Development of a GC-MS methodology for determining the adulterant 2,4-dinitrophenol in weight loss food supplements

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Abstract

Currently, the pursuit of a body that meets beauty standards has become a major goal for a large part of the population, greatly intensified by pictures of perfect bodies shared on social networks. This has impelled the growth of the "fitness" market, which offers a wide range of products for weight loss, including plant food supplements (PFS). Since overweight currently affects millions of individuals worldwide, weight-loss products are undoubtedly one of the most popular types of PFS. However, different reports, including from the Food and Drug Administration (FDA) and the EU Rapid Alert System of Food and Feed (RASFF), have raised concerns about fraudulent practices in this sector, particularly the addition of pharmaceutical drugs to boost the PFS effect. Different adulterants have been reported in PFS supplements, including 2,4-dinitrophenol (2,4-DNP), a compound used in the 1st World War to produce ammunition that showed weight loss as a side effect. Although 2,4-DNP is able to cause weight loss, it is associated with a high rate of dangerous effects since the drug causes the uncoupling of mitochondrial oxidative phosphorylation, leading to hyperthermia and diaphoresis, which, together with tachycardia and decreased blood pressure, can lead to death. Nevertheless, due to its thermogenic capacity, 2,4-DNP is a potential adulterant of weight loss PFS. Therefore, this work aimed at developing a methodology using gas chromatography coupled with mass spectrometry (GC-MS) to identify and quantify this compound if present in commercial samples of PFS. After optimization of the GC-MS operational parameters and derivatization procedure, the methodology was validated following the general guidance "ICH Q2 Validation of analytical procedures" of the European Medicines Agency (EMA) for method validation. All the parameters followed the EMA guideline including specificity, linearity, range of the curve, precision, accuracy, detection and quantification limit, and matrix effect. The calibration curve was shown to be linear within the range of 5 ppm to 0.05 ppm, with a limit of quantification of 0.05 mg/L and a limit of detection of 0.015 mg/L. Subsequently, the methodology was applied to a small set of commercial supplements, with the compound not being detected in any of the analyzed products.

Keywords: Plant food supplements, Weight-Loss, 2,4-Dinitrophenol, Method Validation, Adulteration.

Resumo

Atualmente, a procura por um corpo que corresponda aos padrões de beleza tornou-se um objetivo importante para grande parte da população, muito intensificado pelas fotografias de corpos perfeitos partilhadas nas redes sociais. Este fato impulsionou o crescimento do mercado "fitness", que oferece uma vasta gama de produtos que ajudam na perda de peso, incluindo os suplementos alimentares à base de plantas (PFS). Uma vez que o excesso de peso afeta atualmente milhões de pessoas em todo o mundo, os produtos para perda de peso são, sem dúvida, um dos tipos de suplementos mais populares. No entanto, diferentes relatórios, incluindo os da Food and Drug Administration e do Sistema de Alerta Rápido da UE para Alimentos para Consumo Humano e Animal, suscitaram preocupações acerca de práticas fraudulentas neste setor, em particular a adição de medicamentos para aumentar o efeito dos PFS. Diferentes adulterantes foram relatados em PFS, incluindo o 2,4-dinitrofenol (2,4-DNP), um composto usado na Primeira Guerra Mundial para produzir munições e que apresentou perda de peso como efeito colateral. Embora o 2,4-DNP seja capaz de causar perda de peso, está associado a uma elevada taxa de efeitos perigosos, uma vez que a droga provoca o desacoplamento da fosforilação oxidativa mitocondrial, levando a hipertermia e diaforese, que, juntamente com taquicardia e diminuição da pressão arterial, podem levar à morte. No entanto, devido à sua capacidade termogênica, o 2,4-DNP é um potencial adulterante de PFS para perda de peso. Assim, este trabalho teve como objetivo desenvolver uma metodologia utilizando a cromatografia gasosa acoplada à espectrometria de massa (GC-MS) para identificar e quantificar este composto quando presente em amostras comerciais de PFS. Após a otimização dos parâmetros operacionais do GC-MS e do procedimento de derivatização, a metodologia foi validada seguindo as orientações gerais "ICH Q2 Validation of analytical procedures" da Agência Europeia de Medicamentos (EMA) para validação de métodos. Todos os parâmetros seguiram as diretrizes da EMA, incluindo especificidade, linearidade, intervalo da curva, precisão, exatidão, limite de deteção e quantificação, e efeito da matriz. A curva de calibração mostrou-se linear no intervalo de 5 ppm a 0,05 ppm, com um limite de quantificação de 0,05 mg/L e um limite de deteção de 0,015 mg/L. Posteriormente, a metodologia foi aplicada a um pequeno

conjunto de suplementos comerciais, não sendo detectado o composto em nenhum dos produtos analisados.

Palavras-Chave: Perda de Peso, 2,4-Dinitrofenol, Validação de Método, Adulteração, Suplementos à Base de Plantas.

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Acronyms and Abbreviations

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
COVID	Coronavirus Disease
CV	Coefficient of Variation
DSHEA	Dietary Supplement Health and Education Act
DAD	Diode Array Detector
DNP	Dinitrophenol
DCP	Dichlorophenol
EFSA	European Food Safety Authority
ECD	Electron Capture Detector
EU	European Union
FDA	Food and Drug Administration
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography Coupled to Mass Spectrometry
INTERPOL	The International Criminal Police Organization
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Coupled to Mass Spectrometry
LD	Limit of Detection (mg/L)
LQ	Limit of Quantification (mg/L)
MSD	Mass Spectrometry Detector
MSTFA	N-Trimethylsilyl-N-methyl trifluoroacetamide
NPD	Nitrogen-Phosphorus Detector
PFS	Plant Food Supplements
RASFF	Rapid Alert System for Food and Feed
RPM	Revolutions Per Minute
SIM	Selected Ion Monitoring
SD	Standard Deviation

THMP	Traditional Herbal Medicinal Products
TMCS	Trimethylchlorosilane
TNT	Trinitrotoluene
TOF-MS	Time of Flight Mass Spectrometry
UK	United Kingdom
US	United States
σ^2	Variance

1. Introduction

From ancient times to the present, humanity has dealt with images, paintings, and photographs that portray "ideal" body figures and beauty standards. These representations often convey subliminal messages that promote the idealization of certain body standards, which are frequently unrealistic and can have detrimental effects by leading people to have a dissatisfied view of their own bodies. Different evidence, including research literature, has shown that body dissatisfaction can lead to extreme behaviors in the search for the ideal physique, such as the development of eating disorders and the use of anabolic steroids to increase muscle tone (McCabe and Ricciardelli, 2004). Currently, we are much more exposed to ideal bodies due to social networks, which can potentiate the number of individuals with dissatisfaction about their image (Tiggemann and Slater, 2017). The utilization of social networks has witnessed significant growth in recent years, driven by technological advancements and the widespread accessibility of digital devices. This surge is further propelled by globalization and the proliferation of various social networking platforms. The COVID-19 pandemic has notably accelerated this trend, with social distancing measures prompting a substantial shift towards online interactions via social networks (Mota, 2022). This surge in the use of Social Network Services has brought an inundation of images portraying unrealistic body standards, a phenomenon previously confined to magazines, television programs, and advertisements. As a consequence, a pressing issue has been unveiled, namely the challenge of self-acceptance concerning the own body image (Franchina and Coco, 2018). According to Tiggemann and Slater (2017), the escalating use of social networks, particularly among young people and adolescents, directly correlates with the degree of dissatisfaction regarding their physical appearance.

On the other hand, research indicates also a direct correlation between the surge in body dissatisfaction and the increased demand for fitness facilities, gyms, and, notably, dietary supplements such as nutrient supplements, amino acids, and fat-burning supplements (Yager and O'Dea, 2014). This poses a significant issue since dietary supplements are legally regarded by the Food and Drug Administration (FDA) and European

Union (EU) as foods. Therefore, they undergo no safety evaluation before being introduced into the market (Rocha et al., 2015). In this regard, several reports from the literature and governmental agencies have highlighted the presence of pharmaceutical adulterants in several of these products to boost their effects. Among these products, one category reported as one of the most affected by this problem is food supplements marketed for weight-loss (Rocha et al., 2015). Several different substances illegally added to this type of supplement have already been described, notably the presence of banned anorectics such as sibutramine, but also antidepressants, laxatives, stimulants, and diuretics, among others. One other notable example is 2,4-dinitrophenol (or simply 2,4-DNP). This substance can be employed as an adulterant in dietary supplements for weight loss purposes due to its activity in increasing metabolism rate. However, its usage has proven highly toxic, potentially resulting in fatal consequences depending on the dosage administered (Fernandes and Izidoro, 2022).

Therefore, the main objective of this work was the development and validation of a methodology based on GC-MS analysis for the specific detection of 2,4-DNP if illegally added to food supplements. In addition, this work also had the specific objective of applying the proposed methodology for the analysis of commercial samples of weight-loss supplements to check label compliance respecting the absence of this compound.

2. State of the art

2.1. Food supplements legislation

According to Directive 2002/46/EC of the European Parliament, food supplements are defined as *"foodstuffs intended to complement and/or supplement the normal diet and which constitute concentrated sources of certain substances nutrients or others with nutritional or physiological effect, alone or in combination, sold in dosed form, such as capsules, lozenges, tablets, pills and other similar forms, powder sachets, liquid ampoules, dropper bottles and other similar forms of liquids or powders that are intended to be taken in measured units of reduced quantity."* In other words, these prod-

ucts may contain a wide range of nutrients and other ingredients such as vitamins, minerals, amino acids, essential fatty acids, fiber and various plants or herbal extracts, which are intended to complement the diet, adjusting it to individual biological needs. In the EU, harmonized legislation regulates the vitamins and minerals, and the substances used as their sources, that can be added in food supplements, with a list of these compounds being described in annexes I and II of Directive 2002/46/EC. Later on, following a comprehensive assessment carried out by the European Food Safety Agency (EFSA) between 2005 and 2009, this directive was amended by Commission Regulation (EC) No 1170/2009 as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements. In 2008, the EU commissioned a study on the use of substances with nutritional or physiological effects other than vitamins and minerals in food supplements, which resulted in a report to the Council and the European Parliament (Commission of the European Communities, 2008). In this report, the problem of certain herbal extracts being used both as ingredients in food supplements and in medicinal products (e.g., as traditional herbal medicinal products) was highlighted. In addition, it was mentioned that in borderline cases, this could result in situations where a given product is considered a food supplement in certain Member States while a medicinal product in others. Unfortunately, the study and the report did not result in new regulations being implemented for harmonization purposes in the EU. Currently, with regards to other substances to be used as ingredients of food supplements, it is considered that those may be governed by national rules or may be subject to other specific EU legislation (EFSA, 2024). In this last case, for example, if a substance intended to be used in food supplements does not have a history of safe use in the EU before 1997, it is considered as a novel food, thus according to Regulation (EC) No 2015/2283 an approval must be requested and EFSA should provide a scientific opinion on its safety. In what concerns national rules, it should be mentioned that several Member states have drawn up positive or negative lists, particularly focusing on medicinal plants and their extracts. For example, the competent authorities of Belgium, France and Italy, have created a common list of botanicals allowed for use in food supplements, the so called BELFRIT list (Ministero della Salute, 2024). In fact, it is interesting to notice that plant food supplements (PFS) may include medicinal plants in their composition that can be poten-

tially used in medicines production. The same ingredients can be employed in both products, which are considered in distinct groups (foods and medicines) and, therefore, under different legislation. In particular, Traditional Herbal Medicinal Products (THMP) are regulated by Directive 2004/24/EC and under the guise of the European Medices Agency (EMA). Directive 2004/24/EC is a crucial regulation for THMP, regulating their launch on the European market for human use. This document meticulously outlines the steps to be followed from production to the sales authorization granted by the government. According to this legislation, the product in question must undergo an internal evaluation by the producers themselves, who take responsibility for conducting all the necessary tests to quantify and qualify the components present in the product. After this internal assessment, the documents are forwarded to a committee of representatives from each member state of the European Union (EU). At this stage, the evaluation committee analyzes the documentation and may request additional information in case of lack or errors in the information provided. In addition, the regulatory authorities have the prerogative to ask for further safety assessments based on the data provided by the manufacturers. This may include reviews of toxicological studies, stability data, and other information relevant to ensuring the product's safety for human consumption. If all the requirements are met, a sales authorization is granted for EU member countries.

Contrary to what happens in THMP, in food supplements, as they are considered foods, the responsibility for the safety of these products lies with the food business operator placing the product on the market. Thus, food supplements do not require a safety assessment or a pre-approval for market placement, and manufacturers only need to inform the relevant authority about their introduction to the market by submitting a copy of the label along with a duly completed notification table (in Portugal this submission is made to the Direção-Geral da Alimentação e Veterinária, DGAV). The competent authority is responsible for checking if everything declared in the label agrees with the directive 2002/46/EC (Ribeiro, 2015).

The scenario in the United States (US) is very similar to that in the EU since dietary supplements are also considered foods being regulated by the Dietary Supplement Health and Education Act of 1994 (DSHEA) which states that “a dietary supplement is a

product (other than tobacco) intended to supplement a diet, as long as it bears or contains 1 or more of the following dietary ingredients: vitamins; minerals; herbs or other botanicals; amino acids; dietary substances used by man to supplement a diet by increasing the total dietary intake; concentrates, metabolites, constituents, extracts, or a combination of the ingredients referred to above, and is intended to be taken by mouth as a pill, capsule, tablet, or liquid". This law establishes dietary supplements as a distinct category of foods with its own requirements for safety and labeling and limits the regulatory oversight by the US Food and Drug Administration (FDA) as these products do not need previous approval from the FDA before being commercialized (Rocha et al., 2015).

In contrast to the regulatory approach for drugs, FDA regulations for dietary supplements do not impose strict quality control or standardization of the components used to manufacture the final product, nor do they require proof of efficacy. Similar to the EU, The FDA only requires a notification informing about the ingredients used in the dietary supplements. Nevertheless, for ingredients without a history of use in the FDA's records prior to 1994, the manufacturer is responsible for conducting safety studies and submitting documentation to the FDA to demonstrate their safety (Santos, 2017). In addition, the manufacturer must report any adverse effects related to the use of the product after it has been marketed (McWhorten, 2023).

2.2. Adulteration of supplements

In recent decades, there has been a steady growth in the consumption of food supplements, prompting producers to increase both their production capacity and the diversity of options available on the market (Yager and O'Dea, 2014). This competitive scenario encourages manufacturers to explore different options toward offering a diverse range of supplements designed to capture consumer interest. Among these options, plant food supplements (PFS) have gained considerable popularity. Their attractiveness is largely based on the perception that they are "natural", given their association with plants/products derived from nature, conveying a (false) sense of security to consumers (Rocha et al., 2015). However, in addition to the potential risks intrinsic to botanical's consumption (e.g. possible side effect or interaction with prescription drugs) in the last decade several reports have highlighted cases of PFS adulteration, either due

to botanical swaps of higher cost medicinal plants by lower cost ones or to the illegal addition of pharmaceuticals (or their analogs) to potentiate the product's effect (Grazina et al., 2023; Rocha et al., 2015). Because dietary supplements are categorized as food products, they are exempt from undergoing safety analyses beyond those required by the food legislation before being introduced to the market. Therefore, the detection of frauds generally occurs after the product being marketed, either during controls carried out by governmental agencies (in Portugal by the ASAE) or after investigation of situations that led to hospitalization, or during controls at the EU borders. The illicit addition of prohibited substances for quicker effects aims to boost results and increase product acceptance and sales and, consequently, profits for the producer, however it constitutes a major food safety and public health concern, particularly due to the growth of consumption of these product and the associated risks of inadvertently consuming adulterant substances. Another aspect to highlight is the ease with which these products can be marketed. Food supplements in general, including PFS, are widely used on a global scale and, in several developed regions such as the European Union and the United States, these products are easily found in supermarkets, pharmacies (without the need for a medical prescription), health food stores, among other establishments. In addition, the e-commerce of PFS is growing due to the convenience of purchasing these products via the internet without leaving home. However, controls are even more difficult to implement in this type of commerce. This wide dissemination further contributes to the increase in sales of these supplements, as well as the potential for frauds.

Over the last years, different studies and control agencies reported a significant increase in cases of adulteration in food supplements of various kinds (Jairoun et al., 2021; Shin et al., 2022; Amidžic et al., 2023). In Europe, there is a control system established by the European Commission, known as RASFF (Rapid Alert System for Food and Feed). This system is composed of the member countries of the European Union (EU) and is responsible for consolidating all the data related to the information provided by authorities of the member countries of the European Union about any type of safety problem, including adulterations, associated with food, feed and related products. The system was established to enable food safety authorities to quickly share information

on health risks posed by food and feed, allowing them to take immediate action to prevent these risks. RASFF provides a 24-hour service to ensure that the food authorities receive urgent notifications about food and feed, so that they can take further action. The information provided by RASFF results in products being recalled from the market preventing them from coming into contact with the end customer (European Commission, 2024). A quick search on this site, using the following criteria: "*2019-2024; All EU countries; dietetic foods, food supplements, and fortifying foods; Hazard category*", reveals the existence of 564 notifications linked to these criteria (RASFF, 2024). This data highlights the importance of closely monitoring the safety and integrity of these products to protect public health and guarantee transparency in the food market. There are also several reports about the adulteration of food supplements since the beginning of the data collection. According to Koncz (2021) since 2003, the data from RASFF corresponding to quality issues in food supplements has included 2559 entries. Among these, 319 are specifically associated with problems in weight-loss food supplements. Notably, within this subgroup, 202 out of the 319 reports are linked to slimming products that contain unapproved synthetic drugs in their composition, such as 2,4-DNP, sibutramine, phenolphthalein, and other compounds containing similar organic groups. Similarly in the US, the FDA has reported several tainted dietary supplements. According to Rocha et al. (2015), from 2010 to 2015, from a total of 416 public alerts released by the FDA, 155 corresponded to adulterated weight-loss products, from which most cases (87%) involved the illegal addition of sibutramine, although other adulterants such as phenolphthalein or fluoxetine, were also reported (Rocha et al., 2015).

2.3. Dinitrophenol origin and use

2,4-Dinitrophenol (1-hydroxy-2,4-dinitrobenzene), is an organic compound with the formula $C_6H_4N_2O_5$, presented as a solid crystal with a yellowish color and a characteristic musty smell (Sigma-Aldrich, 2023). In the last century, this compound had wide application in various industrial areas, serving as a dye, pesticide, photographic developer and wood preservative, among other uses. Its use dates back some 80 years ago, when it was historically used in munitions and explosives factories in Europe during the two world wars, marking the beginning of the first studies on dinitrophenol (Fernandes and Izidoro, 2022).

France used a mixture of 40% dinitrophenol and 60% trinitrophenol as an explosive, unlike other Western countries, which predominantly used TNT (trinitrotoluene) (Parascandola, 1974). Employees exposed to this mixture showed rapid weight loss, initially with no apparent side effects. This phenomenon sparked interest in the potential use of dinitrophenol in the treatment of obesity, given the perception of weight loss in factory workers, without the need for additional treatments (Fernandes and Izidoro, 2022).

As the curiosity about the compound grew, the study by Dr. Maurice Tainter, a renowned researcher at Stanford University in the United States, stood out. His research involved adults who had tried thyroid treatments and diets without success. Tainter observed that the continuous intake of 2,4-dinitrophenol increased basal metabolic rate by 50%, resulting in increased calorie expenditure and consequent weight loss. The study involved 113 participants, 15 men and 98 women. The drug, in the form of 2,4-DNP or its associated salt, was administered in doses of around 75 mg per capsule (or 100 mg of the associated salt, corresponding to 75 mg of the compound), once or twice a day, before the most important meals, varying according to the patient's degree of obesity. After 125 days of treatment, the average weight loss was 1.5 pounds (0.7kg) per week. Side effects such as increased body temperature, excessive sweating, vasodilation, tiredness, and nervousness were reported, although there were no deaths or extreme side effects (Tainter et al., 1933).

However, before this study was carried out, there were already records of several deaths associated with exposure to 2,4-DNP, especially in occupational contexts, characterized by exposure in the workplace, where there is direct contact between the worker and the compound. The first documented case of death from 2,4-DNP poisoning dates back to 1918, respective to a worker in a French explosives and munitions factory during the First World War (Fernandes and Izidoro, 2022).

According to Grundlingh (2011), after the publication of Tainter's study in the 1930s, there was an exponential increase in the use of this drug, accompanied by the significant emergence of side effects associated with the use of 2,4-DNP as a weight loss agent. The increase in the use of this compound resulted in several documented cases

of death and intoxication, prompting the need for further investigation into its mechanism of action, especially concerning weight loss in patients who used it. In the review-carried out by Grundlingh (2011), cases of reported deaths since the compound began to be used industrially until 2010 were compiled. According to this article, the first decades, from 1910 to 1920, marked by the predominant use of the compound in industries, mainly for war purposes, represented the period with the highest rate of deaths due to 2,4-DNP intoxication. Remarkably, no deaths related to the consumption of the compound were reported from 1920 to 1930, which remained the case until the 1930s, coinciding with the completion of Tainter's study.

As a result of Tainter's research, the use of the substance expanded considerably, and it was marketed in the form of pills to treat obesity under popular names such as "Dinitriso", "Nitromet", "Dinitrenal" and "Alpha-Dinitrophenol". Promoted as a drug capable of increasing the basal metabolic rate by 50%, with no apparent health risks, its popularity grew even more due to other contemporary studies suggesting the compound's safety and efficacy in weight loss. Despite warnings issued by the US Board of Pharmacy and Science about potential adverse effects and fatal risks, it is estimated that the substance was prescribed to more than 100,000 people by around 20 companies during the period between the release of Tainter's study and the compound's ban in 1938 (Bleasdale et al., 2018). Figure 1 illustrates the significant increase in the number of deaths related to consumption of the compound in the 1930s, coinciding with the marketing of weight-loss drugs.

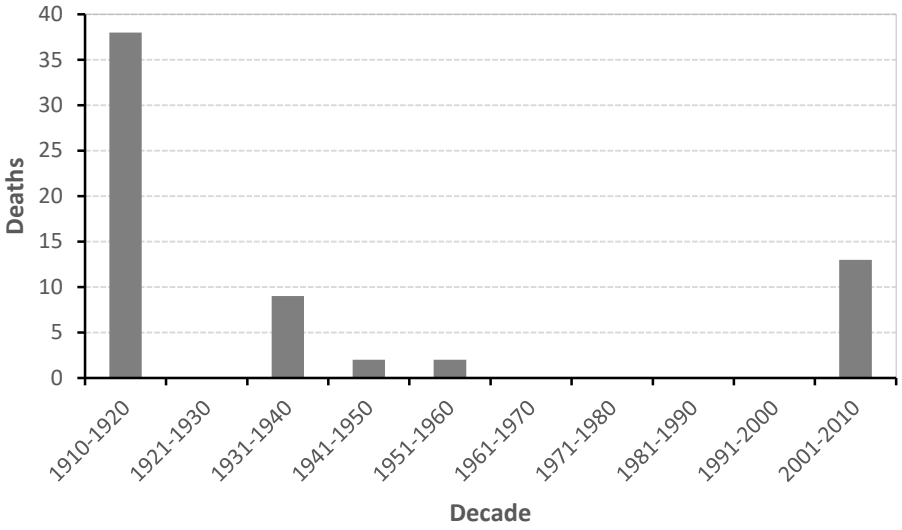

Figure 1. Number of deaths attributed to contact with 2,4-DNP by decade (Adapted from Grundlingh et al., 2011).

After five years of use for aesthetic purposes, sufficient evidence, added to the increase in deaths and adverse effects, led to the conclusion that 2,4-DNP posed a threat to public health. In 1938, the FDA banned the compound due to the danger it presented (Fox et al., 2005). However, in the 1980s, there was a resurgence in the use of the agent, resulting in new cases of poisoning. After a period of a few decades with no reports of deaths caused by 2,4-DNP, since 2000 there has been an increase in records, highlighting the need to raise awareness about the use of this drug. It was only in 2003 that the compound was finally banned in the UK, and in 2015, The International Criminal Police Organization issued a warning about the risks associated with ingestion and contact with the agent (The International Criminal Police Organization, 2015).

In the last two decades, 12 cases of poisoning and death from 2,4-DNP have been reported. This increase can be attributed to social pressure related to beauty standards, leading people to look for quick solutions to achieve their ideal weight. In addition, with the ease of buying products online and the widespread use of social media to promote these products, the accessibility of food supplements and non-prescription drugs has increased significantly (Von Ende and Oliveira, 2021). In addition to cases of death, symptoms such as increased body temperature, intense sweating, nausea, vomiting,

coma, convulsions, multiple organ failure, and tachycardia, among others, have also been documented (Fernandes and Izidoro, 2022).

2.3.1. Dinitrophenol as an adulterant

The pursuit of rapid loss of weight has fueled the demand for substances like 2,4-DNP, known for its quick effects. This demand has led to sales of this compound online on dubious websites and the emergence of fraudulent practices by the illegal addition of this compound into food supplements, which are frequently marketed via the internet (Sousa, 2020).

According to Amidžic et al. (2023), who conducted a review of data based on RASFF reports spanning from 2011 to 2022, among 68 reports of unauthorized pharmaceuticals identified in food supplements for weight-loss, 45 were related to sibutramine, 18 to phenolphthalein, 7 to *N*-didesmethyl sibutramine and 6 related to 2,4-dinitrophenol. Notably, only 16 out of the 45 reports involving sibutramine (35.6%) were classified as "*serious risk notifications*" (35,6%), while for 2,4-DNP, all 6 reports were reported as "*serious risk notifications*" (100%).

Koncz et al. (2021) also conducted an evaluation of the most frequently used synthetic adulterants and the trends of adulterated food supplements within the European Union based on warnings from the RASFF over the period from 1988 to 2019. The study provided valuable insights into the prevalence of unauthorized substances in slimming products and the associated health risks posed to consumers. A total of 2559 records of food supplements with quality issues were identified in the RASFF database, of which 319 (12.5%) were marketed for weight loss. Among these, 202 (63.3%) were found to contain unapproved synthetic drug ingredients. The most common adulterant was 2,4-dinitrophenol (DNP), present in 113 products (35.4%), followed by sibutramine, which was found in 69 products (21.6%). It is noteworthy that reports of sibutramine adulteration were found almost every year, and 2,4-DNP was reported in just four different years, namely in 2003, 2017, 2018, and 2019, as shown in Figure 3 (Koncz et al., 2021).

Besides RASFF reporting these issues, in 2016, the FDA issued a report addressing the sale of illicit medications on the internet. As part of their investigation, 4,402 websites

were suspended for engaging in the unauthorized sale of medicines. Notably, the research also uncovered 110 websites selling 2,4-dinitrophenol as a purported weight-loss product (Food and Drug Administration, 2016).

Recently, Sousa et al. (2020) conducted a review focusing on 2,4-DNP and its use as a weight loss agent. In this study, 14 online websites selling weight-loss products containing 2,4-DNP as a "fat burner" supplement were highlighted as shown in Figure 2.

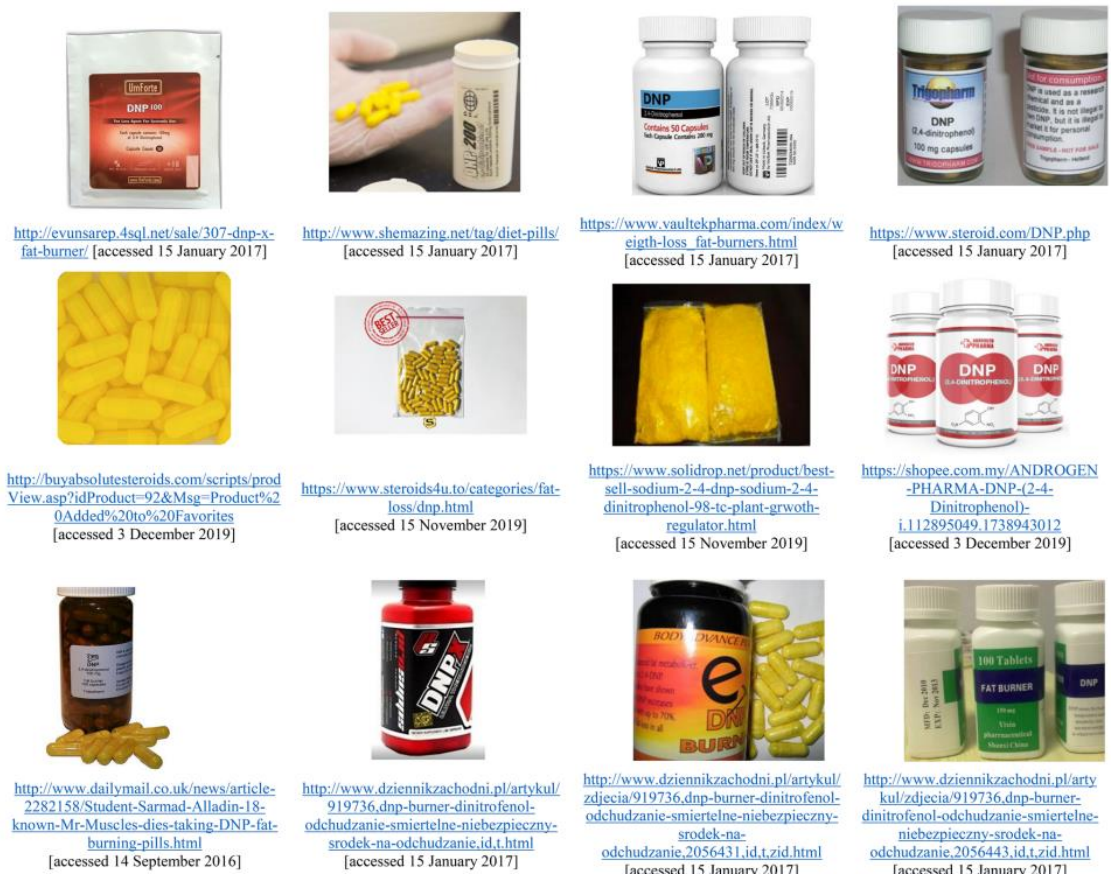

Figure 2. Examples of products marketed online containing 2,4-dinitrophenol (Adapted from Sousa et. al, 2020).

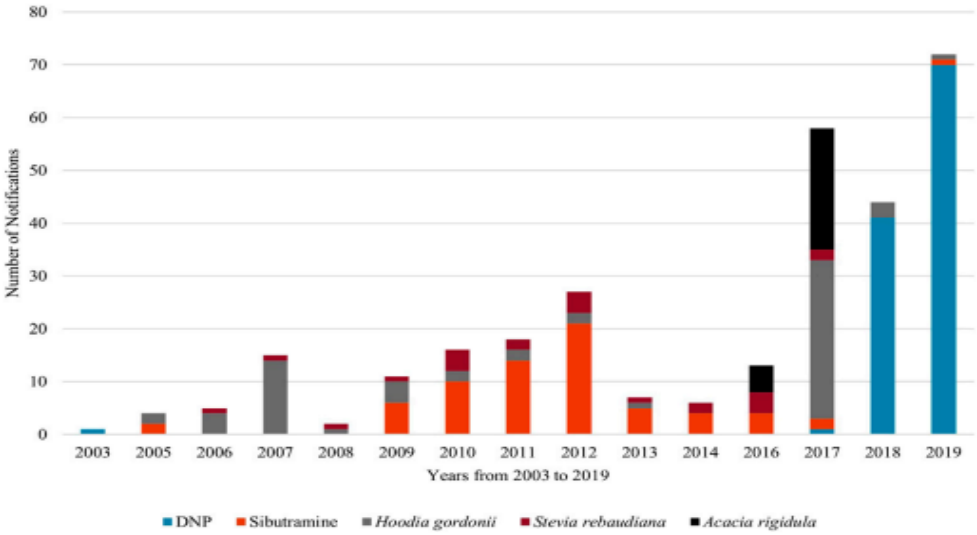

Figure 3. RASFF notifications on weight-loss food supplements (Adapted from Koncz et al., 2021).

In addition to data on the number of products identified as illegally containing this substance, in 2011, Grundlingh and co-workers reviewed the information regarding the documentation of deaths related to 2,4-DNP and found 62 reports in the medical literature. However, these numbers probably do not fully represent reality and could be even higher, as they refer just to reported situations.

2.3.2. Cases of dinitrophenol poisoning

Several studies have investigated the effects of 2,4-DNP exposure not only through ingestion, but also via skin contact and inhalation. One of the first studies to examining the dangers of this substance was conducted in 1919 by the physician Roger G. Perkins. The study took place in a factory of munitions and explosives in France, where many workers presented symptoms such as gastrointestinal problems, anorexia, nausea, vomiting, tachycardia, diarrhea, and cramps. Acute intoxication present symptoms of numbness and tingling in the arms and legs, intense sweating, agitation, anxiety, shortness of breath, and dyspnea. In addition, there was an increase in body temperature and accelerated heartbeat. The study also reported cases of fulminant intoxication, which were more frequent in workers with a history of alcohol, kidney, and liver prob-

lems. In these cases, death occurred within a few hours, with adynamia, intense diarrhea, and initial cramps. Workers often returned home after the onset of the symptoms but quickly deteriorated, developing a high fever of over 40˚C, intense sweating, tachycardia, yellowing of the skin in the areas of contact with the chemical, and intense thirst. Following a brief period of apparent improvement, the condition deteriorated with convulsions, unconsciousness, coma, and death within a few hours. Some workers continued working as usual, but at the end of the working day, they were found gasping for breath in the streets, experiencing fevers as high as 43°C, and ultimately succumbing before medical assistance (Perkins, 1919).

Another significant study into the potential danger of 2,4-DNP and its derivatives dates back to 1885, when two French researchers, P. Cazenueve and R. Lépine, reported the thermogenic effects of salts of dinitrophenols, commonly used as dyes. At the time, these chemicals were used as food dyes and in the silk and wool industry. The compounds were administered to dogs, resulting in symptoms similar to those observed in the studies of the following century: vomiting, diarrhea, short and heavy breathing, and, most notably, a significant increase in the animal's body temperature, reaching 44˚C. During this episode of hyperthermia, no increase in blood oxygenation was observed, indicating that the increase in temperature was not related to increased muscle activity (Parascandola, 1974).

At the beginning of the studies of the compound, it was believed that the compound was responsible for increasing the metabolic rate or stimulating the metabolism, causing weight loss (Tainter et al., 1933). But over the years, it was discovered that 2,4-DNP affects the cell's mitochondrion, an organelle crucial for cellular respiration. This drug functions as a mitochondrial uncoupler, disrupting oxidative phosphorylation in the mitochondria and interfering with the Krebs cycle's oxidative phosphorylation. Mitochondrial uncouplers, such as 2,4-DNP, are weak acids that are lipid-soluble and can traverse the inner membrane of the mitochondria. They transport protons into the mitochondrial matrix, prompting substrate oxidation even in the absence of adenosine diphosphate (ADP). By transporting protons across biological membranes, 2,4-DNP disrupts the electrochemical proton gradient. Consequently, the energy released during the oxidation-reduction reactions in the electron transport chain becomes insufficient

to facilitate adenosine triphosphate (ATP) production. Instead, this energy is dissipated as heat, resulting in a rapid increase in metabolism and higher caloric expenditure than usual, effectively causing more caloric consumption. This is phenomena is known as uncoupling oxidative phosphorylation (Sousa et al., 2020).

Since Tainter's disclosure in 1933, several cases of intoxication and death associated with the use of 2,4-DNP have been recorded, with a remarkable consistency in symptoms among the majority of patients. Nausea, vomiting, excessive sweating, fever, tachycardia, short wheezing breaths, hyperthermia, tachypnea, coma, adynamia, and other symptoms are widely reported by patients, as detailed in Table 1.

Table 1. Symptoms and outcome of hospitalization due to the ingestion 2,4-dinitrophenol (Adapted from Fernandes and Izidoro, 2022).

Author/ year publication	Symptoms	Outcome of hospitalization
Mcfee et al., 2004	Excessive sweating, fever, agitation, delirium, bradycardia.	Patient died
Hsiao et al., 2005	Fever, tachycardia, agitation, confusion, cramps.	Patient died
Miranda et al., 2006	Fatigue, tiredness, myalgia, nausea, vomiting, tachypnea.	Patient died
Tewari et al., 2009	Fatigue, nausea, excessive sweating, tachycardia, fever, agitation.	Patient died
Le et al., 2015	Fever, headache, skin irritation, swelling in certain parts of the body	Recovered
Zack et al., 2016	Nausea, vomiting, excessive sweating, tachycardia, tachypnea	Patient died
Perez et al., 2017	Shortness of breath, palpitations, nausea, vomiting, fever, tachycardia, hypertension, excessive sweating.	Patient died
Patankar et al., 2020	Fatigue, excessive sweating, myalgia, vomiting, tachycardia.	Patient died
Sarwar et al., 2020	Palpitation, excessive sweating, nausea, vomiting, shortness of breath, agitation, tachycardia.	Patient died
Jung et al., 2020	Patient found dead after taking pills to treat obesity	Patient died
Freeman et al., 2021	A traumatized patient was taken to emergency surgery but died quickly	Patient died

2.4. Analytical techniques for detecting 2,4-dinitrophenol

Aware of all the problems related to the use of 2,4-DNP, methodologies have been developed to detect and quantify this compound, with most of them being developed for post-mortem identification of the chemical in blood, urine, or in contaminated water. A commonly used for method for detecting the compound 2,4-dinitrophenol and its derivatives is gas chromatography coupled with mass spectrometry detection (GC-MS) (Campos et al., 2019).

Chromatography is a highly effective technique for the separation of compounds, working as a previous step in the identification and quantification of chemical components in complex mixtures (Skoog et al., 2006). This approach is based on the difference in interactions between the various components present in a complex mixture with the mobile phase and the stationary phase, which constitutes the substrate on which the compounds interact to achieve the separation. Due to its notable efficacy, chromatography has been extensively applied, covering a wide range of sectors, from pharmaceutical to environmental analysis. The versatility of chromatography makes it an indispensable method in research and development laboratories. Another crucial aspect is that this separation technique can be hyphenated with different detectors, such as mass spectrometry (MS), achieving high sensitivity and allowing detecting the compounds at very low concentrations. This ability to analyze traces is especially valuable in fields such as toxicology and quality control, where accuracy and sensitivity are imperative. In addition, chromatography coupled to MS detection offers the advantage of being selective, allowing the effective separation and identification of similar compounds, which is crucial in complex samples, such as food supplements that may include several medicinal plants and corresponding phytochemicals. The separation, towards improved selectivity, is achieved by choosing the appropriate combination of stationary phase and operating conditions. Skoog et al. (2006) highlight the uniqueness of chromatography by stating that "*no separation method is as powerful and has such widespread application as chromatography*". This statement underscores the prominent position that this analytical technique occupies in chemical analysis, highlighting its robustness, versatility, and significant contribution to understanding and characterizing complex mixtures.

Gas chromatography (GC), in line with the fundamental principles of chromatography, is distinguished by the use of a gaseous mobile phase. This analytical method involves the injection of the sample in the injector, which is responsible for the vaporization of the sample's compounds. The vaporized sample mixture is eluted using a mobile phase composed of inert gas, which takes the sample through the column. Because the compounds need to be vaporized before being inserted into the chromatographic column, this technique is not adequate for thermally unstable compounds as they can degrade when subjected to high temperatures. Non-volatile compounds can be derivatized to volatile derivatives, as long as they remain stable. The separation of compounds in the mixture is achieved due to the different interactions between these compounds and the stationary phase, resulting in different retention times. This effective separation is fundamental to obtaining accurate results, and it is monitored at the end of the chromatographic column, with the aid of a detector that is appropriate for the type of compounds/sample being analyzed. The correct choice of detector is essential for the sensitivity and selectivity of the technique. Detectors such as flame ionization detectors (FID) for organic compounds and electron capture detectors (ECD) for halogenated compounds are commonly used. However, using MS detectors improves the accuracy in identifying compounds while achieving a higher sensitivity. (Skoog, et al. 2006).

Mass spectrometry is an analytical technique used to identify and quantify atoms and molecules based on the mass to charge of the ions generated from the sample. The ions are separated and detected, allowing for the determination of molecular structure, composition, and abundance. Widely applied in various scientific fields, such as chemistry and biochemistry, this technique operates through several stages. Initially, the sample is ionized, converting atoms or molecules into ions. The ions are then accelerated through an electric field, acquiring kinetic energy proportional to their charge. The accelerated ions are subjected to a magnetic field, where the deflection is inversely proportional to their mass/charge. This results in the separation of the ions based on their masses. The separated ions reach a detector, which measures the intensity of the ionic current as a function of position, thus generating the mass spectrum. The spectrum analysis reveals peaks corresponding to the different ions present in the sample. Each peak represents an ion with a specific mass, the position of the peak being related to the mass

of the ion and the height of the peak to the relative abundance of the different ions. The spectrum is interpreted by comparing the experimental patterns with known data on molecules or ions.

The most commonly used mass spectrometer type is the quadrupole mass analyzer, mainly due to its size, price and robustness compared with the other types of mass spectrometers. The main component of a quadrupole instrument consists of four parallel cylindrical rods (originally hyperbolic) that act as electrodes. Opposite rods are electrically connected, with one pair connected to the positive terminal of a variable direct current (DC) source and the other pair connected to the negative terminal. In addition, variable high-frequency alternating current (AC) voltages with a phase difference of 180° are applied to each pair of rods. To obtain a mass spectrum with this instrument, ions are accelerated into the space between the rods by a potential difference of 5 to 10 V. During this process, the AC and DC voltages on the rods are simultaneously increased, while the ratio between them is kept constant. At a given moment, all ions except those with a specific mass-to-charge (*m/z*) value collide with the rods and become neutral molecules. Only ions with specific *m/z* values are able to reach the detector, generating the mass spectra. This type of mass spectrometer has an advantage that it scan with a time lower than 100ms (UFJF, 2013). There are 2 types of scan analysis using this type of mass spectrometer, the full scan, which consists of a scanning of all *m/z* that reaches the detector, and the SIM (Selected Ion Monitoring), which focuses only on user-selected *m/z* values. The SIM mode allows the user to select the known ions from a compound, producing a cleaner chromatogram (Worsfold et, al. 2005).

The triple quadrupole mass spectrometer is a variation of the quadrupole mass analyzer that consists of three quadrupole analyzers arranged in sequence. In this setup, the first quadrupole (Q1) selects ions with a specific mass-to-charge (*m/z*) ratio. The second quadrupole (Q2) functions as a collision cell rather than a mass analyzer. Within Q2, ions from Q1 are accelerated into a high-vacuum region where they undergo collisions with an inert gas, causing fragmentation and forming product ions. These product ions then enter the third quadrupole (Q3), which performs a mass analysis of the ions generated in the collision cell (Chiaradia et, al., 2008).

These two types of mass spectrometers allow for the identification of components in a sample, determination of their masses, and, using reference standards, quantification of their relative concentrations. Mass spectrometry, therefore, plays a crucial role in the characterization of chemical substances, biomolecules, and organic compounds (Skoog et al., 2006). Complementing this, gas chromatography is valued for its speed and efficiency in the separation of compounds, enabling rapid and accurate analysis across diverse fields, including environmental, pharmaceutical, and food analysis.

The methodologies outlined in this chapter represent the most commonly employed techniques for detecting the target compound of this work, 2,4-dinitrophenol. Table 2 provides an overview of the existing methods used to determine the concentration of this compound in various sample types, including capsules, contaminated water, blood, and urine.

Table 2. Methods reported in the literature for determining 2,4-dinitrophenol (Adapted from Sousa, et al. 2020).

Sample	Separation Method	Detection Method	References
Capsule	LC	DAD	Lee, et al. (2014)
	GC	MS	
	LC	TOF/MS	
Capsule	GC	MS	Miranda, et al. (2006)
	LC	DAD	
Capsule	LC	DAD	Rebiere, et al. (2012)
Blood	LC	DAD	Miranda, et al. (2006)
Blood	LC	MS	Politi, et al. (2007)
Blood	GC	MS	Robert and Hagardorn (1983)
Urine	GC	MS	Miranda, et al. (2006)
Urine	LC	MS/MS	Politi, et al. (2007)
River Water	GC	MS	Nakamura et al. (2001)
Rain Water	GC	NPD	Nick and Schöler (1992)
	GC	MS	
Water	GC	MS	Lu et al. (1999)

2.4.1 Derivatization

To perform an analysis by gas chromatography, the compound of analysis must be thermally stable and have sufficient volatility to be volatilized by the injector. For compounds that have a high molar mass or compounds that are strongly polar, it may be necessary to perform the derivatization process in order to increase its volatility and stability, and consequently improve the performance of the quantification for the compound's study (Collins et al. 2006).

As shown in Table 2, there have been some methods previously proposed to measure the concentration of 2,4-DNP by using different separation and detection techniques. Some of them, included the derivatization of the sample containing 2,4-DNP, which is a strongly polar substance since it has two nitro and one hydroxyl group linked to its benzene ring. Derivatization (e.g., silylation) replaces the hydroxyl group with a more volatile functional group, improving the compound's chromatographic behavior in gas chromatography and improving the molecule's ionization efficiency in the mass spectrometer, leading to better sensitivity.

Derivatization is a set of reactions that is capable of modifying the compound of analysis in order to generate products with higher volatility. For gas chromatography, some molecules could be problematic due to their functional groups, such as –COOH, -OH, -NH, -SH. These functional groups could form hydrogen bonds that can affect the volatility and thermal stability of the compounds, but also causing its adsorption to the column resulting in lower detectability and deficient peak separation. In this case, the derivatization process is strongly recommended as it increases volatility while reducing polarity, leading to the reduction of analyte adsorption on the column, enhancing detector response and providing more efficient separation of chromatographic peaks (Halket and Zaikin, 2003).

Some derivatization reactions can be used to improve the detectability of compounds by GC, including alkylation, acetylation, silylation and methylation. In the case of the study compound of this work, the most common derivatization reaction used is the silylation. Silylation is an important chemical process that involves the introduction of a silyl group in a certain compound, the silyl derivative is formed by an active proton

displacement in the functional groups (-OH, -NH, -SH) by an alkyl silyl group as it is represented in Equation 1. Functional group responses to silylation are different, as follows: alcohols>phenols>carboxylic acids>amines>amides (Segura et al., 1998). There are several reagents available that can be used to perform the silylation procedure, such as hexamethyldisilazane (HDMS), trimethylchlorosilane (TMCS), N-Methyl-N-trimethylsilylacetamide (MSA), trimethylsilyl-alditol (TMSA), N-trimethylsilyl-diethyl-amine (TMSDEA), N-trimethylsilyl-dimethyl-amine (TMSDMA), N-methyl-N-trimethyl-silyl-trifluoroacetamide (MSTFA), N,O-bis(trimethylsilyl)-acetamide (BSA), N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), N-trimethylsilyl-imidazole (TMSI). The ability to react with the compounds is approximately (depending on the studied compound) shown as follows: HMDS<TMCS<MSA<TMSA<TMSDEA<TMSDMA<MSTFA<BSA<BSTFA<TMSI (Moldoveanu and David, 2018).

$$R_3Si\cdots X \; + \; Y\cdots H \; \rightarrow \; R_3Si\cdots Y + H\cdots X$$

Silyl Reagent	Compound w/ Active hydrogen	Silylated Compound	Hidrogen w/ Reagent Leaving Group

Equation 1. Generic silylation representation.

2.5 Method Validation

An analytical method is a methodology that may present an error, may it be systematic or random. These types of errors may cause significant variations in the final results. Therefore, is essential to demonstrate that the method generates a reliable and aligned result with the designated purpose (Eurachem, 2014). To ensure the reliability of the method and decrease the chance of these errors to occur, many tests need to be carried out, producing and treating enough data to give the required robustness to the proposed methodology.

There are different guidelines providing step-by-step guidance to validate an analytical procedure. These guidelines outline the necessary parameters for validating an analytical procedure, including specificity, linearity, range, accuracy, precision, quantification limit and detection limit. Each of these parameters has specific criteria that must be met to consider the procedure valid.

Specificity is the capacity of an analytical procedure to differentiate, identify, and quantify the interest analyte from the others in a complex matrix without interference from others that may be present in the sample. A specific method is an instrumental methodology capable of providing a response for a single substance (Pereira, 2015).

The matrix effect is defined by the United States Food and Drug Administration agency (FDA) as “direct or indirect alteration or interference in response because of the presence of unintended analytes or other interfering substances in the sample”. It can be described as difference in response generated by the GC-MS in a standard solution with the analyte, and a response generated by the same solution, but in presence of other matrices, in same analytical conditions (Food and Drug Administration, 2018).

Linearity refers to the ability of a method to provide analytical responses (dependent variable) that are directly proportional to the analyte concentration (independent variable) within a specific concentration range, resulting in a calibration curve. The calibration points must be prepared within this calibration range. The majority of the international conventions indicate that the calibration curve must have between five and eight points in the calibration curve (Ribani et al., 2004). For the acceptance criteria of the calibration curve linearity, the determination coefficient (R^2) must be higher than 0.99.

The range corresponds to the interval of the lowest value of the analyte and the highest value of the analyte that can be calculated using the analytical method. Normally, this parameter is studied concomitantly with the linearity study, to choose the best fit using the calibration curve, determination coefficient, range and concentrations of the studied samples (Pereira, 2015).

To determine the quantity of compound present in a sample, it is necessary to build a calibration curve. The first task is preparing standard solutions with known concentrations of the compound of interest. The statistical analysis of the calibration curve can allow also for estimating the linearity of the method, the limit of detection (LOD), and the limit of quantification (LOQ).

The external standard calibration method involves preparing solutions of the target compound at various known concentrations. These solutions are analyzed, and the equipment's responses are recorded for later processing using statistical software. The calibration data collected by the instrument is then plotted in graphics software, creating a concentration *vs.* signal curve. This curve is adjusted using linear correlation to ensure that the signal response is accurate enough to quantify the compound in the sample under study. However, in real analyses, the signal may not always follow a linear relationship with concentration. In such cases, the least squares method can approximates the data to a straight line, plotting the best-fit line through the measured signals. However, it is advised to change the range to obtain a linear relation. The regression analysis of this line, represented by the correlation coefficient (R^2), provides a measure of the quality of the calibration curve. This calibration curve also defines the mathematical function that correlates concentration with signal. In the graph, the y-axis represents the signal of the detected compound, while the x-axis corresponds to its concentration. Once all calibration points are read, the unknown samples are analyzed, and the signals obtained are compared with the calibration curve function to determine the concentration of the unknown samples (Skoog et al., 2006).

In addition, the calibration curve allows the calculation of some evaluation parameter and analytical limits of the method, as quantification and detection limits.

The limit of detection (LOD) is the lowest concentration of the target analyte that can be detected, using the analytical method. LOD can be measured in 3 different ways, the visual method, signal/noise (S/N) method, and can be calculated based on the calibration curve parameters (European Union Reference Laboratory, 2016).

The method based on the calibration curve parameters is calculated using the values given by the calibration curve parameters, using equation 2:

$$LOD = 3 \times \frac{\text{SD}}{\text{Slope}}$$

Equation 2. Limit of detection.

where SD is the standard deviation of the intersection of the calibration curve and slope is the slope of the calibration curve.

For LOD determination, a calibration curve must be done with the analyte in the range of sample analysis.

The limit of quantification is the lowest concentration of the compound that can be measured and quantified by reaching with a suitable level of precision and accuracy. The same fundamentals used in LOD calculation can be used in LOQ calculation. The LOQ can be measured by visual estimation, which is very subjective, thus the statistical method is the most frequently used, once this takes into consideration the parameters of the calibration curve (European Union Reference Laboratory, 2016). The calculation to LOQ can be defined using equation 3:

$$LOQ = 10 \times \frac{\text{SD}}{\text{Slope}}$$

Equation 3. Limit of quantification.

Using the calibration curve parameters to calculate the LOD, the lowest value of the calibration curve is considered the LOQ, and the ratio between LOD and LOQ is given by equation 4:

$$LOD = 0.3 * \text{LOQ}$$

Equation 4. LOQ and LOD ratio

The capacity of an analytical method to produce consistent results is revealed by its precision. In specific conditions, it allows an estimate of the variation between results in different analyses performed with the same or similar samples (with the same concentration). The lower the dispersion between the readings of the same sample, the higher the precision of the method, and its precision can be shown by two dispersion parameters: coefficient of variation (CV), as shown in Equation 6, and standard deviation (SD), as shown in equation 5. The repeatability allows the characterization of the method in terms of variation between different readings, evaluating the repeatability of the results found with the same method conditions.

$$SD = \sigma = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n}}$$

Equation 5. Standard Deviation.

$$CV(\%) = \frac{SD}{\bar{x}} \times 100$$

Equation 6. Coefficient of Variation.

Accuracy is a relation between reference concentration and calculated concentration. It can be measured by comparing the calculated values with the reference ones. This comparison will result in a deviation that should be no higher than 10% for each sample analyzed. The lower the value of this difference, the higher the accuracy. Accuracy can be determined using equation 7 (Ribani et al., 2004).

$$E\% = \frac{|T.C - C.C|}{T.C}$$

Equation 7. Sample Relative Error Calculation

where T.C is the theoretical concentration and C.C is the calculated concentration.

3. Objectives

As mentioned and shown in Table 2, different methodologies have been proposed in previous studies for the analysis of 2,4-DNP. However, most of those concerned samples obtained in post-mortem cases, such as contaminated water, urine, and blood. This work aims to develop a methodology capable of detecting the presence of this compound in food supplements, thus detecting possible adulterations and ensuring greater control of food supplements for weight-loss purposes.

The selection of the method for determining 2,4-DNP is influenced by the characteristics of the initial sample. The choice between GC-MS and LC-MS must consider the sample's complexities and the specific advantages of each method to ensure efficient and accurate analysis of 2,4-dinitrophenol in food supplements. Considering that PFS are generally composed of a wide variety of chemical compounds, frequently due to the presence of different medicinal plants, each with their own complexity, the selection of the technique to be used must consider the matrix's complex nature. Although both GC-MS and LC-MS can be used effectively (Table 2), the application of the LC-MS quantification method stands out for its specificity and accuracy. However, GC-MS has the advantage of being a method in which the thermolabile molecules present in the mixture (e.g., phenolic compounds from medicinal plants) can be decomposed before entering the chromatographic column, which may confer a significant advantage by reducing the number of compounds entering the chromatographic column. If derivatization is not used, this approach can result in a cleaner chromatogram with fewer peaks, as fewer compounds reach the detector. Thus, in this work, the use of GC-MS is selected due to its robustness, sensitivity, precision, speed of analysis, and the possibility of analyzing complex samples, as the case of PFS with several compounds in their composition.

Therefore, the main objective of this work was the development and validation of a methodology based on GC-MS analysis for the specific detection of 2,4-DNP if illegally added to food supplements. In addition, this work also had the specific objective of applying the proposed methodology for the analysis of commercial samples of weight-loss supplements to check label compliance respecting the absence of this compound.

4. Materials and Methods

4.1. Chemicals

The 2,4-DNP standard solutions were prepared using 2,4-dinitrophenol (moistened with water, ≥98.0%) obtained from Sigma-Aldrich (Merck, Darmstadt, Germany) and methanol (≥99.8%, HPLC grade) acquired from VWR Chemicals. For the GC derivatization procedure, *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Lichropur ≥99.0%) , N-Trimethylsilyl-N-methyl trifluoroacetamide with 1% trimethylchlorosilane (MSTFA w/ 1% TMCS, LiChropur) and chlorotrimethylclorosilane (TMCS, for GC derivatization, LiChropur™, ≥99.0%) were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany). As the cleaning solvent for GC syringe, dichloromethane (HPLC grade) from Fisher Scientific, was used.

4.2. Equipment

All the analyses were performed using a SHIMADZU (QP2020 model) Quadrupole GC-MS system equipped with an AOC-20i Plus autosampler and a 10 µL syringe. The carrier gas was helium, and the chromatographic column was a SHIMADZU Low Bleed SH-Rxi-5ms 30 m (0.25 mm inner diameter and 0.25 µm film thickness) capillary column.

4.3. Standard solutions, derivatization procedure and samples

The stock solution was prepared by measuring 50 mg of 2,4-DNP into a 50 mL volumetric flask and completing the remaining volume with methanol, reaching a final concentration of 1000 mg/L. An analytical balance from Adam Equipment, model ADA-210-C, was used.

The standard solutions with concentrations of 0.01, 0.05, 0.10, 0.50, 1.0, 2.0, 4.0, 5.0, 25.0 and 50 mg/L were prepared by dilution of the stock solution using volumetric flasks of 10 mL. For this purpose, three pipettes with different capacities were used: an AVANTOR single-channel pipettor with a range of 100-1000 µL, a BIOPIPETTE Plus single-channel pipettor for 20-200 µL, and a GILSON single-channel pipettor for 2-20 µL. All

prepared standard solutions were transferred to 2 mL or 4 mL flat-based clear and amber glass vials with septa from Alwsa Technologies and stored at 4˚C until the moment of analysis.

For the derivatization procedure, 1 mL of each individual standard solutions was measured to a glass vials and taken to dryness using a laminar flow cabin. After dryness, 100 µL of the derivatizing reagent (MSTFA with 5%TMCS) was added and the vial was placed in an oven at 90°C for 60 min. To incubate all samples requiring derivatization, a desktop drying oven from SCIENTIFIC (Series 9000) was used. Then, 100 µL of the final solution was measured to a micro-insert for short thread vials ND9 from Avantor and analyzed by GC-MS.

For the preparation of the derivatizing mixture, an Sterican® intramuscular needle with a long bevel and an ECOJECT 2 mL sterile disposable syringe were used to collect TMCS from the stock reagent. For drying the vials containing the prepared methanol solution, a heating plate from IKA was utilized. For sample extraction, a BANDELIN SONOREX RK 52 ultrasonic bath, an orbital shaker-incubator from BIOSAN (model ES-20/60), and an Eppendorf centrifuge (model 5810 R) were employed.

4.4. GC-MS operating conditions

The initial GC-MS operating conditions, both for chromatographic separation (GC) and mass spectrometry (MS) were obtained from literature (Geraldo de Campos et al., 2019). The developed method from Geraldo de Campos and collaborators was used for the quantification of 2,4-DNP (after derivatization) in blood and urine of death patients. In the present work, due to the fact that 2,4-DNP is present in a different matrix (food supplements samples), several modifications to the operating conditions must be made.

The auto sampler configuration was set with 0 rinses with solvent pre-run, and 10 rinses of solvent post-run. The injection mode used was split with a ratio of 1:5, and a 1 µL volume of injection. The carrier gas (mobile phase) used was helium. The flow control mode used was linear velocity with a column flow of 1.60 mL/min. The injector

temperature was operated at 280°C. The oven temperature was set with an initial temperature of 140°C for 1.0 min, followed by a temperature ramp of 15°C/min ratio until 250°C, with a final hold time of 2.50 min totalizing 10.83 min of analysis.

The mass spectrometer was operated in selected ion monitoring mode (SIM), using the 2,4-DNP target selected ions of 137, 195 and 241. By choosing the SIM mode, the selectivity and sensibility were improved, once just the selected ions were chosen to be analyzed, making the baseline noise decrease, and increasing the intensity of the analyte peak. Therefore, SIM allowed to reach lower levels for the quantification and detection limits. The start time of the MS scan was set to 3.00 min, and the end time was set to 10.50 min. The ion source temperature was set to 230°C and the interface temperature was set to 250°C.

The data acquisition and treatment were made in the Shimadzu LabSolutions software (Ver. 4) and for data analysis Microsoft Excel was used.

4.5. Method Validation

The validation of the developed GC-MS methodology to detect and quantify 2,4-dinitrophenol in food supplements was based on the European Medicines Agency " ICH Q2 Guideline on validation of analytical procedures". This guideline defines the necessary elements and steps to perform during the validation process of analytical procedures necessary for the registration applications submitted within the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) member regulatory authorities. The guideline can also be applied to other analytical procedures as part of quality control and other quantitative or qualitative measurements (European Medicines Agency, 2024). Several parameters are included in the guideline, namely, specificity, response (linearity, range), accuracy, precision, quantification limit and detection limit.

For specificity purposes, the GC-MS program was first optimized to ensure the separation of the target compound from some peaks associated with the derivatization process, and 2,4-DNP was evaluated using the selected ion monitoring (SIM) as MS mode of operation. Namely, the ions 137, 195 and 241 were selected as target ions.

To evaluate the matrix effect, a calibration curve in the validated linear range (from 0.05 to 5 mg/L) was determined using a mix of all commercial samples of Plant Food Supplements. The matrix effect is frequently referred in different papers and/or guidelines. The matrix effect is not a validation parameter of the European Medicines Agency guideline "Validation of Analytical Procedures: Text and Methodology Q2", but a parameter of some international regulatory institutions as FDA to methods that use LC-MS (Paschoal et, al. 2008). This parameter evaluated to ensure that the matrix were not causing any interferences in the methodology, ensuring that the values calculated were correct. To evaluate the matrix effect, all the samples and a known concentration of analyte was mixed.

Moreover, using the final developed methodology, 8 commercial samples with different compositions were analyzed before and after being spiked with 2,4-DNP to confirm the absence of the matrix effect.

For response (linearity and range), different ranges of concentration in the calibration curve were tested. The best fit for the R^2 and the expected concentration of the analyte in the real samples was chosen to be validated. For linearity determination, a 6 points calibration curve was determined in a range from 0.05 mg/L to 5.00 mg/L. Five readings of the curve were made in different days, the results were treated and the determination of the coefficient of correlation (R^2) must be higher than 0.99 in all the readings to be considered valid, and the coefficient of variation between the readings of the same standard point in these different days must not exceed 15% for calibration standards and 20% for the LOQ calibration standard. Higher and lower concentrations were also tested, but linearity was affected.

For precision determination, one intermediate point of the calibration curve was set to be used in the repeatability determination, this point was read in 6 different days for 6 times in each day. The variation coefficient was calculated intraday (6 readings per day) and inter-day (36 readings in total) and the value, both intra and inter day, must not exceed the value of 15% as described in EMA guideline "ICH guideline M10 on bioanalytical method validation and study sample analysis".

Since certificate materials of reference do not exist for the compound in evaluation. For accuracy determination, samples containing known amounts of the compound were prepared in the laboratory. For this purpose, the commercial samples were spiked with solid 2,4-DNP and analyzed using the proposed methodology. The obtained results for each of them were then compared with the theoretical amount (added quantity). In general, the recovery of the spiked amount should be between 90% and 110%. In addition, the relative error can be calculated, which value should be lower than 10%. The calculation of the relative error was performed using Equation 7.

For LOQ and LOD determinations, the calibration curve parameters were used. The lowest point of the calibration curve was considered the LOQ of the methodology, since the readings of standards with concentrations below 0.05 mg/L resulted on coefficient of variations exceeding 20%. The LOD was calculated using the equation 4.

4.6. Analysis of commercial samples

Eight different commercial samples were purchased from different brands and with different composition. The composition of the samples is presented in Table 3.

Table 3. Commercial Samples Composition

Sample	Ingredients	Amount per portion (mg)
#1	L-Tyrosine	500
	L-Carnitine tartrate	300
	Green coffee bean extract	200
	Green leaf tea extract	200
	Ginger root extract	200
	Caffeine anhydrous	200
	African mango seed extract DER 10:1	150
	Panax ginseng leaves and stems extract	50
	Black pepper fruits extract	20

	Chromium	0.10
#2	Guarana extract	450
	L-Carnitine tartrate	400
	Caffeine	152
	Green tea extract	150
	L-tyrosine	81.20
	Green coffee extract	60
	Grapefruit extract	50
	Black pepper extract	5
	Chromium	0.40
#3	*Garcinia cambogia* Extract	375
	N-acetyl L-Carnitine	375
	Green coffee	169
	White bean	150
	Cocoa	113
	L-Carnitine-L-tartrate	75
	L-tyrosine	75
	L-5-HTP	24
	L-methionine	19
	L-theanine	19
	Ginger	15
	Black pepper	3.80
	Ginkgo biloba	3.80
	Zinc	3.80
	Niacin	7.50
	Vitamin B6	1.60
	Vitamin B12	0.73
	Chromium	0.14
#4	L-Carnitine	300
	Bitter orange fruit extract	300
	Green tea leaf extract	200
	Caffeine	200
	Garcinia cambogia fruit extract	150
	L-tyrosine	100
	Cayenne pepper	100
	Black pepper extract	5
	Chromium	0.40

#5	L-Carnitine	500
	Taurine	500
	FOS	200
	Caffeine	156
	L-tryptophan	100
	L-tyrosine	150
	L-phenylamine	200
	Inulin	200
	Alpha-lipoic acid	10
	Inositol	50
	Bitter orange fruit extract	335
	Green tea leaf extract	223
	Garcinia cambogia fruit extract	167
	Guarana seed extract	200
	Dandelion root extract	150
	Horsetail herb extract	150
	Bladderwrack extract	150
	Iodine	0.15
	Black pepper extract	5.3
	Piperine	5
	Cayenne pepper	100
	Capsaicin	0.3
	Thiamine	1.1
	Riboflavin	1.4
	Niacin	16
	Pantothenic acid	6
	Vitamin B6	1.4
	Biotin	0.5
	Folic acid	0.2
	Vitamin B12	0.025
	Chromium	0.2
#6	Oat fiber	3.33
	Artichoke Extract	20
	Garcinia cambogia fruit extract	12.5
	Sene extract	133
	Plum extract	5
	Ginger extract	5
	Kiwi extract	5
	Chromium	0.4
#7	Caffeine	101.5

	Garcinia cambogia fruit extract	80
	N-acetyl L-Carnitine	70
	Carmellia sinensis extract	50
	Black pepper	2.5
	Vitamin B6	1
#8	Artichoke Extract	600
	Citrus peel extract	200
	Vitamin C	80
	Chromium	0.1

Before GC-MS analysis all the commercial samples were prepared using the following procedure. The solid pills were macerated using a mortar and pestle, and the encapsulated pills were opened and the powder obtained were used to do the preparation. Using an analytical balance, precisely 50 mg of each individual sample was measured and placed into a 15 mL centrifuge tube. Then, 5 mL of methanol was added, and the tube was placed in an ultrasonic bath for 5 min. After this time, the tube was agitated (300 rpm) for 30 min using an orbital shaker incubator (Biosan ES-20/60). The samples were centrifuged for 10 min at 9000 rpm and finally the final obtained supernatant solution was diluted. For the dilution, 125 μL of the supernatant was measured to a volumetric flask of 10 mL and completed with methanol. Then, 120 μL of this solution was pipetted to a 2 mL glass vial, and then submitted to a drying procedure. After drying, 100 μL of the derivatizing reagent, MSTFA w/ 5% TMCS, was added into the vial which was placed in incubator at 90°C for 60 min. Finally, 100 μL of the final solution was transferred to a micro-insert and submitted to GC-MS analysis. The same sample preparation was used during the evaluation of the accuracy of the methodology.

5. Results and Discussion

5.1. Method Development

As a first step, a methanolic solution of 2,4-DNP was prepared and analysed by GC-MS using the conditions adapted from Campos et al. (2019) with the operation program set up in the equipment being designated as “Method_V1”. This method was selected due to its similarity to the analysis to be conducted throughout this work, as well as the complexity of the matrix involved. Therefore, the first operating conditions set in the GC-MS equipment were the following: helium was used as carrier gas with 1.6 mL/min of flow rate, splitless injection mode, the initial temperature programmed in the GC was 100°C with 1 min of hold time and then the temperature increased to 250°C with a rate of 30°C/min and a final hold time of 2.5 min. The temperature of the injector, MS source and transfer line were set to 265°C, 230°C and 150°C, respectively. When the splitless mode was used, the peaks in the chromatogram usually showed ion saturation in the MS spectrum, resulting in a loss of linearity of the curve. Therefore, as described in material and methods, the method was changed to use a split ratio of 1:5.

Initially, dilutions of the non-derivatized methanolic standard solution were analyzed, with concentrations of 100 ppm, 80 ppm, and 60 ppm. Dilutions with lower concentrations (20 ppm, 10 ppm and 5 ppm) did not evidence peaks in the chromatograms. The correlation between the mentioned concentrations that originated a visible peak (100 ppm, 80 ppm and 60 ppm) and the obtained areas was calculated to assess the analytical response, evidencing a weak correlation with an R^2 value of 0.9101. Moreover, the analysis of the non-derivatized compound resulted in non-defined peaks with tailing as shown in Figure 4. Consequently, it was decided to undertake a derivatization procedure.

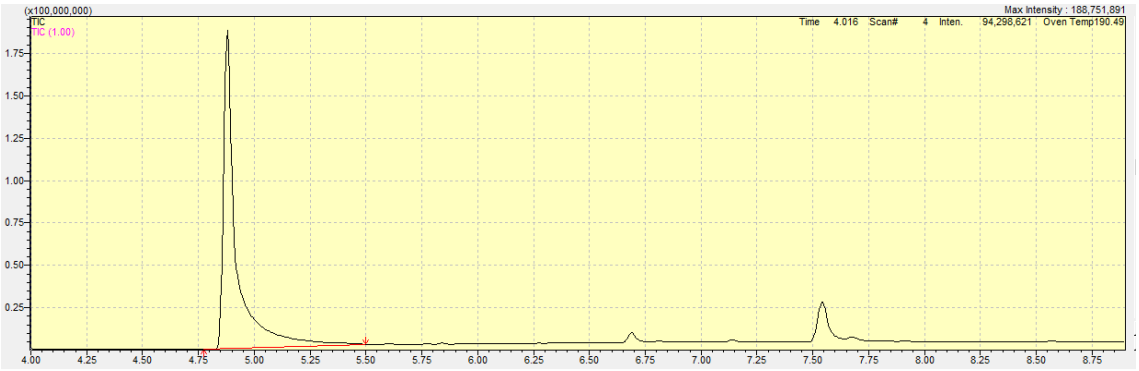

Figure 4. GC-MS chromatogram of 2,4-DNP standard (100 ppm) without derivatization, analyzed using the conditions of method #1.

The first derivatization attempt was carried out using a derivatizing solution containing BSTFA with 1%TMCS, and 3 different concentrations of 2,4-DNP, namely 20 ppm, 10 ppm, and 5 ppm. After analyzing the derivatized standard solutions, the correlation analysis between the areas and concentration of these 3 points corresponded to a R^2 value of 0.9917, evidencing the importance and necessity of carrying out the derivatization procedure in the development of the work. After this test, all the standards/samples were derivatized before being analyzed.

In this first analysis of the derivatized standard, the presence of 2 peaks was observed in the higher concentrations. Doing a similarity search against NIST 17, a library with more than 1.000.000 mass spectra, it was concluded that the peaks corresponded to the derivatized and underivatized 2,4-DNP, therefore suggesting that the derivatization reaction was not complete (as will be discussed in subsection “Derivatization procedure”).

Optimization of the operating conditions

In what regards the operation conditions of the GC-MS equipment, after testing the conditions proposed by Campos et al. (2019), named as Method#1, several changes were implemented aiming for improvements. First, to avoid the possible condensation of the compounds in the transfer line, this temperature was increased. Knowing that in a GC-MS method, the temperature of the transfer line should generally be set close to the temperature of the end of the GC column and the MS ion source to ensure efficient

transfer of analytes from the column into the MS without condensation, the interface temperature was changed from 150°C to 250°C (Method #2).

Moreover, it was determined that additional configurations/changes could be implemented to further improve the analysis time, selectivity (e.g. eliminate or minimize the minor peaks from the baseline) and further enhance the 2,4-DNP analysis. Because minor peaks appeared along the chromatogram due to the use of the derivatizing solution and Method#2 corresponded to a short chromatographic run, to guarantee that none of these minor interferents was being retained in the column, a second temperature ramp in the oven program was introduced. In Method#3, the initial temperature started in 100°C for 1 min, then the temperature increased 30°C/min until reach 250°C with a hold time of 6.50 min, and the final temperature was set to 270°C with a rate of 30°C/min. The total time of the run was 12.5 min. After, considering the retention time of the standard and aiming to improve the baseline and the peak shape, the temperature of the oven was set at 140°C, and the injector temperature was set in 280°C (Method #4). The decision to increase the injector temperature was based on the study of Kim et al. (1993), who performed a systematic study of flash heater derivatization (FHD) of nitrophenols using different derivatizing reagents and conditions. The authors concluded that FHD conducted at high temperature (280 °C) could be successfully used for nitrophenolic compounds not easily derivatized with conventional derivatization. Therefore, to further ensure the full derivatization of 2,4-DNP, it was decided to use the same injector temperature proposed by Kim et al. (1993), namely 280°C. This modification resulted in a faster chromatographic run and a better peak shape with improved resolution. After performing several analyses with different concentrations of standard (and different derivatizing reagents), it was observed that after 8 min, no other relevant peaks were present. Therefore, to reduce the duration of each reading, the final oven temperature in the ramp was eliminated, and the hold time at 250°C was adjusted for 4.50 min. These operating conditions, named Method#5 showed a better resolution of the peak of the target compound, and a shorter time of analysis

Simultaneously to these tests, the cut off time for the MS detector was reduced to 3 min to guarantee that the presence of any underivatized 2,4-DNP could be observed. In fact, depending on the concentration of the tested standard solution, two

peaks could be observed in the chromatogram using method #5: one at approximately 3.5 min and another at 4.5 min. The peak at 3.5 min corresponds to the non-derivatized 2,4-DNP, while the peak at 4.5 min corresponds to the derivatized 2,4-DNP as shown in Figure 5. The mass spectra of non-derivatized and derivatized 2,4-DNP is shown in Figure 6.

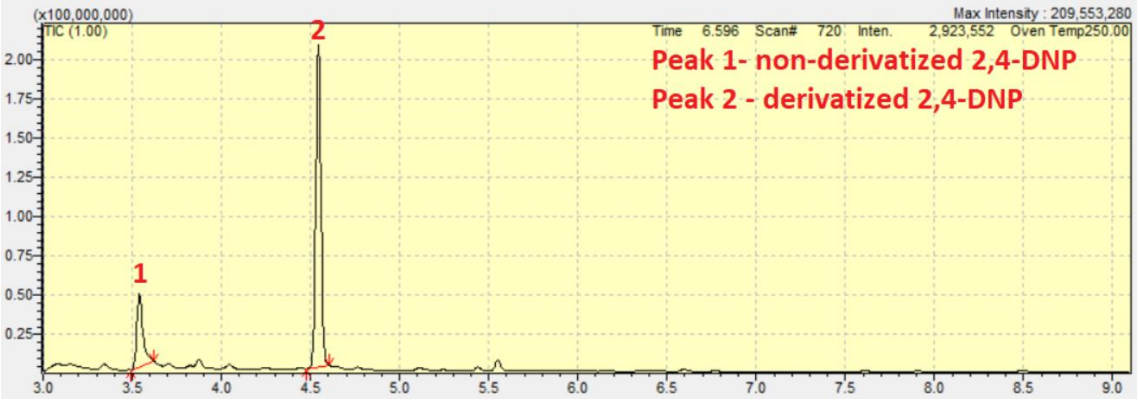

Figure 5. GC-MS chromatogram of 2,4-DNP standard (25 ppm) after derivatization with MSTFA w/ 1% TMCS analyzed using the conditions of method #5.

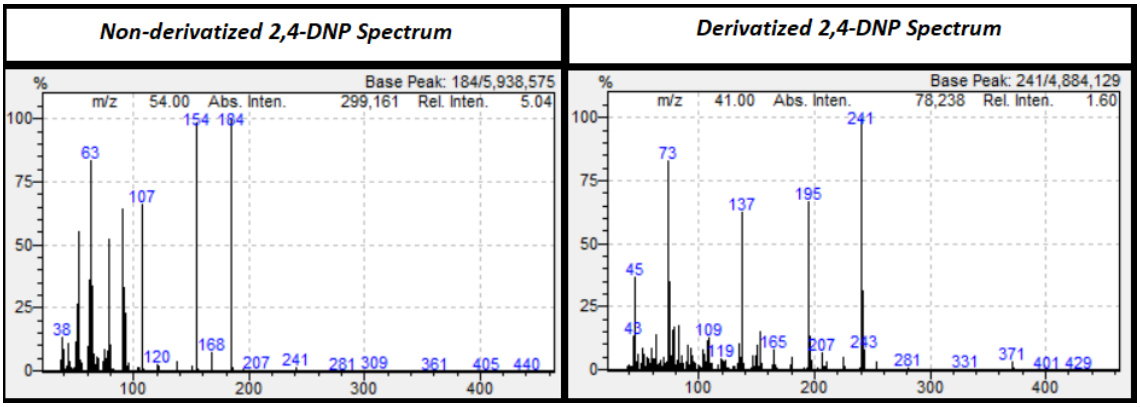

Figure 6. Non-Derivatized and Derivatized 2,4-DNP MS spectrum.

Derivatization procedure

Simultaneously with the instrument method development, tests with different derivatizing agents and different derivatization conditions were carried out. The other agent used to do the derivatization process was MSTFA w/ 5% TMCS and the change in derivatization conditions was using a higher temperature and time of derivatization.

Initially, derivatization tests were performed using the same derivatizing agent (BSTFA w/ 1% TMCS) and a temperature of 60 °C for 30 min, the tests were made equally, in the same day, and using the same method (method #5). As mentioned, that approach resulted in 2 peaks in the chromatogram, evidencing the incomplete derivatization of the compounds. Therefore, the temperature was raised to 90 °C and tested for 30 min and 60 min. To perform this comparison test, Full SCAN spectrometer configuration was used.

Using higher temperature and time of derivatization, and the same derivatizing agent, the 2,4-DNP peak shows a higher area in the chromatogram in the same concentration, as shown in Figures 7 and 8. So it was chosen to perform the derivatization with a higher temperature and time of derivatization, namely 90°C for 60 min.

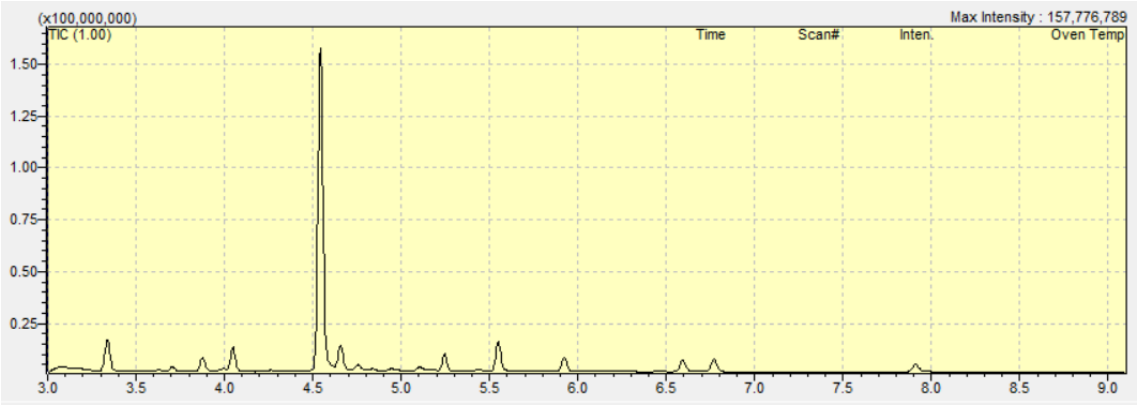

Figure 7. GC-MS chromatogram of 2,4-DNP standard (7.5 ppm) after derivatization with BSTFA w/ 1% TMCS using 30 minutes and 60°C derivatization

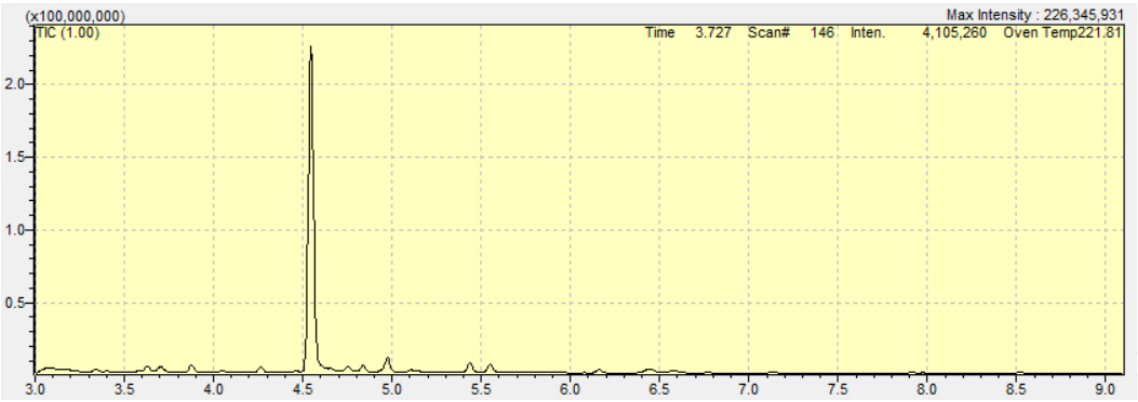

Figure 8. GC-MS chromatogram of 2,4-DNP standard (7.5 ppm) after derivatization with BSTFA w/ 1% TMCS using 60 minutes and 90°C derivatization.

In addition to the temperature and time conditions, another derivatizing agent was tested, namely MSTFA w/ 5% TMCS. MSTFA was selected according to the study of Kim et al. (1993) that showed the best performance of this agent in the derivatizing of different nitrophenols, including 2,4-DNP, when compared to several others.

To perform the comparison of derivatizing agents, all the tests performed were made using the same derivatization conditions (90°C for 60 min) and using the same standard concentration (0.5 ppm). The analysis of this standard, including the derivatization process and GC-MS analysis, was made in parallel on the same day and using the same method (method #6). Using BSTFA w/1%TMCS, the mean value of the peak area was 13,072,762 while using MSTFA w/5% TMCS, the mean peak area of the analyte was 38,857,539. Considering that the area of the 2,4-DNP peak was 197% higher using MSTFA w/5% TMCS, as shown in Figures 9 and 10, the derivatizing agent chosen to perform all the derivatization during the method validation was MSFTA w/5% TMCS.

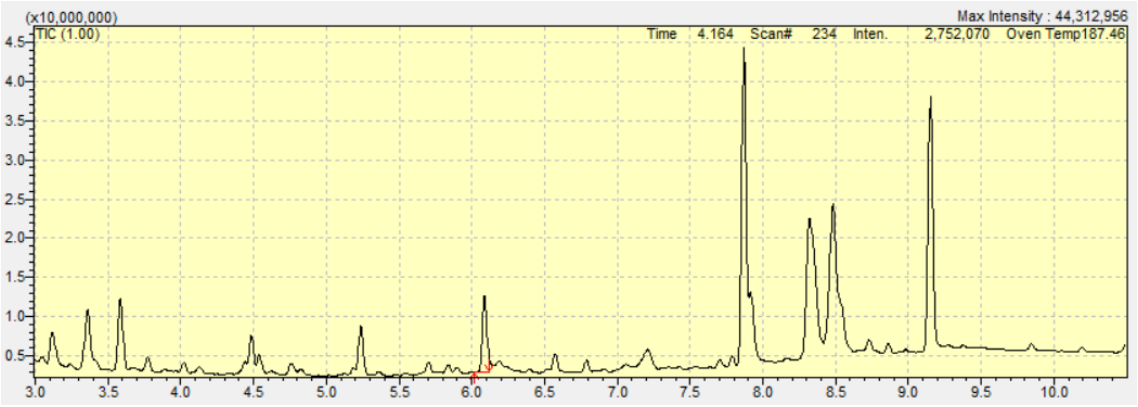

Figure 9. GC-MS chromatogram of 2,4-DNP standard (0.5 ppm) after derivatization with BSTFA w/ 1% TMCS using 60 minutes and 90°C derivatization.

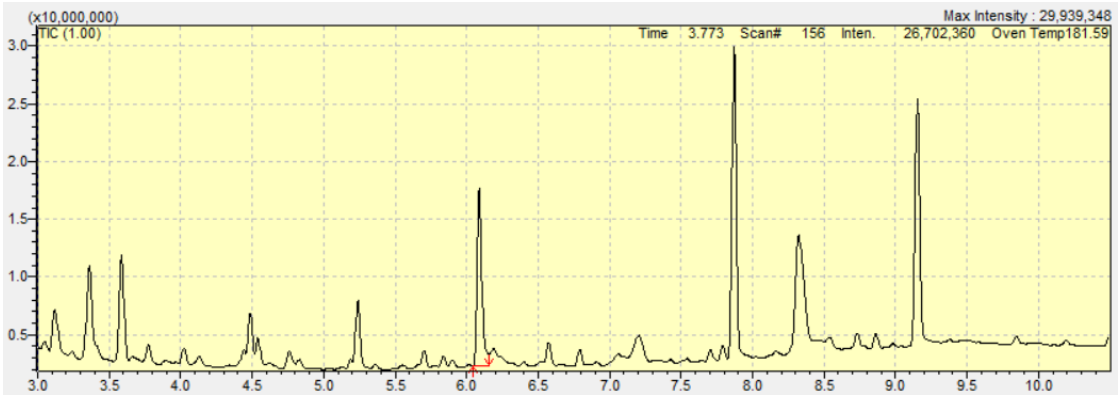

Figure 10. GC-MS chromatogram of 2,4-DNP standard (0.5 ppm) after derivatization with MSTFA w/ 5% TMCS using 60 minutes and 90°C derivatization.

Finally, it should be mentioned that during the analysis aiming for method optimization, it was noticed that the solvent used to clean the needle of the injector (methanol) could interfere with the derivatized compound, causing a shift in the reaction, i.e causing the reverse reaction to occur and originating the peak at 3.5 min (corresponding to the non-derivatized compound). The solvent that caused this interference was methanol, thereby leading to the conclusion that the solvent used to clean the injection needle could not have hydroxyl groups or other that could interfere with the derivatizing agent. In order to solve this problem, the solvent was changed to dichloromethane, once it was realized that hexane was not able to do a proper cleaning of the needle. Using hexane, sometimes the error "-03" appears in the injector, corresponding to the indication of plunger error/plunger being stuck. Another thing to highlight, is that the catalyst agent used in the derivatization process (TMCS) is an extremely oxidant compound, so metallic surfaces are susceptible of oxidation in contact with this liquid. Therefore, the process of needle cleaning turned out to be extremely important, to avoid the automatic injector error. As curiosity, Figure 11 is shown the results of metal contact with TMCS namely of the needle used to withdraw small volumes from the original container of the reagent to a vial to allow pipetting.

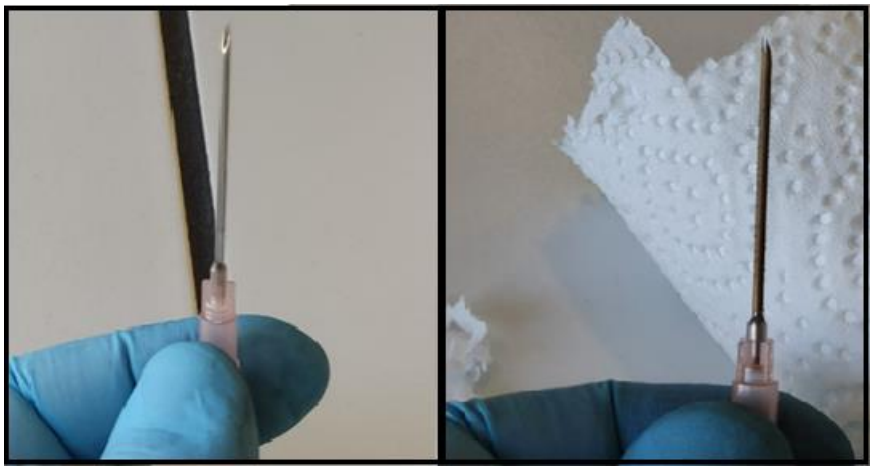

Figure 11. TMCS metal oxidation

MS acquisition mode

As can be observed in Figure 13, after derivatization (MSTFA w/5% TMCS) a variety of small peaks appear in the chromatogram. Therefore, to avoid the co-elution of these peaks with the analyte peak, another modification was made to the oven program, aiming for a better separation of the small peak shoulder near the target compound (2,4-DNP). Thus, the rate of temperature increase in the ramp was decreased from 30°C to 15°C per minute to enhance the separation of compounds. This resulted in a total run time of 10.5 min, with this method being designated as Method#6.

Method #6 was then used to compare the performance of different MS acquisition modes, SCAN and SIM. Using the SIM mode, the peaks related with the derivatizer were not detected/shown in the chromatogram, so this approach shows less peaks, cleaning the chromatogram of unnecessary peaks. The ions selected for the SIM mode were the 3 most abundant ions present in the spectra of the derivatized 2,4-DNP molecule. As shown in the Figure 6, the selected ions presented *m/z* of 241, 195 and 137.

Overall, the method used has the same configuration of Method #6, but this time, the SIM configuration in MS window was chosen, using the ions 241, 195 and 137. To distinguish from the method using SCAN mode, this was designated as Method #7. The result is shown in Figure 12.

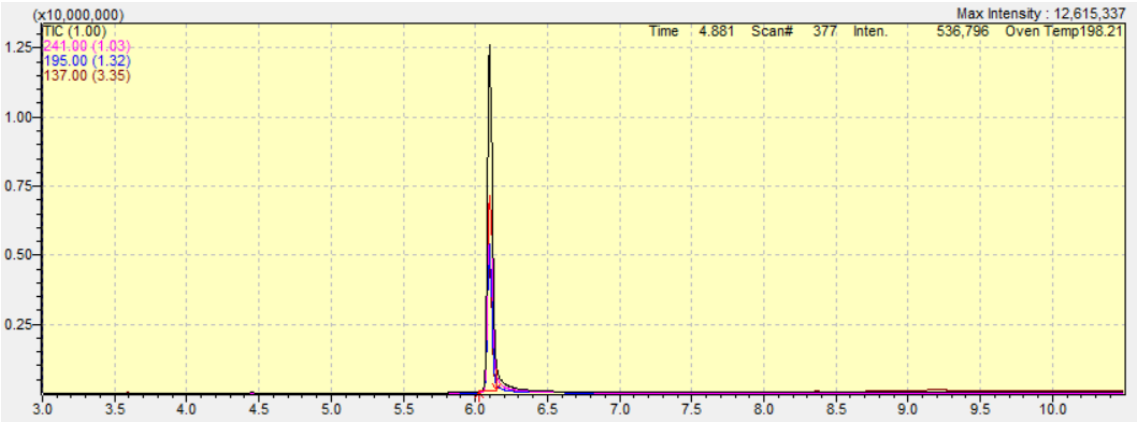

Figure 12. Method #7 Chromatogram of 2,4-DNP standard (1 ppm) after derivatization with MSTFA w/5% TMCS.

Another reason for choosing the SIM configuration is that the blank (MSTFA with 5% TMCS) produces a variety of peaks, which can make it challenging to detect low concentrations of 2,4-DNP due to the prominence of these peaks in the chromatogram. Therefore, to accurately detect low concentrations and ensure reliability, the SIM configuration was selected. The blank reading of MSTFA with 5% TMCS obtained using the Scan MS configuration is shown in Figure 13.

Figure 13. Blank MSTFA with 5% TMCS SCAN Chromatogram.

The two most significant peaks, according to the NIST17 library, correspond to palmitic acid (95% similarity) with retention time of approximately 7.85 min and stearic acid (94% similarity) elluting approximately at 9.15 min. The analysis time is maintained with more than 10 minutes to ensure that these 2 compounds do not interfere with the next injections. To exclude the possibility of the material being the source of these lipids, new glass material and discardable plastic pipet tips were used as well as a new bottle of

solvent. Thus, the origin of these peaks can possibly be from the compounds used during the derivatization procedure as they present a purity of 99.0%.

5.2. Method Validation

5.2.1 Specificity

For specificity purposes, the method must be performed in a manner that ensures the peak obtained corresponds to the compound under study (in this case, 2,4-DNP). Initially, the method was tested using SCAN mode because this mode allows for a wide range qualitative and exploratory analysis since the obtained spectra can be matched with spectra libraries, such as the NIST 17, using the "Similarity Search" function of the software. Nevertheless, after testing the method in SCAN mode, the SIM mode was selected due to its several advantages, such as improved sensitivity and selectivity. In particular, considering that PFS are complex matrices, using the SIM mode approach can help minimize matrix interferences since it focuses on specific ions of the target analyte, allowing for its more reliable detection, particularly in samples with challenging backgrounds. Although phenolic compounds present in the PFS are known to be thermolabile, as they contain hydroxyl groups, they can also be derivatized. In this scenario, using the SIM approach would allow for a “cleaner” baseline of the chromatogram and, expectably, a lower number of peaks in the samples, then increasing the selectivity of the methodology, once just the most abundant ions from the derivatized 2,4-DNP are targeted. The chromatogram comparison between the SCAN and SIM modes is shown in Figure 14.

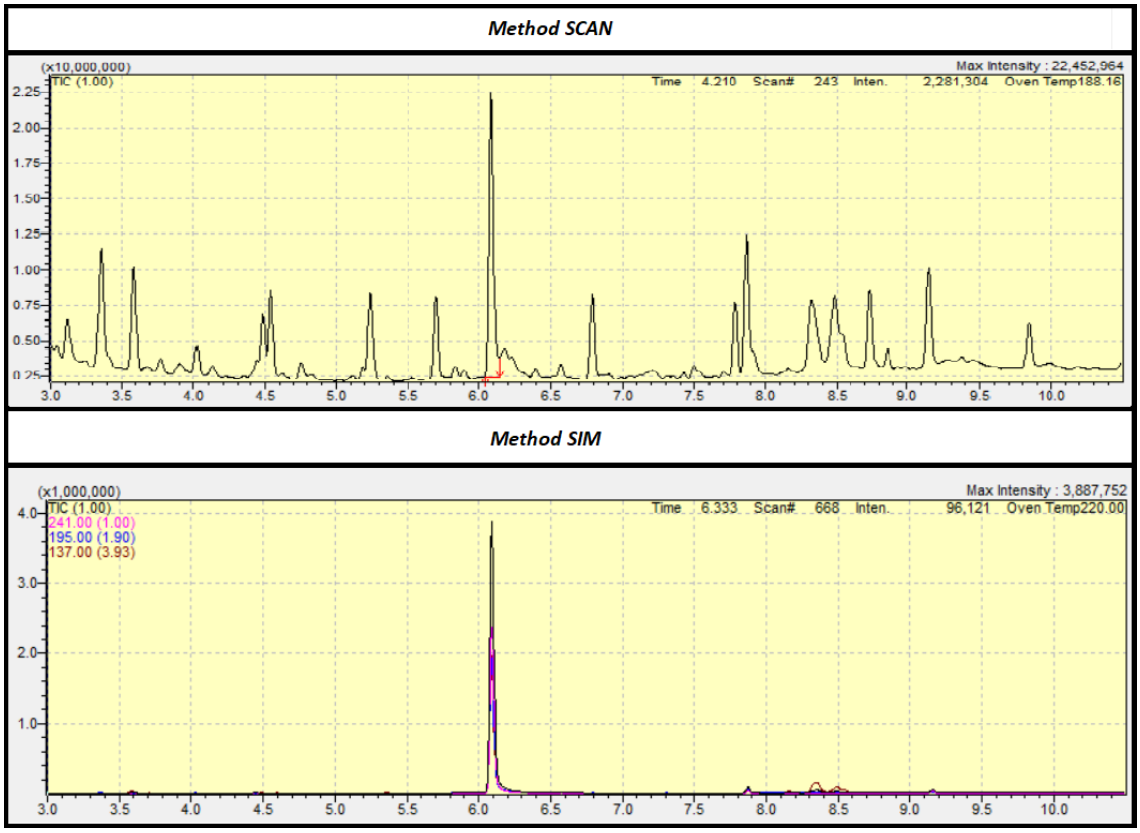

Figure 14. Comparison of the same analysis of derivatized 2,4-DNP using the SCAN (upper figure) and SIM method (lower figure).

In the chromatogram acquired in the SCAN mode, the peak present around 6 min is identified as the peak of 2,4-DNP based on the "similarity search" feature of the lab solutions software, which indicates a similarity of over 90% to 2,4-DNP as shown in figure 15 thereby confirming that the major peak at 6 min corresponds to 2,4-DNP.

Similarity Search Results

Report View Compound Info Process Help

Hit	Similar	Regi	Compound Name	Mol Wt	Formula	Library
1	91	☑	Phenol, 2,4-dinitro- $$ .alpha.-Dinitrophenol $$	184	C6H4N2O5	NIST17s.lib
2	90	☐	Phenol, 2,4-dinitro- $$ .alpha.-Dinitrophenol $$	184	C6H4N2O5	NIST17s.lib
3	89	☐	Phenol, 2,4-dinitro- $$ .alpha.-Dinitrophenol $$	184	C6H4N2O5	NIST17-1.lib
4	88	☐	Phenol, 2,4-dinitro- $$ .alpha.-Dinitrophenol $$	184	C6H4N2O5	NIST17s.lib
5	80	☐	Phenol, 2,4-dinitro- $$ .alpha.-Dinitrophenol $$	184	C6H4N2O5	NIST17s.lib
6	80	☐	Phenol, 2,6-dinitro- $$ o-Dinitrophenol $$ Phe	184	C6H4N2O5	NIST17-1.lib
7	80	☐	Phenol, 2,6-dinitro- $$ o-Dinitrophenol $$ Phe	184	C6H4N2O5	NIST17s.lib
8	80	☐	Benzene, 1-ethoxy-2,4-dinitro- $$ 2,4-Dinitroph	212	C8H8N2O5	NIST17s.lib
9	78	☐	Phenol, 2,5-dinitro- $$ Phenol, .gamma.-dinitro-	184	C6H4N2O5	NIST17s.lib
10	76	☐	3,4-Dinitrophenol	184	C6H4N2O5	NIST17-1.lib
11	75	☐	Benzenesulfonic acid, 2,4-dinitro- $$ 2,4-Dinitr	248	C6H4N2O7S	NIST17s.lib
12	75	☐	Benzene, 1-ethoxy-2,4-dinitro- $$ 2,4-Dinitroph	212	C8H8N2O5	NIST17-1.lib
13	71	☐	Phenol, 2,5-dinitro- $$ Phenol, .gamma.-dinitro-	184	C6H4N2O5	NIST17-1.lib

Figure 15. 2,4-DNP identification by carrying out a Similarity Search in NIST17 with Lab Solutions.

As mentioned, for the SIM mode approach, the three most abundant fragments of the compound were selected. Upon examining the spectrum of 2,4-DNP, it was noted that these three ions have *m/z* 241, 195, and 137. By using these three ions for the MS configuration in SIM mode, the chromatogram displayed a single relevant peak corresponding to all three ions, thereby confirming that this peak is from 2,4-DNP, as shown in Figure 14 (lower figure), and the specificity of the analysis.

Evaluation of Matrix Effect

Another test made to evaluate the specificity of the method, was the evaluation of the matrix effect, intending to access the interference of the matrix in the calibration curve parameters (slope, intercept, R^2). The test was performed by mixing/homogenizing all the samples, weighting the same mass used in a sample analysis and submitting it to the solid-liquid extraction process. The extract was added to each point of the calibration curve, and the the obtained solution evaporated, submitted to derivatization and analyzed using the optimized GC-MS methodology. The area values obtained from the calibration curve determined by adding the standards to the matrix extract were compared to the area values obtained by using a conventional calibration curve. The obtained result is shown in Table 4.

The calculated R^2 was 0.993, showing that the background matrix/compounds of the samples do not interfere in the calibration curve parameters. Another parameter evaluated was the percentage difference of the readings of the same standard point between the calibration curve constructed with the standards added to the matrix (spiked) and with the conventional calibration curve.

Table 4. Results of the obtained areas in the matrix effect analysis.

Concentration	ppm						
	0.05	0.1	0.5	2	4	5	R^2
Spiked	369113	755474	6745421	16237864	29048258	36725658	0.9933
Non-Spiked	403202	712378	7302650	17257226	29877168	38166353	0.9911
D%	8.82%	5.87%	7.93%	6.08%	2.81%	3.84%	

5.2.2 Linearity and range

For the assessment of linearity, the method must be executed in such a way that the linearity of the calibration curve remains above 0.99 in all analysis, both intra- and inter-day. For this purpose, the different points of the calibration curve are analyzed six times on different days and three times on the same day, with each reading yielding a correlation coefficient greater than 0.99. In the intraday testing, the readings for the same calibration point must not exceed a coefficient of variation of ≤15% for curve standards and ≤20% for the LOQ standard.

For this test, in addition to intra- and inter-day curve assessments, calibration curves were generated on different days to ensure the reliability of the method. The calibration curve was prepared and analyzed in different days, totalizing 5 days of analysis. The results are presented in Table 5.

Table 5. Results of the linearity analysis.

Day	0.05ppm Signal	0.1ppm Signal	0.5 ppm Signal	2 ppm Signal	4 ppm Signal	5 ppm Signal	R^2
1	356913	648718	6428522	21308456	44701520	52748046	0.9980
2	372760	645470	6950074	20669125	46967671	52174596	0.9924
3	298364	715689	7302650	27967734	54639570	60693581	0.9924
4	345903	822208	7588003	23995991	45177038	52471761	0.9949
5	226130	785134	7888121	22995148	44256566	50355884	0.9929
CV%	18.55%	10.98%	7.84%	12.32%	9.14%	7.49%	

All readings achieved the minimum correlation coefficient (R^2) value of 0.99, and the coefficient of variation of replicate analysis of the same calibration point did not exceed the value of 15% for all calibration points of the curve, with the exception of the LOQ which was lower than 20%.

Regarding the intraday precision assessment, the average of the readings reached a R^2 value of 0.995, and the coefficient of variation of the readings was no higher than 10% for all of the calibration curve points as shown in Table 6, demonstrating the linearity of the method in the considered range.

Table 6. Linearity analysis performed in triplicate.

Concentration	0.05ppm	0.1ppm	0.5 ppm	2 ppm	4 ppm	5 ppm	R^2
Signal	569758	974629	6430387	29158797	55111746	63687743	0.9955
	501489	868519	7162095	26888476	55736598	63794245	0.9964
	520717	850159	6543812	28226001	56721606	62961683	0.9930
Average	530654	897769	6712098	28091152	55856650	63481223	0.9953
CV%	6.63	7.48	5.86	4.06	1.45	0.71	

For the range parameter, the method must be developed such that the concentrations used in the calibration curve show a good linearity by yielding a correlation coefficient (R^2) greater than 0.99. Another factor to consider is the usability of this range, as the selected range must be compatible with the samples to be tested.

The selected range was from 5 ppm to 0.05 ppm, and the calibration curve provided a correlation coefficient greater than 0.99 for all analyses. Higher concentrations were tested but not considered because, at concentrations higher than 5 ppm, all 2,4-DNP selected ions in the mass spectrometer were saturated, as shown in Figures 16 and 17. This saturation caused a variability in the obtained areas, resulting in a decrease in the correlation coefficient. The correlation coefficient obtained respecting a range of 0.05 to 10 ppm of 0.8844, reaffirming the exclusion of the point 10 ppm and the choice of range from 0.05 to 5 ppm. Therefore, 5 ppm was established as the being the higher

concentration in the defined range. Moreover, the range chosen has a difference of 100 times from the lowest to the highest value in the curve, making it a valid range for studying the samples.

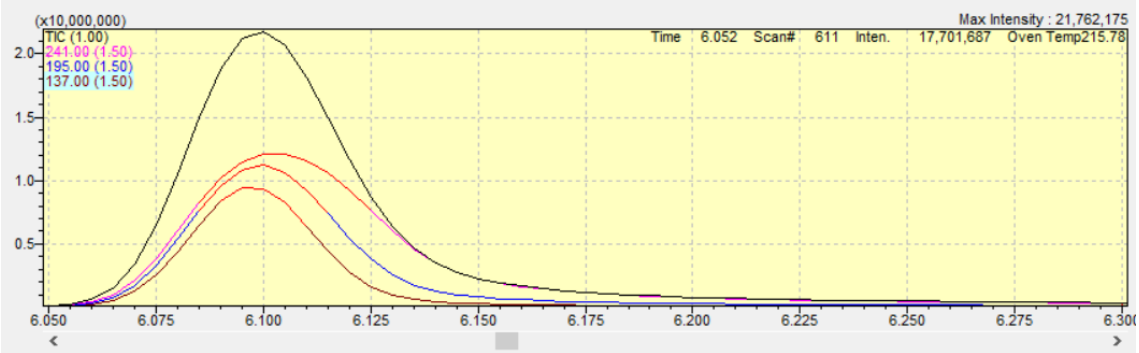

Figure 16. Analysis of 2,4-DNP standard (10 ppm) using the SIM method.

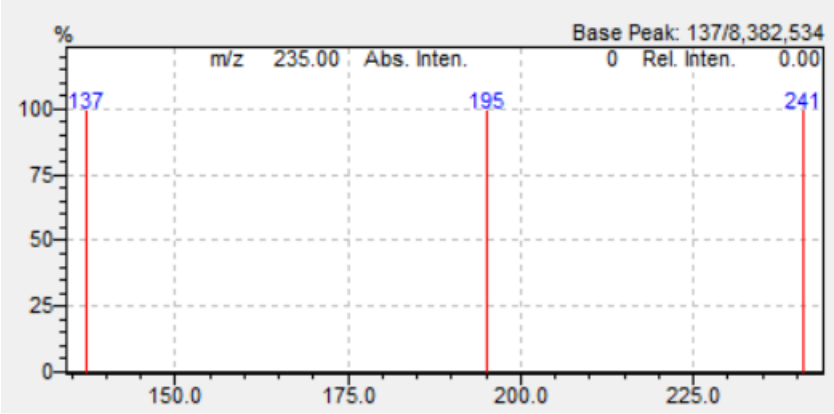

Figure 17. Mass Spectra of 2,4-DNP standard (10 ppm) acquired in the SIM mode.

5.2.3 Detection and Quantification Limit

For the detection limit there is no value required, but the value is calculated according to the calibration curve. The methodology used to estimate the value of detection limit was based on calibration curve parameters. Using this method, the lowest point of the calibration curve is considered the limit of quantification of the methodology.

As seen in Figure 18, the 2,4-DNP peak in the lowest point of the calibration curve is a peak with good signal and is differentiable from the noise of the equipment, being suitable to be considered the LOQ. Another parameter used to consider the LOQ as 0.05 mg/L was the coefficient of variation at this point. As shown in Table 6, the coefficient was less than 20% but very close to the limit. Lower concentrations were tested in replicates and showed a high variation in the areas of the 2,4-DNP peak (>20%), therefore demonstrating they were not suitable for use as a LOQ.

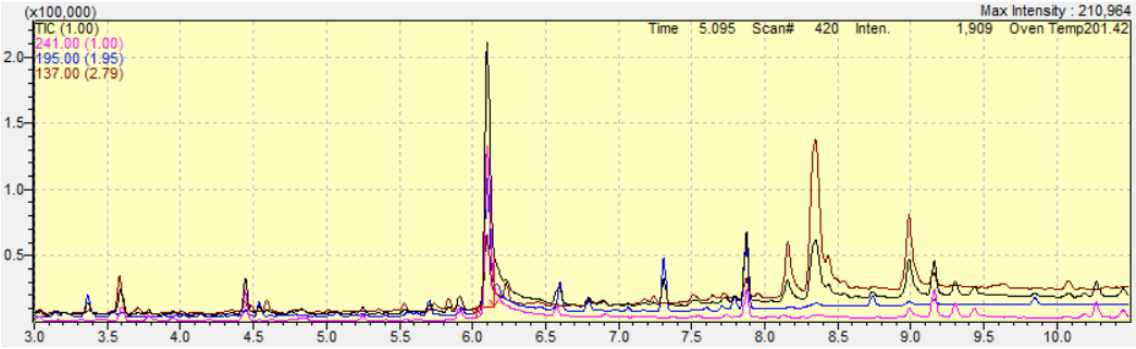

Figure 18. Chromatogram obtained for the analysis of 0.05ppm calibration point.

In this work, the lowest point of the calibration curve was determined as being the LOQ of the method and equation 4 was used to determine the LOD. Therefore, the LOQ value of the method was determined as 0.05 ppm of 2,4-DNP and the LOD was established as 0.015 ppm of 2,4-DNP.

5.2.4 Precision and Repeatability

For the evaluation of precision, one intermediate point of the curve was chosen and analysed 6 times, in 6 different days. The coefficient of variation in each day and putting all the readings together should not trespass the value of 15%. The results of the GC-MS analysis regarding to point 0.5 ppm (intermediate point of the calibration curve) inter and intraday are shown below on Table 7.

Table 7. Coefficient of variation calculation.

Date of Analysis	Reading	Peak Area	CV%
27/06/2024	1	6821470	2.24
	2	6826096	
	3	6977038	
	4	7237029	
	5	6980023	
	6	7069601	
28/06/2024	1	7312612	2.86
	2	7763474	
	3	7922630	
	4	7605446	
	5	7810177	
	6	7839359	
04/07/2024	1	7888121	6.15
	2	8658305	
	3	7654088	
	4	7487604	
	5	7435412	
	6	7405389	
05/07/2024	1	7758618	7.49
	2	6986064	
	3	6869650	
	4	6792776	
	5	7922755	
	6	7956668	
08/07/2024	1	7588003	3.86
	2	8275541	
	3	7952484	
	4	7592640	
	5	7563858	
	6	7537760	
10/07/2024	1	7778366	1.96
	2	7647314	
	3	7761035	
	4	7725904	
	5	7764217	
	6	7385855	

The calculated coefficient of variation for interday precision was 5.68%. Considering this value and that none of the intraday coefficients of variation surpassed the value of 15%, the method shows a good repeatability and can be considered precise.

5.2.5 Accuracy

For the evaluation of the accuracy, the method must be developed to ensure that the samples analyzed after the calibration curve show no more than 10% relative error from the true value (i.e., samples with known concentrations prior to testing). To achieve this, various samples with different matrices are spiked with known amounts of the adulterant and analyzed after the calibration curve. The concentration of target compound in each sample is calculated, and the error, based on the theoretical concentration, should not exceed 10%.

For this test, samples from different brands and with various matrices were selected (Table 3). The samples were first extracted and tested without the addition of the studied compound (2,4-DNP), aiming to evaluate and detect the eventual presence of the compound and, in case of absence to confirm that none of the brands were adulterating their products. After analysing all the extracted commercial samples, the samples were adulterated (spiked) with 2,4-DNP at different concentrations and tested again to assess the accuracy of the methodology in determining the concentration of the adulterant.

The analysis conduceted before spiking with 2,4-DNP, also allowed to infer if the product had any component in its composition that could interfere in the 2,4-DNP peak (presenting the same retention time). The chromatograms of the supplements are shown in Figures 19 to 26.

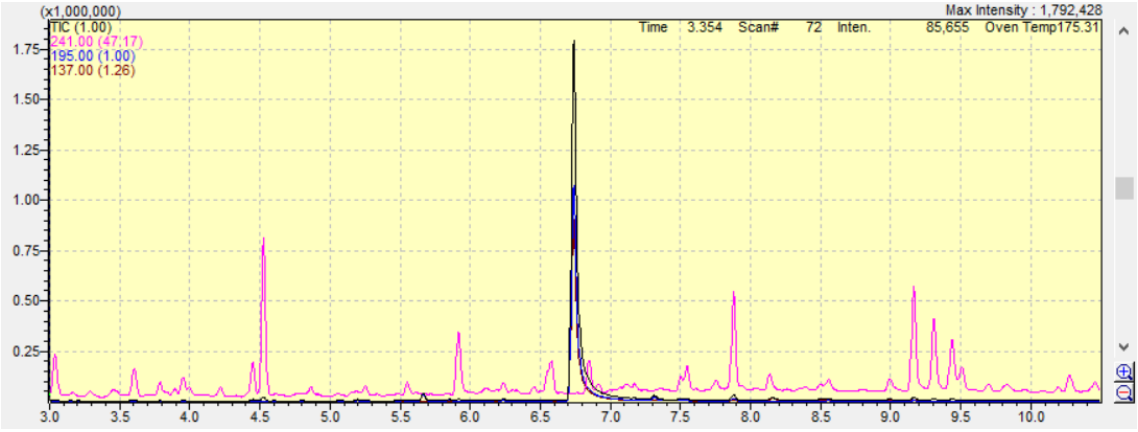

Figure 19. Chromatogram obtained for sample #1 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

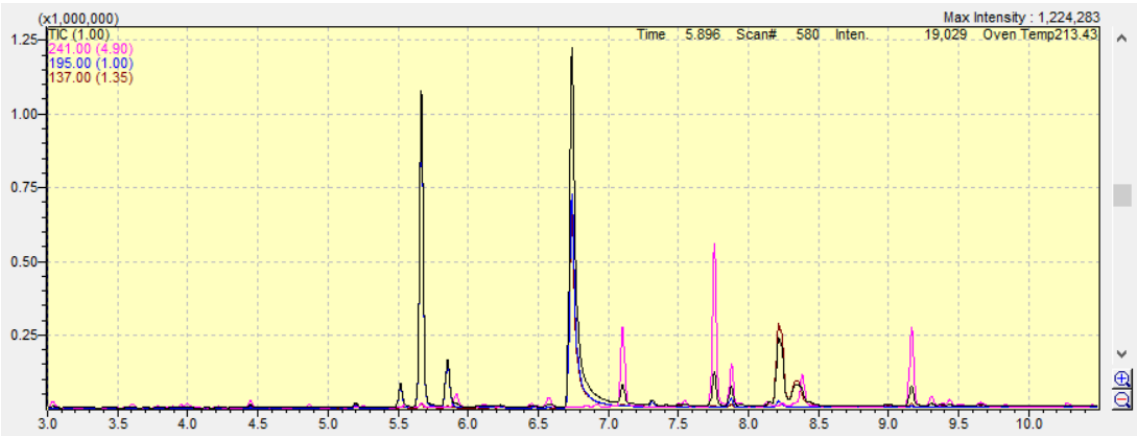

Figure 20. Chromatogram obtained for sample #2 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

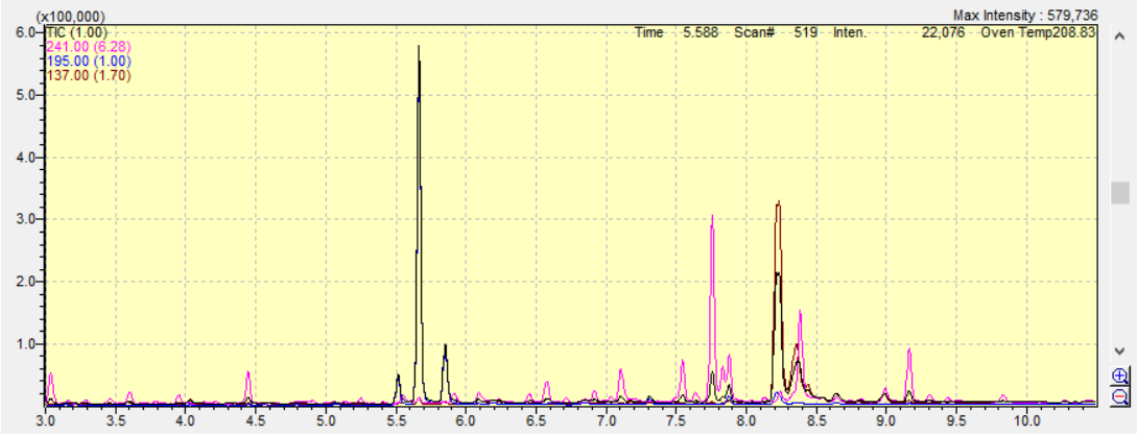

Figure 21. Chromatogram obtained for sample #3 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

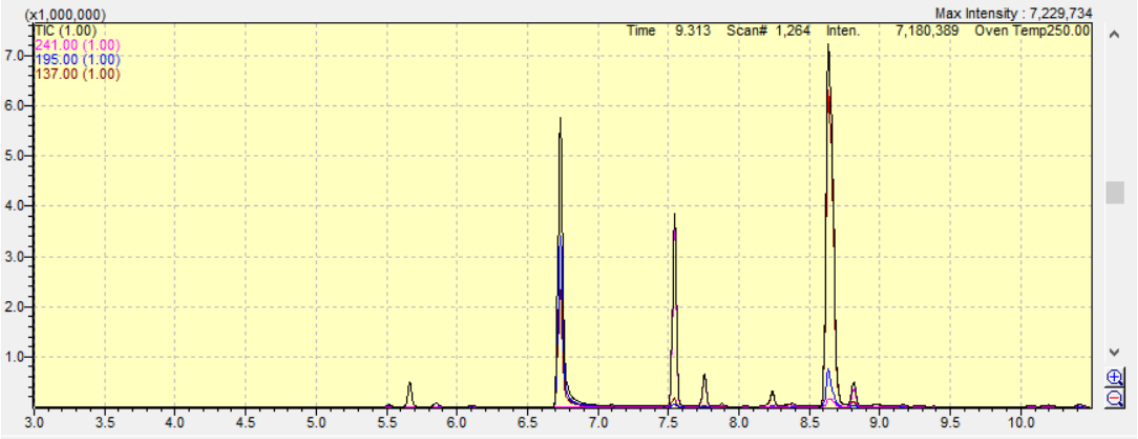

Figure 22. Chromatogram obtained for sample #4 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

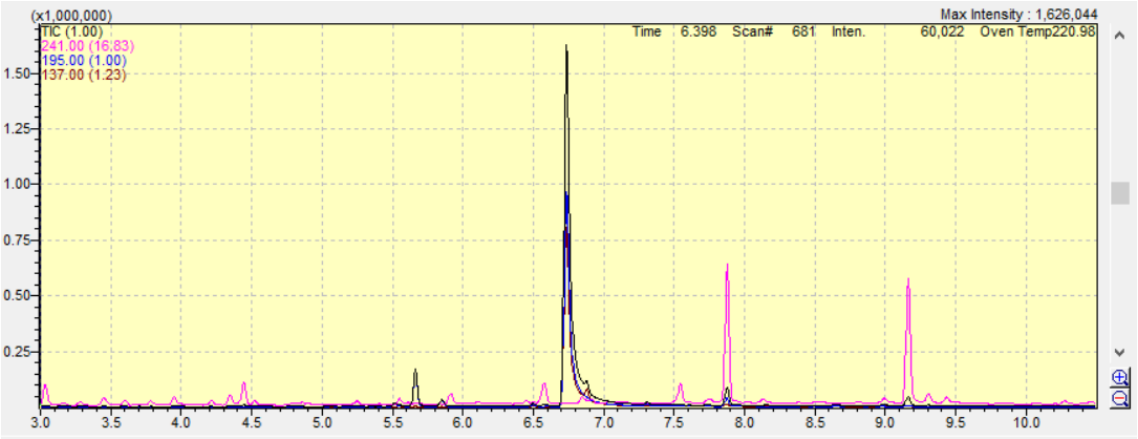

Figure 23. Chromatogram obtained for sample #5 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

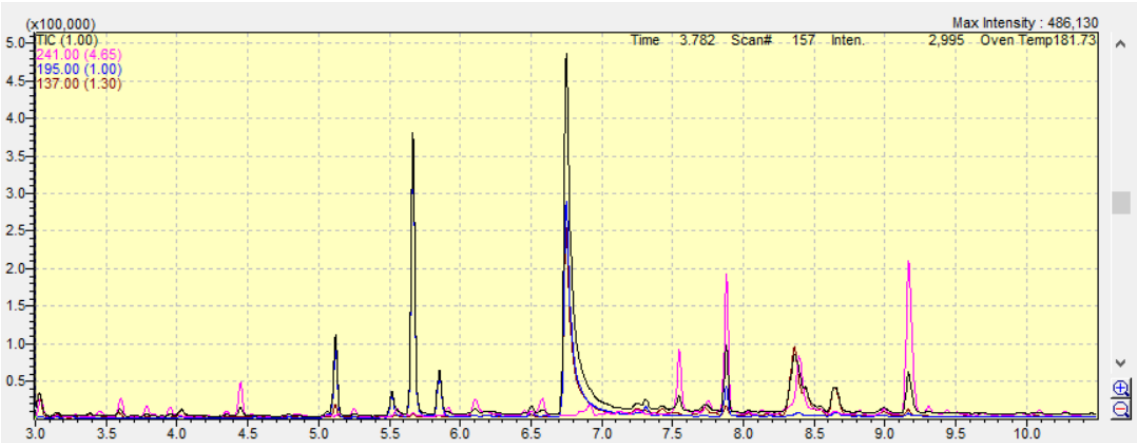

Figure 24. Chromatogram obtained for sample #6 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

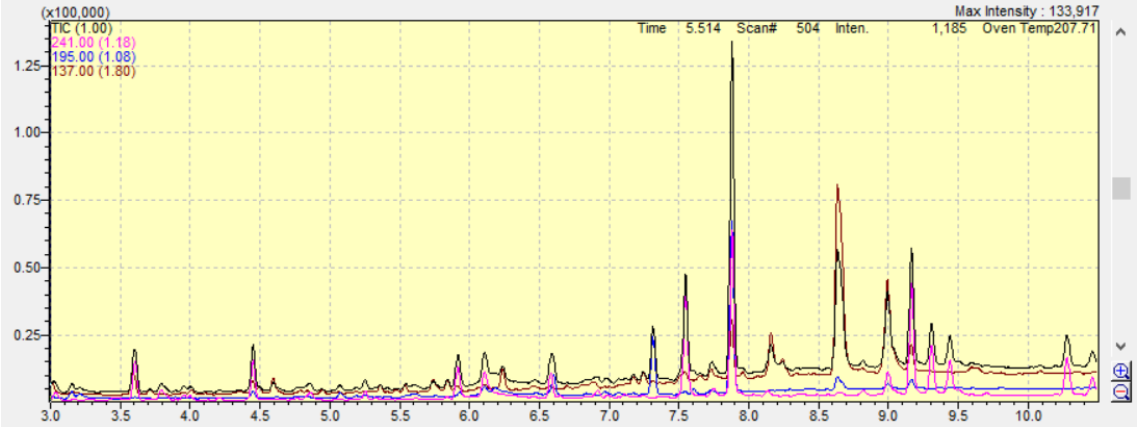

Figure 25. Chromatogram obtained for sample #7 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

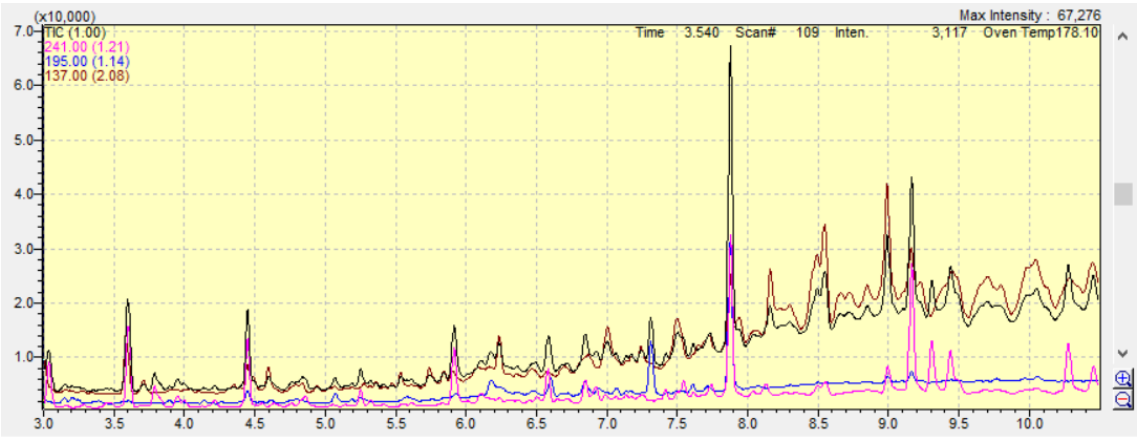

Figure 26. Chromatogram obtained for sample #8 derivatized with MSTFA w/5% TMCS analyzed using conditions of method #7.

As can be observed, none of the supplements has a peak in the 2,4-DNP peak area (approximately from 6.05 to 6.20 min), so the components of each supplement do not interfere on the area of the 2,4-DNP peak, moreover, none of the supplements has 2,4-DNP in its composition.

The samples were tested using method #7, derivatized with MSTFA w/5% TMCS. The procedure of derivatization was the same for all the samples. The tests were carried out in different days, so the calibration curves used were derivatized in different days. The weight of 2,4-DNP spiked in the samples was selected in order to be below and above the described daily maximum amount that could cause death (approximately 150mg per

day), in order to have samples in all the intervals of the range. The weight of 2,4-DNP in each sample, the weighed mass of the sample, and the amount of 2,4-DNP theoretically in the sample are shown in Table 8.

Table 8. Samples spiked with 2,4-DNP used in the evaluation of accuracy.

Sample/type	Replicate identification	Sample Weighed (mg)	2,4-DNP spiked (mg)	2,4 DNP per capsule (mg)	Amount per day
#1/Capsule	1	58.3	13.0	123.27	3 capsules
	2	55.0	5.07	50.7	
	3	50.4	11.93	130.18	
#2/Capsule	1	48.1	10.1	188.98	4 capsules
	2	49.7	2.57	46.54	
	3	49.9	8.69	156.73	
	4	56.6	17.53	278.75	
#3/Capsule	1	51.8	5.79	106.66	1 capsule
	2	48.9	8.63	135.89	
	3	57.5	12.39	157.08	
#4/Tablet	1	53.7	2.13	28.56	1 capsule
	2	45.9	7.29	114.35	
#5/Capsule	1	51.0	12.07	146.73	3 capsules
	2	49.8	12.77	221.23	
#6/Capsule	1	55.4	8.43	78.52	4 capsules
#7/Tablet	1	52.1	1.36	7.96	1 tablet
	2	47.2	4.53	29.27	
#8/Tablet	1	49.8	4.27	102.89	4 tablets
	2	54.3	11,39	251.71	

After the treatment of the data obtained for each sample, the concentration was expressed in ppm. Table 9 shows the concentrations calculated based on the weight of 2,4-DNP added to the samples, as well as the concentrations determined through testing using the GC-MS methodology.

Table 9. Results of the evaluation of the accuracy of the method (as relative error, E%).

		2,4-DNP		
Sample	Sample Identification	Theoretical Concentration (ppm)	Calculated Concentration (ppm)	E%
#1	1	3.87	3.54	9%
	2	1.52	1.38	9%
	3	3.57	3.67	3%
#2	1	3.01	2.98	1%
	2	0.77	0.81	5%
	3	2.61	2.74	5%
	4	5.26	5.06	4%
#3	1	1.74	1.82	5%
	2	2.59	2.70	4%
	3	3.72	4.07	9%
#4	1	0.64	0.69	8%
	2	2.19	2.30	5%
#5	1	3.62	3.83	6%
	2	3.83	4.16	9%
#6	1	2.53	2.71	7%
#7	1	0.41	0.37	10%
	2	1.36	1.22	10%
#8	1	1.28	1.35	5%
	2	3.41	3.64	7%

After the preparation and determination of the concentrations in the samples, the E% (relative error) was measured. The results are shown in the last column of Table 9, and none of them exceeded a 10% variation. This coefficient is calculated based on the expected result (theoretical 2,4-DNP concentration) and the calculated result (calculated 2,4-DNP concentration).

Based on the obtained results (not surpassing the value of 10% of E%), the method shows to be accurate.

5.3. Final Considerations

During the method development, some considerations were made about the methodology, related to the derivatization process, solvent use, derivatization temperature, and storage of the samples.

About the derivatization procedure, 2 different derivatizing reagents were used, MSTFA w/ 5% TMCS and BSTFA w/ 1% TMCS, both of them generated good chromatograms in GC-MS system, but one of them performed better in what concerns the range and quality of the chromatogram. The MSTFA w/ 5% TMCS generated better resolution in the chromatogram, as well as a lower detectable concentration of 2,4-DNP. Because of this, MSTFA w/ 5% TMCS was chosen and used during the method validation.

About the solvent used, 3 principal errors were realized, first of them was the needle cleaning with methanol, it causes a reverse reaction of the derivatizer, causing 2 peaks in the chromatogram, one referring to the non-derivatized 2,4-DNP and the other related to derivatized 2,4-DNP. After using others solvent cleaners of the injectors, the non-derivatized peak disappears, showing that the methanol cannot be used as solvent cleaning. The other problem related to solvent cleaner in the injector is related to poor cleaning of the injector. After the use of methanol to clean the samples, hexane was used, but the error “-03” started to appear in the auto sampler, an error referent to a problem in cleaning of the auto sampler. After testing with other solvents, the better reaveled to be dichloromethane, since it provided a proper cleaning of the injection needle while avoiding the the error “-03” due to the automatic injector being stuck. Additionally, it was verified that before sample injection, the solvent cleaning of the needle could cause higher coefficients of variation, particularly for lower concentrations. Therefore during the validation, this step was avoided and instead a higher number of solvent cleaning cycles was programed after the sample was injected.

About the derivatization temperature, the study of Campos et al. (2019) use the temperature of derivatization of 60°C for 30 min and BSTFA w/ 1% TMCS as derivatizing agent. For the current study, it was noticed that using these derivatization conditions, the derivatization was not complete, generating a visible peak of non-derivatized 2,4-DNP. Therefore, the derivatization conditions were changed to 90°C for 60 min, allowing

for a complete reaction and no longer causing the non-derivatized peak in the chromatogram.

6. Conclusion and Future Perspectives

In this study, a methodology to determine the concentration of 2,4-DNP in plant food supplements was developed and validated based on the criteria of EMA guideline "ICH method validation procedure". The final optimized methodology was able to determine the target compound (2,4-DNP) in concentrations ranging between 5 ppm and 0,05 ppm with a mean correlation coefficient of 0.995. All the necessary validation specifications were met, including specificity, linearity, precision, and accuracy , according to the proposed values. The limit of quantification (LOQ) of the method was established as being the calibration point with the lowest concentration, corresponding to a value of 0.05 ppm, and the limit of detection (LOD) was calculated to be 0.015 ppm. The method demonstrated robustness, accuracy, and repeatability through extensive testing. The complex matrices present in the plant food supplements did not interfere with the detection of 2,4-DNP, indicating the method's suitability for such samples. Considering all validated parameters, the method can be considered reliable for determining the presence and concentration of 2,4-DNP in plant food supplements. This methodology can be applied across various sectors, including industry, supplement manufacturing, regulatory agencies, and government institutions, to help prevent consumer exposure to 2,4-DNP. In this work, to demonstrate the practical applicability of the method, it was further applied to 8 commercial samples of PFS for weight loss. The method demonstrated the absence of the target compound in the commercial samples.

Several opportunities for future improvements and studies have been identified. One possible enhancement would be testing the method on different GC-MS systems to confirm its validity across varying equipment. Further interlaboratory studies would be valuable to ensure the method's repeatability in different laboratory environments, as well as with different analysts, thereby increasing confidence in its robustness. Compared to existing methodologies, this newly developed method stands out due to its ability to detect 2,4-DNP in complex matrices. The methodology developed here can inspire further research into detecting this compound in other complex samples.

Future studies could focus on the use of GC with a triple quadrupole MS, since that would allow establishing qualifier and quantifier ions and undoubtedly identify the

compound while further improving the sensibility and selectivity of the method. This approach would further improve accuracy, and the limits of detection and quantification, once the triple quadrupole permits the use of the multiple reaction monitoring during the analysis.

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