrupole mass spectrometer based on its mass spectra is shown in Figure 2. The mass spectrum was observed in NIST MS Interpreter. Structures for fragment ions are proposed in Figure 2.

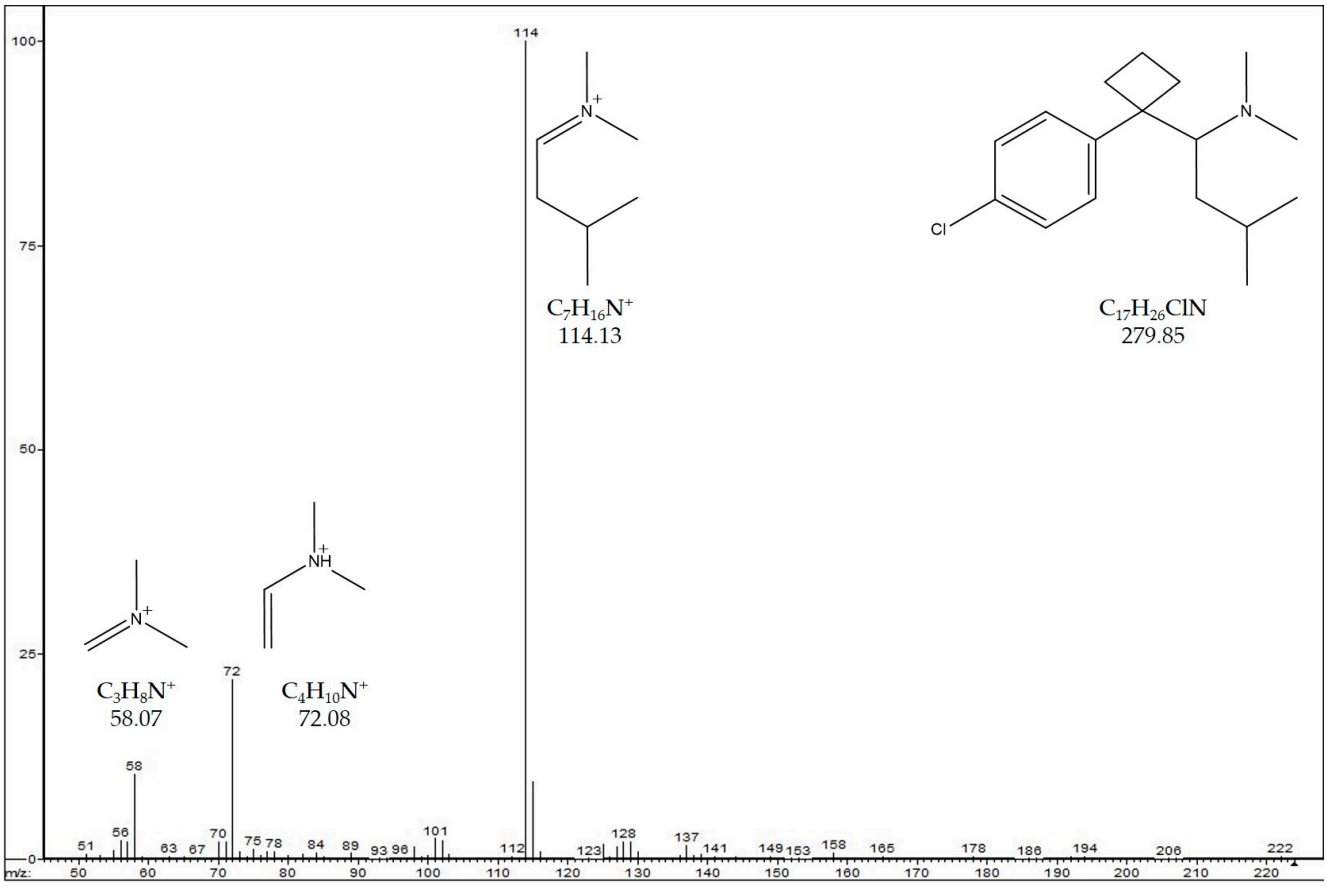

Figure 2. Fragmentation pattern of ions for analysis and identification of sibutramine based on MS spectrum in full scan mode.

The conditions described in Table 1 were used to obtain the results. The retention time for sibutramine was 6.71 min.

Table 1. GC-MS chromatography conditions.

Gas Chromatograph	Tested Parameters	Chosen Parameters
Carrier gas Flow rate	- 1.0 ± 0.2 mL/min	Helium 1.0 mL/min
Injection mode	-	Splitless
Injector temperature	200–250 °C	250 °C
Injection volume	-	1 µL
Oven temperature	Initial temperature 60 ± 10 °C 40 °C /min ± 10 °C	60 °C held for 1 min, then increased to 160 °C at 40 °C/min, and after that increased to 225 °C at 10 °C/min and held for 2 min

Table 1. *Cont.*

Gas Chromatograph	Tested Parameters	Chosen Parameters
Mass Spectrometer		
Ionization mode		Electron ionization
Acquisition mode		Selected ion monitoring (SIM)

3.1. Method Validation

3.1.1. Linearity

The ability of an analytical procedure to produce assay results that correspond to the analytic concentration in the sample range is termed linearity. To establish linearity, at least five concentrations are required, and an external standard curve was used to accomplish that. Nine concentrations and their corresponding peak areas were measured in order to evaluate the linearity of the developed method. The linearity ranged from 0.3 to 30 µg/mL. The linear regression line for sibutramine hydrochloride was y = 10,527,465.47x − 1,924,703.44, with R^2 = 0.9999, as shown in Figure 3.

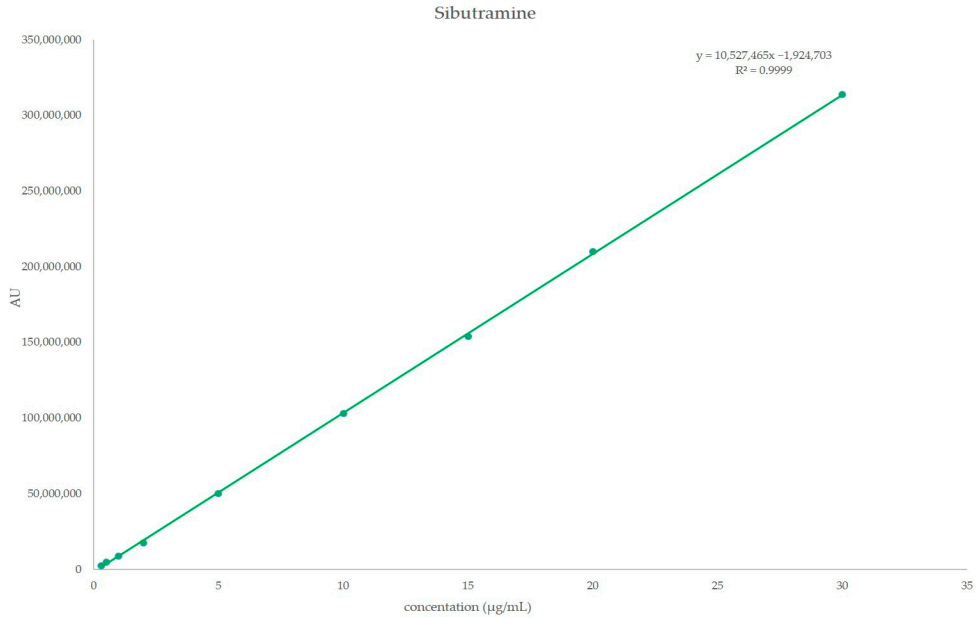

Figure 3. The calibration curve of the sibutramine standard.

A representative chromatogram of the standard solution of 10 µg/mL sibutramine hydrochloride monohydrate is represented in Figure 4.

3.1.2. Accuracy and Precision

Accuracy was expressed as a percentage of recovery that measured the discrepancy between the mean and the true value that has been assumed. The accuracy test of the developed method was carried out at three known concentration levels. The concentration levels used were 20, 10, and 1 µg/mL.

The level of similarity or consistency among multiple measurements of the same sample obtained under the same conditions is referred to as precision in analytical procedures. By calculating the coefficient of variation for the three known concentration levels, each with six replicates, the precision of the method was calculated as intra-day and inter-day precision. The accuracy and precision results are presented in Tables 2 and 3.

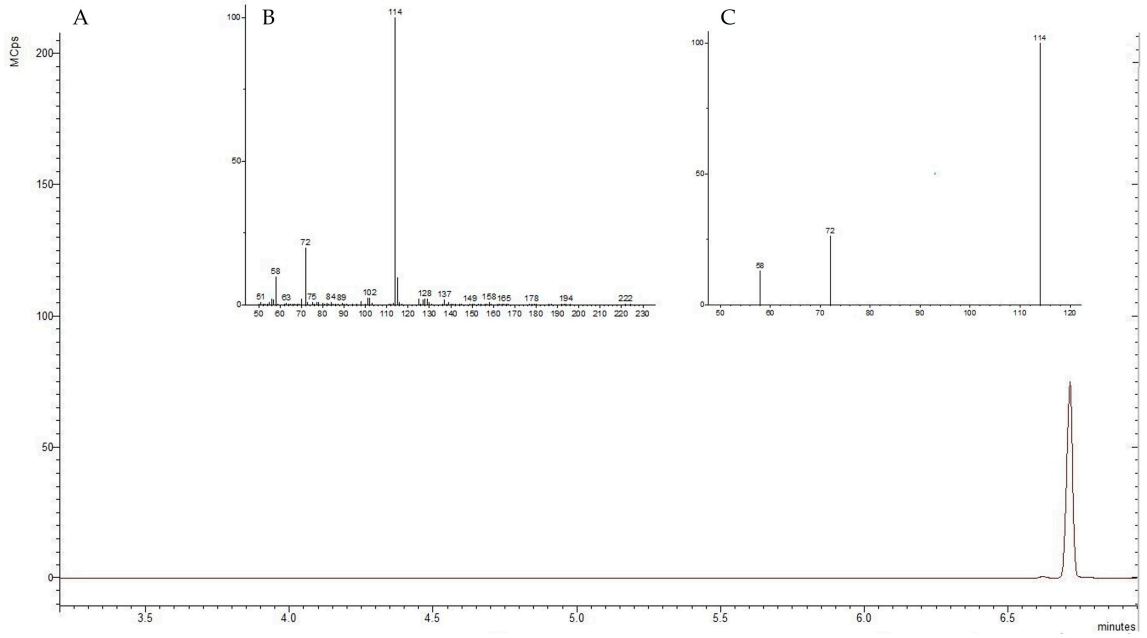

Figure 4. GC-MS chromatogram of the standard solution of 10 mg/mL sibutramine hydrochloride (**A**) according to MS spectrum in full scan (**B**) and SIM mode (**C**) with a retention time of 6.71 min.

Table 2. Results on the accuracy and precision of the developed GC-MS method in methanol.

Accuracy				Precision					
Concentration (μg/mL)	Mean (μg/mL) ± SD	Recovery%	CV%	Intra-Day Precision			Inter-Day Precision		
				Mean (μg/mL) ± SD	Standard Error	CV%	Mean (μg/mL) ± SD	Standard Error	CV%
20	20.46 ± 0.23	102.30	1.11	20.25 ± 0.37	0.15	1.84	20.91 ± 0.50	0.20	2.37
10	10.06 ± 0.14	100.55	1.41	10.11 ± 0.12	0.05	1.23	10.09 ± 0.20	0.08	1.95
1	1.026 ± 0.004	102.57	0.40	1.02 ± 0.005	0.002	0.52	1.02 ± 0.011	0.004	1.03

Table 3. Results on the accuracy and precision of the developed GC-MS method in blank dietary supplements.

Sample Matrix	Spiked at 1 μg/mL			Spiked at 10 μg/mL			Spiked at 20 μg/mL		
	Recovery%	Intra-day Precision (CV%)	Inter-Day Precision (CV%)	Recovery%	Intra-Day Precision (CV%)	Inter-Day Precision (CV%)	Recovery%	Intra-Day Precision (CV%)	Inter-Day Precision (CV%)
Sample 25	102.38	2.19	2.09	100.22	2.23	2.99	100.14	2.55	2.96
Sample 35	102.43	102.71	2.59	100.10	100.78	2.87	101.31	100.82	2.79

3.1.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were estimated by multiplying the standard deviation by factors of three and ten, respectively, and dividing by the slope of the calibration curve. They were found to be 0.181 μg/mL and 0.5488 μg/mL.

3.1.4. Robustness

Robustness refers to the ability of the GC system to maintain consistent and reliable performance despite variations in operating conditions or external factors. It is an important characteristic that ensures the accuracy and reproducibility of analytical results. It was evaluated by determining the variations in column flow rate and oven temperature. The

initial oven temperature was changed in the range of 60 ± 2 °C, and the flow rate was varied in the range of 1.0 ± 0.1 mL/min. Results are presented in Table 4. The RSDs were less than 5%. This method was robust to small variations in GC conditions.

Table 4. Results on the robustness of the developed GC-MS method.

Change in Parameters	Average%	SD	RSD
Standard method	100.38	0.242	2.409
Flow rate 1.1 mL/min	100.96	0.496	4.911
Flow rate 0.9 mL/min	101.74	0.405	3.978
Oven temperature 62 °C	97.24	0.300	3.082
Oven temperature 58 °C	98.39	0.141	1.43

3.2. Method Application

The developed and validated method was successfully utilized to quantify levels of sibutramine in 50 randomly selected dietary supplements. The identification of sibutramine in tested samples was carried out by comparing their retention time and their mass spectra with those of reference standard. The dietary supplements that resulted in a high area under the peak but did not correspond to the calibration curve were further diluted. Concentrations of sibutramine in analyzed dietary supplements are presented in Table 5 as the mean of three different replicates.

Table 5. GC-MS chromatography results.

Sample No.	Country of Origin of the Manufacturer	Place of Purchase	Sample Type	Product Description	Daily Intake Recommenda-tion	Sibutramine Detected Per Capsule	Sibutramine Daily Intake
1	Bulgaria	Pharmacy	Capsule	Chitosan	6–9 capsules per day	<LOQ	-
2	Bulgaria	Pharmacy	Capsule	Chitosan L-carnitine Chromium	6 capsules per day	<LOQ	-
3	USA	Online bodybuilding and fitness store	Capsule	Green Tea Extract. Green Tea Leaf	2 capsules per day	<LOQ	-
4	Bulgaria	Internet	Capsule	Green Coffee Extract, Choline, L-Methionine, Green Tea Extract.	2 capsules per day	<LOQ	-
5	Bulgaria	Internet	Capsule	Garcia Cambodgia Extract	1 capsule per day	<LOQ	-
6	Bulgaria	Online bodybuilding and fitness store	Capsule	L-carniteine, Tribulus Terrestris Extr.	2–4 capsules per day	<LOQ	-
7	Bulgaria	Pharmacy	Capsule	L-carnitine tartarata Garcinia cambodja extract Raspberry Ketones Green Coffee Bean Extract Chromium picolinate	1 capsule per day	75.95 µg	75.95 µg
8	Bulgaria	Pharmacy	Capsule	Herba Polygonum hydropiper, Cortex Erhamni frangulae, Folia Senna, Radix Ononidis, Herba Alchemilla vulgaris, Radix Reven, Fructus Foeniculi, Folia Uve ursi	2–4 capsules per day	<LOQ	-
9	United Kingdom	Drug store	Capsule	Curcuma longa, Piper nigrum	2 capsules per	<LOQ	-

Table 5. *Cont.*

Sample No.	Country of Origin of the Manufacturer	Place of Purchase	Sample Type	Product Description	Daily Intake Recommendation	Sibutramine Detected Per Capsule	Sibutramine Daily Intake
10	India	Pharmacy	Capsule	Balsamodendron mukul, Garcinia cambogia, Gymnema sylvestre, Terminalia chebula, Trigonella foenum graeceum	4 capsules per day	<LOQ	-
11	Bulgaria	Bodybuilding and fitness store	Capsule	L-lysine, Inositol Acetyl L Carnitine, L-Carnitine Tartarate, Chromium Picolinate, Citrum Auranticum Extract, Guggul Extract, Yohimbe Extract, White Willow Bark Extract, Caffeine, Gardinia Cambogia Extract	2 capsules per day	<LOQ	-
12	Bulgaria	Internet	Capsule	Coffea canephora	2 capsules per day	<LOQ	-
13	Canada	Drug store	Capsule	Garciania cambogia	2–4 capsules per day	<LOQ	-
14	Turkey	Internet	Capsule	Frangula bark L-carnitine Cardamom Ginger Garcinia cambodja Lepidium Galangal Fennel Cinnamon Clove Dill	1 capsule per day	9655.68 µg	9655.68 µg
15		Internet	Capsule	L-carnitine, Gamelia sinensis, Panax ginseng, Chromium Picolinate	2 capsules per day	<LOQ	-
16	Poland	Online bodybuilding and fitness store	Capsule	Green coffee extract, Green tea extract (Camelia sinensis), L-Tyrosine, Yerba mate extract, Caffeine, Pantothenic acid, Ginger Root Extract, Cayenne pepper extract, Black pepper extract	4 capsules per day	<LOQ	-
17	Canada	Bodybuilding and fitness store	Capsule	Green Tea Extract (Camellia sinensis), Patented Phytosome® process binds green tea extract to phosphatidylcholine, Caffeine	2 capsules per day	<LOQ	-
18	Bulgaria	Drug store		Activated Carbon, St. John's Wort, Flax, White horehound, Thyme Lemon balm, Mallow Hawthorn flower, European hop, Anise, Verbena, Fennel, Agrimony, Nettles, Mint, Chicory	3 capsules per day	<LOQ	-
19	Bulgaria	Internet	Capsule	Green Tea Extract (Camellia sinensis)	1 capsule per day	<LOQ	-
20	USA	Online bodybuilding and fitness store	Capsule	Hoodia, Linoleic Acid, Cinnamon, Raspberry Ketones, Green Coffee Beans, Chitosan, Evening Primrose, Bitter Orange, Chromium Picolinate	2 capsules per day	3712.66 µg	7425.32 µg

Table 5. *Cont.*

Sample No.	Country of Origin of the Manufacturer	Place of Purchase	Sample Type	Product Description	Daily Intake Recommendation	Sibutramine Detected Per Capsule	Sibutramine Daily Intake
21	Bulgaria	Drug store	Capsule	Momordica charantia L. Extract	1 capsule per day	<LOQ	-
22	Bulgaria	Pharmacy	Tablet	Green Tea Extract (Camellia sinensis)	3–6 capsules per day	<LOQ	-
23	Romania	Internet	Capsule	Asplenium trichomanes L., Zeolite, Glucomannan, Green tea, Garcinia Cambogia	2 capsules per day	<LOQ	-
24	Bulgaria	Online bodybuilding and fitness store	Capsule	L-Lysine Methionine Acetyl L-carnitine L-carnitine Chromium picolinate Phenylalanine White Willow Bark Ext. Caffeine	4 capsules per day	21.88 µg	87.52 µg
25	Bulgaria	Pharmacy	Capsule	L-Taurine, L-Tryptophan, Hb. Equiseti arvensis, extr., Cort. Betulae pendulae, extr., Hb. Centellae asiaticae, extr., Rad. Zingiberi officinalis, extr., Manganese sulfate, Nicotinacide, Pyridoxin hydrochloride, L-Selenomethionine	2 capsules per day	<LOQ	-
26	China	Pharmacy	Capsule	Momordica balsamina, Aloe Vera, Garcinia Cambogia, Cassia Tora, Crataegus, Water lily leaves	2 capsules per day	<LOQ	-
27	Italia	Pharmacy	Tablet	Patented complex of polysaccharide macromolecules (Policaptil Gel Retard®); Orange, Apple, and Lemon flavors	6 capsules per day	<LOQ	-
28	Poland	Pharmacy	Capsule	Bitter orange (Citrus aurantium) Extract, Blueberry powder, Urtica dioica Extract, Apple cider vinegar powder, Zinc	2 capsules per day	<LOQ	-
29	India	Online pharmacy store	Tablet	Mahasudarshan churna, Pterocarpus marsupium, Clerodendrum phlomidis, Boerhaavia diffusa, Tinospora cordifolia, Cyperus rotundus, Embelia ribes, Bauhinia variegate, Curcuma longa, Terminalia chebula, Emblica officinalis, Plumbago zeylanica, Ziginber officinale, Piper nigrum, Piper longum	6 capsules per day	<LOQ	-
30	Moldova	Internet	Tablet	Metandrostenolon	2–5 capsules per day	<LOQ	-
31	Moldova	Internet	Tablet	Stanozolol	5–10 capsules per day	<LOQ	-
32	United Kingdom	Internet	Capsule	Green Tea Extract, L-Tyrosine, Caffeine, 50 mg Naringin, L-Tryptophan, Citrus Aurantium, Vitamin B6	2 capsules per day	<LOQ	-

Table 5. *Cont.*

Sample No.	Country of Origin of the Manufacturer	Place of Purchase	Sample Type	Product Description	Daily Intake Recommendation	Sibutramine Detected Per Capsule	Sibutramine Daily Intake
33	France	Pharmacy	Capsule	Clarinol G-80, CLA, Camellia sinensis extract, Guarana Extr. Chromium (III) Chloride	3 capsules per day	<LOQ	-
34	Bulgaria	Pharmacy	Capsule	Garcinia Cambogia, Chromium picolinate	2 capsules per day	<LOQ	-
35	Bulgaria	Pharmacy	Capsule	L-Tryptophan, L-Taurin, Bromelain, Hb. Equiseti arvensis, extr., Tub. Helianthi tuberosi, extr., Hb. Centellae asiaticae, extr., Coenzyme Q-10, Pyridoxin hydrochloride, L-Selenomethionine, Manganese sulfate	2 capsules per day	<LOQ	-
36	Bulgaria	Internet	Capsule	Dehydroepiandrosterone	2 capsules per day	<LOQ	-
37	Bulgaria	Online bodybuilding and fitness store	Capsule	Dehydroepiandrosterone	1 capsule per day	<LOQ	-
38	Bulgaria	Online bodybuilding and fitness store	Capsule	L-Carnitine Tartarate	3 capsules per day	<LOQ	-
39	USA	Online bodybuilding and fitness store	Capsule	L-Carnitine Tartarate, Garcinia Cambogia, Caffeine	2 capsules per day	<LOQ	-
40	USA	Drug store	Capsule	Gabonensis Irvingia	2 capsules per day	<LOQ	-
41	Bulgaria	Internet	Capsule	Chlorodehydromethyltestosterone	3–5 capsules per day	16.59 μg	82.95 μg
42	Poland	Bodybuilding and fitness store	Tablet	Black Peper Fruit Extract	1/4 tablet	<LOQ	-
43	United Kingdom	Bodybuilding and fitness store	Capsule	Garcia Cambogia Extract	3 capsules per day	<LOQ	-
44	United Kingdom	Bodybuilding and fitness store	Capsule	CLA (Conjugated Linoleic Acid)	1 capsule per day	<LOQ	-
45	United Kingdom	Bodybuilding and fitness store	Capsule	Citrus Sinensis Extract, Grapefruit Extract, Blood orange concentrate, Guarana seed extract, Green Coffee Bean Extract, White Willow Bark Extract, Citrus Aurantium Extract, Bioperine® (black pepper extract)	3 capsules per day	<LOQ	-
46	Poland	Bodybuilding and fitness store	Capsule	Malabar Tamarind Fruit Extract, White Bean Extract, Apple Cider Vinegar, Chromium, Vitamin B12	2 capsules per day	<LOQ	-
47	Poland	Bodybuilding and fitness store	Capsule	L-Carnitine, Green Tea Extract	4 capsules per day	<LOQ	-
48	Bulgaria	Pharmacy	Capsule	CLA (Conjugated Linoleic Acid)	2–4 capsules per day	<LOQ	-

Table 5. *Cont.*

Sample No.	Country of Origin of the Manufacturer	Place of Purchase	Sample Type	Product Description	Daily Intake Recommendation	Sibutramine Detected Per Capsule	Sibutramine Daily Intake
49	USA	Bodybuilding and fitness store	Capsule	Garcinia cambogia Extract	1 capsule per day	<LOQ	-
50	USA	Online bodybuilding and fitness store	Capsule	Sibutramine Chromium picolinate Vitamin C	1 capsule per day	14,854.94 µg	14,854.94 µg

Six dietary supplements were detected to contain sibutramine in the range of 16.59–14,854.94 µg per single dosage. The exemplary chromatograms of the dietary supplements containing sibutramine are given in Figure 5.

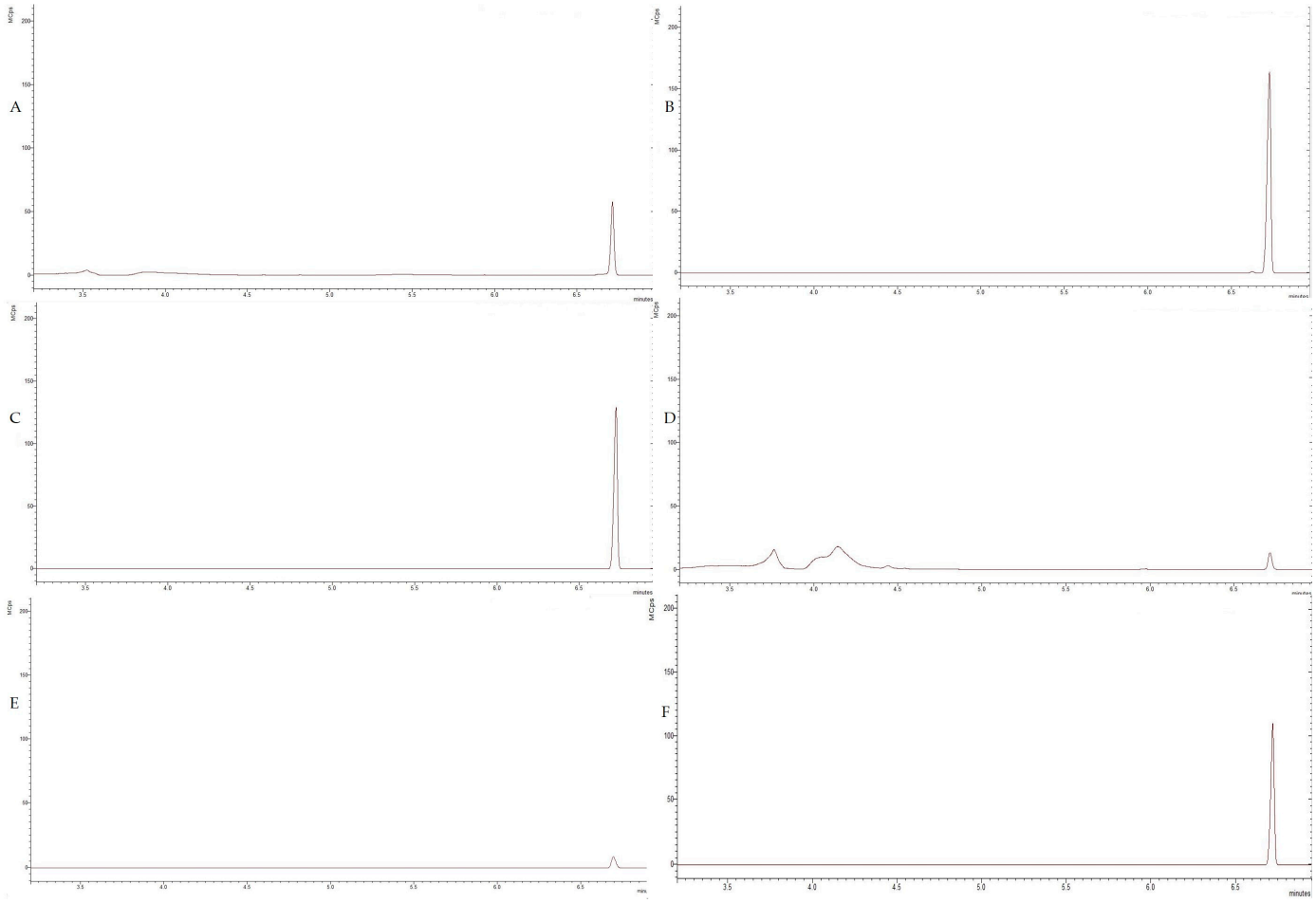

Figure 5. GC chromatograms of the six weight loss dietary supplements that contain sibutramine. (**A**) GC chromatogram of Sampe 7. (**B**) GC chromatogram of Sample 14. (**C**) GC chromatogram of Sample 20. (**D**) GC chromatogram of Sample 24. (**E**) GC chromatogram of Sample 41. (**F**) GC chromatogram of Sample 50.

Higher amounts of sibutramine were detected in Samples 14, 20, and 50, with concentrations of 9655.68 µg, 3712.66 µg, and 14,854.94 µg per single dose, respectively. Previously, similar alarming results had been reported using different techniques such as HPLC, TLC, HPTLC-UV, GC-MS, LC/MS/MS, and electrochemiluminescence [17,22,47,48,50–55]. For the purpose of finding sibutramine in DSs, the research from Khazan et al. was followed

using GC-MS. Eight DSs were analyzed. The method was maintained for 36 min and was used for screening three possible adulterants, i.e., sibutramine, phenolphthalein, and phenytoin. The retention time of sibutramine was approximately 18.5 min. Brief descriptions of accuracy and precision are presented as average values of all compounds as follows: 95.6% for accuracy and 6.7% RSD for precision [22]. Sibutramine was found in six DSs, which compares to 75% of all dietary supplements studied, but the exact values are not presented. In another study from Popescu et al., ten DSs were analyzed and the GC-MS method was used as a conformation of FTIR analysis. The total runtime of the analysis was 48 min. The retention time of sibutramine was 8.86 min. Two DSs were adulterated with sibutramine [56]. In comparison with the previous method for analyzing sibutramine with GC-MS, our method is time-saving and was completed in 12 min. The retention time for sibutramine was set at 6.71 min. Moreover, the method presents more detailed information about precision and accuracy, and the results are as follows: precision up to 2.99% and accuracy in the range of 100.10–102.71%, suitable for quantification and qualification analysis. Analysis of more DSs—in our case, 50—shows a more realistic picture of adulteration with sibutramine present in DSs. SIM mode offers several benefits compared to full scan mode in GC-MS. One major advantage is the enhanced sensitivity and signal-to-noise ratio achieved through SIM mode. By minimizing background noise and targeting specific ions relevant to the analyte, SIM mode boosts sensitivity, making it particularly advantageous for trace-level compounds and complex matrices. With reduced interference from unrelated ions, SIM mode provides more accurate quantification of target compounds—in our case, sibutramine—and ensures reliable and precise results. This improved sensitivity also translates to better detection limits and increased accuracy in quantification. SIM mode is not ideal for screening or profiling complex samples because it only monitors pre-selected ions. In contrast, full scan mode offers a broader view of the entire mass range, making it better suited for exploratory analyses and comprehensive characterization of complex samples. On the other hand, potential interferences that could affect the accuracy of results in GC-MS in scan mode analysis include matrix effects, co-elution of compounds, ion suppression or enhancement, isobaric compounds, background noise, and complex fragmentation patterns in mass spectra, which can make compound identification challenging, especially for structural isomers or compounds with similar mass spectra. Furthermore, the matrix of a dietary supplement can have a significant impact on the accuracy and reliability of gas chromatography–mass spectrometry (GC-MS) analysis in SIM mode. Dietary supplements often contain a wide range of compounds, as can be seen in Table 5. Some dietary supplement components may suppress or enhance the ionization of the sibutramine, leading to biased quantification results.

An unsettling pattern regarding dietary supplements sold in Croatia was noted in 2016 by Adela Krivohlavek et al. [21]. They reported that 20% of the 123 examined supplements contained sibutramine, and the maximum amount was 26.41 mg/g using LC-ESI-MS/MS [21]. Kim et al. examined 188 dietary supplements in 2014 and discovered that 29 of them contained sibutramine in amounts ranging from 0.03 to 132.40 mg/g [16]. The highest amount of sibutramine detected in this method is approximately ten times more than the amount reported in our method, which is 14.85 mg. Huang et al. reported that using HPLC-UV-ESI-MS, sibutramine was detected in two samples with concentrations of 0.51 mg/mL and 2.17 mg/g, respectively [57]. Mathon et al. reported that amounts of sibutramine reached up to 35 mg per capsule [54]. The study conducted by Hachem et al. examined 164 dietary supplements, where 43 samples contained sibutramine in concentrations ranging between 0.1 and 22 mg [58]. A similar alarming study was reported by Zeng et al., where 447 dietary supplements for weight loss were tested, and 55 out of 119 adulterated samples contained sibutramine, which is approximately 12% [59]. According to our study, also 12% of DSs were adulterated with sibutramine, while amounts up to 75% were reported in the study of Khazan et al. [22]. Various amounts of sibutramine and percentages of contaminated DSs in reported results can occur as a consequence of different regulations among countries and varied access to a wide range of DSs.

Despite many regulations and various methods of detection, sibutramine still remains a major undeclared substance in DSs in many countries. Insufficient analytical oversight of dietary supplements before market introduction and the adoption of substandard manufacturing practices have resulted in the proliferation of numerous unsafe products [8]. To mitigate the risks of sibutramine contamination or adulteration in DSs, obligatory GMP, GLP control, and modifications of regulations are needed. Without proper quality control measures, manufacturers may use contaminated raw materials or ingredients, leading to unintentional adulteration of dietary supplements. In analyzed Samples 7, 24, and 41, the possibility of unintentional adulteration occurs. Furthermore, some manufacturers may intentionally add sibutramine to their products to enhance the products' weight-loss effects. Due to the good accuracy, precision, and rapidity of the developed GC-MS method, it could be used in doping control analysis.

3.3. Undeclared Sibutramine and Overweight/Obese Consumers

According to the World Health Organization (WHO), obesity and overweight have reached pandemic levels nowadays and are considered among the most urgent social health concerns [60–62]. Based on data from the World Obesity Federation, it is estimated that 2.7 billion adults could be considered obese by 2025 [60]. Overweight adults are defined as those with a body mass index (BMI) above 25 and obese adults as those with BMI above 30. The etiology of obesity is complicated and complex. Normally, it involves dysregulation of the body's energy balance system and excess calorie consumption. It could be caused by hormonal imbalance, medication intake, sedentary lifestyle, genetic predisposition, or other factors [11]. Overweight and obesity are associated with many serious chronic medical conditions including cardiovascular diseases, dyslipidemia, diabetes, osteoarthritis, cancer, etc. [63].

Typically, obese and overweight adults have a daily intake of many medicines for treating their chronic diseases. Weight management in such patients is a complex, complicated, multistep, and long process. It must be carefully planned and consistent with their specific health conditions and medication intake. The multistep weight management practice is based on several stages: dietary intervention (restriction of calorie consumption), lifestyle modifications, increased physical activity, DS intake, and pharmacological treatment [11]. In some cases, surgical procedures may also be recommended [11]. All stages of this process could be well planned and successfully controlled. However, the intake of DSs for promoting weight loss may expose these patients to risk. The lack of mandatory analytical control of these products is a prerequisite and a possibility of the presence in them of an unknown and unlabeled compound with serious pharmacological activity. According to our findings, Samples 14, 20, and 50 contain significant amounts of sibutramine, with concentrations of 9655.68 μg/per dose (9.6 mg/per dose), 3712.66 μg/per dose (3.7 mg/per dose), and 14,854.94 μg/per dose (14.8 mg/per dose), respectively. In the recent past, the recommended initial dose of sibutramine as a medication was 10 mg/day. This recommendation was that the daily dose should be increased by 5 mg if weight loss was insufficient [64]. Although sibutramine concentrations were lower than 10 mg in some samples, we consider that these DSs expose consumers to serious health risks. Moreover, this intake of sibutramine is unintentional and users are not supposed to be exposed to serious side effects and incidents such as heart attack, stroke, and arrhythmia.

3.4. Undeclared Sibutramine as a Source of Unintentional Doping

Unintentional doping refers to the use of banned substances by athletes without their knowledge or intent to enhance performance. Sibutramine is included on the WADA Prohibited List as a specified stimulant [65]. Athletes are strictly prohibited from using sibutramine both in and out of competition. The presence of sibutramine in an athlete's system during a doping test can lead to serious consequences, including suspensions, loss of titles or medals, and damage to an athlete's reputation. The issue of undeclared sibutramine arises when athletes unknowingly consume it through contaminated or adul-

terated dietary supplements or products. Some supplement manufacturers may include sibutramine or other prohibited substances in their products without listing them on the label. This can occur due to mislabeling, cross-contamination during the manufacturing process, or intentional adulteration to enhance the product's effects. The burden of proof lies with the athlete to demonstrate unintentional ingestion, which can be challenging. Recently, there have been many reports of the unlabeled presence of sibutramine in dietary supplements and evidence of potentially serious risk to athletes' health and reputation [16,17,20,22,51,54,58,66–69]. According to our findings, of 50 analyzed DSs, six contained sibutramine in the range of 16.59 μg/ per capsule to 14,854.94 μg/per capsule. These dietary supplements can reach levels higher than the WADA-regulated minimum required performance level (MRPL) of 50 ng/mL [70].

One of the analyzed samples (Sample 41) contained not only undeclared sibutramine, but also an anabolic steroid, which is also considered as a doping compound. There is limited scientific research specifically addressing the interaction between anabolic steroids and sibutramine. However, based on their individual effects on the body, combining these substances can pose significant risks: both anabolic steroids and sibutramine have the potential to increase blood pressure and heart rate. This combination may amplify these effects, leading to a greater strain on the cardiovascular system. Intake of Sample 41, which contains chlorodehydromethyltestosterone and sibutramine, can increase the risk of heart-related complications, including heart attack, stroke, and cardiac arrhythmia [24,25,71–73]

Unintentional doping with sibutramine is a complex issue that highlights the challenges faced by athletes in maintaining a clean and drug-free sporting environment. Stricter regulations, improved supplement testing, and enhanced education can help athletes protect themselves from unintentional doping incidents and maintain the integrity of sports.

4. Conclusions

In recent years, the use of DSs for weight loss has been on the rise, driven by factors such as the introduction of new products, liberal legislation, the perception of safety, and the association with a healthy lifestyle. However, the use of DSs poses significant challenges in terms of definition, regulation, and safety. Sibutramine, a previously approved anti-obesity drug, is one of the most frequently detected unlabeled compounds in weight loss DSs. Despite the withdrawal of sibutramine, the problem of adulterated weight-loss dietary supplements remains, with many of these products being labeled as natural. Therefore, the development of effective screening methods for the detection of sibutramine in DSs is crucial to ensure consumer safety. In this study, a gas chromatography–mass spectrometry method was developed and validated for the screening of sibutramine in weight loss supplementation. The method demonstrated good linearity, accuracy, precision, and robustness. The limit of detection and limit of quantification of sibutramine were determined as 0.181 μg/mL and 0.5488 μg/mL, respectively. The validated method was then applied to analyze 50 weight loss DSs and 6 of these supplements were found to contain sibutramine in varying amounts. The high concentrations of sibutramine detected in some samples raise concerns about the potential health risks associated with the use of these adulterated DSs. The developed GC-MS method provides a sensitive, reliable, and efficient approach for the screening of sibutramine in DSs. This method can contribute to ensuring consumer safety by enabling the identification of adulterated dietary supplements and the prevention of their side effects.

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