Chemical identification of 2,4-dinitrophenol (2,4-DNP) by GC-MS in 'fat burners' and studies of its *in vitro* hepatotoxicity

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I certify that the research described herein is original.

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ABSTRACT

During the last decades, we have witnessed unparalleled changes in human eating habits and lifestyle, intensely influenced by culture and society pressure. Sports practice has been strongly implemented in daily routines and visits to the gym peaked driven by the indulge in intensive 'weight loss programs'. Also, the pledge of boasting a healthy and beautiful body, all instigate the use of very attractive 'fat burners', which are products easily available in market, called safe, and known as a quick way to reduce body weight. Along with the boosted consumption of all those new formulations that daily appear on the market claiming high efficacy on weight loss, the number of reported poisonings also increase. A major reason for concern is the fact that the substances listed on these product labels are not effectively present in the supplements and it is also frequently observed the presence of substances in the formulations that are not labelled at all. One eminent example of such substances is 2,4-dinitrophenol (2,4-DNP).

2,4-DNP is well-known for its weight loss enhancer properties and has been associated with a class of the dietary supplements called 'fat burners'. By interacting with the cellular metabolism and increasing muscle definition, 2,4-DNP became very attractive as an adjuvant in weight loss processes and attracted the attention of the bodybuilder's community. Its interest and use is of highest concern also among adolescents and adults who fight overweight.

Since 1938, 2,4-DNP has been banned as a product for human consumption in many countries, due to its adverse effects. The main symptoms associated with 2,4-DNP intoxication include hyperthermia, tachycardia, decreased blood pressure and acute renal failure, which motivated a worldwide warning issued by the Interpol Anti-Doping Unit, reinforcing its dangerousness. Information on the effects of 2,4-DNP mainly derive from the intoxication cases reported by the emergency care units, and there is no specific antidote or treatment. For these reasons, the development of analytical methods that quickly allow the detection and identification of this substance in commercial products and in intoxicated individuals is therefore of utmost importance both for forensic and clinical purposes.

In this study, we intended to chemically identify the components of two products marketed online as 'fat burners' (Supplement 1 and Supplement 2) by gas chromatography coupled to mass spectrometry (GC-MS) analysis and to verify the presence or absence of 2,4-DNP in these formulations. In addition, we aimed to evaluate

the potential hepatotoxicity of these commercial products and compare the elicited effects to those of 2,4-DNP.

Representative samples of capsules of Supplement 1, as well as the capsules included in the commercial small plastic bags of Supplement 2 were analysed. The content of the capsules was extracted with methanol and subsequent GC-MS investigations identified caffeine, glycerol, fatty acids (including 2-palmitoylglycerol or glycerol monostearate) and sugars (glucose, sucrose and lactose) as the main constituents. No significant qualitative variability was observed in products sold with the same trade name. The comparison with the GC-MS data with the authentic analytical standard excluded the presence of 2,4-DNP in the tested products. Results from the quantitative analysis of caffeine contained in the Supplement 2 products show that the daily supplementation indicated by the manufacturer exceeds the recommended daily dosage (approximately 400 mg *versus* 848 mg of caffeine per day).

Furthermore, *in vitro* bioassays performed for toxicological investigations revealed that primary hepatocytes of Wistar rats isolated through a two-step collagenase perfusion exhibited a concentration-dependent mortality when exposed to 2,4-DNP at concentrations ranging from 549 nM to 10 mM, at 37° C for 24 h, as assessed by three viability assays [neutral red uptake assay: EC_{50} 98.05 µM; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay: EC_{50} 96.82 µM; lactate dehydrogenase leakage assay: EC_{50} 174.83 µM]. These results indicate that the mitochondria was the most sensitive organelle to 2,4-DNP-induced toxicity, followed by lysosome and by the cytoplasmic membrane. Then, the mechanisms contributing to the observed cytotoxicity were scrutinized by exposing cells at 1 µM, 10 µM, 50 µM, 100 µM, and 200 µM of 2,4-DNP for 24 h. A significant concentration-dependent increase of intracellular reactive species ($p<0.01$, at ≥50 µM; Dunnett's test) concurrent to a concentration-dependent decline in antioxidant defences [decreased reduced glutathione (redGSH; $p<0.01$, at ≥100 µM; Dunnett's test); decreased total glutathione ($p<0.01$, at ≥100 µM; Dunnett's test); increased oxidized glutathione (GSSG; $p<0.05$, at ≥1 µM; Dunnett's test); and decreased redGSH/GSSG ($p<0.001$, at ≥1 µM; Dunnett's test)]. Significant increase of mitochondrial membrane potential was observed at 10 µM and 50 µM ($p<0.05$; Holm-Sidak's test), which was in accordance with the exacerbated synthesis of adenosine triphosphate (ATP; $p<0.0001$, at 10–100 µM; Dunnett's test). Energy-dependent instigation of apoptosis was also confirmed by activation of pro-caspase-8 at all tested concentrations ($p<0.05$; Dunnett's test) and by the increased activities of caspase-3 and -9, at a concentration of 10 µM on higher ($p<0.05$; Dunnett's test).

In summary, the results presented herein reveal the absence of 2,4-DNP in both tested commercially available ‘fat burners’, suggesting that these new formulations of Supplement 1 and Supplement 2 are safer from this component. In fact, we prove in this work that the consumption of weight loss products containing 2,4-DNP produce toxicological effects. According to our results, 2,4-DNP seems to accelerate hepatocellular metabolism through instigation of ATP formation at the mitochondria. This effect might be responsible by the observed oxidative burst that in turn could activate cell death signalling pathways through intrinsic and extrinsic pro-apoptotic mechanisms. This information is crucial to reinforce the dangers associated with 2,4-DNP as a substance used for weight loss.

Keywords:

‘Fat Burner’; 2,4-dinitrophenol (2,4-DNP); GC-MS; *in vitro* hepatotoxicity; primary rat hepatocytes; oxidative stress; apoptosis.

RESUMO

Nas últimas décadas, os hábitos alimentares têm vindo a sofrer enormes alterações, influenciados pelo estilo de vida e devido à pressão exercida por parte da sociedade para a obtenção de um corpo aparentemente saudável. O desporto tem sido fortemente implementado na rotina diária das pessoas que optam por estilos de vida saudáveis e as idas ao ginásio tornaram-se frequentes, muito motivadas por programas intensivos de "perda de peso". Hoje em dia, o prazer de ter um corpo saudável e bonito começa a prevalecer sobre as simples "dietas". Deste modo, e a par com os avanços da indústria, todos os dias surgem no mercado novas formulações rotuladas como seguras que prometem total eficácia nos resultados obtidos. Estes produtos tornaram-se muito aliciantes e o seu consumo tem vindo a aumentar juntamente com o número de relatos de intoxicações. O seu perigo é potenciado por não constarem do rótulo os verdadeiros constituintes do produto ou por conterem outros que não constam do rótulo.

Atualmente, o 2,4-dinitrofenol (2,4-DNP) tem vindo a ser associado a uma classe de suplementos dietéticos denominados de "queimadores de gordura", sendo conhecido como uma substância potenciadora da perda de peso. Os seus efeitos de interação com o metabolismo e o aumento da definição de massa muscular têm estimulado o interesse da comunidade de fisiculturistas. No entanto, a sua procura tem-se intensificado por parte de adolescentes e adultos que combatem o excesso de peso. Desde 1938 que a sua comercialização enquanto produto destinado ao consumo humano foi proibida devido aos seus efeitos secundários. Os principais sintomas que estão associados à intoxicação por este composto incluem hipertermia, taquicardia, diminuição da pressão arterial e falha renal aguda. Recentemente, foi lançado um alerta mundial por parte da Unidade Contra a Dopagem da Interpol reforçando a sua perigosidade, atribuindo maior ênfase à proibição de comercialização. Os dados existentes sobre os efeitos do 2,4-DNP provêm apenas dos casos de intoxicação que chegam às unidades de saúde hospitalar, não existindo ainda um antídoto ou um protocolo de tratamento estabelecido que se possa aplicar. Assim, justifica-se a necessidade de aprofundar o conhecimento sobre este composto tornando-se essencial o desenvolvimento de metodologias analíticas que permitam a sua deteção e quantificação neste tipo de produtos comerciais, para fins clínicos e forenses.

Um dos objetivos deste estudo passou por identificar quimicamente os componentes presentes em dois produtos comercializados *online* como "queimadores de gordura" (Suplemento 1 e Suplemento 2), recorrendo à técnica de cromatografia gasosa acoplada à espetrometria de massa (GC-MS), de modo a verificar a presença ou ausência de 2,4-

DNP nestas formulações. Este trabalho destinou-se ainda a avaliar os efeitos hepatotóxicos destes mesmos produtos e do 2,4-DNP individualmente, usando cultura primária de hepatócitos de rato Wistar como modelo *in vitro*.

A análise por GC-MS é considerada uma metodologia analítica altamente sensível e foi aplicada para avaliar a composição real destes dois "queimadores de gordura". Foi analisado o conteúdo de uma amostra representativa das cápsulas de Suplemento 1 bem como de algumas das cápsulas contidas nas saquetas de Suplemento 2. O conteúdo das cápsulas foi extraído com metanol e os principais compostos identificados por GC-MS foram: cafeína, glicerol, ácidos gordos (incluindo 2-palmitoilglicerol e glicerol monostearato) e açúcares (glucose, sacarose e lactose). O conteúdo dos produtos mostrou-se semelhante entre eles, não apresentando variabilidade entre amostras do mesmo produto. A possibilidade da existência de 2,4-DNP foi excluída por comparação com a análise do respetivo padrão analítico. Considerámos relevante efetuar a quantificação da cafeína e os resultados mostraram que a toma diária sugerida no rótulo do produto Suplemento 2 excede a dose diária recomendada (aproximadamente 400 mg *versus* 848 mg de cafeína por dia).

Os ensaios *in vitro* destinaram-se a avaliar a citotoxicidade e revelaram que os hepatócitos primários de rato *Wistar*, isolados através de uma perfusão com colagenase, exibiam uma mortalidade dependente da concentração quando expostos a concentrações de 2,4-DNP que variaram entre 549 nM e 10 mM, a 37°C durante 24 h, conforme avaliado em três ensaios de viabilidade [ensaio de absorção de vermelho neutro: EC_{50} 98.05 µM; ensaio de redução do brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio: EC_{50} 96.82 µM; ensaio da lactato desidrogenase: EC_{50} 174.83 µM]. Estes resultados indicam que a mitocôndria foi o organelo mais sensível à toxicidade provocada pelo 2,4-DNP, seguido do lisossoma e da membrana plasmática. Seguidamente, os mecanismos que contribuíram para a citotoxicidade observada foram examinados por exposição das células a concentrações de 1 µM, 10 µM, 50 µM, 100 µM e 200 µM de 2,4-DNP, durante 24 h. Verificou-se um aumento significativo da dependência da concentração de espécies reativas intracelulares ($p<0.01$, ≥50 µM; teste de Dunnett) com consequente diminuição das defesas antioxidantes dependente da concentração [diminuição da glutationa reduzida (GSH reduzida; $p<0.01$, ≥100 µM; teste de Dunnett), diminuição da glutationa total ($p<0.01$, ≥100 µM; teste de Dunnett), aumento da glutationa oxidada (GSSG; $p<0.05$, ≥1 µM; teste de Dunnett) e diminuição da razão GSH reduzida/GSSG ($p<0.001$, ≥1µM; teste de Dunnett)]. Observou-se um aumento significativo do potencial de membrana mitocondrial nas concentrações de 10 µM e 50 µM ($p<0.05$; teste de Holm-Sidak) que se apresenta de acordo com a síntese

aumentada de adenosina trifosfato (ATP; $p<0.0001$, 10–100 µM; teste de Dunnett). O aumento da apoptose, dependente de energia, também foi confirmado pela ativação da pró-caspase-8 em todas as concentrações testadas ($p<0.05$; teste de Dunnett) e pelo aumento da atividade das caspases -3 e -9 para concentrações superiores a 10 µM ($p<0.05$; teste de Dunnett).

Em resumo, estes resultados revelam a ausência de 2,4-DNP em ambos os "queimadores de gordura" analisados. De acordo com os resultados obtidos neste estudo, o 2,4-DNP parece acelerar o metabolismo hepatocelular atuando como promotor da formação de ATP nas mitocôndrias. Este efeito pode justificar o dano oxidativo observado *in vitro* que, por sua vez, poderá ativar os processos de sinalização de morte celular através de mecanismos pró-apoptóticos intrínsecos e extrínsecos. Esta informação é crucial para reforçar os perigos associados ao 2,4-DNP enquanto substância usada para a perda de peso.

Palavras-chave:

"Queimador de gordura"; 2,4-dinitrofenol (2,4-DNP); GC-MS; hepatotoxicidade *in vitro*; hepatócitos primários de rato; stress oxidativo; apoptose.

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2,4-DNP	2,4-Dinitrophenol
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
Ac-DEVD-pNa	Acetyl-Asp-Glu-Val-Asp-p-Nitroanilide
Ac-IETD-pNA	Acetyl-Ile-Glu-Thr-Asp-p-Nitroaniline
Ac-LEHD-pNa	Acetyl-Leu-Glu-His-Asp-p-Nitroanalide
β-NADH	β-Nicotinamide adenine dinucleotide
BSA	Bovine Serum Albumine
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CAS	Chemical Abstracts Service
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DAD	Diode Array Detetor
DCFH-DA	2′,7′-Dichlorofluorescin diacetate
ΔΨm	Mitochondrial membrane potential
DGAV	*Direção Geral de Alimentação e Veterinária*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis[2-nitrobenzoic acid]
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
EI	Electron Ionization
EPA	Environmental Protection Agency
F	Female
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug, and Cosmetic Act
FID	Flame Ionization Detetor

FSA	Food Standards Agency
GC	Gas Chromatography
GHP	GH Polypro (membrane filter)
GR	Glutathione Reductase
GSSG	Glutathione disulfide
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IS	Internal Standard
LC	Liquid Chromatography
LDH	Lactate dehydrogenase
M	Male
MEM	Minimum Essential Media
MS	Mass Spectrometry
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass to charge ratio
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NIST	National Institute of Standards and Technology
NPD	Nitrogen Phosphorus Detetor
NR	Neutral Red
PFBBr	Pentafluorobenzyl bromide
RASFF	Rapid Alert System for Food and Feed
redGSH	Reduced glutathione
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Retention Time
SEM	Standard Error of the Mean
SPE	Solid-phase Extraction

SPME	Solid-phase Microextraction
tGSH	Total glutathione
TMCS	Trimethylchlorosilane
TMRE	Tetramethylrhodamine ethyl ester
TMS	Trimethylsilyl
TNB	5-Thio-2-nitrobenzoic
TOF	Time of Flight
UK	United Kingdom
UNEP	United Nations Environment Program
USA	United States of America
UV/Vis	Ultraviolet–visible
WHO	World Health Organization

PART 1 – INTRODUCTION

1.1. The problem of 'fat burners'

Several changes have been occurring in society regarding dietary habits. Physical exercise and a strong adhesion to sports requiring strength have promoted the search for all types of supplementation that may include drugs and drugs of abuse (Petróczi et al., 2015). The constant dissatisfaction with physical stature and body is proving to be an 'epidemic' issue. This happened with the concern to fight obesity, which has also increased. Therefore, with the advances of the industry and the constant need to respond to the search for these supplements, new formulations labelled as safe and promising total effectiveness in the reduction of body weight appear on the market. These products have become very attractive to be included in weight loss programs, and to be used by those who practice sports and competitions that require greater body strength and to bodybuilders.

The most popular supplements for weight loss are the 'fat burners' that are used as weight loss promoters by inducing a greater oxidation of fat during the performance of physical exercise. The main physiological effects induced by 'fat burners' include an increase of fat oxidation during exercise, suppress appetite, increase weight loss, prevent weight gain after weight loss, impair fat absorption and increase fat metabolism and energy expenditure (figure 1).

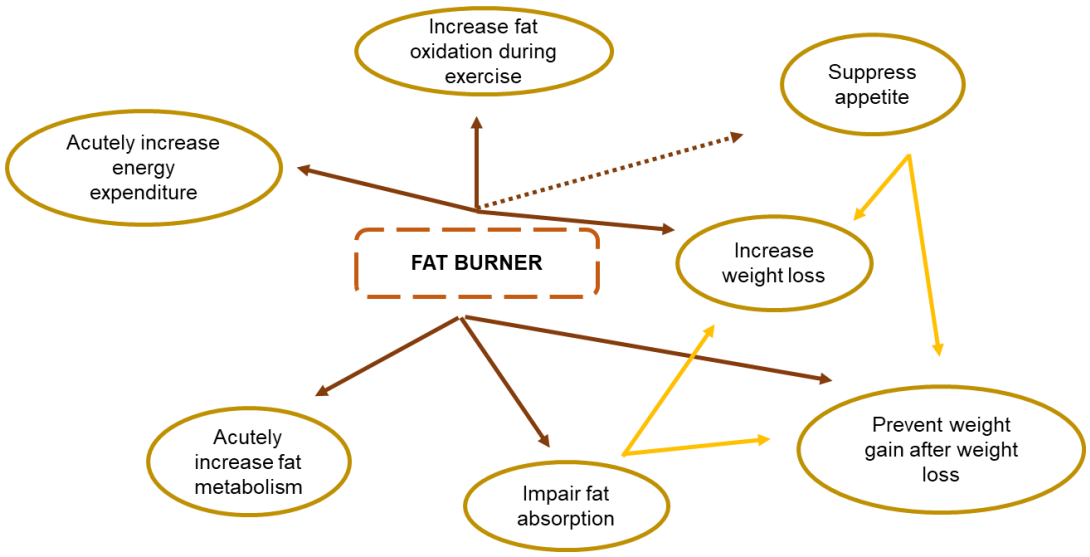


Figure 1: The interaction of 'fat burners' on increasing fat metabolism and promoting weight loss, adapted from Jeukendrup and Randell (2011).

However, many users of these type of supplements often believe that there is no need to associate the consumption of these substances with any type of physical activity or diet. In addition to their potential in fat loss, they have also been attributed the ability to promote muscle gain mass (Lee et al., 2014). These supplements can be purchased on the internet or in stores that sold sports supplementation.

These products can be combined with plans for physical exercise, diet and behavioural therapies, and are often integrated into a therapeutic scheme that involves a reduction in appetite, a false increase in satiety and a reduction in nutrient absorption (Ioannides-Demos et al., 2011). Generally, these supplements contain a combination of substances whose main action is the increase of the metabolic rate. The most popular products include caffeine, carnitine, green tea, conjugated linoleic acid (mixture of isomers), *Coleus forskohlii* extract, chromium and fucoxanthin (Jeukendrup and Randell, 2011). However, it has often been found that not all constituents of commercial products are mentioned on the label. In fact, labelling problems have been reported in many supplements available because they are adulterated with mixtures of other substances (da Justa Neves and Caldas, 2015). Many of these substances are of legal use but are unsafe at the concentrations in which they are used, others are of illegal use, as steroids (Pomeranz et al., 2015). Therefore, there is uncertainty and danger regarding the use of these supplements that refrain health professionals from their prescription. On the other hand, their commercialization is not restricted to medical prescription, providing the opportunity to commercialize non-prescription products (Blanck et al., 2007).

1.2. Trends in 2,4-dinitrophenol (2,4-DNP) use

Dinitrophenols are a family of synthetic organic compounds that exist in the solid state, with crystalline structure and yellow color (Harris and Corcoran, 1995; Miranda et al., 2006). They have a high degree of toxicity and can be absorbed by the skin, oral, and inhalation routes (Risikobewertung, 2015). 2,4-Dinitrophenol (2,4-DNP) is one of the isomers belonging to this family of compounds. It began to be applied in the manufacture of ammunition and explosives during the First World War, having been used as food and textile dye, wood preservative and agricultural pesticide. It was in the year of 1933 that its potentialities associated with the loss of weight were discovered, having begun its commercialization without medical prescription (Grundlingh et al., 2011; Janz, 2005; Lee et al., 2014). However, in 1938 it was considered unfit for human consumption because of its toxicity by the Food and Drug Administration (FDA) in the United States of America (USA) and afterwards, only in 2003, by the Food Agency in the United Kingdom (UK)

(Türkay et al., 2014). An alert was issued by the Interpol in 2015 in collaboration with the World Anti-Doping Agency which reinforced the dangers associated with the use of this compound (Agency, 2015; Costa, 2015; INTERPOL, 2015). Over all these years, deaths have been attributed to the ingestion of this dangerous substance, and in many cases were associated with the ambition of body fat loss (Veloso, 2013).

The following sections present a bibliographic review of 2,4-DNP about legislation and epidemiology; chemical structure, physicochemical properties and commercial presentation; pharmacokinetics; pharmacodynamics; toxic effects, diagnosis and treatment associated with intoxication, as well as analytical methodologies for the determination of 2,4-DNP in commercial products and biological matrices. The literature search was carried out using the PubMed database using the words *2,4-dinitrophenol weight loss*, obtaining a total of 35 articles and *2,4-dinitrophenol toxicity* with a total of 264 articles, until January 2017. Only papers related to exposure and effects in humans were considered as well as those that included analytical methods of analysis. Information (up to the same dates) was also collected on websites using the Google search engine.

1.3. Legislation and epidemiology

The data on the prevalence of use of 2,4-DNP are scarce, especially at the European level. In Portugal, legislation prohibits its use in cosmetic products but authorizes its commercialization as a pesticide and it is included in the 'List of pesticide products' authorized for sale (Veterinária, 2015). Most of the information available in the literature on the use of this substance and associated toxicity comes from the USA and UK. In the USA, reports of the occurrence of death from intoxication with 2,4-DNP, in 1938 led to the first alert that contemplated this compound and labelled it as 'unfit for human consumption', labelling it as 'extremely dangerous'. Also in the same year, the US Environmental Protection Agency (EPA) published it on the list of hazardous air pollutants, and warned of the need to prohibit its commercialization in pesticides products, since this compound could be legally marketed as a pesticide (Harris and Corcoran, 1995; Zaharia et al., 2016). Currently, this supervision is performed by the FDA (Administration, 2008). In Europe, only in 2003, the UK Food Standards Agency (FSA) issued a warning to the public not to consume any product containing 2,4-DNP in its constitution, also labelling it 'unfit for human consumption' (Zaharia et al., 2016).

Recently, 110 websites, among a reference of 4402, that commercialize 2,4-DNP as a 'fat burner' have been detected. This inspection action was carried out by the FDA and its main objective was to combat the illicit manufacture and marketing of this product in order to remove it from the market (Administration, 2016). Despite all this supervision and successive warnings and bans already issued, this compound is not yet listed on international regulatory bodies such as the World Health Organization (WHO) or the United Nation Environment Program (UNEP) (Zaharia et al., 2016). Table 1 lists the entities that have regulated this compound for human use.

Table 1: Entities that regulated the use of 2,4-DNP (2,4-dinitrophenol).

Year	Country	Entity	Regulation
1938	USA	FFDCA	Prohibition
1938	USA	US EPA	Prohibition
2003	UK	UK FSA	Prohibition
2008	Portugal	Department of Health (Decreto-Lei n.° 189/2008)	Prohibition
2015	Portugal	Department of Agriculture – List of pesticide products (Veterinária, 2015)	Authorization

Legend: FFDCA – Federal Food, Drug, and Cosmetic Act; UK – United Kindom; USA – United States of America; EPA – Environmental Protection Agency; FSA – Food Standards Agency.

Regarding the available data on the epidemiology and number of deaths related to this compound, figure 2 represents data between the years of 1910 and 2010, reported in a study that compiled all cases of fatal poisonings until 2010 described in the literature (Grundlingh et al., 2011).

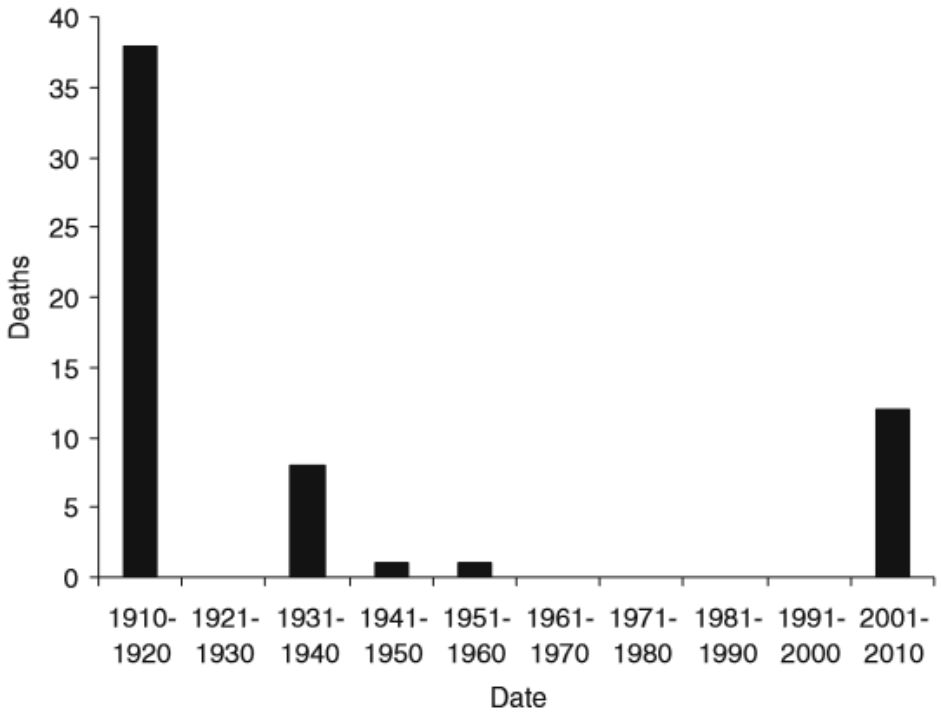


Figure 2: 2,4-DNP (2,4-dinitrophenol) related deaths, between 1910 and 2010, obtained from the research of all cases reported in the literature up to 2010 (Grundlingh et al., 2011).

As can be depicted in figure 2, the highest prevalence occurred between 1910 and 1920, which coincides with its use as a dye in the textile industry, as a pesticide in agriculture, and as a wood preservative. The routes of exposure, in these cases, are dermal and inhalation, and are therefore associated with unintentional exposures. After this period, and in the subsequent decade, there was no report of 2,4-DNP inducing intoxications (Grundlingh et al., 2011). From 1931 and in the following two decades there was a record, although not so significant, of deaths associated with this compound, after Maurice Tainter discovered its potential as a 'fat burner' (Grundlingh et al., 2011; Tainter et al., 1933). In 1981, in Texas, the physicist Bachynsky developed a formula named 'Mitcal', whose main constituent was 2,4-DNP, and began marketing it at the clinic where he worked. This product was intended to be part of an intracellular temperature elevation therapy. Its adverse effects on the patients were reported, with the first death occurring in 1984. Consequently, the FDA banned the sale of this formulation (Grundlingh et al., 2011). In the first decade of the 21st century, 2,4-DNP appears again in media such as the internet, blogs dedicated to bodybuilding, but also blogs dedicated to nutrition. Initially it began to be referred to as 'promise for weight loss and for increasing muscle definition' but quickly its harmful effects on health and its relationship with irreversible intoxications became relevant. In 2015, the Interpol issued an alert to reinforce the dangers associated

with the ingestion of this compound (INTERPOL, 2015). Despite all the prevention measures and alerts issued, the demand for this type of products has increased. Table 2 summarizes the cases of death from intoxication with 2,4-DNP, occurring between 1916 and 2016, available in the scientific literature.

Table 2: Summary of published fatalities between 1916 and 2016, relating to exposure to 2,4-DNP (2,4-dinitrophenol), adapted from Grundlingh et al. (2011).

Sex	Age	Type of exposure	Dose	Time to death	Maximum temperature (°C)	Year	Reference
M	Unknown	Occupational	Unknown	14 h	40.5	1916	(B., 1916)
M	Unknown	Occupational	Unknown	Unknown	Unknown	1918	(Warthin, 1918)
36 workers	Unknown	Occupational	Unknown	Unknown	43	1919	(Roger, 1919)
M	Unknown	Weight loss	2.5–5 g	10 h	>43	1933	(Geiger, 1933)
F	46	Weight loss	0.3 g over 6 weeks	Unknown	40.4	1934	(William and L., 1934)
F	31	Weight loss	6.06 g in 4 days	16 days after start of trial	38.9	1934	(Masserman and Goldsmith, 1934)
F	25	Weight loss	2.88 g over 5 days	7 days after first dose	38.9	1934	(Poole and Haining, 1934)
F	Unknown	Weight loss	10 g over 6 weeks	5 days after admission	41	1934	(Silver, 1934)
M	37	Weight loss	9 g over 7 days	11 h	40.9	1934	(Tainter, 1934)
F	13	Weight loss	5.4 g over 46 days	20 h after admission	40.9	1936	(Goldman and Haber, 1936)
F	21	Weight loss	4.5 g	9 h	41.1	1936	(Purvine, 1936)
M	Unknown	Intentional	Unknown	12 h	Unknown	1953	(Swamy, 1953)
M	61	Accidental	Unknown	24 h	Unknown	1960	(Cann and Verhulst, 1960)
F	17	Weight loss	Unknown	28 h	38.5	2002	(Pace and Pace, 2002)
M	22	Weight loss	2.4 g over 4 days	17 h after last dose	38.9	2004	(McFee et al., 2004)
F	17	Intentional	2.3 g–2.88 g	10 h	>40	2005	(Hsiao et al., 2005)
M	24	Bodybuilding	Unknown	Unknown	40.8	2005	(Suozzi et al., 2005)

Sex	Age	Type of exposure	Dose	Time to death	Maximum temperature (°C)	Year	Reference
F	17	Bodybuilding	Unknown	3 h after presentation	39.4	2006	(Miranda et al., 2006)
M	28	Bodybuilding	Unknown	50 min after presentation	41.1	2006	(Miranda et al., 2006)
M	30	Bodybuilding	Unknown	Unknown	Unknown	2007	(Politi et al., 2007)
F	27	Weight loss	Unknown	8 h after presentation	38	2009	(Tewari et al., 2009)
M	46	Intentional	2.8 g	21 h	37.8	2010	(Bartlett et al., 2010)
M	Unknown	Intentional	2.8 g	15 h	39.5	2010	(Siegmueller and Narasimhaiah, 2010)
F	49	Occupational	Unknown	15 h	38.9	2011	(Jiukun et al., 2011)
M	41	Occupational	Unknown	9 h	38.5	2011	(Jiukun et al., 2011)
F	23	Weight loss	Unknown	Unknown	Unknown	2012	(Brooke, 2013; Zaharia et al., 2016)
M	18	Weight loss/Bodybuilding	Unknown	Unknown	Unknown	2013	(Watson, 2013; Zaharia et al., 2016)
M	30	Weight loss/Bodybuilding	≤1.8 g	15 h	42.9	2014	(Türkay et al., 2014)
F	21	Weight loss	Unknown	Unknown	Unknown	2015	(Ward, 2015; Zaharia et al., 2016)
M	50	Weight loss	12.3 g	9 h	Unknown	2016	(Zack et al., 2016)

By analysing the table, one can associate the demand for this compound with the purpose of weight loss and to a younger aged group. However, there is also some adherence by individuals of other ages in the process of weight loss.

A study published by Kamour et al. in 2014, which collected data from 1 January 2007 to 31 December 2013 in the UK, based on consultations carried out by telephone and through the accesses registered on the TOXBASE platform (toxicological database), revealed the growing trend for interest and search for information on 2,4-DNP that probably increased the number of cases of toxicity. Figure 3 shows these data (Kamour et al., 2014).

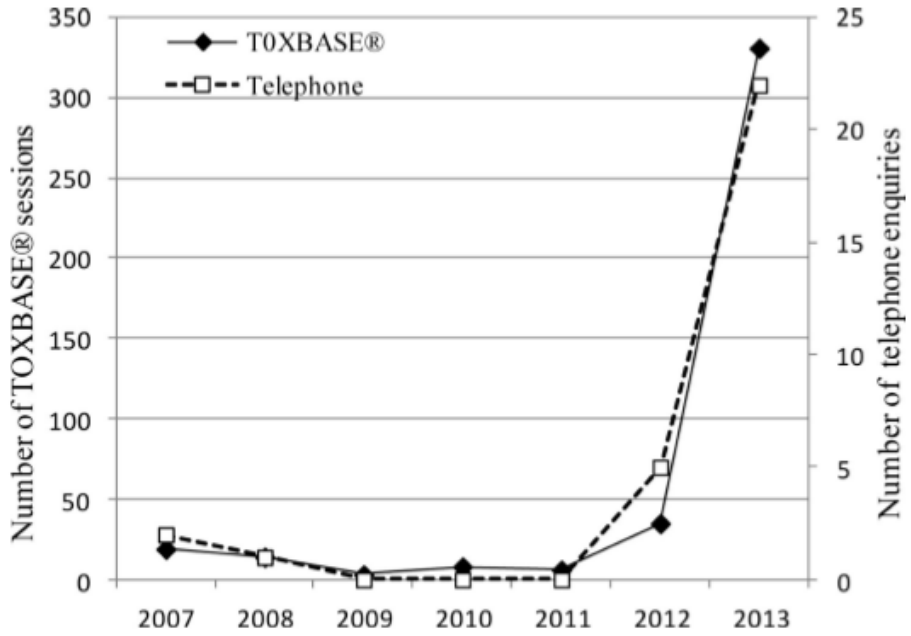


Figure 3: Registration of telephone consultations and access data for TOXBASE between January 2007 and December 2013 for 2,4-DNP (2,4-dinitrophenol) (Kamour et al., 2014).

To counteract the number of intoxications, authorities in the UK have taken many preventive actions in 2013, consisting of police warnings, local authorities control of sales on the internet, public outreach actions at gyms, and also by sending letters addressed to family doctors and emergency medical departments with information on 2,4-DNP. Because of these measures, the number of cases of intoxication by 2,4-DNP apparently decreased between the end of 2013 and the beginning of 2014, alongside with a decrease in the number of telephone and database information search records (figure 4). In the period of 2014/2015 there were 13 cases related to the toxicity of 2,4-DNP, at

least one of these cases being a fatal poisoning. However, the chart shows a new trend for the increase in the number of 2,4-DNP-related surveys (telephone and database) from 2015, which may mean that its use will again increase (Thomas and Duarte-Davidson, 2015).

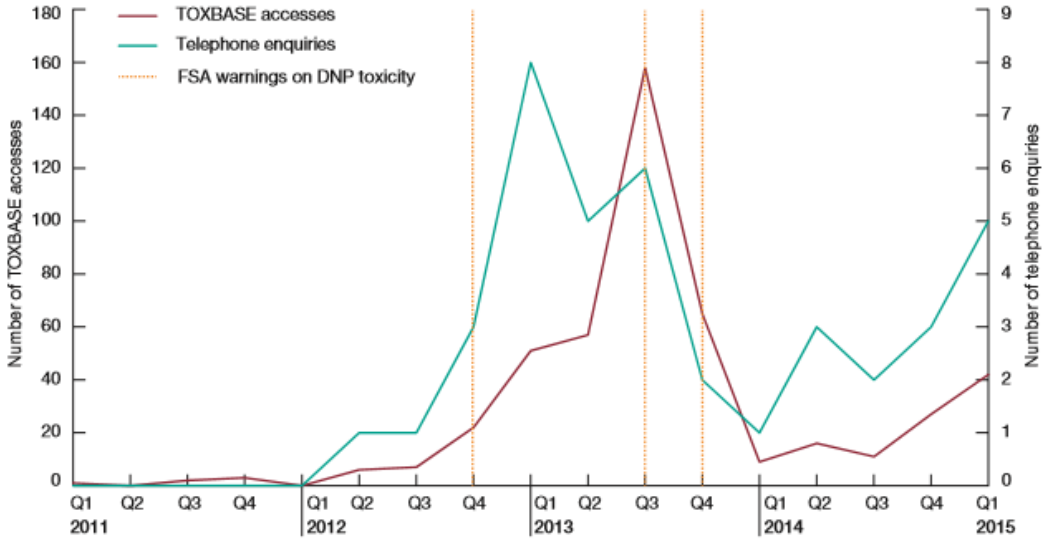


Figure 4: Registration of telephone consultations and TOXBASE access data of 2,4-DNP (2,4-dinitrophenol) from January 2011 to March 2015 (Thomas and Duarte-Davidson, 2015).

During the year of 2015, the authorities continued the warning measures already promoted in 2013, with a greater focus on physicians of the emergency medical departments and the supervision of internet sales. Because of these initiatives, the number of surveys (telephone and database) declined again, meaning a possible decrease in use (figure 5) (Thomas and Duarte-Davidson, 2016).

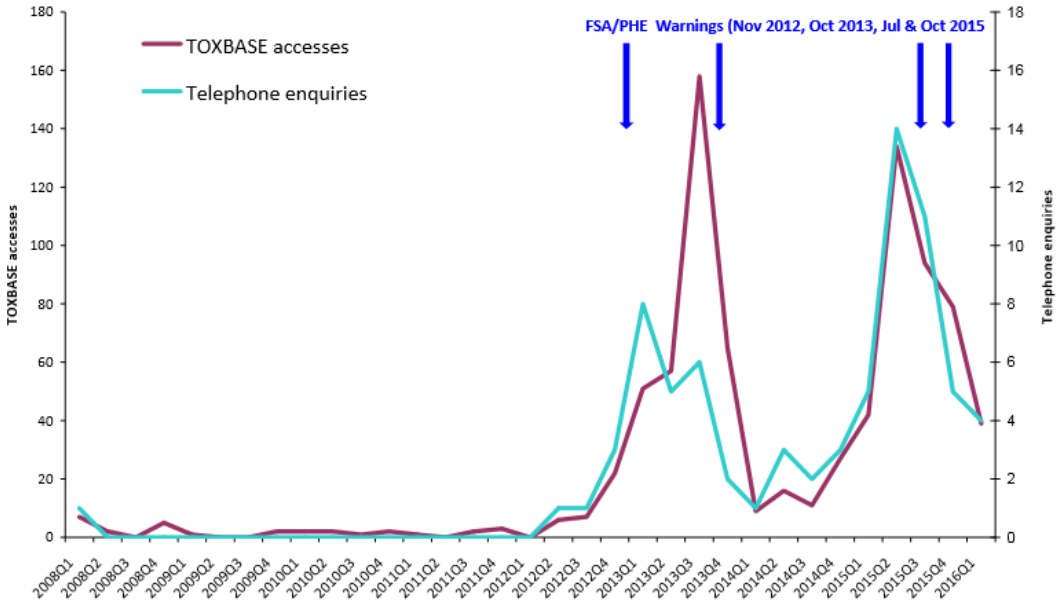


Figure 5: Registration of telephone consultations and access data to TOXBASE, with relevance starting in the first quarter of 2015 (Thomas and Duarte-Davidson, 2016).

From the data published in this study it is concluded that in the UK, since 2008, at least 77 cases of intoxication associated with telephone contacts have occurred, 11 of which corresponded to fatal poisonings and, of these, 6 occurred in 2015. These data confirms the increase in the use of 2,4-DNP in 2015 in the UK (Thomas and Duarte-Davidson, 2016). It is generally concluded from all these reports that preventive and warning measures for the most vulnerable population, as well as for health professionals, have a positive effect on trends in the consumption of potentially hazardous substances such as 2,4-DNP.

1.4. Chemical structure, physicochemical properties and commercial presentation

The molecular formula of 2,4-DNP is $C_6H_4N_2O_5$ and its chemical structure is represented in figure 6 (Petróczi et al., 2015; TOXNET).

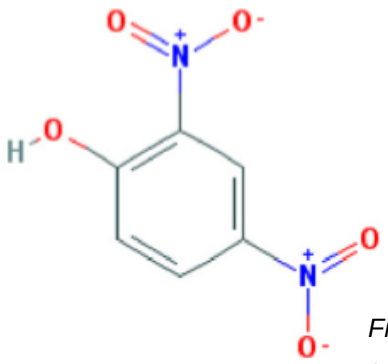


Figure 6: Chemical structure of 2,4-DNP (2,4-dinitrophenol) (TOXNET).

As mentioned before, 2,4-DNP is one of the isomers belonging to the family of dinitrophenols, a class of organic compounds of synthetic origin (Miranda et al., 2006). Table 3 summarizes some of the physicochemical properties of 2,4-DNP (TOXNET).

Table 3: Physicochemical properties of 2,4-DNP (TOXNET).

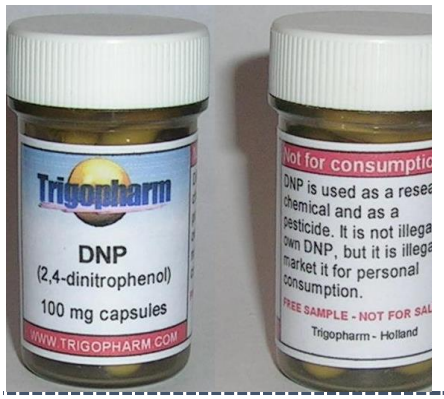
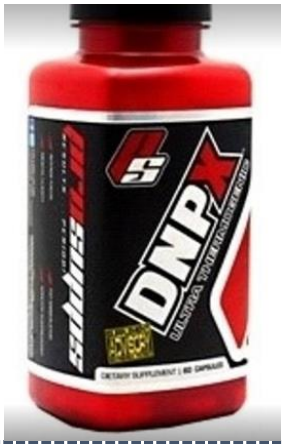
Physicochemical Properties	
Molecular weight	184.106 g/mol
Melting point	114.8 °C
Dissociation constant	pKa = 4.09
Partition Coefficient	logP = 1.67
CAS number	51-28-5

Legend: CAS – Chemical Abstracts Service.

In addition to this designation, this substance can also appear as 2,4-DNP, dinitrophenol or, simply, DNP. It is marketed in the form of cream, powder, tablets or capsules. In this last case, it is common for capsules to contain between 100-200 mg of compound or to be mixed with thyroxine or anabolic steroids (Grundlingh et al., 2011; INTERPOL, 2015; Türkay et al., 2014). Online sales began by being masked with different names associated, such as Sulfo/Solfo Black, Nitro Kleenup or Caswell No.392, Aldifen, Chemox, Nitophen, Dinofan, Dinosan, Dnoc, Osmotox PRB, Fenoxyl PRB or Tertosulphur PRB. Some of these variants may also be listed on the label of dietary supplements intended for weight loss (Petróczi et al., 2015). Nowadays, when searching the internet through the Google search engine, it is verified that these designations are associated with the theme of weight loss and muscle mass definition. Table 4 shows some examples of commercial products allegedly containing 2,4-DNP in their composition.

Table 4: Products containing 2,4-DNP (2,4-dinitrophenol) commercialized online.

Product	Webpage address
	http://evunsarep.4sql.net/sale/307-dnp-x-fat-burner/ [accessed 15 January 2017]
	http://www.shemazing.net/tag/diet-pills/ [accessed 15 January 2017]
	https://www.vaultekpharma.com/index/weigth-loss_fat-burners.html [accessed 15 January 2017]

Product	Webpage address
	https://www.steroid.com/DNP.php [accessed 15 January 2017]
	http://www.dailymail.co.uk/news/article-2282158/Student-Sarmad-Alladin-18-known-Mr-Muscles-dies-taking-DNP-fat-burning-pills.html [accessed 15 January 2017]
	http://www.dziennikzachodni.pl/artykul/919736,dnp-burner-dinitrofenol-odchudzanie-smiertelne-niebezpieczny-srodek-na-odchudzanie,id,t.html [accessed 15 January 2017]
	http://www.dziennikzachodni.pl/artykul/zdjecia/919736,dnp-burner-dinitrofenol-odchudzanie-smiertelne-niebezpieczny-srodek-na-odchudzanie,2056431,id,t,zid.html [accessed 15 January 2017]

Product	Webpage address
	http://www.dziennikzachodni.pl/artykul/zdjecia/919736,dnp-burner-dinitrofenol-odchudzanie-smiertelne-niebezpieczny-srodek-na-odchudzanie,2056443,id,t,zid.html [accessed 15 January 2017]

1.5. Pharmacokinetics

2,4-DNP may be absorbed in the gastrointestinal tract, when ingested, in the lungs, when inhaled, and through the skin. After absorption, it is transported through the bloodstream to organs and tissues such as the liver and kidneys. Because it is a lipophilic and low molecular weight compound it is rapidly absorbed by passive diffusion. In the stomach, this process occurs in its non-ionized form because it is an acidic compartment (pKa of 2,4-DNP: 4,09). In the bloodstream of 2,4-DNP binds in part to plasma proteins while another portion remains in circulation. It is this fraction that reaches the organs and tissues, exerting toxic effects.

It is mainly metabolized by a reduction reaction that occurs in its nitro groups, forming metabolites of lower toxicity in rat liver homogenates represented in figure 7 (Harris and Corcoran, 1995).

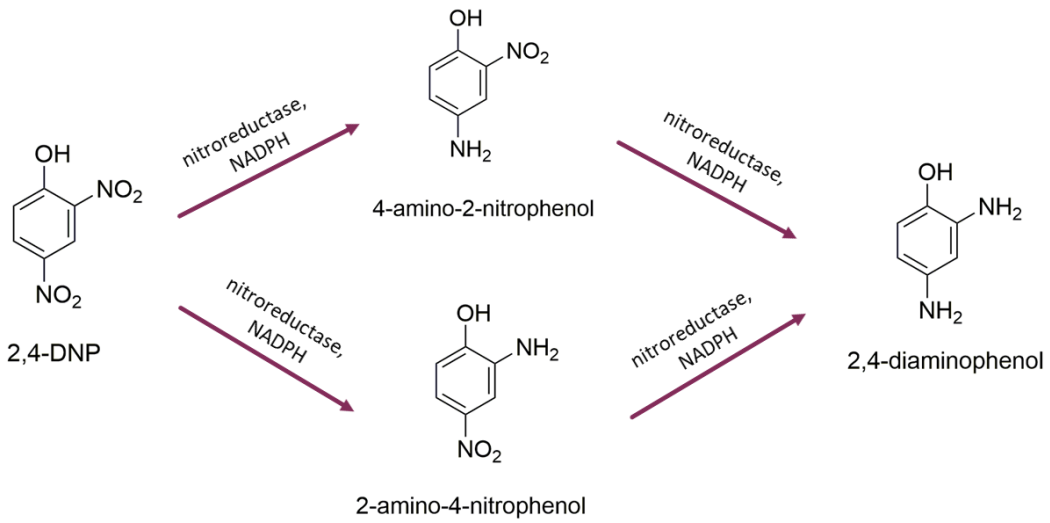


Figure 7: Metabolic pathway of 2,4-DNP (2,4-dinitrophenol) in rat liver homogenates, adapted from Harris and Corcoran (1995). Legend: NADPH – Nicotinamide Adenine Dinucleotide Phosphate.

The nitroreductase enzymes responsible for this process are microsomal and cytosolic (where they have the highest activity) and use nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. The resulting metabolized compounds are 4-amino-2-nitrophenol, 2-amino-4-nitrophenol and 2,4-diaminophenol (Harris and Corcoran, 1995). A study performed by Eiseman et al. indicates that 2-amino-4-nitrophenol is the most abundant metabolite in rat liver homogenates (Eiseman et al., 1972).

The elimination of the metabolites and of part of the main compound that did not undergo metabolization occurs preferentially through the urine and it is excreted by passive diffusion (Harris and Corcoran, 1995).

1.6. Pharmacodynamics

2,4-DNP acts as an uncoupler agent for mitochondrial oxidative phosphorylation. Its intracellular target is the mitochondria, the organelle responsible for cellular respiration, interfering with oxidative phosphorylation in the Krebs cycle. In the presence of 2,4-DNP, the energy released during the oxidation-reduction reactions, by the consequent transport of electrons, is not able to potentiate the process of adenosine triphosphate (ATP) production (Zaharia et al., 2016).

2,4-DNP acts to block the absorption of inorganic phosphate (PI) which, consequently, prevents its addition by ATP synthase to adenosine adipose phosphate (ADP) molecules (figure 8) (Hargreaves et al., 2016).

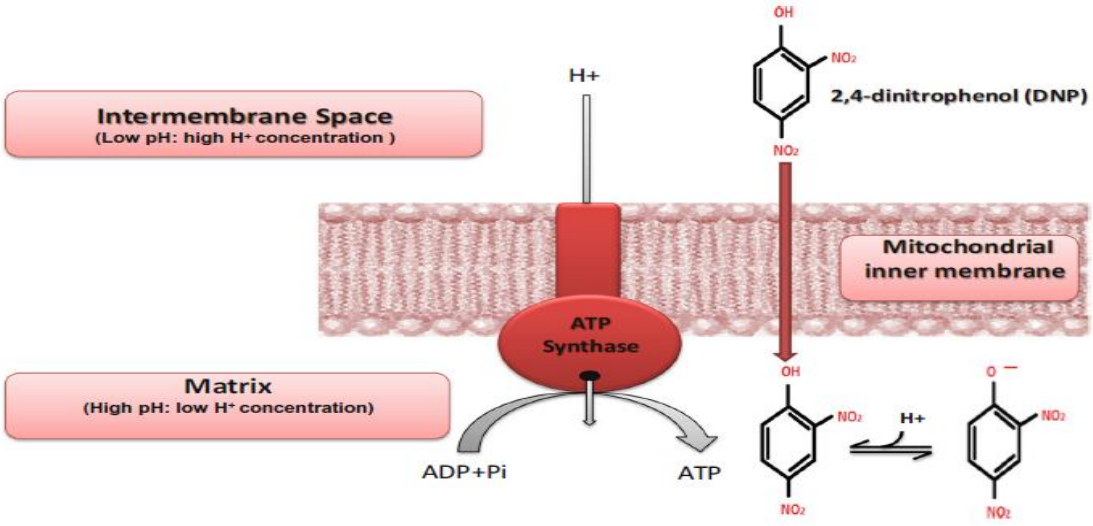


Figure 8: The mechanism by which 2,4-DNP (2,4-dinitrophenol) induces uncoupling of oxidative phosphorylation at the inner mitochondrial membrane (Hargreaves et al., 2016). Legend: ADP – Adenosine Diphosphate; ATP – Adenosine Triphosphate; Pi – Inorganic Phosphate.

The final production of ATP by the cell is negatively influenced and leads to an inhibition of all biological processes that require energy. Additionally, it causes a change in the electrochemical proton gradient by increasing permeability for its passage out of the cell (Grundlingh et al., 2011). Thus, there is a decrease in energy efficiency, potentiated by an increase in the permeability of the membrane to the protons and by the impossibility of converting ADP to ATP. Energy dissipation is then promoted in the form of heat which enhances a rapid increase in metabolism and a higher calorie consumption than usual. Thus, there is a strong inhibition of the cellular respiration process. However, the accumulation of potassium continues and the inorganic phosphate ceases to be absorbed by the mitochondria and remains accumulated (Zaharia et al., 2016).

1.7. Toxic effects, diagnosis and therapy associated with intoxication

Available data on the effects of 2,4-DNP come from reports of patients arriving at emergency medical facilities. The most frequently described symptoms are nausea, vomiting, sweating, tachycardia, hyperthermia, confusion, agitation, breathing difficulties,

back and abdominal pain (Bartlett et al., 2010; Grundlingh et al., 2011; Siegmueller and Narasimhaiah, 2010; Zack et al., 2016). However, there are other visible external signs related to the change in color and texture of the skin, namely, the appearance of a yellow color and rashes. Acute renal failure is another serious consequence associated with this level of toxicity, evidenced by the existence of tubular necrosis observed in the autopsy of fatal cases (Grundlingh et al., 2011).

There is yet no guideline available for the treatment of 2,4-DNP poisoning. This treatment should therefore be performed symptomatically taking into account the symptoms that the patient is manifesting (Zack et al., 2016). To facilitate the elimination of the compound, the administration of activated charcoal can be considered (Grundlingh et al., 2011; Türkay et al., 2014). Hyperthermia can be minimized by cooling the body through cold baths, administration of benzodiazepines, or dantrolene (currently under study) (Lee et al., 2014; Zack et al., 2016). Anxiety and agitation can also be treated with the administration of benzodiazepines (Grundlingh et al., 2011; Türkay et al., 2014). To verify possible changes in the state of consciousness, it is common to use the Glasgow Coma Scale (Türkay et al., 2014). In cases involving cardiorespiratory arrest situations, professionals may follow the guidelines of the European Resuscitation Council (Nolan et al., 2015).

1.8. Analytical methods for 2,4-dinitrophenol (2,4-DNP) determination in commercial products and biological matrices

The determination of 2,4-DNP in commercial products is possible using liquid or gas chromatography separation techniques and using different detectors [Flame Ionization Detector (FID), Ultraviolet-visible (UV-VIS), Diode Array Detector (DAD), Mass Spectrometry (MS)]. Samples of suspected tablets may be analyzed by High Performance Liquid Chromatography coupled to DAD (HPLC-DAD), Liquid Chromatography coupled to MS and Time-of-Flight (LC-MS-TOF) or Gas Chromatography coupled to MS (GC-MS) to identify and quantify 2,4-DNP in the presence of other compounds (Lee et al., 2014) (Miranda et al., 2006). The determination of 2,4-DNP in biological matrices (blood and urine) is usually carried out by LC-MS (Miranda et al., 2006; Politi et al., 2007; Robert and Hagardorn, 1983). Table 5 summarizes the most used analytical techniques.

Table 5: Analytical methods used to determine 2,4-DNP (2,4-dinitrophenol) in different samples.

Sample	Sample preparation	Separation method	Detection method	Reference
Capsule	---	LC GC LC	DAD MS TOF/MS	(Lee et al., 2014)
	SPE with methanol solution (1 mg/mL).	GC LC	MS DAD	(Miranda et al., 2006)
	Stock solutions were prepared in methanol (0.5 mg/mL). Dilution of standard working solution was prepared in methanol (12.5 µg/mL). Example: powder was prepared in pure acetonitrile. The suspension obtained was mechanically stirred, sonicated and centrifuged. Filtration: through a 0.45 µm pore size GH Polypro (GHP) membrane filter.	LC	DAD	(Rebiere et al., 2012)
Blood	**Liquid/Liquid extraction:** extraction with n-butyl chloride.	LC	DAD	(Miranda et al., 2006)
	Without previous sample preparation. Addition of internal standard (3,5,6-d_3-2,4-dinitrophenol) solution in methanol; drying under nitrogen flow; deproteinization with acetonitrile; vortex mixing and centrifugation. Dilution of the supernatant with formic acid (0.1%).	LC	MS	(Politi et al., 2007)
	Liquid/Liquid extraction: dilution of samples with saline; addiction of distilled water containing of 3,5,6-d_3-2,4-dinitrophenol; acidification with citrate buffer (pH = 3, citrate/phosphate buffer) and extraction with diethyl ether; counter-extraction with 5% aqueous $NaHCO_3$. Acidification with HCl and re-extraction with diethyl ether. The organic phase was dried with anhydrous sodium sulphate; evaporation of diethyl ether at 60ºC under nitrogen flow. Reconstitution with benzene.	GC	MS	(Robert and Hagardorn, 1983)

Sample	Sample preparation	Separation method	Detection method	Reference
Blood	**Liquid/Liquid extraction** with 3,5,6-d_3-2,4-dinitrophenol as internal standard. **Derivatization** with BSTFA + 1% TMCS.	GC	MS	(Fogelberg, 2012)
Urine	---	GC	MS	(Miranda et al., 2006)
	Without previous extraction. Addiction of urine to internal standard (3,5,6-d3-2,4-dinitrophenol). Dilution of the sample in formic acid (0.1%).	LC	MS	(Politi et al., 2007)
River water	**SPE:** filtration of the river water through a 0.45 µm filter; acidification to pH 2 with hydrochloric acid (1 M). Elution with acetone and evaporation under a nitrogen flow. **Derivatization:** addiction of 10% aqueous potassium carbonate and acetone solution of 5% PFBBr reagent. Evaporation to 100 mL under a nitrogen flow. Addiction of dichloromethane and the organic phase was washed with mili-Q-purified water.	GC	MS	(Nakamura et al., 2001)
Precipitation water	**Liquid/Liquid extraction:** acidification with H_2SO_4 to pH 2, extraction in a rotary perforator with a pentane/ether mixture (5:1) (v/v). Separation of organic phase, dried over Na_2SO_4 and concentration to 0.5 mL. **Derivatization:** addition of cooled etheric diazomethane solution. Concentration in the rotary evaporator.	GC	NPD MS	(Nick and Schöler, 1992)
Water	**SPME:** extraction for 30 min with a polyacrylate coated fiber (desorption time was 3 min at 270°C); acidification with H_2SO_4 to pH 2.	GC	MS	(Lu et al., 1999)

Legend: BSTFA – N,O-Bis(trimethylsilyl)trifluoroacetamide; DAD – Diode Array Detector; GC – Gas Chromatography; GHP – GH Polypro; LC – Liquid Chromatography; MS – Mass Spectrometry; NPD – Nitrogen Phosphorus Detector; PFBBr – Pentafluorobenzyl Bromide; SPE – Solid/Liquid Extraction; SPME – Solid-phase microextraction; TMCS – Trimethylsilyl chloride; TOF – Time-of-Flight.

In general, when the GC technique is used, 2,4-DNP should be extracted from the matrix, either by liquid-liquid extraction (Fogelberg, 2012; Mathers et al., 2007; Nick and Schöler, 1992; Politi et al., 2007; Robert and Hagardorn, 1983), or by solid-phase extraction (SPE) (Nakamura et al., 2001). The compound will have to be extracted with an organic solvent to be injected into the GC. To improve the chromatographic performance, it is possible to use derivatization by fluoroacylation with N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as reported by Fogelberg et al. (2012) and Mathers et al. (2007). Another methodology recommended in GC is the solid-phase microextraction (SPME), where the compound passes to the gas phase and it is subsequently adsorbed onto a fiber with a certain polarity, and then desorbed in the chromatograph injector (Lu et al., 1999). In the case of the use of the HPLC technique, a prior concentration of the compound using SPE before injecting it directly into the LC.

In summary, and as can be seen in Table 5, to be able to apply these analytical methods it is essential to carry out a previous process of treatment of the sample when analyzing the compounds of this family. This is mainly due to its high polarity and volatility that affect its chromatographic resolution. Thus, when the compounds have these characteristics they will have to undergo a pretreatment process (Mahugo Santana et al., 2009), which is usually carried out using extraction techniques followed by a derivatization procedure (Nick and Schöler, 1992) in which the most widely used derivative agent is BSTFA (Fogelberg, 2012; Mathers et al., 2007) although pentafluorobenzyl bromide (PFBBr) may also be used as an alternative (Nakamura et al., 2001). SPE (Nakamura et al., 2001); SPME (Lu et al., 1999) and liquid-liquid extraction (Fogelberg, 2012; Mathers et al., 2007; Nick and Schöler, 1992; Politi et al., 2007; Robert and Hagardorn, 1983) after acidification, are the most common extraction methodologies found in the literature.

PART 2 – OBJECTIVES

The ‘fat burners’ belong to a popular group of nutrition supplements that improve fat metabolism, energy expenditure and increase fat oxidation during exercise. These supplements may contain many ingredients, each one with its own mechanism of action, the combination of these substances is claimed to have additive effects. One of such constituents used in weight loss products is 2,4-DNP. Although it has been banned as a product for human consumption, one must consider the possibility of 2,4-DNP being illicitly included in ‘fat burners’ formulations. Therefore, **one of the main objectives** of this work was to unveil the presence of 2,4-DNP in two ‘fat burners’ bought online, the Supplement 1 and the Supplement 2.

Since many of these supplements have not yet been evaluated by the FDA it is possible that the ingredients that are included in package label do not correspond to the real content of the formulation. Thus, our **second aim** was to chemically characterize the products purchased and to compare their real composition with the labelled composition. A comparison among different capsules of the same product was also performed.

The **last objective** was to elucidate the *in vitro* hepatotoxicity of these supplements and, in particular, of 2,4-DNP, which was deemed responsible for several intoxications associated with weight loss product users. In case of apparent citotoxicity, the mechanisms involved were elucidated.

PART 3 – CHEMICAL CHARACTERIZATION OF COMMERCIAL WEIGHT LOSS PRODUCTS

CHAPTER 1: MATERIALS AND METHODS

3.1.1. Reagents

All the reagents used were of analytical grade. methanol and dichloromethane were obtained from Fisher Chemicals (Loures, Portugal). All other reagents were acquired from Sigma-Aldrich (Saint Louis, Missouri) unless stated otherwise. The purity of all standards is known and was always greater than 98.0% (w/w).

3.1.2. 'Fat Burners'

Two commercial formulations, Supplement 1 and Supplement 2, marketed for weight loss were purchased from Prozis website (www.prozis.com/pt), which offers a wide range of products related to supplementation and functional feeding mainly used for human consumption. Table 6 lists the characteristics of these products used for analyses.

Table 6: Commercial formulations analysed in the study.

	Formulation	Expiration date	Manufacturer	Place of manufacturing	Product form
1	Supplement 1	4th Feb, 2018	International Ingredients	USA	White capsules contained in a black plastic bottle
2	Supplement 2	Nov, 2018	Universal Nutrition	New Brunswick, New Jersey, USA	Multicolour capsules contained in small plastic bags* sold in a metallic box

* Each plastic bag contains 9 capsules: 2 white capsules + 1 red capsule + 2 blue capsules + 1 orange capsule + 1 yellow capsule + 1 red/white capsule + 1 blue/white capsule. Legend: USA – United States of America.

Supplement 1 is considered a super-thermogenic product. The labelled composition was divided in three groups of ingredients, based on the claimed three benefits provided. Accordingly, *Super-thermogenesis Complex* includes *Coleus forskohlii* and L-theanine; *Extreme Energy Complex* includes caffeine anhydrous; and *Powerfull Weight Loss Complex* includes green coffee extract (*C. canephora robusta*). The label also indicates other ingredients, namely L-carnitine/ L-tartrate, cocoa extract (standardized for 6% theobromine), bioperine (black pepper 95% piperine), cayenne pepper (*Capsicum*

annuum), veggie complex (hypromellose, red iron oxide and titanium dioxide), vegetable cellulose, silicon dioxide and magnesium stearate. Recommended dosage by the manufacturer is one capsule for the first day; two capsules once daily for the second and third days; two capsules twice daily for the fourth day and beyond. In addition, the manufacturer recommends, for best results, using the product for 60 days, combined with diet and training.

The Supplement 2 formula includes ingredients designed to stimulate metabolism, thermogenesis, diuresis, muscle preservation and thyroid function, as well as to provide extra energy and to suppress appetite. The eight different components described in the Supplement 2 label are: *Stimulant/Thermogenic Complex* that includes caffeine anhydrous, kola nut (seed), guarana seed, yerba mate (leaf) and raspberry extract; *Metabolic Complex*, which only includes polyphenols, such as green tea extract, oolong tea extract, black tea extract, coffee bean extract and white tea extract; *Thyroid Complex* that includes L-tyrosine, olive leaf extract (15% oleuropein) and *Salvia officinalis* (leaf); *Water Shedding Complex* that includes dandelion root (taraxol/taraxerol), nettle root, burdock, *Arctium tappa* root, buchu leaf, juniper berry fruit and celery seed; *Nootropic Complex* that includes gotu kola extract, choline bitartrate and *bacopa monniera* (leaf; bacopasides A & B); *Cortisol Inhibiting Complex* that includes ashwangandha extract (withanolides, panax ginseng – root, phosphatidylserine and magnolia bark extract), *Cholecystokinin (CCK) Boosting Complex* that includes *Gymnema sylvestre* leaves, apple pectin and jojoba seed extract (simmondsin); and *Bioavailability Complex* that includes ginger root (gingerols/shogaols), cayenne fruit, grapefruit (seed; 6,7-dihydroxybergamottin), quercetin, citrus fruit extract (naragin) and *Pepper nigrum* seed extract. Recommended dosage by the manufacturer was two plastic bags (nine capsules each) a day, one pack on waking and the other one 4 to 6 hours later, in cycles of three weeks followed by interruption of one week.

3.1.3. Standards and samples preparation

Three individual capsules of Supplement 1 and all capsules from three Supplement 2 plastic bags were used. The husks were first removed and all the fine powders of each capsule were individually transferred into glass vessels and dissolved in methanol at a concentration of 1 mg/mL. The obtained suspensions were then sonicated for 15 minutes. Two microliters of each solution were injected directly in the GC-MS equipment. Solutions of all standards (including, 2,4-DNP and caffeine) and the internal standard (IS) 4-hydroxy-3-methoxybenzylamine hydrochloride were also prepared at a 1 mg/mL

in and stored at -20ºC. All intermediate dilutions and working solutions were made up in methanol.

3.1.4. Derivatization and injection

Solutions were analysed by GC-MS directly after the addition of 5 µL of 200 µg/mL IS to 1 mL of samples and standards at 1 mg/mL. Alternatively, was also made an analysis of the samples and standards after derivatization with N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). In the latter case, derivatization was performed according to the method developed by Silva et al. (2010) with some modifications. Accordingly, 50 µL of 2,4-DNP standards and samples at 1 mg/mL were added of 5 µL of 200 µg/mL IS and 945 µL of methanol. Then, solutions were evaporated to dryness under nitrogen flow. Fifty microliters of dichloromethane and 50 µL of MSTFA were then added to the dried residue. Samples were vortex mixed and further incubated at 80°C for 30 min. After cooling to room temperature, 2 µL of sample were automatically injected into the GC-MS equipment. A summary of the procedures is shown in figure 9.

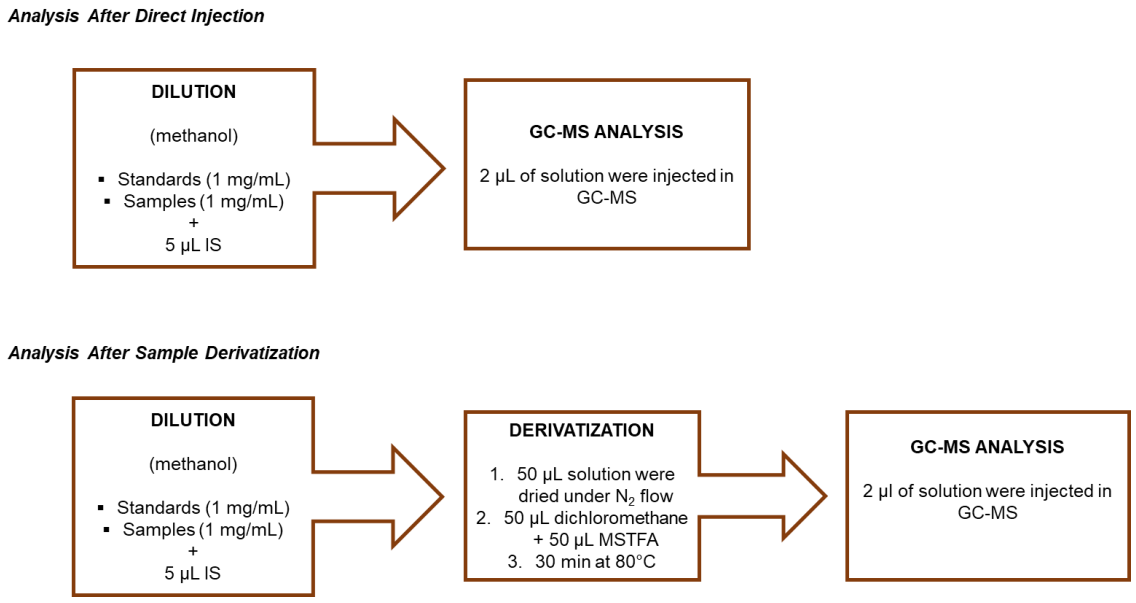


Figure 9: Summary of sample preparation procedures for GC-MS analysis. Legend: GC-MS – Gas Chromatography coupled to Mass Spectrometry; IS – Internal Standard (4-hydroxy-3-methoxybenzylamine hydrochloride); MSTFA – N-methyl-N-trimethylsilyl-trifluoroacetamide; N2 – Nitrogen.

3.1.5. GC-MS conditions

GC-MS analysis was performed on a SCIONTM TQ (triple quadrupole) 436-GC-MS system (Bruker Daltonics, Fremont, CA) equipped with a SCION TQ mass detector and a Bruker Daltonics MS workstation software (version 8.2). A capillary column Rxi-5Sil MS (30 m × 0.25 mm × 0.25 µm) from RESTEK was used for the chromatographic separation. Helium C-60 (Gasin, Portugal) was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injection was in split mode (1:10) and the injector temperature was 250°C (held for 20 min.). As for the oven temperature, it was held for 1 min at 80°C, followed by an increase at a rate of 20°C/min until reaching 250°C (held for 2 min) and finally an increase of 10°C/min to 300°C (held for 20 min). The MS detector was operated in electron ionization (EI) mode. Data acquisition was performed in full scan mode with a mass range between 50-500 m/z at a scan rate of 6 scan/s.

3.2.1. Research of 2,4-dinitrophenol (2,4-DNP) in weight loss commercial products

As 2,4-DNP was categorised as a weight loss drug, a notorious growth in its demand was observed. Nevertheless, after the first fatalities, it was banned by FDA for human consumption. Despite its legal regulation and all reported toxic effects, 2,4-DNP-containing products have still been commercialised, often sold under different names (Zaharia et al., 2016). These slimming products are not generally subjected to control by regulatory authorities and, as a consequence, they recurrently lack quantitative and/or qualitative standardization of the chemical content. Often, many substances that are not labelled on the package appear in the formulation, and the contrary also occurs, raising serious concerns regarding safety and marketing of these products. Furthermore, as many dietary supplements derive from plants, they are frequently considered as 'natural' products (Rocha et al., 2016), which transmits a false sense of safety to the consumers. Surveillance on this type of product is therefore of utmost relevance. So, we investigated the possibility of 2,4-DNP to be present as an adulterant in slimming supplements, even at very low detectable levels.

We started this work by analysing and comparing the chromatographic profile and mass spectra of 2,4-DNP. Methanol was the solvent chosen for analysis. This choice was based on the fact that 2,4-DNP is a polar molecule that can be more easily extracted by polar solvents. The data obtained herein is shown in figure 10 and is line with the 2,4-DNP data [retention time (RT) and mass to charge ratio (m/z)] already described in literature (Miranda et al., 2006; Robert and Hagardorn, 1983). Our data confirmed the characteristic mass spectra of 2,4-DNP with ion peaks of m/z 63, 53, 154, 184 and 91 after fragmentation.

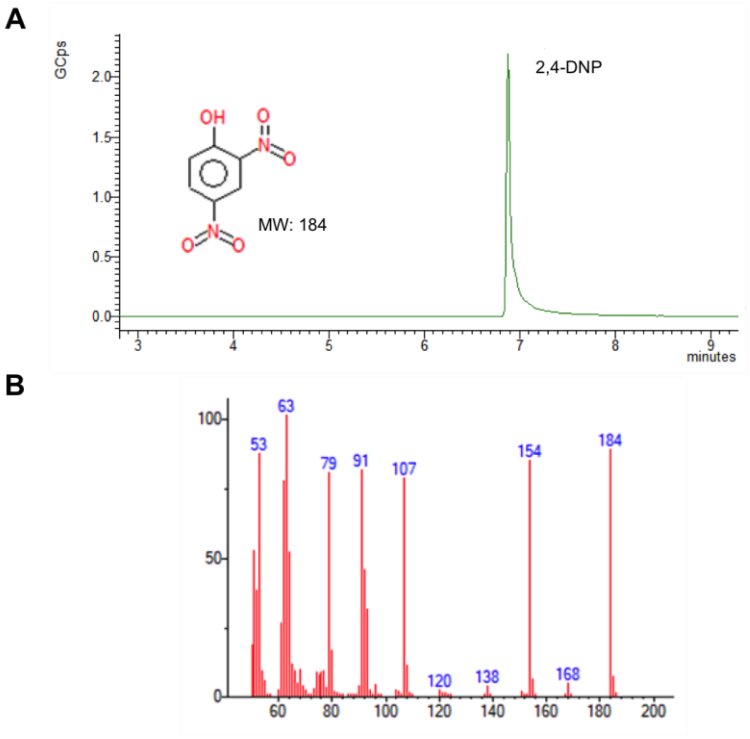


Figure 10: Full scan chromatographic profile (A) and mass spectra (B) of 2,4-dinitrophenol (2,4-DNP) directly injected into the GC-MS after dissolution in methanol.

Due to the low volatility of 2,4-DNP, which could affect its chromatographic definition we also analysed the chromatographic profile and mass spectra of 2,4-DNP following derivatization with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). Derivatization reactions are usually performed to increase volatility, decrease the polarity, and to make compounds more stable (Mahugo Santana et al., 2009). The introduction of a silyl group (R_3Si) to a molecule (silylation) through derivatization with MSTFA is a process frequently used to enhance GC performance. The silylation involves the nucleophilic attack upon the silicon atom of the silyl donor, producing a bimolecular transition state. This mechanism involves the replacement of the active hydrogen on –OH, -SH, -NH, -NH_2, -COOH groups by a trimethylsilyl group, and occurs in molecules that display a good leaving group, which means molecules with low basicity, structure with ability to stabilize a negative charge in the transitional state, and low affinity between the leaving group and silicon atom. The reaction for silylation is show in the below equation (Orata, 2012; Sigma-Aldrich).

$$Sample - OH + (CH_3)_3 - Si - X \rightarrow Sample - O - Si - (CH_3)_3 + HX$$

From the comparison of both methods, results obtained after derivatization of our 2,4-DNP standards (figure 11) did not present greater sensitivity or increased resolution of the peaks, when compared with data from direct injections. Then, further analyses were performed with non-derivatized samples.

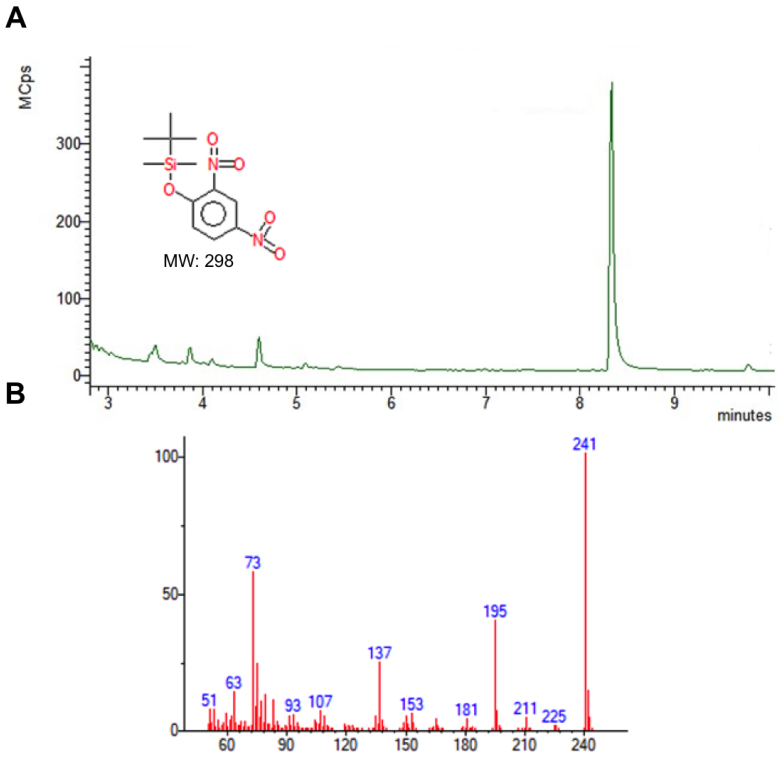


Figure 11: Full scan chromatographic profile **(A)** and mass spectra **(B)** of 2,4-dinitrophenol (2,4-DNP) injected into the GC-MS after dissolution in methanol and derivatization with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA).

A survey was conducted regarding all products sold as 'fat burners' in websites dedicated to bodybuilding (for example, bodybuilding.com). It was notorious the diversity of products consumed with the purpose of body fat loss. Evidence indicated that in Portugal Prozis (prozis.com) is the most popular website to purchase such type of products. So, motivated by the variability of the products offered online, compared to that available in traditional stores, and by the information offered by these websites on the most commercialized and popular products, we decided to purchase online the two slimming products to be test in the current study. Supplement 1 and Supplement 2 formulations were selected and acquired through Prozis website.

We further investigated the presence of 2,4-DNP in these two different commercial supplements with alleged thermogenic properties. For this, a comparison of

chromatograms, in terms of RT and mass spectra of 2,4-DNP chromatographic peak, between standards and samples was carried out.

First, data from GC-MS analysis of methanol solutions of 2,4-DNP and Supplement 1 were compared and revealed the absence of the drug, as no match between 2,4-DNP and Supplement 1 chromatographic profiles (RT and m/z) could be established (figure 12).

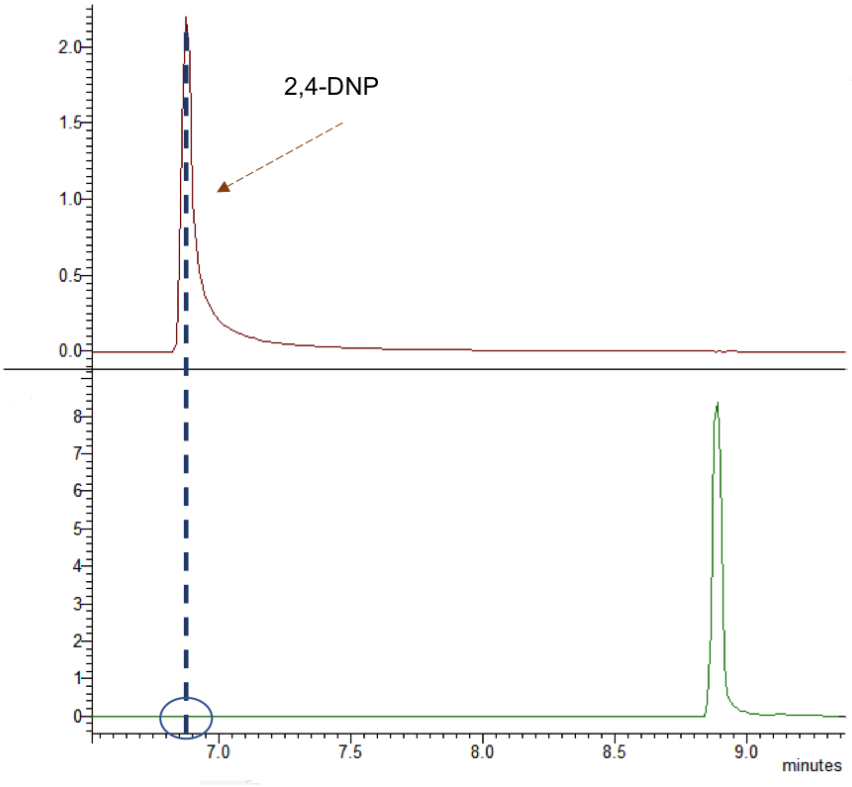


Figure 12: Chromatographic profiles of 2,4-dinitrophenol (2,4-DNP; brown) and Supplement 1 (green).

Similar results were obtained for Supplement 2, since 2,4-DNP was not detected in any of the capsules tested (figure 13).

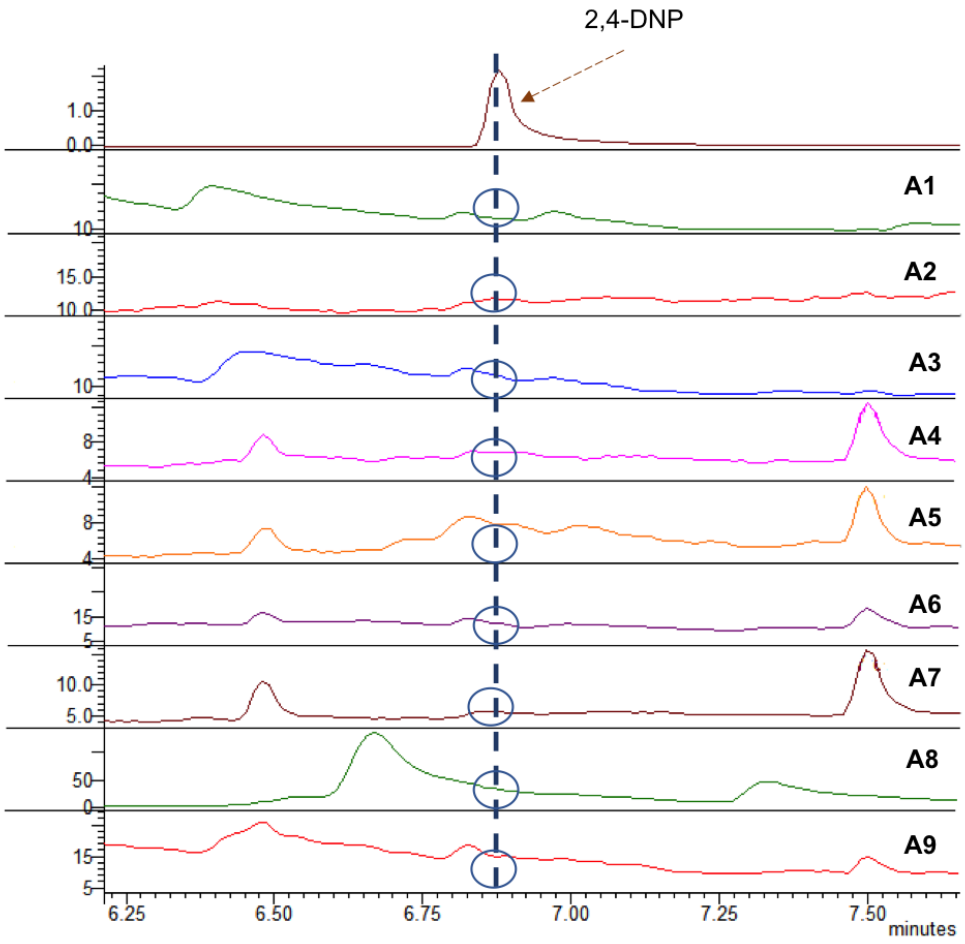


Figure 13: Chromatographic profiles of 2,4-dinitrophenol (2,4-DNP) and each of the capsules contained in a plastic bag of Supplement 2.

Rebiere et al. (2012) investigated the presence of several hazardous weight loss substances in adulterated slimming formulations. Although several illegal substances, which are regulated because of side effects or toxicological concerns, were detected, such as sildenafil, synephrine, clenbuterol, nicotinamide, phenolphthalein, rimonabant, sibutramine, N,N-didesmethylsibutramine, and yohimbine. All these substances are considered the most frequently found undeclared pharmacological ingredients in food supplements by FDA (Walker et al., 2016). On the contrary, and similar to our results, 2,4-DNP was not found in the different dietary supplements analysed. In 2016, 2,4-DNP was also included in the List of undeclared and unauthorized pharmacological substances frequently reported in food supplements by the Rapid Alert System for Food and Feed (RASFF).

In a study that investigated the characteristics of slimming products on the Brazilian clandestine market, Justa Neves and Caldas (2015) observed that sibutramine was the most referred substance in adulterated products whose declared origin was Brazil. This

occurrence was similar to that published by Sadaka et al. (2011) concerning composition of weight loss products sold the Lebanese market. Sildenafil is also a very common substance in illicit dietary supplements, particularly in Italy (Damiano et al., 2014).

3.2.2. Characterization of commercial products

As we did not detect 2,4-DNP in the purchased products, we proceed to the investigation of the chemical profiles of the referred thermogenic formulations. The GC-MS method was again chosen to identify the various compounds present in this slimming formulation. Spectra of unidentified peaks observed in GC-MS data of Supplement 1 and Supplement 2 samples were further searched through spectra databases supported by National Institute of Standards and Technology (NIST14). The identification process was based on EPA methods for determination of volatile/semi-volatile compounds, according to the respective RT, kovats indices and RMatch values (Milman, 2005). The methods used to characterize the weight loss dietary supplements are summarised in figure 14.

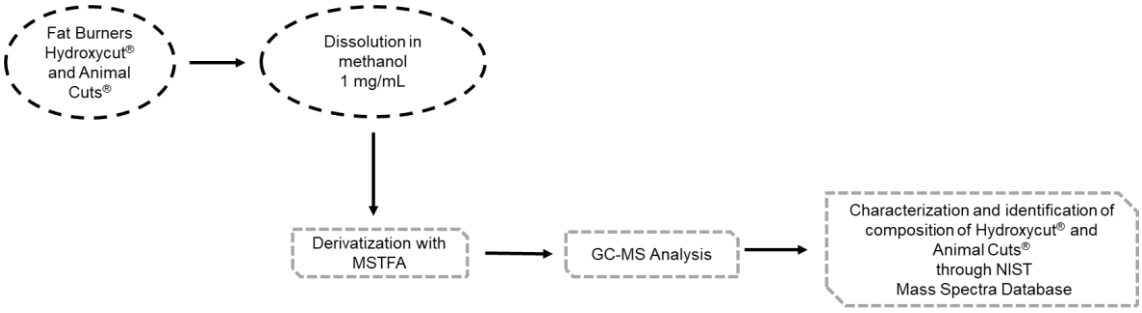


Figure 14: Schematic representation of the procedure used for the chemical characterization of 'fat burners'. Legend: GC-MS – Gas Chromatography coupled to Mass Spectrometry; MSTFA – N-methyl-N-trimethylsilyl-trifluoroacetamide; NIST – National Institute of Standards and Technology.

The main difficult in the characterization and detection of adulterants in these products was that the matrices are often very different and compounds exhibit rather different physicochemical characteristics (Rebiere et al., 2012). The dissolution of compounds was also performed in methanol. This choice is in accordance with methodologies previously described in literature (Chen et al., 2009; Rocha et al., 2016; Vaysse et al., 2010). Nevertheless, some problems of solubility had arisen due to elevated amount of

plant extracts present in these formulations, but after testing alternative solvent systems, we considered that this would still be the most suitable solvent for this type of analysis.

3.2.2.1. Supplement 1

Supplement 1 was introduced in the market in 2002 as a weight loss supplement but shortly thereafter the first reports of acute hepatocellular injury appeared. The original formulation was then changed by the manufacturer but these types of intoxications continue to occur (Araujo and Worman, 2015; Garcia-Cortes et al., 2016). In 2009, FDA warned the public about the severe risk of liver injury attributed to the 'fat burner' and the manufacturer withdrew it (Avigan et al., 2016; Garcia-Cortes et al., 2016; Stickel and Shouval, 2015). Nowadays, Supplement 1 is marketed under a few different variants. Supplement 1 was listed as one of the most frequent products seized by Brazilian Federal Police, from 2007 to 2013 (da Justa Neves and Caldas, 2015). We choose another variant of these line of products – Supplement 1 (figure 15).

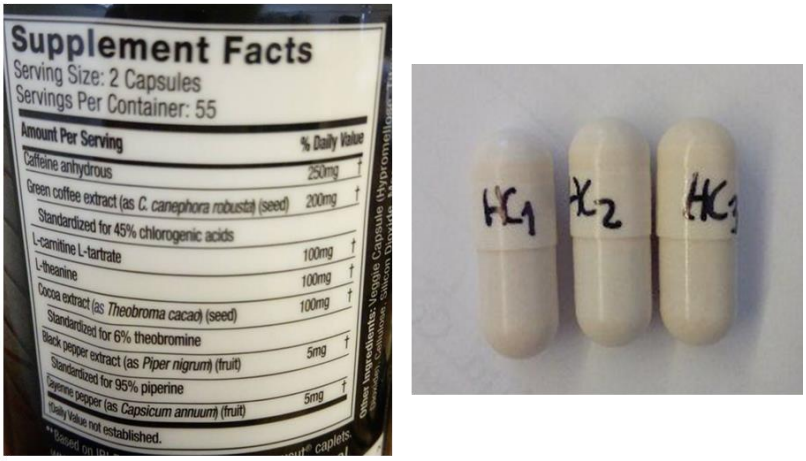


Figure 15: Supplement 1 – Commercial product purchased from prozis.com to carry out this work.

In order to provide a more comprehensive characterization of the composition of the Supplement 1 Supplement 1, GC-MS analysis was carried out with and without derivatization. Accordingly, derivatization with MSTFA was performed due to its usefulness for the detection of early-eluting compounds. Derivatization allowed obtaining more clean chromatograms, better separations with increased resolution and response,

and increased sensitivity of the method, as observed by chromatograms shown in figure 16.

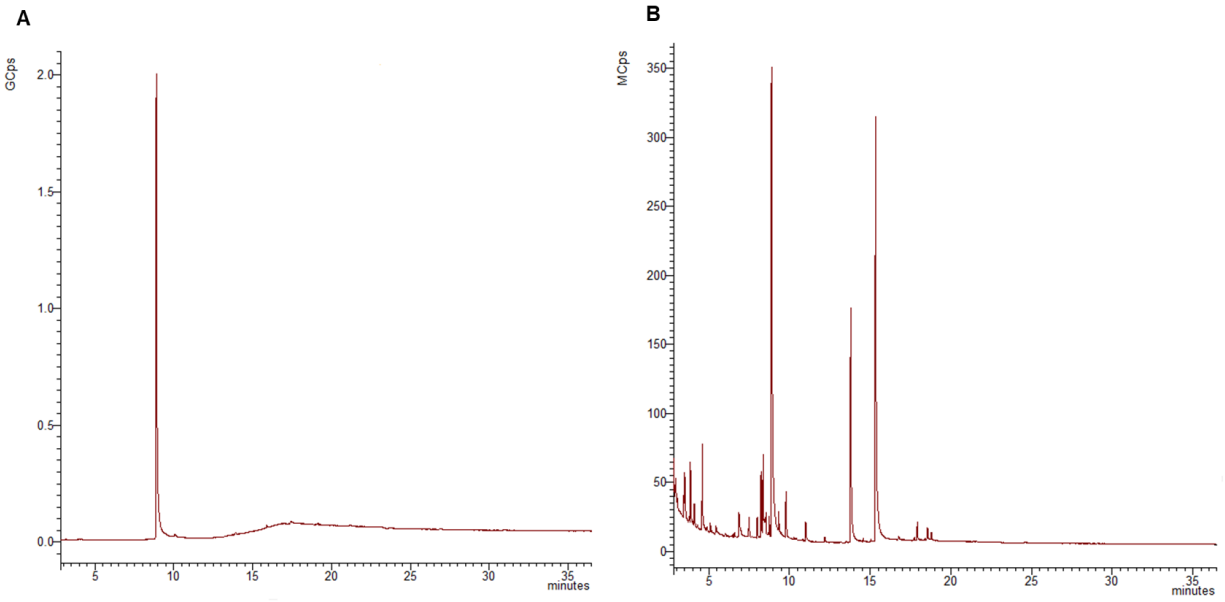


Figure 16: Comparison between Supplement 1 chromatographic profile directly injected (A) into the GC-MS and injected after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (B).

Methanol solutions of three capsules of Supplement 1 were prepared, derivatized, and analysed in GC-MS with the aim of understanding if composition of different capsules of the same package are the same or if there is a lack of qualitative and quantitative uniformity, as described by other authors (Araujo et al., 2015). Similar chromatographic profiles were obtained for all of them (figure 17).

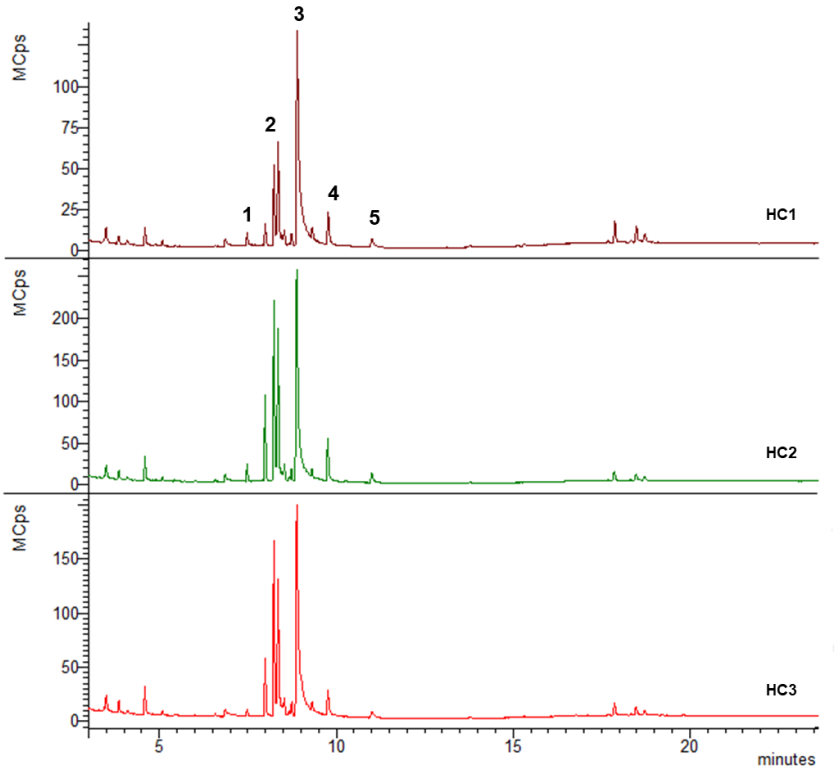


Figure 17: Full scan chromatographic profile of methanol solutions of Supplement 1 (HC) injected after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) of three different capsules (HC1, HC2 and HC3) of the same product, revealing qualitative similarity between them. More relevant peaks: 1 – Tartaric acid, 4TMS; 2 – Unidentified peak; 3 – Caffeine; 4 – Palmitic acid, TMS; 5 – Stearic acid, TMS.

Using the mass spectrum information of each chromatographic peak (*e.g.*, base peak and molecular ion) we could establish relationships with the spectra database. By performing the intersection of information obtained from mass spectra interpretation with mass spectra database (NIST14 library), we could identify the composition of this slimming product. In addition, the identity of lactic acid, oxoproline, tartaric acid, quininic acid, caffeine, palmitic acid and stearic acid was further confirmed. Based on knowledge of the molecular weight after derivatization and ions resulting from fragmentation, comparisons with retention times and the mass spectrum of the derivatized compounds and derivatized standards were established. Positive matches were obtained for all these seven compounds. The other compounds were assigned potential identifications through the analysis of their mass spectra and comparison with database (table 7).

Table 7: Retention time (min), base peak (m/z), other characteristic ions (m/z), calculated kovats, library kovats, match and Rmatch values of active ingredients of Supplement 1 detected after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA).

Compound	Retention time (min)	Base peak (m/z)	Characteristic ions (m/z)	Calculated kovats	Library Kovats	Standard	Match	RMatch
Lactic acid, 2TMS	3.51	73	147+117+75			×	915	960
α-Hydroxyisobutyric acid, 2TMS	3.79	73	131+147+75	1089	1071		795	846
L-5-Oxoproline, 2TMS	6.87	156	73+147+157			×	913	957
1-Dodecanol, TMS	7.16	73	111+75+156	1567	1558		483	627
L-(+)-Tartaric acid, 4TMS	7.49	73	147+292+74			×	866	913
D-(-)-Fructofuranose (isomer 1)	8.43	117	73+217+437	1780	1792		553	614
L-(-)-Sorbofuranose	8.50	73	217+147+75	1791	1779		846	857
D-(-)-Fructopyranose, 5TMS	8.55	73	204+147+217	1800	1802		874	877
Quininic acid, 5TMS	8.75	73	147+345+255			×	812	874
Caffeine	8.87	194	67+109+55			×	949	949
Palmitic acid, TMS	9.77	73	117+75+129			×	906	907
1-Octadecanol, TMS	10.41	75	73+327+58	2148	2152		652	790
Stearic acid, TMS	11.00	73	117+75+129			×	887	944
11-Eicosenoic acid	11.89	57	83+55+75	2348	2360		464	552
Arachidic acid, TMS	12.62	73	117+75+69	2436	2449		471	750
Docosanol, TMS	13.50	75	73+383+57	2543	2542		692	774
Behenic acid, TMS	14.23	117	73+69+95	2633	2644		608	742
5-O-Feruloylquinic acid, 5TMS	17.74	73	75+345+249	3081	3074		712	786
3-Caffeoylquinic acid	17.93	73	345+255+147	3102	3092		838	849
4-O-Feruloylquinic acid, 5TMS	18.40	73	249+255+147	3149	3138		637	750

Legend: m/z – mass to charge ratio; TMS – trimethylsilyl.

A total of 23 different compounds were detected. Most of them belongs to the class of sugars, specifically derivatives of fructose (fructofuranose, sorbofuranose and fructopyranose), as well as other substances such as are fatty acids (behenic acid, arachidic acid, eicosenoic acid, stearic acid and palmitic acid). Substances of other representative classes were also found, namely caffeine, which is a well-known stimulant. Other substances as caffeoylquinic acid and feruloyquinic acid are phenols, which present important antioxidant effects. Apparently, this composition is not associated with the possibility of toxic effects. Notwithstanding, it should be noted that Supplement 1 has already been reported as causing hepatotoxicity due to components such as *Garcinia cambogia*, *Gymnema sylvestre*, chromium polynicotinate, caffeine and green tea (Stickel and Shouval, 2015).

In addition to the qualitative similarity exploited above, it was also noted that there are some differences between the Supplement 1 composition obtained by GC-MS analysis and that labeled on the package. This probably occurs due to the problems associated with low solubility of plant extracts in organic solvents but also due to unspecific information on the supplement label. In addition, we cannot conclude with certainty that a specific compound is not present in the product because it may be present at an insufficient amount to be detected under the conditions used in this study.

3.2.2.2. Supplement 2

Supplement 2 belongs to a set of products that includes hypertrophic supplement, anabolic supplement with essential amino-acids, non-hormonal anabolic supplement, pre-workout muscle volumizing supplement, joint support supplement, essential fatty acids supplement, rest and recovery supplement and multivitamin supplement. Along with Supplement 1, Supplement 2 was also listed as one of the most frequent products seized by Brazilian Federal Police, from 2007 to 2013 (da Justa Neves and Caldas, 2015). Supplement 2 is a thermogenic formulation that was selected among all varieties to the present study (figures 18). The package label of the product presenting chemical composition is displayed in figure 19.

Figure 18: Supplement 2. Commercial product purchased from prozis.com to carry out this work. The capsules were wrapped with plastic bags, which by turn were sold in a metallic container.

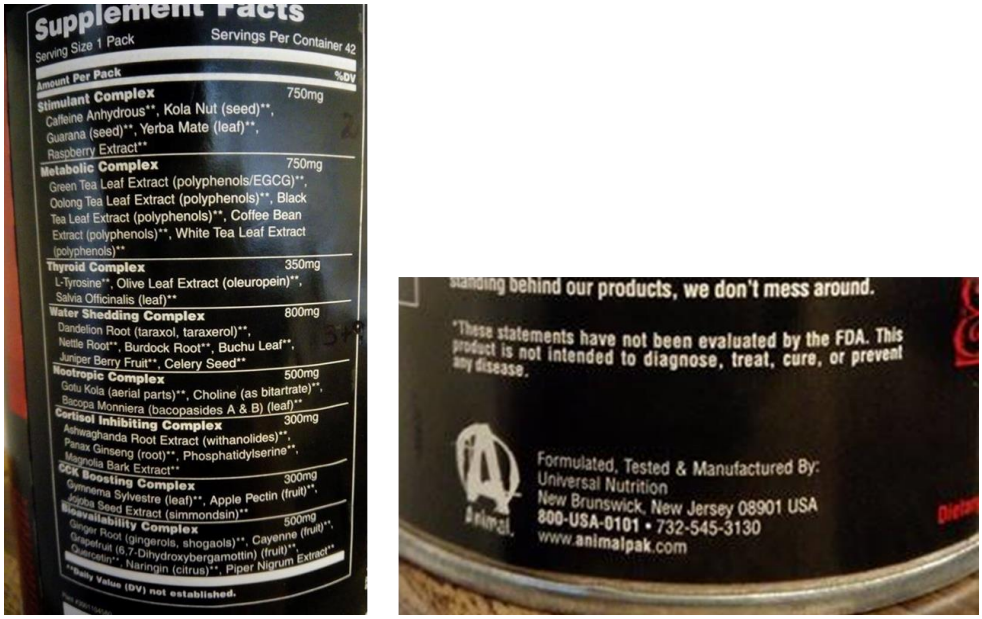


Figure 19: Label of Supplement 2. It is clear on the package label the indication 'these statements have not been evaluated by the FDA'.

Similar to the procedure performed for Supplement 1, nine capsules of Supplement 2 (1–9) contained in each of the three plastic bags (A, B, and C) were dissolved in methanol and directly injected into the GC-MS equipment. In parallel derivatization with MSTFA of these samples was also performed. The obtained chromatograms are shown in figure 20.

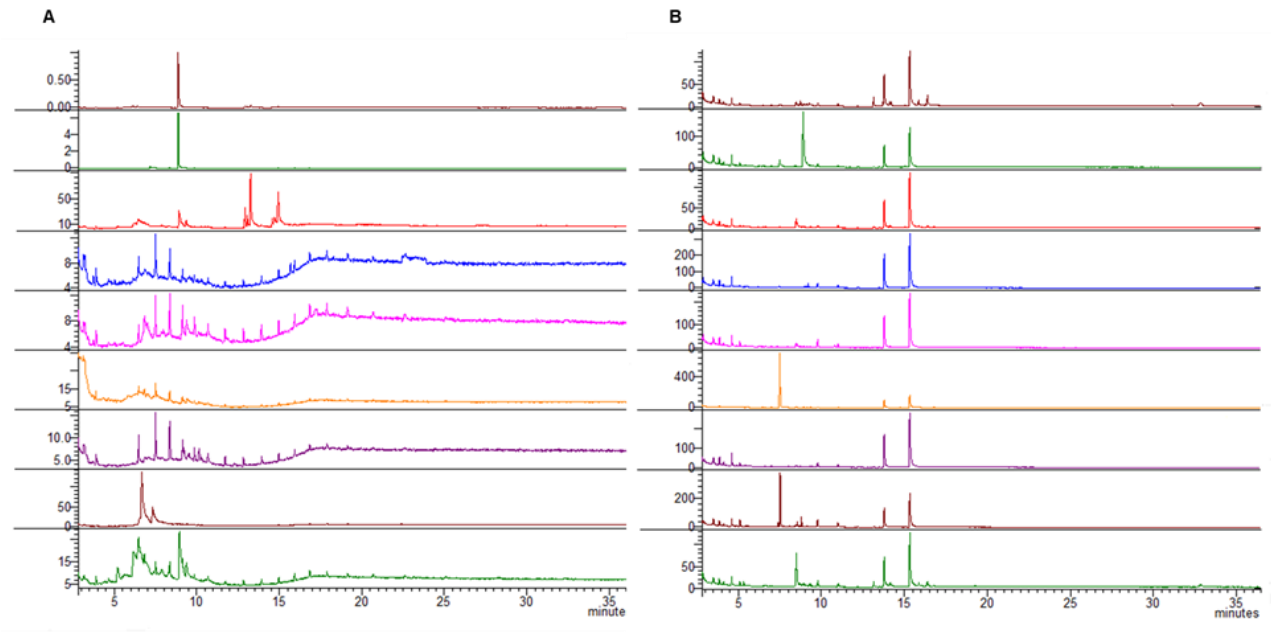


Figure 20: Comparison between Supplement 2 chromatograms obtained without (A) or with (B) derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA).

According to the chromatograms presented, we verify that the derivatization process increased the sensitivity of the GC-MS method and also allowed more clean chromatograms.

Methanol solutions of Supplement 2 capsules (1–9) contained in three different plastic bags (A – C: composed of nine capsules each) were prepared, derivatized, and analysed in GC-MS with the aim of understanding if composition of different capsules of the same package would be the same or lack qualitative and quantitative uniformity.

Results of capsules presenting the same colour from each of the three different plastic packages (A – C), produced similar chromatographic profiles, as exemplified for capsule 1, in figure 21. Similarly, chromatograms of capsules 2–9 revealed homogeneity in qualitative composition among the different plastic packages.

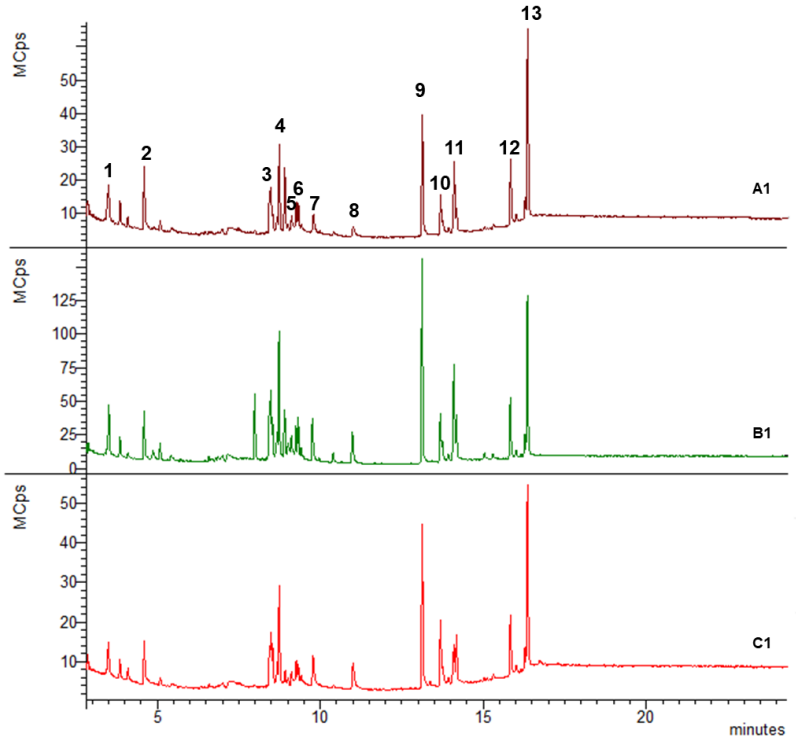


Figure 21: Full scan chromatograms of methanol solutions of capsule 1 (white capsule) from plastic bags A, B and C of Supplement 2, injected after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), revealing similar qualitative composition. More relevant peaks: 1 – Unidentified peak; 2 – Unidentified peak; 3 – D-(-)-Tagatofuranose; 4 – Quininic acid, 5TMS; 5 – Caffeine; 6 – Unidentified peak; 7 – Palmitic acid, TMS; 8 – Stearic acid, TMS; 9 – Unidentified peak; 10 – Lactulose; 11 – Unidentified peak; 12 – Catechine, 5TMS; 13 - Epigallocatechin, 6TMS.

Using the mass spectrum information of each chromatographic peak (*e.g.*, base peak and molecular ion) we could establish relationships between experimental data and the spectra database. The presence of lactic acid, threonine, quininic acid, caffeine, palmitic acid, stearic acid and catechin were confirmed by comparing retention times and mass spectra of each chromatographic peak with the respective standards. Positive matches were obtained for all these seven compounds. The other substances were assigned potential identifications through the analysis of their mass spectra and comparison with compounds of database (NIST14 library) (Table 8).

Table 8: Retention time (min), base peak (m/z), other characteristic ions (m/z), calculated kovats, library kovats, match and Rmatch values of active ingredients of Supplement 2 detected after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA).

Compound	Retention time (min)	Base peak (m/z)	Characteristic ions (m/z)	Calculated kovats	Library Kovats	Standard	Match	RMatch
Lactic acid, 2TMS	3.50	73	147+117+130			×	771	907
L-Threonine	4.10	73	77+116+134			×	437	439
D-(-)-Tagatofuranose	8.49	73	217+147+75	1791	1801		905	908
D-(+)-Talofuranose	8.69	73	217+75+129	1829	1823		718	755
Quininic acid, 5TMS	8.76	73	147+345+75			×	801	861
D-(+)-Galactopyranose, 5TMS	8.93	73	204+191+147	1877	1889		856	870
Caffeine	9.06	194	67+109+55			×	756	864
2,3,4,5,6-pentahydroxyhexanoic acid, 4TMS	9.42	73	217+147+129	1972	1981		553	651
Palmitic acid, TMS	9.78	73	117+75+129			×	829	851
Stearic acid, TMS	11.01	73	117+75+129			×	814	908
2-Palmitoylglycerol, 2TMS	13.50	73	129+75+147	2543	2558		659	711
Lactulose	13.72	73	217+75+147	2569	2587		764	779
Catechine, 5TMS	15.86	73	368+355+369			×	808	859
Epigallocatechin, 6TMS	16.39	73	456+355+457	2914	2904		852	860

Legend: m/z – mass to charge ratio; TMS – trimethylsilyl.

Similarly to Supplement 1, analysis of Supplement 2 revealed high predominance of sugars and fatty acids. A total of 14 different substances were identified; among them, the sugars tagatofuranose, talofuranose, galactopyranose, lactulose and gulonic acid. Fatty acids palmitic acid and stearic acid were also present in all capsules (1–9), along with other substances such as the antioxidant polyphenols catechin and epigallocatechin. Apparently, this composition is not related to the possibility of toxic effects.

We detected the presence of amino acids in some capsules such as, threonine in capsule 1, proline in capsule 3, tyrosine in capsule 4, alanine in capsule 6 and valine, isoleucine, proline and threonine in capsule 9. Also, in capsule 5 linoleic acid and other two fatty acids were noticed. According to Williams (2005), amino acids are involved in the secretion of anabolic hormones, preventing adverse effects of overtraining and preventing mental fatigue.

The lack of consistency in qualitative composition is a serious threat for users and increases the risk of acute toxicity associated with the product consumption. This inconsistency also hampers the assessment of the clinical state of the patient and consequently the application of an appropriate treatment. The supplements that are adulterated may contain only one or more unauthorized substances as described by Vaysse et al. (2010). When a substance is replaced by another on the product label, when it is omitted, or when the amount presented and labeled diverge, the effects can be significantly different. These differences will have an impact on duration of action, on the time required to cause the effects, and on the multiplicity of effects and respective interactions, and could result in severe intoxications. In addition, all these problems demonstrate the lack of control associated to these products regarded as ‘safe’, showing that sellers only concern monetary profits, putting aside the health of users. Worryingly, recent studies on herbal and dietary supplements indicate that herbal-related drug-induced liver injury have increased between 2004 to 2013 (Navarro et al., 2014). As example, another product used for weight loss and improvement of physical performance, was associated to liver pathology (acute and fulminant hepatitis) (Johnston et al., 2016; Stickel and Shouval, 2015).

3.2.3. Caffeine quantitation

Caffeine is frequently present in dietary supplements as a stimulant. According to a study published by Rebiere et al. (2012), the amount of caffeine per capsule of dietary

supplements ranged between 4 mg and 327 mg (per unit). Of concern, even ingredients such caffeine, which is legal and common in supplements, can provoke serious toxicity (da Justa Neves and Caldas, 2015), as supported by case reports of caffeine intoxications (Holmgren et al., 2004). So, we decided to quantify the amount of caffeine present in these two different formulations.

By virtue of the lacking of binding sites for MSTFA, the molecular structure of caffeine, and consequently the originated mass spectrum, is not altered with derivatization. Methanol solutions with different caffeine concentrations were directly injected into the GC-MS and a standard curve calibration was plotted, allowing us to quantify the amount of caffeine present in capsules that proved to contain caffeine in their composition in previous analyses. We verified that Supplement 1 has approx. 45 mg of caffeine per capsule. So, assuming the manufacturer's recommendations of a daily intake of two capsules, consumers will ingest an amount of approx. 90 mg of caffeine per day. For the sake of comparison, a cup of coffee contains about 100 mg of caffeine (Holmgren et al., 2004) or between 40–150 mg, depending on the source (Kerrigan and Lindsey, 2005). Since the amount we achieved in our experimental determination is close to that present in a cup of coffee, it seems quite acceptable, considering that the maximum recommended daily intake is approximately 400 mg (equivalent to the administration of four coffee cups).

Regarding the Supplement 2, only two of the capsules of each plastic bag contained caffeine in their composition, namely capsules 1 and 2. Nevertheless, we obtained the values of 130 mg and 294 mg for quantification of caffeine in these two capsules. Considering that the manufacturer instructions suggest a daily intake of two plastic sachets, the amount of caffeine ingested is about 848 mg of caffeine per day. This value significantly exceeds the recommended daily intake and the dosages commonly observed in numerous dietary supplements, which range from 32 to 200 mg (Kerrigan and Lindsey, 2005). Nevertheless, some studies point out to the discrepancies observed in the real amounts of caffeine present in supplements and the significantly different amounts claimed on the respective labels (Inacio et al., 2016).

Although caffeine intoxications are not very frequent, it has some characteristics that makes it riskier than usually considered. It is easy to obtain, has fast effect and some interactions with pharmaceuticals, such as antidepressants, that substantially raise the plasma level of caffeine after simultaneous intake (Holmgren et al., 2004).

PART 4 – HEPATOTOXICITY OF 2,4-DINITROPHENOL (2,4-DNP)

CHAPTER 1: MATERIALS AND METHODS

4.1.1. Reagents

As referred, the Supplement 1 and Supplement 'fat burners' were purchased from Prozis Portugal. Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (Lisbon, Portugal) and all the cell culture reagents from Gibco (Alfagene, Lisbon, Portugal).

4.1.2. Animals

Male Wistar Han rats with a body weight of 150–250 g were purchased from Charles River Laboratories (Barcelona, Spain) and used in all experiments as the source of hepatocytes. Upon arrival at the Rodent Animal House Facility of our institution, the animals were acclimated in polyethylene cages with wire-mesh at the top, lined with wood shavings, for at least 2 weeks prior to use. The animals were kept under an aseptic environment with controlled temperature (20 ± 2°C), humidity (40-60 %) and lighting (12h light/dark cycle) conditions, and had ad libitum access to sterile water and standard rat chow. Surgical procedures for the isolation of hepatocytes were always conducted between 8 and 10 a.m. and were performed after animal anaesthesia induced by an intraperitoneal injection of a combination of 20 mg/kg xylazine (Rompun® 2%, Bayer HealthCare, Germany) and 100 mg/kg ketamine (Clorketam® 1000, Vétoquinol, France) and maintained by inhalation of isoflurane (IsoVet® 1000, B. Braun VetCare, Germany).

Housing and experimental treatment of animals were approved by the local committee for the welfare of experimental animals and performed in accordance with national legislation by investigators accredited for laboratory animal use by the national authority *Direção Geral de Alimentação e Veterinária* (DGAV), under the terms of the Law No. 113/2013, of 7th August (Decreto-Lei N.º 113/2013, de 7 de Agosto, in Diário da República, 1.ª série – N.º 151).

4.1.3. Isolation and culture of primary hepatocytes

The technique used for the isolation of rat hepatocytes is based on a collagenase perfusion method, as described by Dias da Silva et al. (Dias da Silva et al., 2017). Following a V-shaped incision of the abdomen, the exposed portal vein was punctured with a cannula and the liver perfused in situ with a sterile ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)-buffer at 37°C, for approximately 15 min, at an output flow of 10 mL/min, to allow the cleavage of the hepatic desmosomes

through calcium sequestration by EGTA. The EGTA-buffer was prepared with 248 mL of 9 g/L d-glucose, 40 mL of Krebs-Henseleit buffer (60 g/L NaCl, 1.75 g/L KCl, and 1.6 g/L KH_2PO_4; pH 7.4), 40 mL of 60 g/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8,5), 30 mL of minimum essential media (MEM) non-essential amino acid solution (100×), 30 mL of MEM amino acids solution (10×), 4 mL of 7 g/L l-glutamine, and 1.6 mL of 47.5 g/L EGTA (pH 7.6). Subsequently, hepatic collagen was hydrolysed by perfusion of the liver with a sterile collagenase solution for 10-15 min, at 37°C; this consisted of 155 mL of 9 g/L d-glucose, 25 mL Krebs-Henseleit buffer; 25 mL of 60 g/L HEPES (pH 8.5), 15 mL of MEM non-essential amino acid solution (100×); 15 mL of MEM amino acids solution (10×); 10 mL of 19 g/L $CaCl_2.2H_2O$ solution, 2.5 mL of 7 g/L l-glutamine solution, and 300 U/mL collagenase type IA from *Clostridium histolyticum*. During the two-step perfusion, both solutions were continuously aerated by a stream of carbogen (95% O_2 and 5% CO_2). After perfusion, the liver was dissected, removed from the animal and the hepatocytes were dissociated in a sterile suspension buffer by disruption of the Glisson's capsule. The suspension buffer contained 124 mL of 9 g/L d-glucose, 20 mL Krebs-Henseleit buffer, 20 mL of 60 g/L HEPES buffer (pH 7.6), 15 mL of MEM non-essential amino acid solution (100×), 15 mL of MEM amino acids (10×), 2 mL of 7 g/L l-glutamine solution, 1.6 mL of 19 g/L $CaCl_2.2H_2O$ solution, 0.8 mL of 24.6 g/L $MgSO_4.7H_2O$ solution, and 400 mg bovine serum albumin. As the viable hepatocytes have a higher density than the non-parenchymal cells and dead hepatocytes, the suspension was purified through three cycles of low-speed centrifugation (250 × g, for 5 min, at 4°C) to obtain a highly enriched suspension of hepatocytes. The initial viability of isolated hepatocytes was estimated by the trypan blue exclusion test and it was always above 85%. Trypan blue is an organic negatively charged dye, which is only included inside the cells that present membrane cytoplasmic damage, as a result of the loss of energy-dependent transport and, consequently, of the selectivity in the compounds that traverses the membrane (Strober, 2001).

A suspension of 5×10^5 viable cells/mL in complete culture medium [William's E medium (Sigma-Aldrich, Lisbon, Portugal) supplemented with 10% fetal bovine serum (FBS), 2 ng/mL insulin solution from bovine pancreas (Sigma-Aldrich, Lisbon, Portugal), 5 nM dexamethasone (Sigma-Aldrich, Lisbon, Portugal), 1% antibiotic solution (10.000 U/mL penicillin and 10.000 µg/mL streptomycin), 10 µg/mL gentamicin and 0,25 µg/mL amphotericin B] was seeded onto the central 60 wells of 96-well plates (5×10^4 cells/well) or onto 6-well plates (1×10^6 cells/well), pre-coated with collagen. After seeding, primary hepatocytes were incubated overnight at 37°C, in an atmosphere of 5% CO_2, to allow

cell adhesion. After adhesion, the medium was gently aspirated and the cells were exposed to the test compounds at the selected concentrations.

4.1.4. Incubation of primary rat hepatocytes with test compounds

All drug stock solutions were prepared and stored at -20°C. Subsequent test dilutions were freshly prepared in exposure medium (complete cell culture medium without FBS), immediately before each experiment.

Stock solutions of 2,4-DNP were prepared in ethanol (PanReac AppliChem, analysis 99,8%; Germany). For the cell viability experiments [neutral red (NR), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays], primary rat hepatocytes were exposed to a large interval of 2,4-DNP concentrations (ranging from 0.55 µM to 10 mM). All the other assays that allowed to clarify the mechanisms of hepatotoxicity were performed at five test concentrations: 1 µM; 10 µM; 50 µM; 100 µM; and 200 µM.

Stock solutions of Supplement 1 and Supplement 2 'fat burners' were prepared by extracting the content of each capsule with 20 mL Hank's Balanced Salt Solution (HBSS). These extracts were sonicated for 15 min and the obtained solution was filtered. These solutions were then used for the *in vitro* evaluation of cytotoxicity. One aliquot (1 mL) of each extract was evaporated under nitrogen flow. The obtained residue was resuspended in methanol and characterized by GC-MS. For the evaluation of the elicited mortality by the MTT reduction assay, the cells were exposed to concentrations ranging from 228 ng/mL to 100 µg/mL.

All compound incubations were performed for 24h, at 37°C, in a humidified 5% CO_2 atmosphere.

4.1.5. Cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

The cytotoxic effects of 2,4-DNP and of the fat burner extracts were determined using the MTT reduction assay. This assay measures the succinate dehydrogenase activity, an indicator of metabolically active mitochondria. The MTT, a soluble yellow dye, is reduced to insoluble purple formazan crystals by these mitochondrial reductases. This reaction only occurs in living cells, providing a measure of cell viability that can be

spectrophotometrically quantified. A protocol previously described by Dias da Silva et al. (2014) was used. Briefly, after 24h incubations with the test compounds, the culture medium was aspirated. Then, 100 µL of a fresh solution of 1 mg/mL MTT prepared in cell culture medium was gently added to the attached cells. Plates were incubated at 37°C for 30 min in a humidified, 5% CO_2 atmosphere. After this, the MTT solution was removed and the formed intracellular crystals of formazan were dissolved in 100 µL of dimethyl sulfoxide (DMSO). The absorbance was measured at 550 nm, after 15 min shaking, in a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.). Because MTT is a photosensitive dye, all steps of the procedure were done under light protection.

Data were obtained from four independent experiments for 2,4-DNP or from one independent experiment for 'fat burner' extracts, run in triplicates, with each plate containing increasing concentrations of the test compounds. To reduce inter-experimental variability, data from each plate were normalized and scaled between 0% (negative controls, cells treated only with cell exposure medium) and 100% of the effect (positive controls, cell exposure medium with 1% Triton X-100) and results from four independent experiments were graphically presented as percentage of decrease in MTT reduction *versus* 2,4-DNP concentration (mM).

4.1.6. Cytoplasmic membrane integrity by the lactate dehydrogenase (LDH) leakage assay

The LDH leakage assay was performed to access the cytoplasmic membrane integrity of primary rat hepatocytes after exposure to 2,4-DNP. This is one of the most widely used assays for the determination of the toxicity of drugs *in vitro*. Since LDH is a cytoplasmic oxidoreductase, its presence in the extracellular medium is indicative of alterations in membrane permeability and consequently in cell integrity. Therefore, the assay offers a feasible indication of cell viability. The enzyme LDH catalyses the reversible conversion of pyruvate to lactate in the presence of β-nicotinamide adenine dinucleotide (β-NADH), which in turn is oxidized to nicotinamide adenine dinucleotide (NAD^+).

At the end of the incubation period with the cell treatments, a 10 µL aliquot of cell exposure medium of each well was placed into a new 96-well plate. Then, 40 µL of 0.05 M potassium phosphate buffer (pH 7.4; KH_2PO_4; Merck) and 200 µL of 0.15 mg/mL β-nicotinamide adenine dinucleotide (β-NADH) solution were added. Immediately before

the absorbance reading, 25 µL of 2.5 mg/mL sodium pyruvate solution were added into each well to start the reaction. Both β-NADH and sodium pyruvate solutions were freshly prepared in potassium phosphate buffer. The kinetic of the oxidation of NADH to NAD^+ was followed by measuring the absorbance at 340 nm, every 16 seconds, for 3 min, using an automatic plate reader Power Wave X™ (BioTek Instruments, Inc.), in a kinetic photometric mode. The data obtained in four independent experiments were scaled between positive (culture media with 1% Triton X-100; 100% cell death) and negative (cells treated only with cell culture medium; 0% cell death) controls and results were graphically presented as percentage of extracellular LDH *versus* 2,4-DNP concentration (mM).

4.1.7. Lysosome integrity by the neutral red (NR) incorporation assay

To provide supplementary cell viability data, we performed the NR uptake assay. This assay provides an indirect quantitative estimation of the number of viable cells in culture through the evaluation of the lysosomal integrity. It is based on the ability of live cells to incorporate and bind the weak cationic dye NR, which crosses the cell membrane by non-ionic diffusion, and accumulates in the lysosome of viable cells by interaction with anionic sites in the lysosomal matrix. Dead or damage cells lose their ability to retain the dye and, consequently, lose their colour in the washing procedure.

At the end of 24h of incubation with 2,4-DNP, the exposure medium of the primary rat hepatocytes was aspirated and added 100 µL of 50 µg/mL NR solution, prepared in culture medium. The cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 30 min, allowing the lysosomes of viable cells to incorporate the dye. Then, the cells were carefully washed twice with 200 µL HBSS to eliminate extracellular dye (that was not incorporated in dead or damaged cells) and lysed with 100 µL of lysis solution (50% ethanol, 1% glacial acetic acid, 49% water solution). The absorbance was measured at 540 nm in a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.). The data obtained in four independent experiments were normalised to positive (1% Triton X-100) and negative (only culture medium) controls and results were graphically presented as percentage of cell death *versus* 2,4-DNP concentration (mM).

4.1.8. Measurement of intracellular reactive oxygen (ROS) and nitrogen (RNS) species

The intracellular reactive oxygen (ROS) and nitrogen (RNS) species production was monitored via 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay, as described by Dias da Silva et al. (2014). DCFH-DA is a lipophilic sensitive probe that readily diffuses into the cells, where it is deacetylated by cellular esterases, producing 2',7'-dichlorodihydrofluorescein (DCFH). Then, DCFH is further oxidized by intracellular ROS and RNS, generating the green fluorescent 2',7'-dichlorofluorescein (DCF), which is polar and trapped within the cells.

As DCFH-DA is a non-water-soluble powder, a 4 mM DCFH-DA stock solution was initially prepared in DMSO and stored at -20°C in an opaque airtight container. Immediately before each experiment, the final concentration was made up in fresh cell culture medium (ensuring that the final concentration of DMSO did not exceed 0.05%). On the day of the experiment, hepatocytes in 96-well plates were incubated with 10 µM DCFH-DA, for 30-60 min, at 37°C, previous to the hepatocyte incubations with 2,4-DNP. The cells were then rinsed with HBSS and incubated, at 37°C, during 24h, with the 2,4-DNP test concentrations. A blank control (no cells) was also included in the plate. Fluorescence was measured in a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.) set to 485 nm excitation and 530 nm emission. As DCFH-DA is photosensitive, all steps of the procedure were performed under light protection. The data from four independent experiments are presented as the percentage of negative controls, with each concentration tested in three replicates within each experiment.

4.1.9. Measurement of intracellular total glutathione (tGSH) and oxidized glutathione (GSSG)

Total glutathione (tGSH) and oxidized glutathione (GSSG) levels were determined by the 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB)-GSSG reductase-recycling assay, as previously described by Dias da Silva et al. (2014). For this purpose, primary rat hepatocytes were seeded in 6-well plates and exposed to 2,4-DNP, at 37°C. After a 24h-incubation period, cells were rinsed with 1 mL HBSS, scrapped with 300 µL 5% perchloric acid ($HClO_4$, w/v; Merck) per well, and incubated for 20 min, at 4°C. After centrifugation (13,000g, 5 min, 4°C), the supernatants were collected and stored at -20°C until further determination of GSH and GSSG. The obtained pellet was suspended in NaOH 1 M and used for protein quantification, through the Lowry assay. For tGSH determination, the

supernatants of samples, blank (5% $HClO_4$) and standards were neutralized with 200 µL 0.76 M $KHCO_3$ (vortex mixing until there was no formation of CO_2), on ice. The samples were then centrifuged for 10 min, at 13,000g, at 4°C, and 100 µL of each supernatant were added to a 96-well plate followed by 65 µL of a freshly prepared reagent solution containing 4 mM DTNB and 0.69 mM NADPH in a phosphate buffer (71.5 mM Na_2HPO_4; 71.5 mM $NaH_2PO_4.H_2O$; 0.63 mM EDTA; pH 7.5). Samples were incubated for 15 min at 30°C. Then, 40 µL of 10 U/mL glutathione reductase (GR) solution (freshly prepared in phosphate buffer) was rapidly added to all samples. The consequent formation of 5-thio-2-nitrobenzoic (TNB) by oxidation of GSH was followed for 3 min, every 10 seconds, at 405 nm, using a multi-well plate reader (Power Wave XTM, BioTek Instruments, Inc.) and compared with a GSH standard curve prepared in 5% $HClO_4$. To determine intracellular GSSG, 10 µL of 2-vinylpyridine were added to 200 µL of neutralised supernatants (samples, blank and standards) and mixed for 1h, at 4°C. As 2-vinylpyridine blocks GSH, GSSG will be determined as described for tGSH. The amount of reduced glutathione (redGSH) was calculated by subtracting GSSG from the tGSH [redGSH = tGSH – (2 × GSSG)]. The GSSG standard solutions were prepared in 5% $HClO_4$. The standard concentrations ranged between 0.25 µM and 15 µM for GSH and between 0.10 µM and 13 µM for GSSG. Results from four independent experiments were normalised to the protein content and the final results were presented as nmol per mg of protein.

4.1.10. Assessment of mitochondrial membrane potential (ΔΨm)

Assessment of mitochondrial integrity was performed by measuring tetramethylrhodamine ethyl ester (TMRE) inclusion as described by Dias da Silva et al. (2014). TMRE is a cell permeable fluorescent dye, positively charged, that labels active mitochondria by accumulation as a consequence of to their relative negative charge. Primary rat hepatocytes were seeded onto 96-well plates and incubated with 2,4-DNP for 24h, at 37°C. At the end of the incubation period, the cells were rinsed with HBSS and incubated at 37°C, for 15-30 min, with 100 µL of 2 µM TMRE, in the dark. A 2 mM TMRE stock solution was initially prepared in DMSO (TMRE is a non-water-soluble powder) and stored at -20°C, protected from light. This final 2 µM concentration of TMRE was prepared in fresh culture media, shortly before use (ensuring that the final concentration of DMSO did not exceed 0.05%). Then, the media was gently aspirated and the cells were washed with 200 µL HBSS. Fluorescence was measured on a fluorescence multi-well plate reader (BioTek Synergy™ HT, BioTek Instruments Inc.) set to 544 nm excitation and 590 nm emission. As TMRE is photosensitive, all steps of the

procedure were performed under light protection. The data obtained in four independent experiments with each concentration tested in three replicates were calculated as the percentage of negative controls.

4.1.11. Measurement of intracellular adenosine triphosphate (ATP)

The ATP levels were quantified by a bioluminescence assay that measures the light formed in the oxidation of luciferin, catalysed by luciferase in the presence of ATP, as described by Dias da Silva et al. (2015). Primary rat hepatocytes were seeded in 6-well plates and exposed to 2,4-DNP, at 37°C. After a 24h-incubation period, cells of each well were rinsed with 1 mL HBSS and scrapped with 300 µL 5% perchloric acid (w/v). Samples were incubated for 20 min, at 4°C. After centrifugation (13,000 g, 5 min, 4°C), the obtained pellet was suspended in 1 M NaOH and used for protein quantification, through the Lowry assay. The supernatants were collected and kept at -20°C until further ATP determination. The thawed acidic supernatants were then neutralized with an equal volume of 0.76 M $KHCO_3$ and centrifuged for 10 min, at 13,000g (4°C) and the ATP contents were measured in triplicate by adding 75 µL of the neutralized supernatants, standards or blank (5% $HClO_4$, w/v) to 75 µL of the D-luciferin-luciferase solution [0.15 mM luciferin; 300,000 light units of luciferase from *Photinus pyralis* (American firefly); 50 mM glycine; 10 mM $MgSO_4$; 1 mM Tris; 0.55 mM EDTA; 1% BSA (pH 7.6)]. ATP calibration curve was obtained with standard stock solutions of ATP prepared in 5% $HClO_4$. The emitted light intensity of the samples was determined using a luminescence multi-well plate reader (BioTek Synergy™ HT, BioTek Instruments Inc.) and compared with an ATP standard curve (1.25–20 µM) performed within each experiment. Data from four independent experiments were normalized to the protein content and the results were expressed as nmol per mg of protein.

4.1.12. Determination of protein

The protein content in the samples for ATP and GSH/GSSG determinations was quantified by the Lowry assay (Lowry et al., 1951). Accordingly, 50 µL of each supernatant, standard or blank were transferred in triplicate into a 96-well plate and added with 100 µL reagent A (14.7 mL of 2% Na_2CO_3; 150 µL of 2% $KNaC_4H_4O_6.4H_2O$ and 150 µL of 1% $CuSO_4.5H_2O$), extemporaneously prepared. The plate was incubated for 10 min at room temperature. Then, 150 µL of 15× diluted Folin and Ciocalteu's phenol reagent (Merck Millipore) were added into each well. The plate was again incubated for

10 min at room temperature and the absorbance was measured at 750 nm in a 96-well microplate reader Power Wave XTM (BioTek Instruments, Inc.). Protein bovine serum albumine (BSA) standards (31.25–1000 µg/mL) were prepared in 1M NaOH.

4.1.13. Measurement of caspase-3, caspase-8, caspase-9 activities

Primary rat hepatocytes seeded onto 6 well-plates were incubated with 2,4-DNP for 24h, at 37°C (two wells were used per each treatment condition). After this period, the cell treatment medium was removed. The cells were washed twice and added of 75 µL of complete lysis buffer [50 mM HEPES; 0.1 mM EDTA; 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); supplemented with 1 mM dithiothreitol (DTT); pH 7.4]. Following incubation at 4°C, for 30 min, cells were scrapped and collected into 2 mL centrifuge tubes. After vortex-mixed and incubated, samples were centrifuged at 13,000g, for 10 min, at 4°C. Fifty µL of the supernatant (which contains the cytoplasmic fraction) were placed into a 96-well plate and added of 200 µL of complete assay buffer (100 mM NaCl; 50 mM HEPES; 1 mM EDTA; 0.1% CHAPS; 10% glycerol; supplemented with 10 mM DTT; pH 7.4). This assay was based on the hydrolysis of the peptide substrates specific for each caspase. The reaction was started by adding 5 µL of caspase-3 peptide substrate Acetyl-Asp-Glu-Val-Asp-p-Nitroanilide (Ac-DEVD-pNA, stock concentration 4 mM), caspase-8 peptide substrate Acetyl-Ile-Glu-Thr-Asp-p-Nitroaniline (Ac-IETD-pNA, stock concentration 10 mM) or caspase-9 peptide substrate Acetyl-Leu-Glu-His-Asp-p-Nitroanalide (Ac-LEHD-pNA, stock concentration 10 µM) per well and incubating for 24h, at 37°C, protected from light. The caspase-induced release of the p-nitroanilide moiety of the substrates presents high absorbance at 405 nm, as determined in a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.) (Dias-da-Silva et al., 2015). The absorbance of blanks (non-enzymatic control) was subtracted from each value of absorbance and the data were normalized to the amount of protein of each sample. The protein content in the cytoplasmic fraction was measured using the Bio-Rad DC protein kit as described by the manufacturer, and BSA solutions were used as standards (31.25–1000 µg/mL). Results from four independent experiments were expressed as percentage of negative controls.

4.1.14. Statistical analysis

The normalized MTT, LDH and NR assays data from four were fitted to the dosimetric Logit model, which was chosen based on a statistical goodness-of-fit principle: $\gamma = \theta max$

/ {1 + exp [- θ1 - θ2 × log(*x*)]}, where θmax is the maximal observed effects, θ1 is the parameter for location, θ2 is the slope parameter and *x* is the concentration of test compound. To compare concentration-response curves, the extra sum-of-squares F test was used. Results from ROS/RNS, GSH/GSSG, ΔΨm and ATP assays are presented as mean ± standard error of the mean (SEM) from four independent experiments. Normality of the data distribution was assessed by three methods, the Kolmogorov-Smirnov, D'Agostino & Pearson, and Shapiro-Wilk normality tests. Statistical comparisons between groups were performed by the test that better suited the data set of each assay (see legends of the graph figures), as indicated by the GraphPad Prism software version 7.0 (GraphPad software, San Diego, CA, USA) used to perform statistical calculations (see the legend of the graph figures). Significance was accepted at *p* values <0.05. All were performed using software. Solvent and negative control were included in all experiments and values were compared by the Student's unpaired t test. In all cases, comparison between these controls showed no statistically significant differences (p>0.5).

4.2.1. 2,4-Dinitrophenol (2,4-DNP) reduces cellular viability in primary rat hepatocytes

Since the liver plays a fundamental role in the metabolism of xenobiotics, hepatocytes were of interest to be used in our study. In fact, by virtue of their central function in the metabolic fate of most exogenous and endogenous compounds, they are also of vital importance in the process of (de)toxification (Dias-da-Silva et al., 2015). Hepatocytes are composed of a set of enzymes that intervene in metabolization, a process that mostly occurs in two phases, phase I and phase II. Reactions of phase I involve the oxidation of compounds, resulting in more polar substances that then undergo phase II reactions. Phase II reactions imply the drug conjugation with endogenous molecules by glucuronidation, sulfation, methylation and acetylation (Gomez-Lechon et al., 2007). Most biotransformation processes to which xenobiotics and other endogenous compounds are subjected to, enable them to form more hydrophilic molecules that will facilitate their elimination from body. Hepatocytes have therefore been deemed of enormous importance as an *in vitro* tool for studying hepatotoxicity (Andersson, 2010), supporting our selection of primary rat hepatocytes as the cellular model for the current study.

Primary rat hepatocytes were exposed to a comprehensive range of 2,4-DNP concentrations to enable complete cytotoxic profiles (concentration-dependent responses from 0% to 100% mortality). Cellular viability was then assessed by three different assays, the MTT reduction, the LDH leakage and the NR lysosomal incorporation assay. As shown in figure 22, the observed hepatocyte damage directly increased with the concentration of 2,4-DNP in all cell viability assessments performed herein. However, 2,4-DNP potency was different among the different tests. The most sensitive assays to 2,4-DNP were the MTT (EC_{50} 96.8 µM) and NR (EC_{50} 98.1 µM) assays, which induced very similar responses. On the other hand, the potency of 2,4-DNP to cause cell death in the LDH assay (EC_{50} 174.8 µM) was significantly lower ($p<0.0001$; *F* test), as higher concentrations of drug were necessary to induce the same level of toxicity.

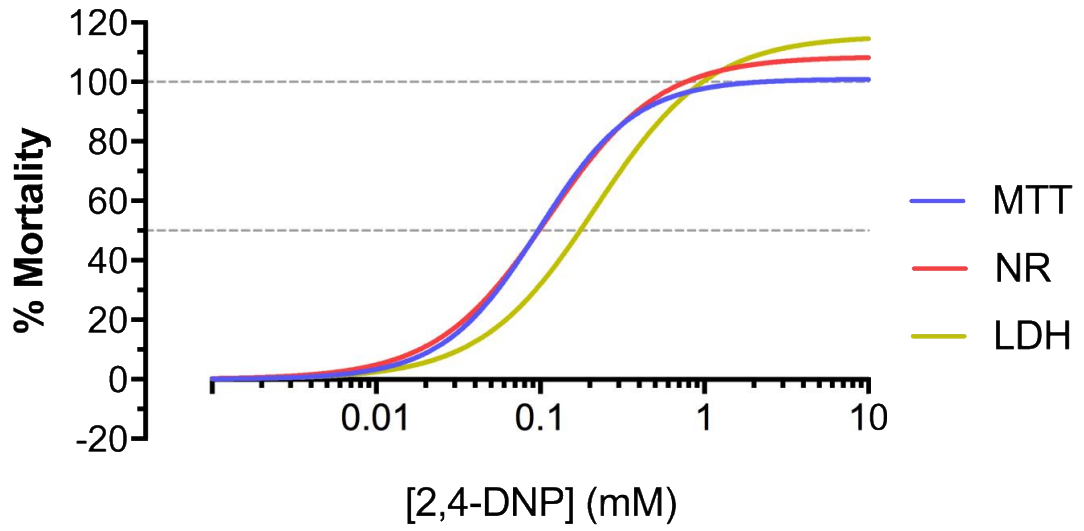


Figure 22: Cell mortality induced by 2,4-dinitrophenol (2,4-DNP) in primary rat hepatocytes after 24h of incubation at 37°C. Data were obtained in three distinct assays: neutral red (NR) uptake assay (data displayed in red), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumreduction (MTT) assay (data displayed in blue) and lactate dehydrogenase (LDH) leakage assay (data displayed in yellow), as indirect measures of cell viability. Data are presented as percentage of cell death relative to the respective negative controls and are from four independent experiments (performed in triplicate for each concentration within each experiment). Curves were fitted using the dosimetric Logit model. The dashed grey lines represent 50% and 100% effect.

The comparison of the obtained results provided information about the relative sensitivity of the target organelles, suggesting that the mitochondria was the most sensitive organelle to 2,4-DNP-induced toxicity, followed by lysosome and by the cytoplasmic membrane. Our data generally agrees well with what has been previously published for 2,4-DNP in vitro cytotoxicity. In mesenchymal stem cells isolated from tibia and femur of Sprague Dawley rats, cells were slightly shrunken after 20 min-exposure at 250 µM and 500 µM of 2,4-DNP, while at 1 mM and 2 mM almost all cells were dead (Ali et al., 2015). In a study conducted in hepatocytes isolated by collagenase perfusion of rat livers, it was observed that exposure at 300 µM of 2,4-DNP for 2 h was required to cause a 50% decrease in cell viability, as assessed by the trypan blue exclusion assay (Moridani et al., 2003). In another previous investigation also using the same time point and cellular model, only slight alterations on cell damage were produced in the presence of the uncoupler; nevertheless, concentrations used in this latter study were much lower than those used (up to 20 nmol per million of freshly isolated hepatocytes, which corresponds to 10 µM in our study) (Letko et al., 1990)

For the sake of comparison with 2,4-DNP results, the cellular viability was also assessed by the MTT assay, after exposure to caffeine, Supplement 1 and Supplement 2 for 24 h. No toxicity was observed at the range of concentrations tested (from 10.7 µg/mL to 4.7 mg/mL, for caffeine; from 228 ng/mL to 100 µg/mL, for Supplement 1 and Supplement 2). This was probably due to the low concentrations used in our experiments. However, the testing of higher concentrations was precluded by the low solubility of these products. From the data obtained, we can only conclude that 2,4-DNP elicited higher toxicity than Supplement 1 and Supplement 2 (100 µg/mL 2,4-DNP corresponds to 543 µM, which elicits 93.58% effect in the MTT assay).

4.2.2 2,4-Dinitrophenol (2,4-DNP) augments production of intracellular reactive oxygen (ROS) and nitrogen (RNS) species

All further assays performed to elucidate the mechanisms responsible for the observed hepatotoxicity were conducted at four 2,4-DNP concentrations: 1 µM, 10 µM, 50 µM, 100 µM and 200 µM. All these concentrations were lower than blood concentrations reported in fatally intoxicated individuals (<262.8 µM) (Politi et al., 2007). Therefore, even the highest concentration used in our study is of biological realistic relevance.

The production of ROS/RNS and the resulting oxidative and/or nitrosative stress was evaluated through the incubation of primary rat hepatocytes with the DCFH-DA cell permeant, following 2,4-DNP incubations for 24 h. As depicted in figure 23, in general, ROS/RNS production increased with the increasing concentration of 2,4-DNP. At 1 µM and 10 µM 2,4-DNP already elicited an increase in ROS/RNS levels by 25%, but the drug-induced effect was only statistically significant ($p<0.01$; ANOVA/Holm-Sidak's) when 2,4-DNP was at ≥50 µM. Likewise, Han et al. (2008b) showed that intracellular H_2O_2 and $O_2^{-\bullet}$ production markedly increased in As4.1 juxtaglomerular cells, when cells were exposed at concentrations higher than 500 µM for 48 h. Studies focussing on measuring the impact of mild mitochondrial uncoupling in mice reported conflicting results. Accordingly, the authors observed a lower release of oxidants, compared to controls, in animals systemically treated with 2,4-DNP (doses ranging between 30–105 µg/kg/day) and the prevention of ROS formation was accompanied by an improvement in the redox state (significant reductions in oxidation of deoxyribonucleic acid (DNA) and proteins in the brain, liver and heart, after treatment for five months) (Caldeira da Silva et al., 2008).

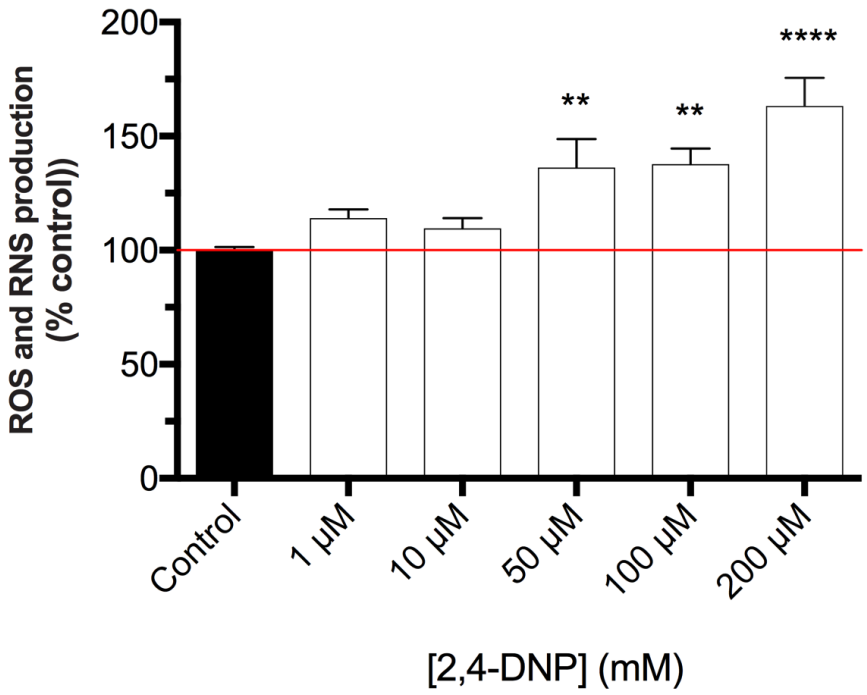


Figure 23: Reactive species (ROS/RNS) production, measured through the 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay, in primary rat hepatocytes, after 24h of incubation with 2,4-dinitrophenol (2,4-DNP), at 37°C. Results are expressed as percentage of control ± standard error of the mean (SEM) from four independent experiments, run in triplicate. Statistical comparisons were made using one-way ANOVA/Dunnett's *post hoc* test. **p<0.01; ****p<0.0001; *versus* control.

Several physiological processes that occur in cell can result in increased oxidant species generation, with consequent decrease of antioxidant protection, and ultimately failure to repair oxidative damage. Particularly, the mitochondrial respiratory chain is the main source of ROS/RNS production in the healthy cell. Besides aerobic respiration, cytochrome P450 metabolism is also a relevant source of prooxidant species. Eiseman et al. (1972) examined *in vitro* metabolism of 2,4-DNP in rat liver homogenates and concluded that 2,4-DNP was metabolized into the metabolites 4-amino-2-nitrophenol and 2-amino-4-nitrophenol. Scarce information on 4-amino-2-nitrophenol toxicity suggests safety concerns, particularly for potential carcinogenicity and mutagenicity of 4-amino-2-nitrophenol (Burnett et al., 2009). In addition, these 2,4-DNP metabolites can enter into redox chain reactions, inducing massive formation of ROS (Eiseman et al., 1972). Excessive production of ROS gives rise to activation of events, which can culminate in cell death (Han et al., 2008a).

4.2.3 2,4-Dinitrophenol (2,4-DNP) disrupts intracellular thiol balance

Since the cell has defence mechanisms against basal production of ROS/RNS, oxidative stress only occurs when there is an imbalance between the oxidants and the antioxidants, in favour of the former species. In this context, redGSH is an important antioxidant that is involved in many cellular processes, including antioxidant defence and drug detoxification (Franco et al., 2008).

As referred, 2,4-DNP suffers hepatic metabolism into 4-amino-2-nitrophenol and 2-amino-4-nitrophenol, and also into 2,4-diaminophenol, which are ROS/RNS generators (Eiseman et al., 1972). In addition to increased oxidative cellular status, redGSH depletion have also been considered a potential biomarker of drug-induced hepatotoxicity (Franco et al., 2008). Therefore, the evaluation of the redGSH and GSSG levels in primary rat hepatocytes exposed to 2,4-DNP was used to provide complementary information on the redox cell status.

As shown in figure 24A, hepatocytes were significantly depleted of redGSH (a potent reactive species scavenger) when exposed at 100 μM ($p<0.01$; ANOVA/Dunnett's) and 200 μM ($p<0.001$; ANOVA/Dunnett's) of 2,4-DNP. Additionally, a significant increase of GSSG was also observed for all concentrations. Cells exposed to high levels of oxidative stress, accumulate GSSG with reduction of the GSH/GSSG ratio, an evidence of oxidative stress. Accordingly, the results of our study on GSH and GSSG intracellular levels were consistent with the significant decrease of GSH/GSSG ratio ($p<0.001$; ANOVA/Dunnett's) after cell treatment with 2,4-DNP, observed for all concentrations (figure 24B).

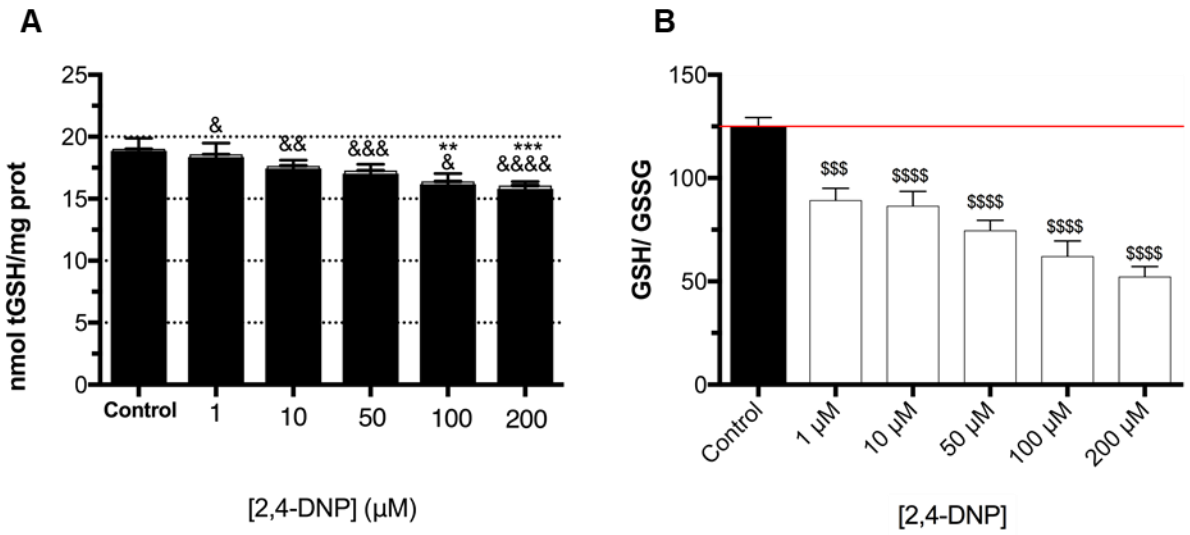


Figure 24: (A) Intracellular contents of reduced (GSH, black bars) and oxidized (GSSG, white bars) glutathione and (B) intracellular GSH/GSSG ratio, in primary rat hepatocytes, after 24h of incubation with 2,4-dinitrophenol (2,4-DNP). Results are expressed as mean ± standard error of the mean (SEM) from four independent experiments. Statistical comparisons were made using one-way ANOVA, Dunnett's *post hoc* test. **p<0.01; ***p<0.001; ****p<0.0001; *versus* GSH control. $^{\&}$p<0.05; $^{\&\&}$p<0.01; $^{\&\&\&}$p<0.001; $^{\&\&\&\&}$p<0.0001; *versus* GSSG control. $^{\$\$\$}$p<0.001; $^{\$\$\$\$}$p<0.0001; *versus* GSH/GSSG control.

4.2.4 2,4-Dinitrophenol (2,4-DNP) hyperpolarizes mitochondria, an effect that was concentration-independent

The total force impelling protons into the mitochondrial matrix (i.e., Δp) is a combination of both Δψm (electrical gradient) and the mitochondrial pH gradient (ΔpHm, an H^+ concentration gradient) (Perry et al., 2011). While Δp affords the energetic force required for ATP production, Δψm provides the electric gradient necessary for calcium sequestration in the organelle, and regulates production of ROS.

2,4-DNP is a lipophilic weak acid that binds hydrogen ions in the intermembrane space of mitochondria, transferring protons back into the mitochondrial matrix by a shuttle-type mechanism and, consequently, dissipating the proton gradient across the mitochondrial membrane. For this reason, 2,4-DNP is considered an 'uncoupler of oxidative phosphorylation' (Hargreaves et al., 2016). Frequently, an increase in protons in the mitochondrial matrix (i.e., loss of the proton gradient) is accompanied by decreased Δψm (depolarization), as observed in Calu-6 and As4.1 cells after exposure to 2,4-DNP (Han et al., 2008a; Han et al., 2008b). However, contrarily to the expected, in primary rat hepatocytes, a significant increase in ΔΨm was observed at 10 µM and 50 µM of 2,4-DNP (p<0.05; ANOVA/Holm-Sidak's), as shown in figure 25.

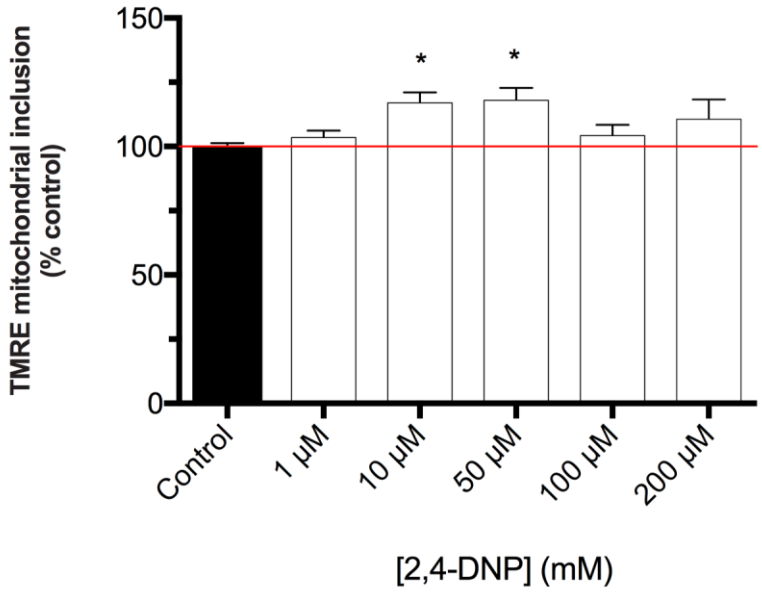


Figure 25: Mitochondrial membrane potential (ΔΨm) measured by the TMRE incorporation in mitochondria of primary rat hepatocytes after 24h of incubation with 2,4-dinitrophenol (2,4-DNP), at 37°C. Results are expressed as percentage of control ± standard error of the mean (SEM) from four independent experiments, run in triplicates. Statistical comparisons were made using one-way ANOVA/Holm-Sidak's *post hoc* test. *p<0.05; *versus* control.

In line with our findings, in some conditions of intracellular stress, increased Δψm (more negative mitochondrial interior) occur parallel to increased proton concentration in the matrix (as that induced by 2,4-DNP) (Perry et al., 2011). In fact, when we assessed the charge gradient Δψm across the inner membrane by the TMRE assay, we did not evaluate the mitochondrial proton gradient, ΔpHm, or the respiratory status. Accordingly, hyperpolarization effects observed herein may be a consequence of dysregulation of intracellular ionic charges, such as increased cytosolic calcium, as already observed by other authors (Arbo et al., 2016). Alterations in cytosolic calcium levels may be induced by mitochondria and endoplasmic reticulum calcium overload and by modulation of calcium reuptake into these organelles. Interestingly, the perturbation of Δψm was not evident at the higher test concentrations, which could indicate that calcium overburden is an initial event of cell stress. This hypothesis awaits experimental confirmation through supplementary mechanistic studies.

4.2.5 2,4-Dinitrophenol (2,4-DNP) alters intracellular energetic stores

Proton gradient across the mitochondrial membrane is of paramount relevance, since complex V requires the proton bypassing as electrochemical energy to synthesize ATP from ADP and Pi. Under these conditions, energy can not be created, which leads to a significant heat production and calorie consumption through stimulation of glycolysis (Hargreaves et al., 2016; Harvey et al., 2004). Some xenobiotics, such 2,4-DNP, that interfere with the mitochondrial breathing, may compromise the production of energy by the cell. Results from evaluation of intracellular ATP levels are represented in figure 26.

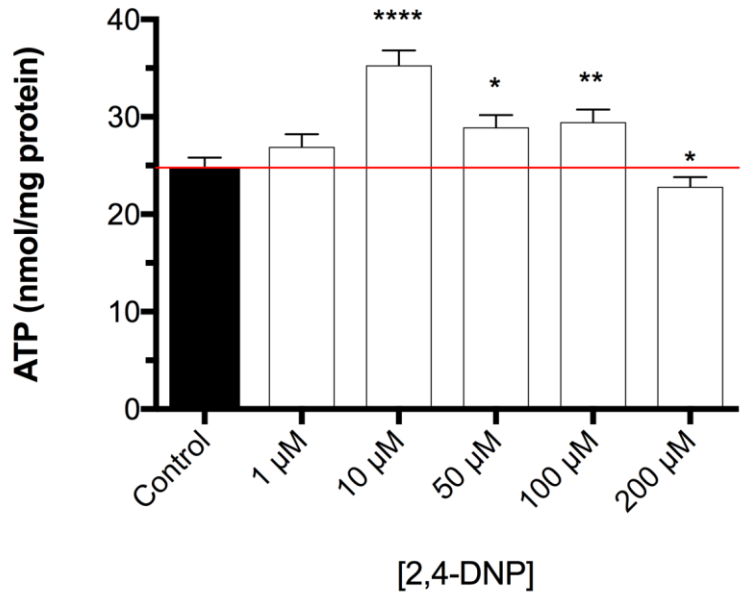


Figure 26: Intracellular ATP levels in primary rat hepatocytes after 24h of incubation with 2,4-dinitrophenol (2,4-DNP), at 37°C. Results are expressed as mean ± standard error of the mean (SEM) from four independent experiments. Statistical comparisons were made using one-way ANOVA/Dunnett's *post hoc* test. *p<0,05; **p<0.01; ****p<0.0001; *versus* control.

When cells were treated with 10–100 µM of 2,4-DNP, ATP levels were significantly higher than control treatments. At the highest concentration of 200 µM cell energetic stores substantially decreased ($p<0.05$; ANOVA/Dunnett's). These results are in line with the mitochondrial hyperpolarization observed, which may lead to the conservation of ATP levels. In previous studies using human male germ cells, a decreased in ATP production was also observed when cells were treated with 2,4-DNP at 200 µM (Erkkila et al., 2006). ATP is used by all living organisms as a form of storage of metabolic energy and as a 'coin' for every energy transfer. ATP generation by the F_0F_1-ATP synthase is crucially

dependent on an active respiratory chain. The respiratory chain generates a transmembrane potential (ΔΨm) across the inner mitochondrial membrane and, consequently, a proton concentration gradient (change in pH), which then provide energy for synthesizing ATP by the ATP synthase. Together, both ΔΨm and Δp help regulate mitochondrial control over energy metabolism, intracellular ion homeostasis and cell death. During cellular stress, Δψm may in turn be altered by dysregulation of intracellular ionic charges, consequently changing Δp and thus ATP production. When ionic fluxes surpass the ability of mitochondria to buffer these changes, ultimately Δp, Δψm, and/or ΔψpHm may collapse, leading to a failure of ATP production, energetic stress, and ultimately cell death. These could help justifying the observed decrease in ATP production, at the highest test concentration.

The higher ATP levels observed at the lower 2,4-DNP tested concentrations may be explained by 2,4-DNP-induced increases in the glycolytic metabolism of glucose (Harvey et al., 2004).

4.2.6 2,4-Dinitrophenol (2,4-DNP) activates extrinsic, intrinsic, and common apoptosis pathways

The mechanism of apoptosis involves mainly two signalling pathways, the mitochondrial pathway (intrinsic pathway) and the cell death receptor pathway (extrinsic pathway). The key element in the mitochondrial pathway is the efflux of cytochrome c (an essential component of the electron transport chain) from mitochondria to cytosol, where it subsequently forms a complex (apoptosome) with Apaf-1 and caspase-9, leading to the activation of the caspase-3. The cell death receptor pathway is characterized by the binding of cell death ligands to cell death receptors (McIlwain et al., 2013), and by activation of caspase-8 and subsequently caspase-3 (Han et al., 2008b). In this case, cell death stimuli is originated from the exterior of the cell following, for instance, drug-induced injury. Both cleavage of pro-caspase-8 (activation of extrinsic apoptotic pathway) and pro-caspase-9 (activation of intrinsic apoptotic pathway) induce the activation of the common apoptotic pathway (cleavage of pro-caspase-3).

As can be seen in figure 27, a significant increase in caspase-8 activity (figure 27A) occurred in primary rat hepatocytes exposed for 24h at all concentrations tested of 2,4-DNP. The activity of this caspase was maximal when cells were treated with 50 µM of 2,4-DNP ($p<0.0001$; ANOVA/Dunnett's). For caspase -9 (figure 27B) and -3 (figure 27C), the activation was concentration-dependent and occurred only at higher concentrations,

as significant differences to controls were verified only from 10 µM on ($p<0.05$; ANOVA/Dunnet's).

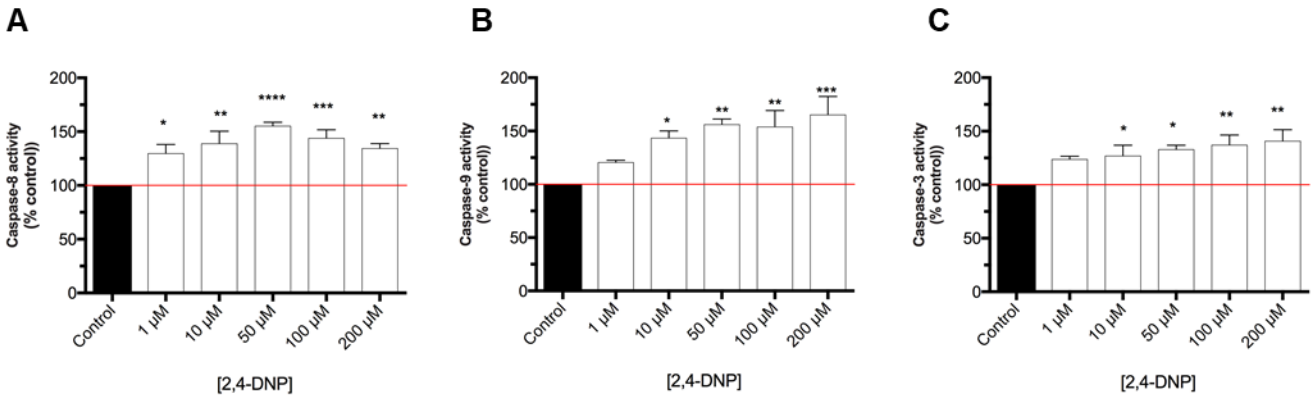


Figure 27: Caspase-8, -9 and -3 activities in primary rat hepatocytes after 24h of incubation with 2,4-dinitrophenol (2,4-DNP), at 37°C. Results are expressed as percentage of control ± standard error of the mean (SEM) from four independent experiments. Statistical comparisons were made using Kruskal-Wallis one-way analysis of variance/Dunnett's *post hoc* test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; *versus* control.

Of interest, the intracellular energetic increase was only significant at concentrations between 10 µM and 100 µM, which could justify the higher activation of caspases at this concentration levels, since apoptosis involves many energy-dependent processes.

Han et al. (2008a; 2008b) showed that 2,4-DNP induced apoptosis in As4.1 juxtaglomerular and Calu-6 cells in a dose-dependent manner. In both these cell lines, treatment with low doses of 2,4-DNP (≤1 mM) induced a G1 phase arrest of the cell cycle after 72 h of exposure. At higher concentrations (2–4 mM), 2,4-DNP induced apoptosis without a specific arrest of the cell cycle (Han et al., 2008a). Interestingly, in As4.1 cell studies, Han et al. (2008b) observed activation of caspase-9 but not caspase-8. Apoptosis via the mitochondrial pathway may increase the permeability of mitochondrial membrane (permeability transition), leading to the leaking of important mitochondrial intermembrane factors to the cytosol, where these proteins activate later apoptotic cascades (common apoptotic pathway through caspase-3 cleavage) and the apoptosome formation (Nicholls, 2002).

The effects of 2,4-DNP on weight loss and its easy access have increasingly attracted people looking for supplementation with 'fat burners', particularly those seeking to combine a healthy lifestyle with diet or the practice of sport. Several competent regulators banned the human consumption of this 2,4-DNP as soon as the first reports of intoxication began to appear. However, the frequent existence of fraudulent labelling and advertising along with the easy access through internet, make 2,4-DNP consumption a reality that remains.

The development of analytical methods that quickly allow the detection and identification of this substance in commercial products is therefore of utmost importance both for forensic and clinical purposes. The work conducted herein concerning the chemical identification of the components included in two dietary supplements commercialized as 'fat burners' (Supplement 1 and Supplement 2) revealed the absence of 2,4-DNP, suggesting that these new formulations are free from this toxic component. However, some other compounds were able to be identified in these formulations namely caffeine, glycerol, fatty acids and fatty acid derivatives (including 2-palmitoylglycerol or glycerol monostearate) and sugars (glucose, sucrose and lactose) as the main constituents. Regarding caffeine, this compound is one of the most commonly detected in this type of products and was also present at high concentrations in our both tested products. Of concern, we noticed that for Supplement 2 users, the daily supplementation with caffeine, considering the amount contained in the product and the daily dose indicated by the manufacturer, far exceeds the recommended daily limit for the substance (approximately 400 mg versus 848 mg of caffeine per day). The high diversity in the composition of these products, which includes plant extracts, amino acids, carnitine, fatty acids, polyphenols among others, is one of the greatest problems associated with these 'fat burner' products. The consumers cannot be sure of what they are ingesting due to the unspecific information containing in the labels. Accordingly, labelling problems have been reported in many supplements available and all too often these products are adulterated with mixtures of other substances.

Information on the effects of 2,4-DNP mainly derive from the intoxication cases reported by the emergency care units, and there is no specific antidote or treatment. Although we did not find 2,4-DNP in our tested products, in-depth toxicological investigations on 2,4-DNP effects are essential to alert the target public to the harm of consuming these products, particularly because over the last decade there has been an increasing on its demand, interest and use, mainly due to all divulgation through the online market. In this

regard, we proved in this work that the consumption of weight loss products containing 2,4-DNP may produce toxicological effects, since the substance exhibited a concentration-dependent decrease in cell viability of the primary hepatocytes isolated from Wistar rats, at 37° C for 24 h, as assessed by three different in vitro bioassays [NR uptake assay EC_{50} 98.05 µM; MTT assay EC_{50} 96.82 µM; and LDH leakage assay EC_{50} 174.83 µM]. These results indicate that the mitochondria was the most sensitive organelle to 2,4-DNP-induced toxicity, followed by lysosome and by the cytoplasmic membrane. In addition, our results, revealed that 2,4-DNP accelerates hepatocellular metabolism through instigation of ATP formation at the mitochondria, at low concentrations (up to 1 µM). This effect might be responsible by the observed oxidative burst, concurrent to a concentration-dependent decline in antioxidant defenses, that in turn could activate cell death signalling pathways through intrinsic and extrinsic pro-apoptotic mechanisms. This toxicological information is crucial to reinforce the dangers associated with 2,4-DNP as a substance used for weight loss.

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