UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Trabalho de Conclusão de Curso de Farmácia

Development of analytical methodology applying GC-FID and GC-MS towards the quality control of nutraceuticals and an update on its regulation

Letícia Guerini

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PLACE OF WORK

This present work have been developed in the Analytical & Technological Research Laboratories – Food and Environment, Di.S.A.A. – University of Milan, Italy.

Statement by the professors of the University of Milan:

"The experimentation object of the thesis was carried out on a nutraceutical prototype destined to be used by people suffering from headaches. The manufacturing company made available to the University of Milan some of the first productions of the nutraceutical speciality, not yet introduced on the market at the time of the trial to which this thesis work refers. The same company made available two extracts of myrrh and an extract of ginger, utilized for the production of the finished product prototypes.

Both the prototype lots and the extracts were the subject of this thesis experimentation. The purpose of the research has been the development of analytical methods for checking the composition of the preliminary prototypes of the finished product and the extracts. Another purpose was to identify the probable cause of differences in the composition of the finished product lots examined by the thesis student.

The finished product, already before the thesis experimentation, did not show any composition deficiency and had been subjected to patent. Analytical methods performed in parallel by the quality control of the manufacturing company and carried out with the same analytical criteria adopted in the thesis, had indeed confirmed that the adopted criteria were correct and suitable to undergo a good quality control.

The work by the thesis student, consisting of the parallel development of the same methods and the verification of the finished product composition, was carried out without the intervention of the quality control of the company, and therefore on an autonomous basis. The results showed that the analytical control criteria tested in the thesis are correct and allow to obtain good results in quality verification.

The samples examined in the thesis were derived from the archive of the manufacturing company. The same company imposed absolute privacy on its identity and on the commercial name of the finished product."

AGRADECIMENTOS

SUMMARY

ABSTRACT

Nutraceuticals are the union of the words 'nutrient' and 'pharmaceuticals', and they represent a link between food and medicine, providing both nutritional and medicinal values, including the prevention and treatment of diseases. With the increasing interest in nutraceuticals and by the fact that they present pharmacological activities and therapeutic indication, it is essential to define appropriate legislation for this sort of product. Besides, it is important to employ analytical methodologies for the quality control of nutraceuticals, in order to guarantee its efficacy and safety. As a result, the reliability of nutraceuticals can be increased, as well as consumers safety and protection against misleadings. With that in mind, preliminary studies of a nutraceutical prototype have been carried out through the evaluation and development of an analytical methodology for quality control. This nutraceutical was designed with myrrh extract (*Commiphora myrrha*), ginger extract (*Zingiber officinale*) and caffeine to counteract the states of headache and migraine, since its bioactive compounds present anti-inflammatory and analgesic activities, as well as vasoconstrictor effect. The analyses were carried out by GC-FID and GC-MS to identify and quantify marker compounds from myrrh extract, ginger extract, caffeine and in different batches of the nutraceutical prototype. Some discrepancies have been found regarding the plant extracts in the nutraceutical extracts, and between different batches of the nutraceutical. The set of results indicates the necessity of establishing parameters for quality control of the raw material and the final product, to ensure consumers a product with high quality standards.

Keywords: nutraceutical, gas chromatography, quality control, *Zingiber officinale*, *Commiphora myrrha*, caffeine.

1. INTRODUCTION

Not only the improvement of health but the state of well-being has become an aim in our modern society. Due to the high costs and the side effects of synthetic curative medicine, the prevention of health problems has led us to be more careful of avoiding or delaying the use of a conventional approach. Therefore, to achieve a healthier lifestyle, the understanding of foods and foodstuffs benefits in our bodies is particularly important and this has been coming to people's attention (Santini et al., 2018; Bimbo et al., 2017; Da Costa, 2017; Goetzke et al., 2014; Goetzke et al., 2013; Barnes et al., 2008). "Let food be thy medicine and medicine be thy food", already said Hippocrates.

In 2013 the global market for foods with health-enhancing features was around $168 billion with an annual average growth rate of 8.5% (Research and Markets, 2014). Observing this considerable and continuous market growth, food industries have been investing in developing new nutrition-modified products to achieve consumer's needs (Boluda and Capilla, 2017; Khan et al., 2014; Goetzke et al., 2013). Nutraceuticals are one kind of these products of substantial interest as a result of its potential nutritional and therapeutic effects. The global nutraceutical market has been exceeding financial forecasts and market growth. Projections show that the global nutraceutical market value will reach $578 billion by 2025, with an annual growth rate of 8.8% (Grand View Research, 2017, Nasri et al., 2014). The European market is the third largest global market for nutraceuticals, while the North American is the first and the Asian-Pacific region the second largest. The growth rates of the European and Brazilian nutraceutical markets are close to the global ones (Mordor Intelligence, 2019).

Nutraceuticals are the union of the words 'nutrient' and 'pharmaceuticals', which means they make a link between food and medicine, providing both nutritional and medicinal value, including the prevention and treatment of diseases (Chanda et al., 2019; Santini et al., 2018; Das et al., 2011; DeFelice, 1995). However well-defined nutraceuticals appear to be, in fact they are still in a grey area among food, food supplement and pharmaceuticals. This is mainly due to the fact that there is no established and specific legislation for this class of products and also due to a lack of nutraceuticals definition (Santini et al., 2018). With the increasing interest in nutraceuticals, both among the population and in the field of research, evidenced by the number of publications over the years (Figure 1), it is essential to implement appropriate legislation for these products and to employ analytical methodologies for the quality control of raw materials and the final product, as well as to guarantee nutraceuticals efficacy.
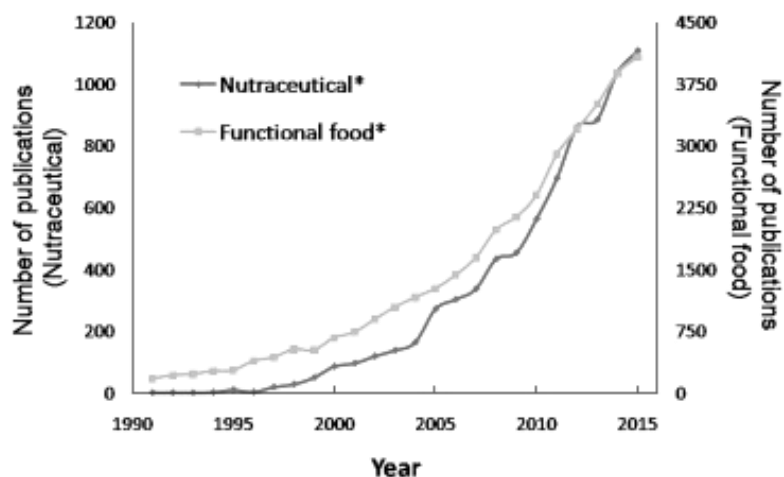
Figure 1. Number of nutraceutical and functional foods publications over the years, from 1990 to 2015. Source Da Costa, 2017.

Quality control is nothing more than a series of procedures designed to guarantee the quality, of whether raw material or final product. All products aiming to improve individual's health need to undergo rigorous quality control. A product's quality is a set of attributes designed and desired to ensure that a specified function is fulfilled. When a quality deviation occurs, it means that there has been a divergence from the quality-related parameters established for its product. This is important to increase the reliability of nutraceuticals, as well as to protect consumers from products that do not guarantee to deliver the expected therapeutic effects. As a result, these products will be able to provide even greater added value with their proven quality. If a nutraceutical claims to prevent and treat a certain disease, it is crucial to prove the products quality and efficacy for the desired purpose.

The present work aimed to elaborate a preliminary quality study of a nutraceutical prototype composed by ginger extract (*Zingiber officinale*), myrrh extract (*Commiphora myrrha*) and caffeine. This study consisted of evaluating and developing analytical methodologies to be applied in the quality controls of both raw materials and the final product. Also, given specific legislation from different regions, the nutraceuticals concepts by several regulatory agencies worldwide have been reviewed, and the challenges to obtain a product with safety and health claims have been discussed.

2. LITERATURE REVIEW

2.1. Nutraceuticals, food supplements, functional foods and herbal medicines

At first sight it seems there are no significant differences among nutraceuticals, food supplements, functional foods and herbal medicines, since they are all vegetable and/or food related. However, there actually are some well-established differences. Beginning with definitions, it is important to start defining food and nutrient. According to the European regulation, food means "any substance or product whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans" (Europe, 2002). Also, it says that "food" does not include items such as medicinal products and cosmetics. In accordance with the Brazilian Decree-Law n° 986 of October 21, 1969, food is "any substance or mixture of substances, whether in a solid, liquid, paste-like or any other appropriate form, intended to provide the human body with normal elements for its formation, maintenance and development" (Brasil, 1969). In 1998, the Ordinance SVS/MS 42/98 brought a new definition of food, which said "food is any substance which is ingested in the natural, semi-finished or prepared form, intended for human consumption, including beverages and any other substance used in their manufacture, preparation or treatment, excluding cosmetics, tobacco and substances used only as medicinal products" (Brasil, 1998b). The term "nutrient", according to the Ordinance SVS/MS 31/98, is "any substance normally consumed as a food constituent and which a) supplies energy; or b) is necessary for the growth, development and maintenance of health; or c) whose deficiency results in biochemical and physiological organism changes (Brasil, 1998a). For the Directive 2002/46/EC of the European Parliament and of the Council, "nutrients" are just vitamins and minerals (Europe, 2002).

Since medicinal products are excluded from the definition of food, it is also important to establish its description. To the European community, according to the directive 2001/83/EC, "medicinal product" is "any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or any substance or combination of substances which may be used in/or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis" (Europe, 2001). According to the Food and Drug Administration (FDA), drug product means "a finished dosage form, for example, tablet, capsule, solution, etc., that contains an active drug ingredient

generally, but not necessarily, in association with inactive ingredients. The term also includes a finished dosage form that does not contain an active ingredient but is intended to be used as a placebo" (USA, 2018). The Brazilian law defines medicinal product as "pharmaceutical product, technically obtained or elaborated, for prophylactic, curative, palliative or diagnostic purposes" (Brasil, 2015; Brasil, 2010b).

There is still a lack of regulated definition for nutraceuticals even though it is a well-spread market and its term has been coined 30 years ago, in 1989, by Stephen DeFelice. He described nutraceuticals as "food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease" (DeFelice, 1995). After him, Zeisel and co-workers in 1999 recommended a new category of dietary supplement called nutraceutical and defined them as "a diet supplement that delivers a concentrated form of a biologically active component of food in a non-food matrix to enhance health" (Zeisel et al., 1999). More recently, in 2016, the European Nutraceutical Association (ENA) established nutraceutical as "products that provide health and medical benefits, including the prevention and treatment of disease" (ENA, 2016). So, as it seems, nutraceuticals are in the middle ground between the definition of food and medicinal products, considering that they are beneficial to health in preventing and/or treating diseases, but also derive from a food source.

Until now, there is an absence of specific legislation for nutraceuticals worldwide. In Brazil, with the Brazilian Health Regulatory Agency (ANVISA); in the USA, with the Food and Drug Administration (FDA); and in Europe, with the European Food Safety Authority (EFSA), and the European Medicines Agency (EMA) nutraceuticals have not been regulated yet. When cited, nutraceuticals fall under the regulation of food supplements, as it happens in the USA, with the FDA, under the authority of the Federal Food, Drug, and Cosmetic Act. In Brazil, for example, a year ago, a resolution (RDC n° 23/2018) on health requirements for food supplements was arranged, but the term nutraceutical does not even appear (Brasil, 2018).

After all, what are the differences among nutraceuticals, food supplements, functional food and herbal medicines? They depend mainly on the product's composition and intended use. Food supplements, also called dietary supplements, are concentrated sources of nutrients and other ingredients with nutritional and/or physiological effects with a view to improving nutritional status or health in dosage forms (i.e. capsules, tablets, liquids). Vitamins, minerals, plants and herbal extracts are an illustration of what might be found in food supplements (IFIS, 2009; Europe, 2006, Europe, 2002; EFSA, 2019). According to the EFSA, "food supplements are intended to correct nutritional deficiencies, maintain an adequate intake of certain nutrients,

or to support specific physiological functions. They are not medicinal products and as such cannot exert a pharmacological, immunological or metabolic action. Therefore, their use is not intended to treat or prevent diseases in humans or to modify physiological functions" (EFSA, 2019). To the Brazilian Health Regulatory Agency (ANVISA), through the RDC n° 243/2018, food supplements are "a product for oral ingestion, presented in pharmaceutical forms, intended to supplement the diet of healthy individuals with nutrients, bioactive substances, enzymes or probiotics, alone or in combination" (Brasil, 2018). To the Dietary Supplement Health and Education Act (DSHEA) (the law that transformed the FDA's authority to regulate dietary supplements in the United States of America), dietary supplement is "a product (other than tobacco) that is intended to supplement the diet, which contains one or more of the following dietary ingredients — a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients; is ingested in pill, capsule, tablet, or liquid form" (USA, 1994). So, as can be seen, food supplements have a supplementary and beneficial roles in nutrition in an already balanced diet and do not have the functionality of preventing and/or treating diseases, such as for nutraceuticals (Cencic and Chingwaru, 2010).

There is no ubiquitous accepted definition of functional food evolving a food categorization. In the USA, for example, functional foods are not officially recognized as a regulatory category for food by the FDA, but some organizations, such as the American Dietetic Association's (ADA's), propose definitions. For them, actually, all foods are functional at some physiological level, because they provide nutrients and substances to give energy to the body. But functional foods exceed necessities as they promote additional health benefits when consumed as part of a varied diet on a regular basis, at effective levels. They include whole foods and fortified, enriched, or enhanced foods (Hasler and Amy, 2009).

For functional foods, as in the USA and Europe, the Brazilian authorities have not proposed a specific category for this type of products. Since the creation of the Technical-Scientific Commission for Advisory in Functional Foods and Novel Foods (CTCAF) in 1998, the first proposal for regulating these products emerged. Although the Commission has in its denomination "functional foods", they are regulated by the same laws as conventional foods, following the world trends. These new foods functionalities may be appropriate for "functional and/or health claims" ruled by the RDC n° 18 and n° 19 of 1999. As in the rules established by the European Parliament, in Brazil, only claims authorised by the ANVISA or the Ministry of Agriculture, Livestock and Supply (MAPA) are allowed, after the examination of the CTCAF

(Nitzke, 2012; Brasil, 1999a; Brasil, 1999b). In accordance with the above-mentioned resolutions: "the food or ingredient that claims functional or health properties may, in addition to basic nutritional functions, produce metabolic and/or physiological effects and/or beneficial health effects, and must be safe for consumption without medical supervision" (Brasil, 1999a. Brasil, 1999b). Thus, functional claims for nutrients and non-nutrients are allowed upon demonstration of efficacy. For nutrients with fully recognised functions within the scientific community, demonstration and analysis of efficacy for functional claims on labelling will not be necessary. It is important to emphasize that the claims cannot refer to the treatment, prevention or cure of diseases. Furthermore, these products should be consumed as long as they are associated with a balanced diet and healthy habits. (Brasil, 1999a; Brasil, 1999b).

Herbal medicines and their derived products are widely used as therapeutic products in many countries over the world. Most herbal medicines and their products are often prepared from the crude plant extracts, which comprise a complex mixture of different phytochemical constituents. As defined by the ANVISA, herbal medicines are "a product obtained from active vegetable raw material, except isolated substances, with prophylactic, curative or palliative purpose, including herbal medicine and traditional herbal product" (Brasil, 2014). An herbal medicine can have an active substance coming from one or more medicinal plant species, but its composition must not include active substances isolated or highly purified, neither synthetic, semi-synthetic or natural, nor the associations of these substances with other extracts, both vegetal and animal. Also, its efficacy and safety, as well as all medicines, should be based in pre-clinical and clinical evidences and technical-scientific literature characterized by a quality consistency. So, for this purpose, the quality must be achieved through the control of raw materials, finished product, packaging materials and stability studies (Brasil, 2015; Brasil, 2014a; Brasil, 2014b; Brasil, 2010b).

To the EMA, herbal medicinal products are "any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations" (Europe, 2004). These herbal substances are those mainly whole, fragmented or plant parts, algae, fungi, without processing, may be fresh, but usually dried. For herbal preparation those obtained by submitting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation, including powdered herbal substances, tinctures, extracts, essential oils, expressed juices and

processed exudates. Substances of synthetic origin remain unacceptable as active ingredients for traditional herbal medicinal products (EMA, 2011; Europe, 2004).

According to the FDA, a botanical drug product is "intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease in humans" (USA, 2016). It consists of vegetable materials, which may include plant materials, algae, macroscopic fungi, or combinations thereof. Botanical drug products may be available as a solution (e.g., tea), powder, tablet, capsule, elixir, topical or injection. They often present unique features, such as complex mixtures, substantial prior human use and lack of active ingredients. Further, their health claims must go through non-clinical and clinical pharmacological and toxicological tests. Fermentation products and highly purified, biotechnology or chemically derived, or other naturally derived drug substances are not considered as a botanical drug product (USA, 2016). Although the three regulatory agencies present some distinct features for herbal medicines, they characterize these products in a very similar way. In summary, a herbal medicine is a product exclusively containing as active ingredients herbal raw materials, substances and/or preparations and they must not present any isolated, highly purified, synthetic, semi-synthetic or natural substances. Moreover, they are medicinal products, intended to be used with prophylactic, curative, diagnostic and palliative purpose in humans diseases.

The different concepts of foods, pharmaceuticals, nutraceuticals, food supplement, functional food and herbal medicine are summarized in the Table 1.

Table 1. Summarized and exemplified concepts of foods, pharmaceuticals, nutraceuticals, food supplement, functional food and herbal medicine.

Food	Pharmaceutical	Nutraceutical	Food supplement	Functional Food	Herbal medicine
Substance intended to be ingested by humans	A finished dosage form that contains an active drug ingredient with properties for prophylactic, curative, palliative or diagnostic purposes	Food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease, and that can present also isolated substances	A product for oral ingestion, presented in pharmaceutical forms, intended to supplement the diet of healthy individuals with dietary ingredients	Provides additional health benefits that may reduce disease risk and/or promote optimal health, and are not pills or capsules, but must remain foods	A product that contains only as active ingredients an herbal substance and/or preparation, without any isolated substance, intended to be uses for prophylactic, curative, palliative or diagnostic purposes
Paullinia cupana (Guaraná) seeds powder	Synthetic caffeine (in pain relievers, cold medicines, over-the-counter medicines for alertness)	Extract of *Zingiber officinale* and *Commiphora myrrha* plus caffeine in a dosage form	*Paullinia cupana* and *Euterpe oleracea* (Açaí) plus vitamins and minerals in dosage forms	Energy drinks	*Paullinia cupana* extract in a pharmaceutical form

2.2. Migraine and tension headache

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (IASP, 1994). Migraine and tension headache are types of localized pain. Tension-type headache (TTH) is the most common form of headache, where women are slightly more affected than men. The life-long prevalence of episodic TTH is almost 80%, while chronic TTH is 3%. TTH is characterized by mild to moderate headache episodes, usually described as nonpulsatile, which do not worsen with routine physical activity, such as walking. The pain lasts from hours to days and is predominantly bilateral. The exact mechanisms are unknown, but are considered to be a combination of myofascial dysfunction (peripheral mechanism) and central nociceptive dysregulation (central mechanism). Usually, the acute pharmacological treatment is with over-the-counter (OTC) analgesics and with non-

steroidal anti-inflammatories (NSAIDs) in combination with preventive and nonpharmacological treatments, and also using tricyclic antidepressants for prevention (Burch, 2019; ICHD-III, 2014; IASP, 2011; Fumal and Schoenen, 2008).

Migraine is a primary, common, incapacitating cephalalgia disorder. Epidemiological studies indicate its high prevalence, with 2% of the global population being affected, where women are slightly more affected than men, classified as the third most prevalent disease and the seventh most disabling disease (ICHD-III, 2014). Migraine is characterized by recurrent, unilateral and pulsatile pain, with variable intensity (moderate to severe) and duration (from two to 72 hours), being aggravated by routine physical activity. Migraine related mechanisms are still controversial. Studies indicate that the brain and its blood vessels respond to an external trigger. Modifications in the pain central modulation combined with the activation of meningeal and vascular nociceptors may be responsible for headaches. Also, the release of peptides that stimulate vasodilatation and cause neurogenic inflammation increases the activation of trigeminovascular neurons by modulating the transmission of pain impulses in the brain (Nattagh-Eshtivani et al., 2018; Wannmacher e Ferreira, 2004). Migraine management involves many factors, both pharmacological and non-pharmacological, and they depend a lot by the type of migraine. A Brazilian migraine protocol of acute pain stipulates to keep the patient at rest under darkness and administer antiemetic and nonsteroidal anti-inflammatory drugs, such as dipyrone and ketoprofen. Triptans are also said to be the mainstay of acute treatment for migraine and may be used in combination with the medicines described before. Plus, medicines to control mild and moderate crises are used in combination with caffeine, which acts by increasing the speed and the beginning of their pharmacological effect (Burch, 2019; Speciali et al., 2018).

2.3. Nutraceuticals prototype for localized pain

A nutraceutical prototype, designed for localized pain, e.g. migraine and tension headache, is being evaluated in the University of Milan. This product is based on plant extracts, such as myrrh and ginger, associated with caffeine. According to the proposal, this product presents anti-inflammatory and analgesic activities and vasoconstrictor effect, which are useful to counteract the states of localized tension (data not shown).

2.3.1. Commiphora myrrha (Nees) Engl.

Commiphora myrrha (Nees) Engl., popularly known as myrrh, is a small tree or shrub located in the tropical and subtropical regions of southern Arabia, India and Africa, especially in dry and arid regions of Ethiopia, Somalia and Kenya (Shen et al., 2012; Su et al., 2011; Baser et al., 2003). Myrrh aromatic gum resin is produced by the secretory tissue in barks plant and has been used as a traditional medicine since ancient times. Among many usages, wound-healing, expectorant, emmenagogue, fever and pain reliever, and also in the treatment of gastrointestinal diseases, tumors, burns, trauma, arthritis and skin infections can be highlighted. Until the discovery of morphine, myrrh was a common analgesic and was also used to clean wounds and sores (Germano et al., 2017; Shen et al., 2012; Su et al., 2011; Hanus et al., 2005; Ubillas et al., 1999).

Phytochemical studies have shown that its main secondary metabolites are flavonoids and terpenoids, such as monoterpenoids, sesquiterpenoids and triterpenoids, found in myrrh extract and gum resin. These phytochemicals can be related to antioxidant, analgesic, antitumoral, anti-inflammatory and antimicrobial activities, which can support myrrh traditional use (Germano et al., 2017; Hamad et al., 2016; Zhaoa et al., 2015; Shen et al., 2012; Su et al., 2012; Hanus et al., 2005). Myrrh's aroma and its analgesic and anti-inflammatory activities are mainly due to curzerene, furanoeudesma-1,3-diene and lindestrene compounds, which have been found as major components in different extract preparations with myrrh resin. There are other species from the genus *Commiphora* knowly used as substitutes and/or adulterants of myrrh and its chemical composition differs from the true myrrh. So, when comparing myrrh extract with other extracts none of the major constituents can be found, that is, its chemical composition differs from the true myrrh (Germano et al., 2017; Su et al., 2011; Dekebo et al., 2002).

In myrrh chemical composition, aromatic sesquiterpenes have been reported, such as furanosesquiterpenes. Furanoeudesma-1,3-diene has been shown as the main compound (34.9 - 27.4 %), which is also very unstable. Also, curzerene (17.9 - 8.5 %) and lindestrene (12.9 - 9.1 %), β-elemene (8.7 - 3.0 %), germacrone (5.8 - 2.8 %), γ-elemene and isofuranogermacrene have been described as major sesquiterpenes from myrrh. However, myrrh essential oil and its resin may present some minor differences. Myrrh essential oil can be obtained by hydrodistillation and supercritical fluid extraction (Germano et al., 2017; Hanus et al., 2008; Hanus et al., 2005; Marongiu et al., 2005; Baser et al., 2003; Brieskorn et al., 1982). Meanwhile,

myrrh resin can be obtained by extraction with different solvents, such as *n*-hexane, water:ethanol (90:10, v/v), water:ethanol (80:20, v/v), water:ethanol:petroleum ether, water:ethanol (15:85, v/v) and absolute ethanol (Ahamad et al., 2017; Germano et al., 2017; Hanus et al., 2008; Hanus et al., 2005; Dekebo et al., 2002; Dolara et al., 2000; Ubillas et al., 1999; Dolara et al., 1996). Previous works have shown myrrh identification and quantification especially by gas chromatography (GC-FID, GC/MS), ^{1}H and ^{13}C NMR and mass spectrometry (Germano et al., 2017; Hanus et al., 2008; Hanus et al., 2005; Baser et al., 2003; Dekebo et al., 2002; Brieskorn and Noble, 1983; Brieskorn and Noble, 1982).

Regarding myrrh analgesic activity, furanoeudesma-1,3-diene and curzerene have been tested for binding to opioid receptors and as a result it was possible to identify analgesic effects from these two compounds and the interaction with brain opioid receptors. Although their effects are less potent than morphine, these components could still be useful (Dolara et al., 1996). Myrrh ethanolic extract has also been tested for analgesic activity and it might be said that peripheral pathways of pain perception are utilized by the components of myrrh extracts to suppress pain perception. Thus, for the anti-inflammatory activity it might be implied that its mechanism is associated with PGE2 decreasing level. In addition, several constituents, such as terpenoids, are found in the analgesic and anti-inflammatory fractions of myrrh extract. These data could indicate that terpenoids play an important role for the anti-inflammatory and analgesic activities, which are reinforced by other literatures (Su et al., 2011). Likewise, the essential oil of *Commiphora myrrha* have been described to have a potent scavenging activity due to the reaction between furan ring, especially from furanosesquiterpenoids, and superoxide anion radical (Ahamad et al., 2017).

Germano et al. (2017) published a clinical double-blind controlled trial on male and female volunteers with a myrrh extract characterized by a high and standardized total content of furanodienes (40.86 ± 0.78 g/kg of extract): curzerene (12.31 ± 0.05 g/kg), furanoeudesma-1,3-diene (18.84 ± 0.02 g/kg) and lindestrene (6.23 ± 0.01 g/kg). This study indicated that this myrrh extract has analgesic activity with a 200 mg dosage for lower-back pain and fever-dependent pain, and a 400 mg dosage for joint and muscle pain, headaches and menstrual cramps. These results had similar effects as the most frequently used analgesic drugs (diclofenac, ketoprofen, ibuprofen, paracetamol, tramadol and ketorolac), but with a much longer course of treatment required, around 20 days (Germano et al., 2017).

Nowadays, myrrh is approved by the FDA to be used as flavouring agent, fragrance and stabilizing ingredient in beverages, cosmetics, drugs and foods (Hamad et al., 2016). Its resin

may also be used as a source of supplement for some inorganic elements in case of deficiency, like zinc, which plays a significant role in immune function, and selenium in antioxidant activities (Ahamad et al., 2017). In Italy, it is possible to find a wide range of myrrh-based products registered as food supplements. Making a quick market research on-line it was possible to find 18 myrrh-based products, all of them in combination with other vegetable extracts, isolated substances, vitamins and minerals. In combination with *Boswellia serrata* and *boswellia* plus black pepper, it is indicated to treat localized tension and joint problems, presenting a 1-4% furanodiene content. Other myrrh-based products with *boswellia* were also found in combination with some minerals, vitamins (vitamin C, D3), turmeric (*Curcuma longa*), and isolated substances such as methylsulfonylmethane, copper, β-caryophyllene and piperine. These products also presented indication as analgesic and anti-inflammatory, and to treat joint problems, with a total content between 200 and 400 mg of *Commiphora myrrha* dry extract in a daily dose. Products with hedge mustard (*Sisymbrium officinale*) and myrrh, sometimes plus propolis and honey, are combined for the beneficial effects on the upper respiratory tract, with a total content of *Commiphora myrrha* dry extract between 32 and 100 mg in a daily dose. There are also products with myrrh in combination with bromelain and turmeric, which are recommended to be used for digestive system and oropharyngeal mucosa problems, and as antioxidant. For these products, the total content of myrrh extract was between 60 and 210 mg. For the bones and cartilage, a combination of myrrh, vitamins (B-complex, C and D vitamins) and collagen has been proposed with 200 mg of myrrh extract per daily dose. These nutraceutical products were presented in chewable tablet, tablet, liquid gel, capsule, sachet, and powder forms.

2.3.2. Zingiber officinale Roscoe

Zingiber officinale Roscoe, commonly known as ginger, is a plant native to Southern Asia, growing also in other tropical and subtropical countries of Africa and South America. The aromatic ginger has been used in the traditional oriental medicine as cold, inflammation and headaches reliever and to treat various diseases and/or its symptoms including nausea, constipation, indigestion, sore throats, muscular aches, rheumatoid arthritis, sprains, fever and infectious diseases. These traditional uses can be related to its bioactive polyphenolic compounds, which have been increasing interests in its anti-inflammatory, antioxidant, anti-tumorigenic, antimicrobial, anti-lipidemic and immune modulatory activities (Krüger et al.,

2018; Sharifi-Rad et al., 2017; Srinivasan 2017; Wang et al., 2017; Prasad et al., 2016; Tanaka et al., 2015; Mukherjee et al., 2014; Dugasani et al., 2010; Jolad et al., 2005; Jolad et al., 2004).

The composition of ginger rhizome is mainly carbohydrates, around 65%, 3-8% fibers, 9% protein, 8% ash, 3 - 6% fatty oil and 2-3% volatile oil (Srinivasan, 2017; Marwat et al., 2015). These volatile oils are responsible for the fragrance found in ginger, which mainly comprise sesquiterpenes and monoterpenes compounds. Instead, ginger oleoresin non-volatile phenolics, i.e. gingerols, shogaols and zingerone, provide its pungent nature (Krüger et al., 2018; Oriani et al., 2018; Srinivasan, 2017; An et al., 2016; Prasad et al., 2016; Yu et al., 2007). Actually, 6-gingerol is the most pungent and the most abundant gingerol constituent, although 4-gingerol, 8-gingerol and 10-gingerol are also found, but in smaller quantities. Gingers oleoresin chemical composition is mainly 6-gingerol (36.6%), 8-gingerol (3.6%), 10-gingerol (5.4%) and 6-shogaol (6.4%) according to Wang and co-workers (2009). Gingerols are the major active compounds in fresh ginger while in dried or cooked ginger shogaols can be named instead. The increased pungent shogaols are dehydrated products from the respective labile gingerols. Therefore, 6-gingerol, 8-gingerol and 10-gingerol concentrations have been found decreasing after drying processing whereas 6-shogaol concentration has been increasing with the same treatments. 6-shogaol, which is shogaols major constituent and the most common dehydration product, has been declared to have superior anti-inflammatory and antioxidant activities than 6-gingerol. In addition to 6-shogaol, zingerone is also a degradation product of gingerols, but converted through the condensation pathway (Ko et al., 2019; Krüger et al., 2018; Oriani et al., 2018; Koch et al., 2017; Srinivasan 2017; An et al., 2016; Semwal et al., 2015; Dugasani et al., 2010; Yu et al., 2007).

As previously discussed, gingerol is one of the most important bioactive components. In addition, there are some differences among gingerols chemical compounds from different sources, even gingerols from the same region can differ significantly. Therefore, it is necessary to establish a qualitative and quantitative method based on the bioactive gingerol-related compounds for quality control of gingers rhizome, since the content of gingerol directly affect the quality and efficacy of the rhizome. Several analytical methods have been reported to determinate the main chemical gingerol components in gingers rhizomes. Among them, gas chromatography coupled with mass spectrometry (GC–MS) is the most used and important ones (Wang et al., 2017).

Ginger has been extensively used as food-flavouring agent, beverage, pickles, chutney, liquors and bakery products, but its biological properties have been exploited and used in the

food processing (such as dietary supplements) and in pharmaceutical industries (Ezzat et al., 2018; Prasad et al., 2016). In Italy, it is possible to find a wide range of ginger-based products registered as food supplements. Making a quick market research it was possible to find 22 products: 16 made only with *Zingiber officinale* extract, three in combination with turmeric (*Curcuma longa)*, one with artichoke (*Cynara scolymus*), one with melissa (*Melissa officinalis*), and one with fennel (*Pimpinella anisum*). All of them showed digestive function, nausea, colic, muscular and articular pains usages. They were presented in chewable tablet, capsule and powder forms, usually with a 5-20% gingerols content in 250 to 1000 mg of *Zingiber officinale* dry extract per daily dose.

In Brazil, *Zingiber officinale* is a commonly used herbal medicine of simplified registration (Brasil, 2014). It presents gingerols and their derivatives as marker compounds (6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol). Gingers therapeutic actions and/or indications are for prophylaxis of nausea caused by movement (kinetosis) and post-surgical nausea, taking orally. The daily dosage indicated is 4 to 16 mg of gingerols in children over 6 years old and 16 to 32 mg of gingerols in adults.

2.3.3. Caffeine

Caffeine (1,3,7-trimethylpurine-2,6-dione) is an alkaloid derived from the group of trimethylxanthines, synthesized in certain plants from adenosine. Caffeine is found in the seeds, nuts, or leaves of guaraná (*Paullinia cupana*), coffee (*Coffea* sp.), green tea (*Camellia sinensis*), matte herb (*Ilex paraguariensis*) and cola (*Cola acuminata*), native from South America and East Asia. Caffeine is the most widely used psychostimulant worldwide, in both food and beverage, and it has a variety of important medical applications (NCATS, 2019; Jovel and Mejía, 2017; Lipton et al., 2017).

The caffeine molecule is structurally similar to adenosine and acts as an adenosine receptor antagonist with psychotropic and anti-inflammatory activities. Upon ingestion, caffeine binds to adenosine receptors in the central nervous system (CNS), which inhibits adenosine binding, inhibiting the adenosine-mediated downregulation of CNS activity. Thus, stimulating the activity of the brain centers and promoting neurotransmitter release that further stimulates the CNS. The anti-inflammatory effects of caffeine are due to the nonselective competitive inhibition of phosphodiesterases (PDEs). Inhibition of PDEs raises the intracellular concentration of cyclic AMP (cAMP), activates protein kinase A, and inhibits leukotriene

synthesis, which leads to reduced inflammation and innate immunity (NCATS, 2019; Lipton et al., 2017).

Caffeine dosing influences its pharmacodynamic effects. Higher doses (75–100 mg/kg) involve central noradrenergic mechanisms, medium doses (10–35 mg/kg) activate central amine systems, and low doses (5 mg/kg) interact with central cholinergic mechanisms. At therapeutic doses (i.e., 100 times higher than those typical in dietary consumption), caffeine can also induce PDEs inhibition, Ca^{+2} release, and GABA-A receptor blockage. Among patients with headache conditions, caffeine is used as an analgesic adjuvant. The analgesic effect of caffeine is based on the potent vasoconstrictor effect, which counteracts the vasodilator effect induced by purines. Studies have shown that caffeine significantly enhanced the analgesic effect of acetylsalicylic acid, acetaminophen or a combination of the two by about 40%. Therefore, caffeine evidently plays a role as an analgesic adjuvant in the acute treatment of primary headache with OTC drugs. A dose of 130 mg enhances the efficacy of OTC analgesics in TTH and a dose of ≥ 100 mg enhances the benefits of OTC analgesics in migraine, explaining why caffeine is typically used in combination with NSAIDs preparations (Burch, 2019; Jovel and Mejía, 2017; Lipton et al, 2017). The recommended consumption of caffeine per day to avoid undesirable effects is approximately 400-450 mg for healthy adults (Jovel and Mejía, 2017).

2.4. Quality Control

According to the Brazilian Pharmacopoeia, the definition of quality control is a set of measures to ensure, at any time, that the production of medicines and other products fulfills the standards of identity, activity, content, purity, efficacy and safety (Brasil, 2010a). The quality control of synthetics and herbal medicines is highly rigorous because it directly affects their curative effect, since they present a pharmacological purpose.

There are some quality guidelines available for pharmaceuticals quality, such as The International Conference on Harmonisation, and some quality standards, such as The International Organization for Standardization to be followed. Besides, there are the Pharmacopoeias, such as the Brazilian Pharmacopoeia (FB), the United States Pharmacopoeia (USP), the British Pharmacopoeia (BP) and the European Pharmacopoeia (Eu. Phr.). They provide a set of technical information with a view to establishing minimum quality requirements and standards for pharmaceutical substances, and medicinal and phytomedicinal products.

Although the lack of a minimum set of requirements for nutraceuticals products, as they are composed of vegetable raw material (food) and/or its biologically active compounds with therapeutical purpose, they should be subjected to well defined quality control processes. Quality control evaluates the chemical, physical-chemical (e.g. solubility, fusion range, loss on drying) and microbiological (biosafety tests) parameters, in order to identify and describe medicines. Therefore, some techniques are used to carry out pharmaceutical products identification and determination.

Some well-known and widely used analytical techniques are Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC), which are often applied tools for separation, and they are attached to detectors (UV, MS, FID, for example) for identification and quantification of chemical compounds.

2.4.1. Gas chromatography (GC)

Gas chromatography is a simple, sensitive, rapid and solvent-free technique capable of separating, identifying, and quantifying even volatile compounds of highly complex mixtures. Its separation principle is based on vapor pressure/boiling point and polarity differences from each compound. This technology was developed in 1952 by J.P. Martin and A.T. James, which awarded Martin the Nobel Prize in Chemistry that year (McNair, 2010; Stauffer et al., 2008; Bartle and Myers, 2002).

Although 67 years have passed and the instrumentation has improved, the basic principles of CG are preserved and remain pretty simple. The GC mechanism and its basic components are shown in the Figure 2.
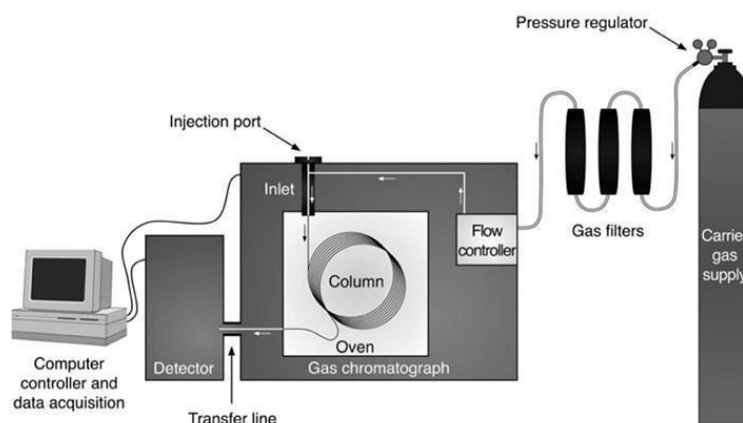

Figure 2. GC apparatus and its components. Source Stauffer et al., 2008.

Initially, a sample is introduced into the injector (or inlet) through the injection port. The injector is programmed with a very high temperature, which leads to the sample evaporation and its conduction to the column (stationary phase) by the carrier gas (the mobile phase). When the sample gets into the column, its compounds begin the chromatographic separation by interacting with the mobile and stationary phases. It means that compounds with less interaction with the stationary phase are the first ones to be eluted out of the column by the carrier gas. On the other hand, components with a greater interaction with the stationary phase are eluted last. But not only by the interaction between the mobile and stationary phases that the compounds elution process happens. As the time goes by the temperature inside the column increases and this heat also moves the components through the column. After leaving the column, the compounds enter a detector that allows the detection of the isolated compounds by an electronic signal proportional to the amount of eluted analytes. This signal is received by a computer where data analysis can be carried out. The magnitude of the signal is plotted versus time and a chromatogram is generated. (Stauffer et al., 2008).

There are some different types of detectors, which respond in different ways to the analyte. The most common ones are the Flame Ionization Detector (FID) and the Mass Spectrometer (MS), both used in this work. Basically, the first one is indicated for chemical compounds quantification, considering the areas of the peaks generated in the chromatogram. The FID features a minimum detectable quantity of approximately 10^{-12} g (for alkanes), an almost universal response, a wide linearity range, is simple to operate and quickly responsive (Visentainer, 2012). The second one is indicated for chemical compounds identification, based on the mass spectrum generated by each fragment of the compound. The analysis of natural products has been especially effective following the interface with a MS detection system in the chromatographic method. Mass spectrometry is the most selective technique for the rapid determination of compounds from the extracts of natural products (Yang et al., 2009).

3. METHODOLOGY

3.1. Reagents and materials

The standard 1-hexanol (99.6% purity) was used as internal standard. HPLC water and absolute anhydrous ethanol (99.9%) were obtained from Carlo Erba reagents (France).

Three different batches (numbers one, two and three) of the prototype nutraceutical capsules were produced. The first batch had 10 capsules, the second one had 100 capsules, and the third one had 80 capsules. The product is composed with myrrh (*Commiphora myrrha*) oleo-gum-resin dry extract, ginger (*Zingiber officinale*) rhizome dry extract 20% gingerols, caffeine and excipients. Two capsules, as a recommended daily dosage, are supposed to have a medium content of 200 mg of myrrh extract, 160 mg of gingers extract and 30 mg of caffeine, which represent 100 mg of myrrh extract, 80 mg of gingers extract and 15 mg of caffeine per capsule.

Samples of standard anhydrous caffeine, *Commiphora myrrha* and *Zingiber officinale* extracts were used for the chemical analysis.

3.2. Capsules content average weight determination

Eight capsules from the batch number one and twenty capsules from the batches numbers two and three have been randomly weighed in an analytical balance with an accuracy of 0.0001 grams. The procedure has been performed first by weighing the capsule intact and then it's shell after removing accurately it's content. At the end, the content mass is the difference between the filled and the empty capsules weighings, according to the Equation 1.

$$\textit{Content mass = filled capsule - empty capsule} \qquad (1)$$

These capsules have been individually weighed as described in the European Pharmacopoeia (Eu. Phr.) 9th edition, method of "uniformity of mass of single-dose preparations". According to this method, not more than two of the individual contents can deviate from the average weight by more than the percentage deviation ($\pm$ 7.5% for uncoated single-dose capsules with an average mass of 300 mg or more and $\pm$ 10.0% for uncoated single-dose capsules with an average mass less than 300 mg) and none by more than twice.

3.3. Capsules content humidity

Five capsules from batches number two (total content mass of 1.6407 g) and three (total content mass of 1.2082 g) have been randomly selected and weighed in a thermobalance Moisture Analyzer MA 60.3Y.WH Series with an accuracy of 0.0001 grams. Its drying profile parameters were programmed as: ten minutes until it reached 105 °C temperature and keeping it to a constant weight.

3.4. Samples preparation

3.4.1. Extraction procedure

The nutraceutical extraction solvent test was performed with the content of one capsule (approximately 300 mg) for each assay. In these assays four different extracts were prepared at room temperature with the following solvents: (1) 10 mL of absolute ethanol:distilled water (80:20, v/v) with 2 mL of dichloromethane, (2) 10 mL of absolute ethanol:distilled water (80:20, v/v), (3) 10 mL of absolute ethanol:distilled water (20:80, v/v) with 2 mL of dichloromethane and (4) 10 mL of absolute ethanol:distilled water (20:80, v/v). After vortexing one minute every ten minutes during one hour, the samples were filtered in a 0.45 µm filter with a PVDF membrane. The process to implement the extraction method continued with the content of two capsules (approximately 600 mg) adopting the proportion of ethanol and water that showed better results, with a 10 mL final volume solution. All the samples were analysed in GC-FID and GC-MS.

In order to analyse the effect of the mixing method, after establishing the best solvent composition, an extract was prepared with ultrasound-assisted extraction instead of vortexing. Therefore, the extract was prepared with the content of two nutraceutical capsules adopting the proportion of absolute ethanol:distilled water (80:20, v/v) with a 10 mL final volume. After one-hour sonication, the samples were filtered in a 0.45 µm filter with a PVDF membrane. The filtered extract was analysed in GC-FID and GC-MS with a 2 µL volume injection.

3.4.2. Samples characterization

These analyses were conducted to characterize the composition of myrrh, ginger extracts, and the nutraceutical prototype. For this purpose, the content of two nutraceuticals

capsules of batches one, two and three were used. All samples were prepared in duplicate. Also, approximately 200 mg of myrrh extract and 160 mg of ginger extract were analysed. All extracts were prepared in triplicate.

All of the extracts were prepared with 10 mL final volume of absolute ethanol:distilled water (80:20, v/v) solution, at room temperature. After vortexing, the extracts were filtered in a 0.45 µm filter. The filtered extracts were analysed in GC-FID and GC-MS, with an injection volume of 2 µL.

3.4.3. Quantitative analyses

3.4.3.1. Standard preparation

1-hexanol was used as standard. The stock solution was prepared dissolving 1-hexanol in anhydrous absolute ethanol 99.9 %, in a proportion of 1:99, reaching a final concentration of 814 mg/L, since 1-hexanol density is 8.14 kg/m³. A diluted solution of 1-hexanol was prepared with ethanol, obtained a final concentration of 387.6 mg/L. For the chromatographic analysis, 1 mL of this diluted solution of 1-hexanol was added to 1 mL of sample extract, resulting in a final concentration of 193.8 mg/L.

3.4.3.2. Compounds quantification

The compounds quantification was performed by adding a known quantity of the 1-hexanol standard solution, prepared as described in the item 3.4.3.1., to the samples. These analyses were conducted with the content of two nutraceuticals capsules of batches two and three. The batch number one was not available. Also, approximately 200 mg of myrrh standard extract, 160 mg of ginger standard extract and 30 mg of caffeine standard were analysed. All of the samples and the extracts were prepared with 10 mL final volume of absolute ethanol:distilled water (80:20, v/v) solution, at room temperature. After vortexing, as described previously, they were filtered in a 0.45 µm filter. Final solutions for GC-FID analyses were prepared with 1 mL of its filtered extract and 1 mL of the 1-hexanol standard solution (preparation in the item 3.4.3). The content injected was 2 µL.

Through chromatograms generated by GC-FID analyses, it was possible to integrate each peak area relating to a specific compound. By the same calculation method, the concentrations of the compounds were obtained in all of the tested samples. The concentration

was calculated through the Equation 2, that relates the integrated area of the internal standard with its concentration and the integrated area of each compound, resulting in the concentration of the respective compound.

$$Cx = \frac{(Ax \; x \; Cst)}{Ast}$$ (2)

Where:

Cx = compound concentration (mg/100 mg)

Ax = integrated compound peak area

Cst = 1-hexanol standard concentration (mg/100 mg)

Ast = 1-hexanol integrated standard peak area

To reach the final concentration in mg of each compound per 100 mg of product, simplifying, the concentration should be multiplied by 20. This is explained because the compound had dilution 1:2 in the solution with the 1-hexanol standard and 1:10 in the initial extract. Finally, the findings must be divided by the total weight of the sample (either myrrh extract, ginger extract or the internal content in the final product capsules). Then the final formula for calculating the compounds concentration is given in the Equation 3.

$$Cx = \frac{(Ax \; x \; Cst)}{Ast} \; x \; 20 \; /Mt$$ (3)

Where:

Cx = compound concentration (mg/100 mg)

Ax = integrated compound peak area

Cst = standard concentration (mg/100 mg)

Ast = integrated standard peak area

Mt = total weight of the substance (mg)

3.5. Preliminary studies of products stability

Twenty capsules were taken from each batches two and three and stored, without its blister, in a laboratory stove at a 40 °C. Twenty other capsules of the same batches were stored without its blister in a laboratory refrigerator at 6 °C. Each analysis was undertaken with two capsules of each batch from each storage temperature. These capsules were weighed and analysed at days: zero, five, twelve, fifteen, twenty, twenty-six, and twenty-nine, applying the same extraction method and analysis described before (Section 3.4.4).

Along with this, the stability analyses were carried out with the capsules inside the blister. One blister with ten capsules of the batch three was stored in the same laboratory stove at a 40°C settled temperature. Another blister with ten capsules of the same batch was stored in the same laboratory refrigerator at a 6°C settled temperature. Each analysis was undertaken with two capsules from each storage temperature. These capsules were weighed and analysed after nine and fourteen days of storage applying the same extraction method and analyses described before (Section 3.4.4).

3.6. Chromatographic analyses

Compounds identification and quantification were based on gas chromatograms and mass spectra, as described above.

3.6.1. Gas Chromatography with Flame Ionization Detection (GC-FID)

All the samples were analysed on a Shimadzu GC-2010 system (Shimadzu Corporation, Japan) equipped with FID and split/splitless injector (split ratio 5.0). Its conditions were programmed from the heating point 60 °C for one minute to 280 °C at 3 °C/min and then 30 minutes under isothermal conditions. The total program time has been stipulated in 104.33 minutes. The injector was kept at 220 °C and the detector at 240 °C. A SPB5 (5 % diphenyl - 95 % dimethylpolysiloxane) column with 60 m length and 0.32 mm inner diameter has been used. Its flow was 1.50 mL/min with a total flow of 12.0 mL/min using helium as carrier gas, at a 94.6 kPa pressure. The column oven has been set up at 60 °C. All the data was carried out in a Shimadzu software GC Solution.

3.6.2. Gas Chromatography - Mass spectrometry (GC-MS)

Some of the samples were also analysed on a Shimadzu GC/MS QP-2010 system (Shimadzu Corporation, Japan) with a Restek Rxi-5ms low-polarity phase crossbond diphenyl-dimethylpolysiloxane column (30 m x 0,25 mm), equipped with a MS and split injector (split ratio 5.0). The GC conditions were programmed from the heating point 60 °C for one minute to 280 °C at 3 °C/min and then 30 min under isothermal conditions. The total program time has been stipulated in 104.33 minutes. The injector was kept at 220 °C and the detector at 240 °C. Helium was used as the carrier gas with a flow rate of 1.50 mL/min.

The extracts and the nutraceutical samples constituents were identified by the Class 5000 Shimadzu software and the NIST (National Institute of Standards and Technology) mass spectra database. All the data was carried out in a Shimadzu software GC Solution and GCMS Solution.

4. RESULTS AND DISCUSSION

4.1. Capsules content average weight determination

As specified in the European Pharmacopoeia (Europe, 2017), twenty units are the required amount of capsules to be weighed to determine the average weight of capsules. However, only ten capsules from the batch number one have been made available, and just eight were possible to analyse at the end.

The values referring to the contents average weight and its Relative Standard Deviations (RSD) are shown in Table 2.

Table 2: Results of the nutraceutical batches content average weights and its RSDs.

	Content Average Weight (mg)	RSD (%)
Batch n° 1	331.35	2.32
Batch n° 2	332.55	3.65
Batch n° 3	224.73	22.94

Batches number one and number two have been shown to be consistent with pharmacopoeial specifications. Neither had a value above or below the acceptance limits ($\pm$ 7.5% for more than 300 mg) nor had a value above or below twice that percentage. On the other hand, batch number three has been shown to be inconsistent with pharmacopoeial specifications ($\pm$ 10% for less than 300 mg).

Meanwhile, for batch number three, just six capsules were within the acceptance limits. Fourteen of those capsules exceed the acceptance limits, where seven of them were between 10.01 to 19.99% above the acceptance limits, four of them were outside between 20.00 to 29.99%, and three of them presented values between 30.00 to 50.41% below the acceptance limits. Therefore, this batch presented more than two values above and below the acceptance limits ($\pm$10% for content weight less than 300 mg) and also presented seven values above and below twice the acceptance limit.

The filled capsules from the three batches have been also analysed. The manufacturer determined ten capsules weighing 3 g, which are 300 mg per whole capsule. The loaded capsules values are shown in the Table 3.

Table 3: Results of the nutraceutical batches filled capsule average weights and its RSDs.

	Capsule Average Weight (mg)	RSD (%)	Deviation from the declared value (%)
Batch n° 1	389.95	2.18	29.98
Batch n° 2	411.01	2.94	37.00
Batch n° 3	300.47	17.21	0.15

Batches number one and number two have shown much higher percentage deviations from the declared value by the manufacturer than batch number three. However, batch number three showed a considerable relative standard deviation, therefore it can be inferred that its uncertainty estimation for the average value is around 20%. Although the mean value may be equal to the declared value, it is quite uncertain and varies a lot.

Analysing the values related to the average internal contents of the three batches, it can be seen that batches 1 and 2 present a very similar average content, but batch 3 differs by more than 100 mg. Therefore, and taking into due consideration the data presented, it is possible to hypothesize some problems related mainly to batch 3, but not only. First of all, the lack of in-processes control, which are analyses undertaken before the production process is finished. Their purpose is to monitor and implement changes if they are necessary. As in cases where automatic encapsulators are unregulated or balances are poorly calibrated, in-processes control could be useful for identifying these irregularities and subsequently correcting them. Another important aspect is choosing both appropriate excipients and their proper amounts for filling the capsules, since they help in distributing the active compounds and, therefore, in the dosage uniformity of the finished product. Also, the quality control of the final product would also identify weight and uniformity problems in the manufactured batches. So, it can be seen that by applying quality control processes, better results for the capsules internal contents could be achieved.

4.2. Capsules content humidity

The humidity content of batch number two was 8.535%, where the initial mass of five capsules was 1.6407 g and the final mass was 1.5007 g. The batch number three presented 6.969% of humidity content, where the initial mass of five capsules was 1.2082 g and the final

mass was 1.1240 g. According to the *Zingiber officinale* monograph of the United States Pharmacopeia (USP), the limit is not more than 10% and according to the *Commiphora myrrha* monograph of the USP, the limit is not more than 15%. The samples are in accordance with this literature (USA, 2012; USA, 2006).

4.3. Extraction procedures

4.3.1. Extraction solvent

Choosing the right extraction method is crucial to obtain the desired compounds in the given quantity. Consequently, it is important to test several methodologies to find the most appropriate. Appropriate in this case means designing the extraction protocol objectively, obtaining the extract in a shortened given time, with the less toxic solvent and with its minimum use, i.e. minimizing the overall economic process and increasing the yield of desired products, which for this matter are polyphenols and sesquiterpenes. Commonly reported extraction solvents used in various proportions for such products are water, ethanol, methanol, acetone, dichloromethane and ether (Mukherjee et al., 2014; Xu et al., 2012; Ubillas et al., 1999).

The extraction protocol was designed based on literature and then adjusted to better fit into the nutraceutical and plant materials to be analysed. The gas chromatograms generated from each extraction solvent method can be seen in the Figure 3.
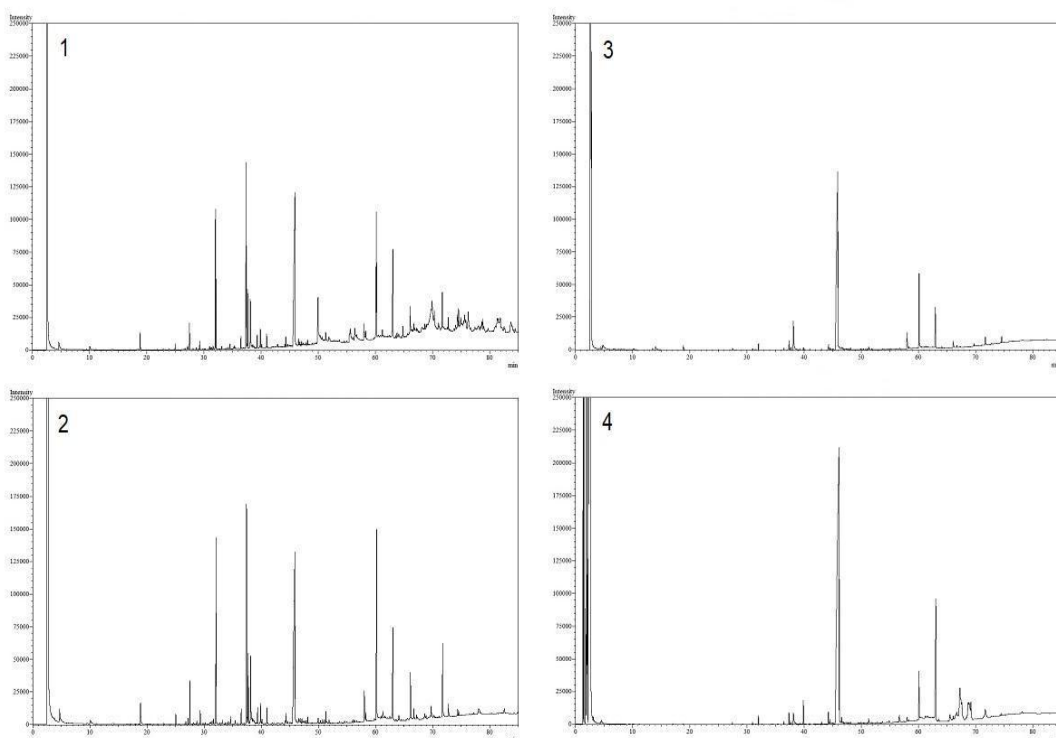
Figure 3: GC-FID chromatograms of different extraction methods of the nutraceutical compounds, batch number one where 1) absolute ethanol:distilled water (80:20, v/v) with dichloromethane; 2) absolute ethanol:distilled water (80:20, v/v); 3) absolute ethanol:distilled water (20:80, v/v) with dichloromethane; 4) absolute ethanol:distilled water (20:80, v/v).

Comparing the respective gas chromatograms extracts prepared with different extraction methods, it is possible to observe that the method 2 with absolute ethanol:distilled water (80:20, v/v) as the solvent extractor is the most effective one. Meaning, ethanol in greater proportion as an extraction solvent is more efficient in compounds extraction. As can be seen in chromatograms 3 and 4, the extraction is not so efficient with water in greater proportion. The addition of dichloromethane presents a lower resolution in the chromatograms 1 and 3.

However, no extraction method can be absolute, that is, it is not possible to extract all of the compounds in the greatest quantities. In this case, the later identified caffeine (retention time at 46.03 min) has lower peak area with the extraction method 2 compared to method 4, which is justified by the fact that caffeine is slightly more soluble in water than ethanol, at room temperature (Committee on FCC, 2004). But the solvent with a higher proportion of ethanol extracted all the other compounds better.

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4.3.2. Extraction mixing conditions

In order to analyse the effect of the mixing method, an extract was prepared with ultrasound-assisted extraction instead and another one with vortexing. They were analysed in GC-FID and their chromatograms can be seen in the Figure 4.
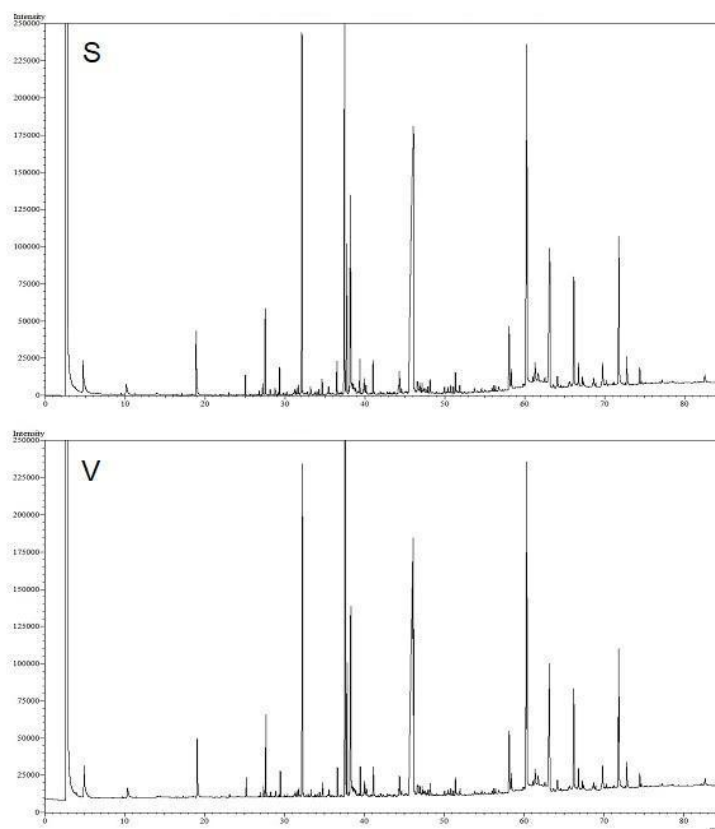
Figure 4: GC-FID chromatograms of different extraction methods of the nutraceutical (batch number one) where S) ultrasound extraction and V) vortex extraction.

When analysing their chromatograms and their peak areas, it is possible to see that they are very similar and present the same proportion among compounds. In conclusion, it can be said that both extraction methods can be applied to the nutraceutical analysis.

4.4. Samples characterization

4.4.1. Extracts characterization

The objective of these analyses was to identify myrrh and ginger compounds to be considered as active and/or analytical markers of myrrh and ginger extracts in the final product.

Likewise, these chemicals were semi-quantified, with a percentage correlation. For this purpose, the analysis of myrrh and ginger dry extracts was performed in GC-FID (triplicate) and also in GC-MS (replicate), which were performed according to item 3.6 of this present work. After that, a duplicate analysis of each one of the nutraceuticals batches (number one, two and three) was performed in order to analyse their compounds in GC-FID and also in GC-MS.

4.4.1.1. Myrrh extract characterization

The characterization of myrrh standard extract has been made possible through the compounds identification by mass spectra. All compounds considered useful as markers are identified in the Figure 5, and indicated in the chromatogram in the Figure 6 with the appropriate numeration, and they correspond to those substances known to be present in *Commiphora myrrha*.

β-elemene (1)   curzerene (2)   γ-elemene (3)

furanoeudesma-1,3-diene (4)   lindestrene (5)

Figure 5: Marker compounds of myrrh hydroalcoholic extract.
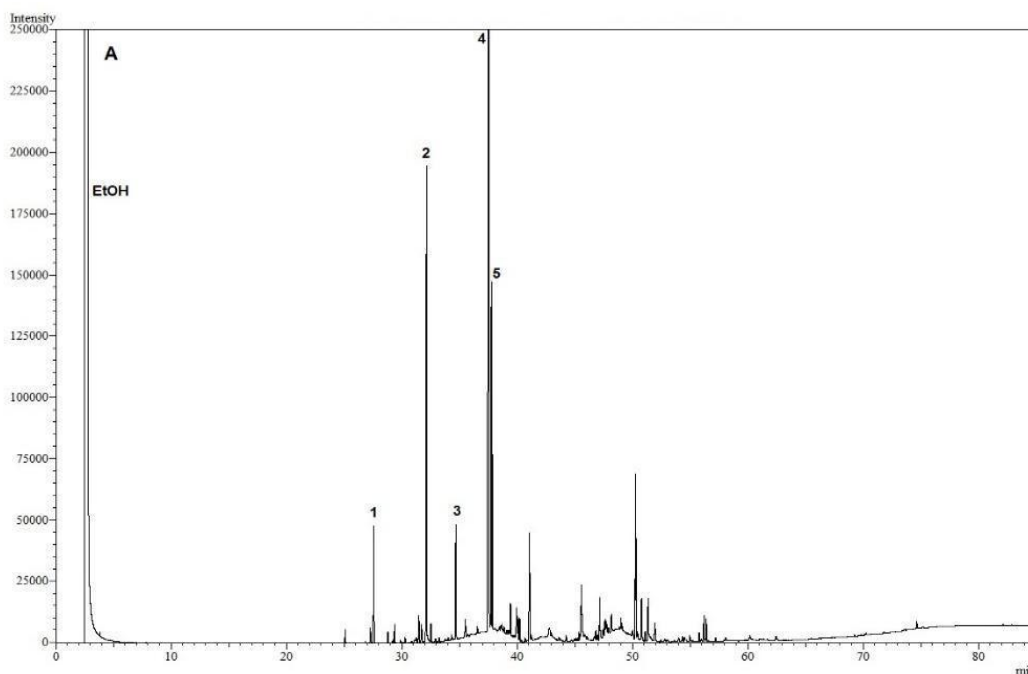
Figure 6. GC-FID of *Commiphora myrrha* extract where 1) β-elemene; 2) curzerene; 3) γ-elemene; 4) furanoeudesma-1,3-diene; 5) lindestrene.

The peaks area integration of each previously listed compounds was performed in three myrrh extracts (A, B and C) and its percentage composition are shown in the Table 4.

Table 4. Main compounds of myrrh extracts percentage composition (%).

Myrrh compounds	Extract A	Extract B	Extract C	Average	RSD (%)
β-elemene (RT: 27.69)	5.48	6.12	6.08	**5.89**	6.0765
Curzerene (RT: 32.23)	26.37	25.85	24.81	**25.68**	3.1451
γ-elemene (RT: 34.79)	5.30	5.88	6.00	**5.73**	6.6420
Furanoeudesma-1,3-diene (RT: 37.57)	46.73	46.19	46.89	**46.60**	0.7737
Lindestrene (RT: 37.85)	16.11	15.96	16.21	**16.09**	0.6211

RT: retention time in minutes. RSD (%): relative standard deviation percentage

With these results, it is possible to establish the coexistence ratio between the five marker compounds of myrrh extract and to conduct the verification and quality control of the raw material.

4.4.1.2. Ginger extract characterization

The characterization of gingers standard extract has been made possible through the compounds identification by mass spectra. All compounds considered useful as markers are identified in the Figure 7, and indicated in the chromatogram in the Figure 8 with the appropriate numeration, and they correspond to those substances known to be present in *Zingiber officinale*:
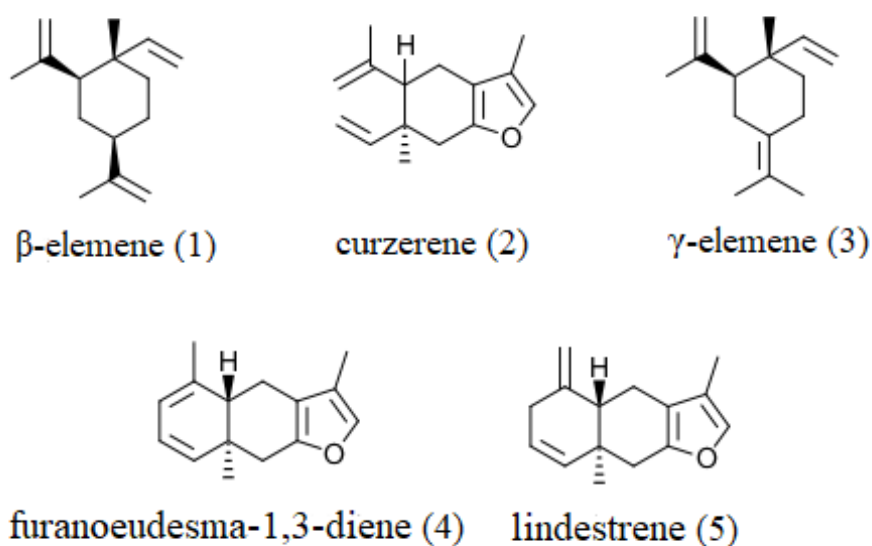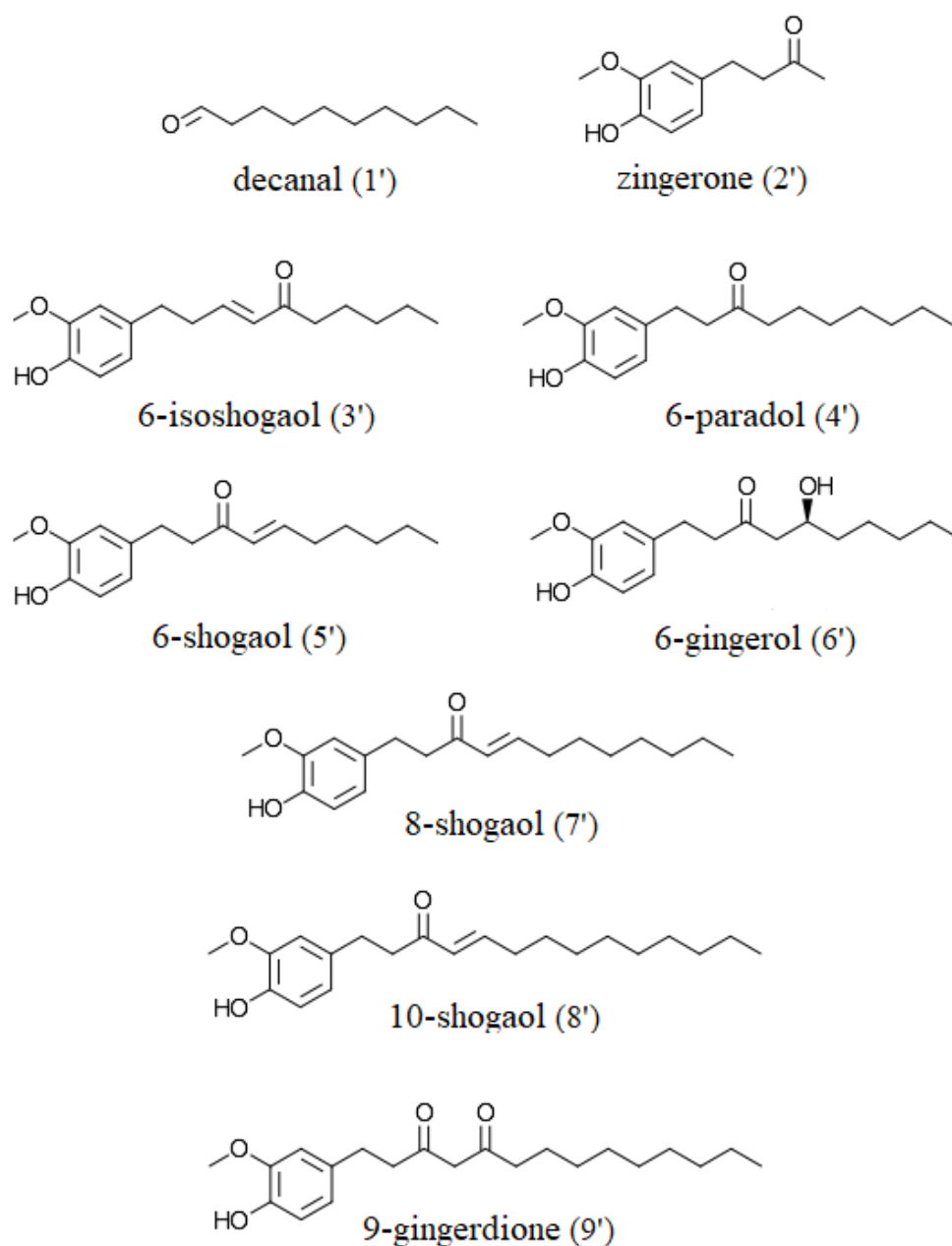

Figure 7: Marker compounds of ginger hydroalcoholic extract.
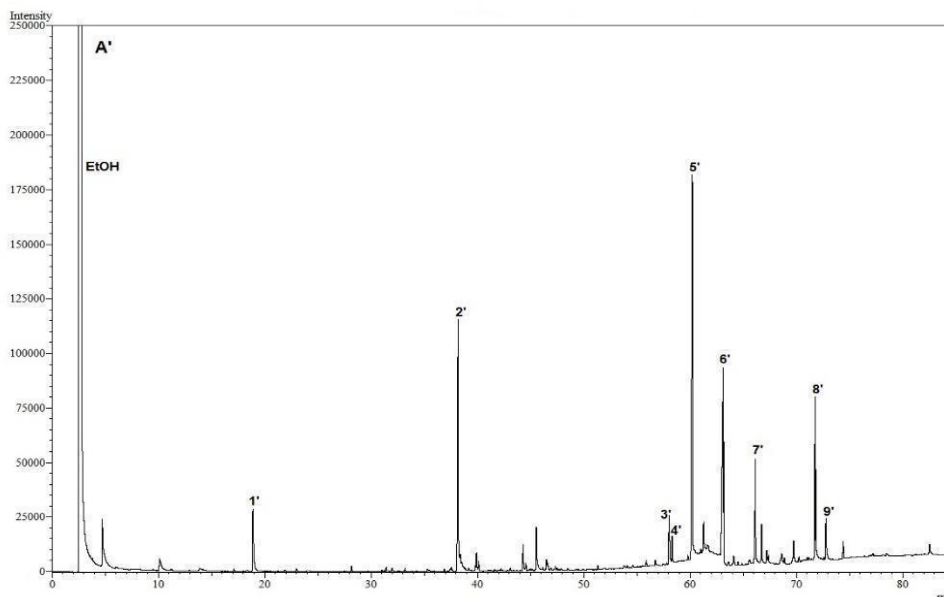
Figure 8. GC-FID of *Zingiber officinale* extract where 1') decanal; 2') zingerone; 3') 6-isoshogaol; 4') 6-paradol; 5') 6-shogaol; 6') 6-gingerol; 7') 8-shogaol; 8') 10-shogaol; 9') 10-gingerdione.

The peak area integration of each previously listed compounds was performed in the three ginger extracts (A', B' and C') and its percentage composition are shown in the Table 5.

Table 5. Main compounds of ginger extracts percentage composition (%).

Ginger compounds	Extract A'	Extract B'	Extract C'	Average	RSD (%)
Decanal (RT: 19.04)	6.02	5.63	5.89	**5.85**	2.7732
Zingerone (RT: 38.31)	18.43	16.07	18.45	**17.65**	6.3301
6-isoshogaol (RT: 58.16)	3.60	3.09	3.93	**3.54**	9.7611
6-paradol (RT: 58.45)	1.36	1.37	1.43	**1.39**	2.2292
6-shogaol (RT: 60.33)	30.32	29.21	30.64	**30.06**	2.0387
6-gingerol (RT: 63.12)	22.32	26.01	22.10	**23.47**	7.6399
8-shogaol (RT: 66.25)	5.80	5.56	5.81	**5.72**	2.0192
10-shogaol (RT: 71.87)	9.66	9.89	9.20	**9.58**	2.9933
10-gingerdione (RT: 72.92)	2.50	3.16	2.56	**2.74**	10.876

RT: retention time in minutes. RSD (%): relative standard deviation percentage

With these results, it is possible to establish the coexistence ratio between the nine marker compounds of ginger extract and to conduct the verification and quality control of the raw material.

4.4.1.3. Nutraceutical extracts characterization

The characterization of the nutraceutical extracts has been made possible through the compounds identification by mass spectra. All compounds considered useful as markers are identified in the Figure 9. The percentage correlation among myrrh marker components presented in the nutraceutical batch number one has been calculated and the results can be seen in Table 6. Also, the percentage correlation among ginger marker compounds presented in the nutraceutical batch number one has been calculated and the results can be seen in Table 7.

Figure 9. GC-FID of the nutraceutical batch number 1 where 1) β -elemene; 2) curzerene; 3) γ-elemene; 4) furanoeudesma-1,3-diene; 5) lindestrene; C) caffeine; 1') decanal; 2') zingerone; 3') 6-isoshogaol; 4') 6-paradol; 5') 6-shogaol; 6') 6-gingerol; 7') 8-shogaol; 8') 10-shogaol; 9') 10-gingerdione.

Table 6. Percentage correlation (%) among myrrh marker compounds in the nutraceutical batch number 1.

Myrrh compounds	Replicate n° 1	Replicate n° 2	Average	RSD (%)
β-elemene	7.53	8.42	**7.98**	7.8912
Curzerene	35.10	35.57	**35.34**	0.9405
γ-elemene	1.34	1.42	**1.38**	4.0992
Furanoeudesma-1,3-diene	43.50	41.97	**42.73**	2.4871
Lindestrene	12.53	12.62	**12.58**	0.5061

Table 7. Percentage correlation (%) among ginger marker compounds in the nutraceutical batch number 1.

Ginger compounds	Replicate n° 1	Replicate n° 2	Average	RSD (%)
Decanal	5.69	5.58	**5.64**	1.3803
Zingerone	17.30	16.40	**16.85**	3.7768
6-isoshogaol	5.42	5.73	**5.58**	3.9319
6-paradol	1.04	1.01	**1.03**	2.0696
6-shogaol	34.88	34.96	**34.92**	0.1620
6-gingerol	16.12	17.06	**16.59**	4.0065
8-shogaol	7.15	6.58	**6.87**	5.8711
10-shogaol	10.59	10.77	**10.68**	1.1918
10-gingerdione	1.82	1.91	**1.87**	3.4123

Likewise, it can be seen the percentage correlation among myrrh marker compounds and ginger marker compounds in the nutraceuticals batch number two in Tables 8 and 9, respectively. All compounds considered useful as markers are identified in the Figure 10.
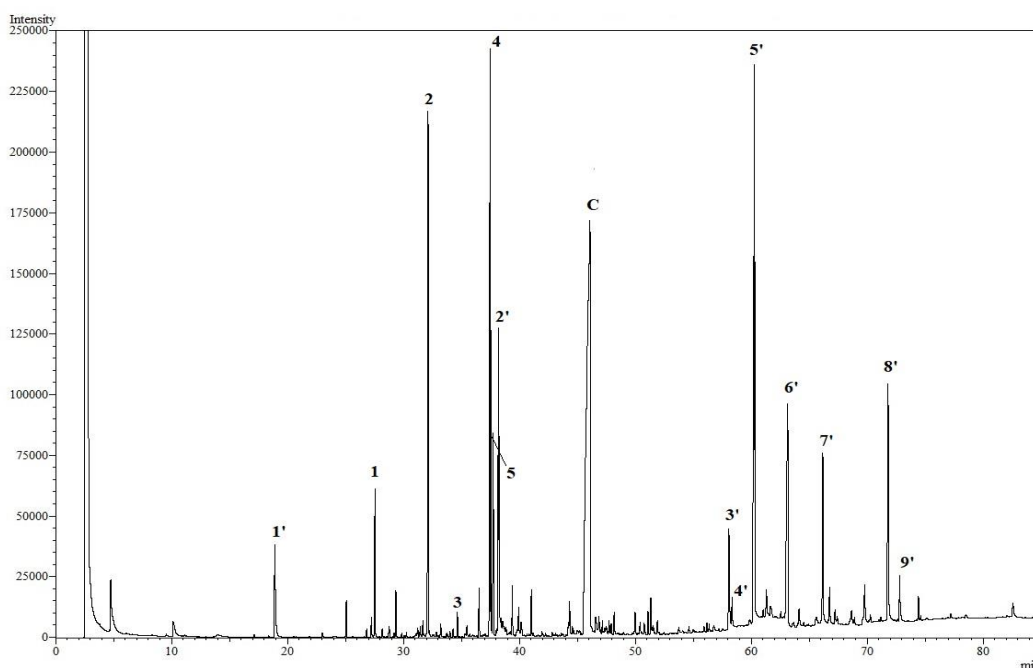
Figure 10. GC-FID of the nutraceutical batch number 2 where 1) beta-elemene; 2) curzerene; 3) gamma-elemene; 4) furanoeudesma-1,3-diene; 5) lindestrene; C) caffeine; 1') decanal; 2') zingerone; 3') 6-isoshogaol; 4') 6-paradol; 5') 6-shogaol; 6') 6-gingerol; 7') 8-shogaol; 8') 10-shogaol; 9') 10-gingerdione.

Table 8. Percentage correlation (%) among myrrh marker compounds in the nutraceutical batch number 2.

Myrrh compounds	Replicate n° 1	Replicate n° 2	Average	RSD (%)
β-elemene	4.73	5.29	**5.01**	7.9038
Curzerene	23.20	24.47	**23.84**	3.7677
γ-elemene	6.13	6.21	**6.17**	0.9168
Furanoeudesma-1,3-diene	49.25	47.93	**48.59**	1.9209
Lindestrene	16.69	16.11	**16.40**	2.5007

Table 9. Percentage correlation (%) among ginger marker compounds in the nutraceutical batch number 2.

Ginger compounds	Replicate n° 1	Replicate n° 2	Average	RSD (%)
Decanal	5.44	5.44	**5.44**	0.0000
Zingerone	16.97	16.85	**16.91**	0.5018
6-isoshogaol	6.11	6.43	**6.27**	3.6088
6-paradol	1.34	1.38	**1.36**	2.0797
6-shogaol	35.04	34.99	**35.02**	0.1010
6-gingerol	16.83	16.48	**16.66**	1.4860
8-shogaol	6.75	6.89	**6.82**	1.4515
10-shogaol	10.16	9.81	**9.99**	2.4786
10-gingerdione	1.36	1.63	**1.50**	12.771

As demonstrated above, the percentage correlation among myrrh marker compounds and ginger marker compounds was also performed in the nutraceuticals batch number three, as shown in Tables 10 and 11, respectively. All compounds considered useful as markers are identified in the Figure 11.
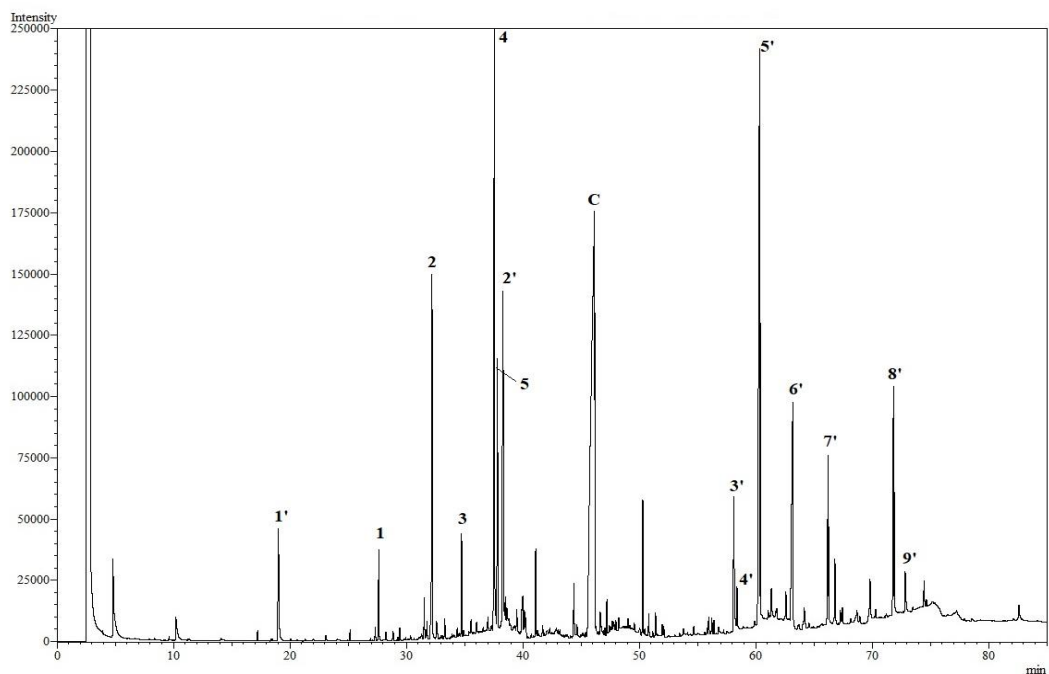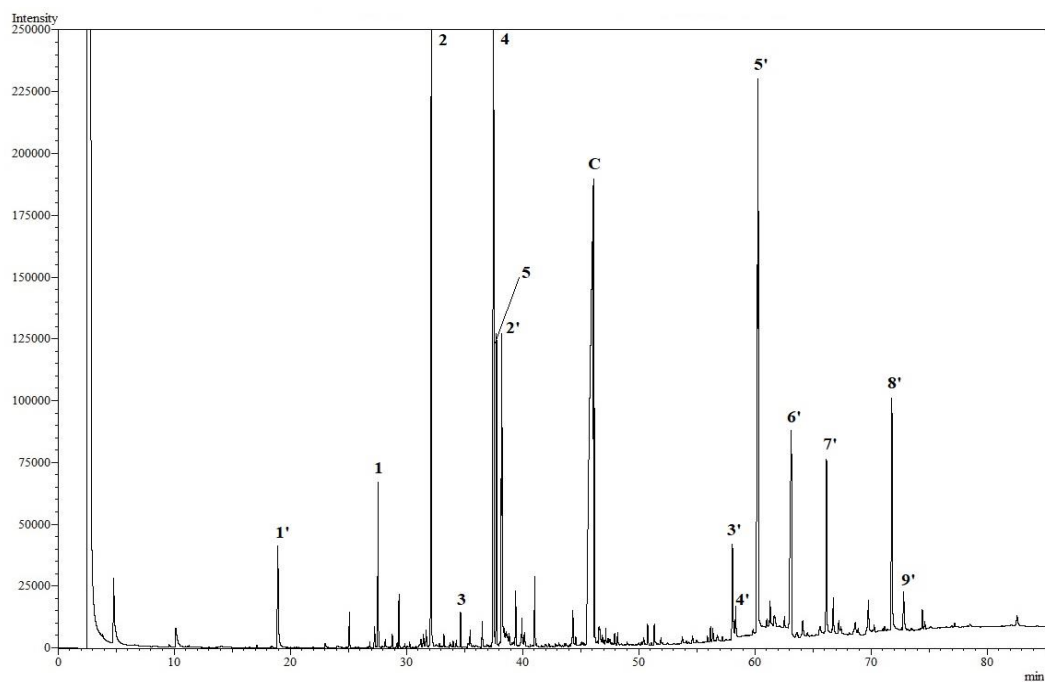
Figure 11. GC-FID of the nutraceutical batch number 3 where 1) beta-elemene; 2) curzerene; 3) gamma-elemene; 4) furanoeudesma-1,3-diene; 5) lindestrene; C) caffeine; 1') decanal; 2') zingerone; 3') 6-isoshogaol; 4') 6-paradol; 5') 6-shogaol; 6') 6-gingerol; 7') 8-shogaol; 8') 10-shogaol; 9') 10-gingerdione.

Table 10. Percentage correlation (%) among myrrh marker compounds in the nutraceutical batch number 3.

Myrrh compounds	Replicate n° 1	Replicate n° 2	Average	RSD (%)
β-elemene	5.70	6.40	**6.05**	8.1814
Curzerene	34.82	34.64	**34.73**	0.3665
γ-elemene	1.41	1.41	**1.41**	0.0000
Furanoeudesma-1,3-diene	45.10	44.81	**44.96**	0.4561
Lindestrene	12.97	12.84	**12.91**	0.7123

Table 11. Percentage correlation (%) among ginger marker compounds in the nutraceuticals batch number 3.

Ginger compounds	Replicate n°1	Replicate n° 2	Average	RSD (%)
Decanal	5.25	5.59	**5.42**	4.4357
Zingerone	17.08	16.23	**16.65**	3.6088
6-isoshogaol	5.62	5.35	**5.48**	3.4807
6-paradol	1.71	1.67	**1.69**	1.6736
6-shogaol	34.91	35.33	**35.12**	0.8456
6-gingerol	16.51	16.09	**16.30**	1.8220
8-shogaol	7.13	7.45	**7.29**	3.1039
10-shogaol	10.09	10.16	**10.12**	0.4889
10-gingerdione	1.71	2.12	**1.92**	15.1391

The average percentage correlation values of myrrh marker compounds in the nutraceutical batches number one, two and three, and also in myrrh standard extract can be seen in the Table 12 below.

Table 12. Summary table of myrrh marker compounds average percentage validation in the analysed prototype product batches one, two and three and the myrrh standard extract.

Myrrh compounds	Batch n° 1 average ± RSD (%)	Batch n° 2 average ± RSD (%)	Batch n° 3 average ± RSD (%)	Myrrh standard extract average ± RSD (%)
β-elemene	7.98 ± 7.89	5.01 ± 7.90	6.05 ± 8.18	5.89 ± 6.08
Curzerene	35.34 ± 0.94	23.84 ± 3.77	34.68 ± 0.37	25.68 ± 3.15
γ-elemene	1.38 ± 4.10	6.17 ± 0,92	1.41 ± 0.00	5.73 ± 6.64
Furanoeudesma-1,3-diene	42.73 ± 2.49	48.59 ± 1.92	44.96 ± 0.46	46.60 ± 0.77
Lindestrene	12.58 ± 0.51	16.40 ± 2.50	12.91 ± 0.71	16.09 ± 0.62

Observing the average percentage correlation values of myrrh marker compounds shown above, it can be inferred that there are some variations among batches itself and between

myrrh standard dry extract. When looking to the batch number two the γ-elemene percentage correlation was six times higher, the proportion of curzerene was around 12% lower, and the proportion of lindestrene and furanoeudesma-1,3-diene was around 4% higher, when compared to the batches number one and three. It can be assumed that batches number one and three contain myrrh extracts with similar typologies, while batch number two shows a different type of myrrh extract, more similar to the dry standard extract of myrrh. It is known that in plant preparations there are variations in their active compounds concentration due to several factors, such as area of cultivation, solar incidence, and other factors. Therefore, it cannot be expected to find a plant preparation, like a dry extract, that is exactly the same as the other, with the same exact composition. Even though, according to the presented data and the acquired results, a better standardization of myrrh extracts obtained from the suppliers is necessary.

The average percentage correlation values of ginger marker compounds in the nutraceutical batches number one, two and three, and also in ginger dry extract can be seen in the Table 13.

Table 13. Summary table of ginger marker compounds average percentage validation. in the analysed prototype product batches one, two and three and the ginger standard extract.

Ginger compounds	Batch n° 1 average ± RSD (%)	Batch n° 2 average ± RSD (%)	Batch n° 3 average ± RSD (%)	Ginger standard extract average ± RSD (%)
Decanal	5.64 ± 1.38	5.44 ± 0.00	5.42 ± 4.44	5.85 ± 2.77
Zingerone	16.85 ± 3.78	16.91 ± 0.50	16.65 ± 3.61	17.65 ± 6.33
6-isoshogaol	5.58 ± 3.93	6.27 ± 3.61	5.48 ± 3.48	3.54 ± 9.76
6-paradol	1.03 ± 2.07	1.36 ± 2.08	1.69 ± 1.67	1.39 ± 2.23
6-shogaol	34.92 ± 0.16	35.02 ± 0.10	35.12 ± 0.85	30.06 ± 2.04
6-gingerol	16.59 ± 4.01	16.66 ± 1.49	16.30 ± 1.82	23.47 ± 7.64
8-shogaol	6.87 ± 5.87	6.82 ± 1.45	7.29 ± 3.10	5.72 ± 2.02
10-shogaol	10.68 ± 1.19	9.99 ± 2.48	10.12 ± 0.49	9.58 ± 2.99
10-gingerdione	1.87 ± 3.41	1.50 ± 12.77	1.91 ± 15.14	2.74 ± 10.88

Observing the average percentage correlation values of ginger marker compounds shown above, it can be inferred that the same type of ginger extract was used in the three batches of the nutraceutical. However, when analysing the values of gingerols and shogaols compounds in the three batches and in the standardized ginger extract, it can be identified that a variation has occurred. The greatest difference can be observed for 6-gingerol, which showed a value around of 16.5% for the nutraceuticals and around of 23.5% for the standardized ginger extract. The same can be seen for 6-shogaol, 6-isoshogaol and 8-shogaol with values around of 35%, 6% and 7%, respectively, for the nutraceuticals and 30%, 3.5% and 5.7%, respectively, for the standardized ginger extract. The increase in shogaols compounds and the decrease in gingerols compounds in the nutraceutical batches (when compared to the standardized ginger extract) can be explained by the fact that shogaols are dehydrated products from the respective labile gingerols upon heating processing, long-term storage and acidic environment (Ko et al., 2019; Semwal et al., 2015). Therefore, it can be assumed that the extract used to prepare the nutraceutical batches could have been stored for longer time or under heating conditions. Nevertheless, a standardization of these preparations is necessary to ensure that the raw material used will necessarily have certain characteristics and in certain ratios.

4.5. Quantitative analyses

4.5.1. Compounds quantitative determination

For quantitative analyses, the internal standard method was implemented. This method is based on adding to the sample a known amount of internal standard, which is a known component different from the investigating analytes. In GC-FID, the internal standard added to a sample represents a peak that is not overlapping the others, is also absent in the sample, has a high purity level, generates a similar detector response and a well resolved peak. Since the internal standard concentration is known, as well as the area of its peak in the chromatogram, it is possible to compare the areas of the standard peak and the analyte peak of interest and calculate its relative concentration. This method is not only less susceptible to errors from injection and sample preparation, but also from instrument instability. Flame ionization detector is suitable to the determination of organic compounds, generating linear signals and low detection limit. A signal is generated from the carbon atoms ionization, and the intensity is proportional to the sample mass (Braun et al., 2017; Visentainer, 2012).

The internal standard chosen for this work was 1-hexanol, for the reasons already described, and also because it is a compound already implemented and standardized as an internal standard in other GC-FID analyses performed by the research group. In addition, studies such as Prasad and co-workers (2016), Marwat and co-workers (2015) and Husain and co-workers (2011) report the presence of 1-hexanol in parts of *Zingiber officinale*, however, the ginger extract provided and previously analysed in this study did not demonstrate this component as a part of it.

The optimal internal standard concentration to be added to the extracts for the quantitative analyses was preliminarily evaluated. It is important to observe a standard peak area and intensity resembling one or more compounds, i.e., that are not much higher or lower than the analytes. Finally, using a standard stock solution (preparation described in the item 3.4.3.1.) with a final concentration of 193.8 mg of 1-hexanol/L.

In order to express the concentration values of myrrh and ginger compounds more accurately, a correction was carried out using the molecular weight of 1-hexanol (102.162 g/mol) as well as the molecular weights of each compound presented in myrrh and ginger extracts. To give an example, the correlation between the concentration of 1-hexanol and the concentrations of furanoeudesma-1,3-diene (214.308 g/mol), which is a compound of myrrh, and of 6-shogaol (276.376 g/mol), which is a compound of ginger, was proposed. As the 1-hexanol standard solution used in the analysis has a concentration of 19.38 mg/100 mL or 0.1938 g/L, and its molecular weight is 102.162 g/mol, it is possible to achieve its molarity as 0.001897 M. Thus, this molar concentration can be multiplied by the molecular weights of the above-mentioned compounds of myrrh and ginger and their concentrations can be obtain, as illustrated in the Equations 4 and 5, respectively.

$$C = 0.001897 \frac{mol}{L} \; x \; 214.308 \frac{g}{mol} = 0.4065 \frac{g}{L} \; of \; furanoeudesma - 1,3 - diene \; (4)$$

$$C = 0.001897 \frac{mol}{L} \; x \; 276.376 \frac{g}{mol} = 0.5243 \frac{g}{L} \; of \; 6 - shogaol (4)$$

So, to express the concentration values as furanoeudesma-1,3-diene instead of 1-hexanol, the calculated concentration of furanoeudesma-1,3-diene was divided by the concentration of 1-hexanol in g/L (6). The given number (correction factor - k) can be multiplied by the concentration of furanoeudesma-1,3-diene expressed in 1-hexanol providing a concentration of its compound as furanoeudesma-1,3-diene (7).

$$\text{Correction Factor (k)} = \frac{0.4065\ g/L}{0.1938\ g/L} = 2{,}0975 = 2{,}10\ in\ furanoeudesma - 1{,}3 - diene\ (6)$$

$$value\ expressed\ as\ 1 - hexanol\ x\ 2{,}10 = value\ expressed\ as\ furanoeudesma - 1{,}3 - diene\ (7)$$

To express the concentration values as 6-shogaol instead of 1-hexanol, the same calculation shown above was conducted by dividing the calculated concentration of 6-shogaol and the concentration of 1-hexanol in g/L (8). The given number (k) can be multiplied by the concentration of 6-shogaol expressed in 1-hexanol providing a concentration of its compound as 6-shogaol (9).

$$\text{Correction factor (k)} = \frac{0{,}5243\ g/L}{0{,}1938\ g/L} = 2{,}7054 = 2{,}71\ in\ 6 - shogaol\ (8)$$

$$value\ expressed\ as\ 1 - hexanol\ x\ 2{,}71 = value\ expressed\ as\ 6 - shogaol\ (9)$$

Other myrrh and ginger compounds correction factors were calculated by the same logic as shown above and they can be seen in the Table 14.

Table 14. Calculation of the myrrh and ginger compounds and caffeine correction factor (k).

Myrrh compounds	Molecular Formula	Molecular Weight (g/mol)	MW x M 1-hexanol[1] (g/L)	k[2]
β-elemene	$C_{15}H_{24}$	204.357	0.3877	**2.00**
Curzerene	$C_{15}H_{20}O$	216;324	0.4104	**2.12**
γ-elemene	$C_{15}H_{25}$	204.357	0.3877	**2.00**
Furanoeudesma-1,3-diene	$C_{15}H_{18}O$	214.308	0.4065	**2.10**
Lindestrene	$C_{15}H_{18}O$	214.308	0.4065	**2.10**
Ginger compounds				
Decanal	$C_{10}H_{20}O$	156.269	0.2964	**1.53**
Zingerone	$C_{11}H_{14}O_3$	194.230	0.3685	**1.90**
6-isoshogaol	$C_{17}H_{24}O_3$	276.376	0.5243	**2.71**
6-paradol	$C_{17}H_{26}O_3$	278.392	0.5281	**2.73**
6-shogaol	$C_{17}H_{24}O_3$	276.376	0.5243	**2.71**
6-gingerol	$C_{17}H_{26}O_4$	294.391	0.5585	**2.88**
8-shogaol	$C_{19}H_{28}O_3$	304.430	0.5775	**2.98**
10-shogaol	$C_{21}H_{32}O_3$	332.484	0.6307	**3.25**
10-gingerdione	$C_{21}H_{30}O_4$	346.467	0.6572	**3.39**
Caffeine	$C_8H_{10}N_4O_2$	194.191	0.3684	**1.90**

[1] Molarity of 1-hexanol standard solution = 0,001897 g/L; [2] Concentration of 1-hexanol standard solution = 0.1938 g/L.

4.5.1.1. Myrrh compounds quantitative determination

The purpose of this determination was to quantify each marker compound previously identified in myrrh extract, by adding a known concentration of 1-hexanol internal standard. These analyses were performed in CG-FID according to the item 3.4.3 of this present work and one of the three resulting chromatograms can be seen in the Figure 12.
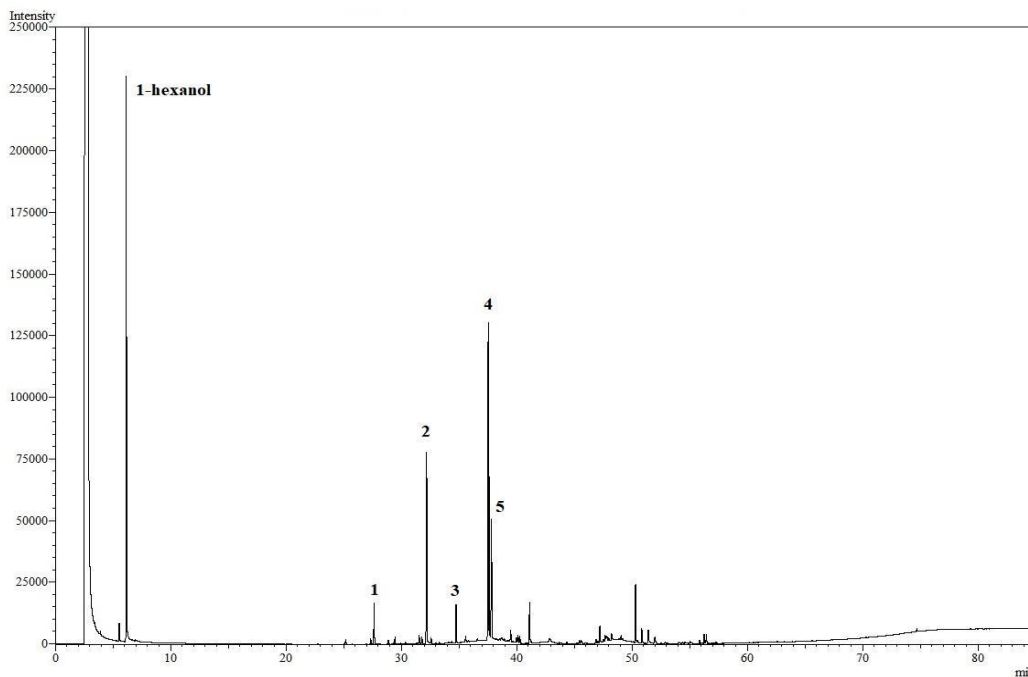
Figure 12. GC-FID of *Commiphora myrrha* extracts where 1) β-elemene; 2) curzerene; 3) γ-elemene; 4) furanoeudesma-1,3-diene; 5) lindestrene and the addition of 1-hexanol.

In order to obtain myrrh compounds concentrations, the areas of each peak marker and the standard were integrated. Its area values were added in the Equation 3 described in the item 3.4.3.2. and the other raw data in the Table 15. To give an example, a calculation will be described below with the first marker compound of myrrh extract.

Table 15. Raw data for the calculation of myrrh compounds expressed in 1-hexanol.

β-elemene peak area (Ax)	62,140.00
β-elemene concentration (Cx)	Cx
1-hexanol peak area (Ast)	565,230.00
1-hexanol concentration (Cst)	19.38 mg/100 mL

Cx = (Ax x Cst)/Ast = (62.140 x 19,38)/565.230 = 2,131 mg of β-elemene /100 mL

100 mL : 2,131 mg = 2 mL : x (mg)

x = 0,04262 mg of β-elemene/mL

0,04262 mg : 1 mL = x (mg) : 10 mL

x = 0,4262 mg β-elemene in 10 mL (final volume extract)

203,5 mg : 0,4262 mg = 100 mg : x (mg)

x = 0,209 mg of β-elemene/100 mg of myrrh extract expressed in 1-hexanol

       The resulting concentration of all compounds expressed in 1-hexanol of each triplicate (A, B and C) can be seen in the Table 16 and with the correspondent correction factors in the Table 17.

Table 16. Concentration of myrrh compounds (in mg/100 mg) expressed in 1-hexanol.

Myrrh compounds	Replicate A [ ]	Replicate B [ ]	Replicate C [ ]	Average [ ]	RSD (%)
β-elemene	0.209	0.202	0.207	0.206	1.71
Curzerene	0.953	0.968	0.968	0.963	0.87
γ-elemene	0.221	0.200	0.200	0.207	5.90
Furanoeudesma-1,3-diene	1.839	1.718	1.688	1.748	3.73
Lindestrene	0.698	0.656	0.648	0.667	4.00
Total compounds	**3.920**	**3.744**	**3.710**	**3.792**	**2.97**

[ ] = concentration.

Table 17. Concentration of myrrh compounds (in mg/100 mg) using the correction factors.

Myrrh compounds	Average Concentration in 1-hexanol	Correction factor (k)	Average Concentration with the correction factor
β-elemene	0.206	x 2.00	0.412
Curzerene	0.963	x 2.12	2.039
γ-elemene	0.207	x 2.00	0.414
Furanoeudesma-1,3-diene	1.748	x 2.10	3.667
Lindestrene	0.667	x 2.10	1.399
Total compounds	**3.792**		**7.931**

4.5.1.2. Ginger compounds quantitative determination

The purpose of this determination was to quantify each marker compound previously identified in gingers extract, by adding a known concentration of 1-hexanol internal standard. These analyses were performed in GC-FID according to the item 3.4.4 of this present work and one of the resulting chromatograms can be seen in the Figure 13.
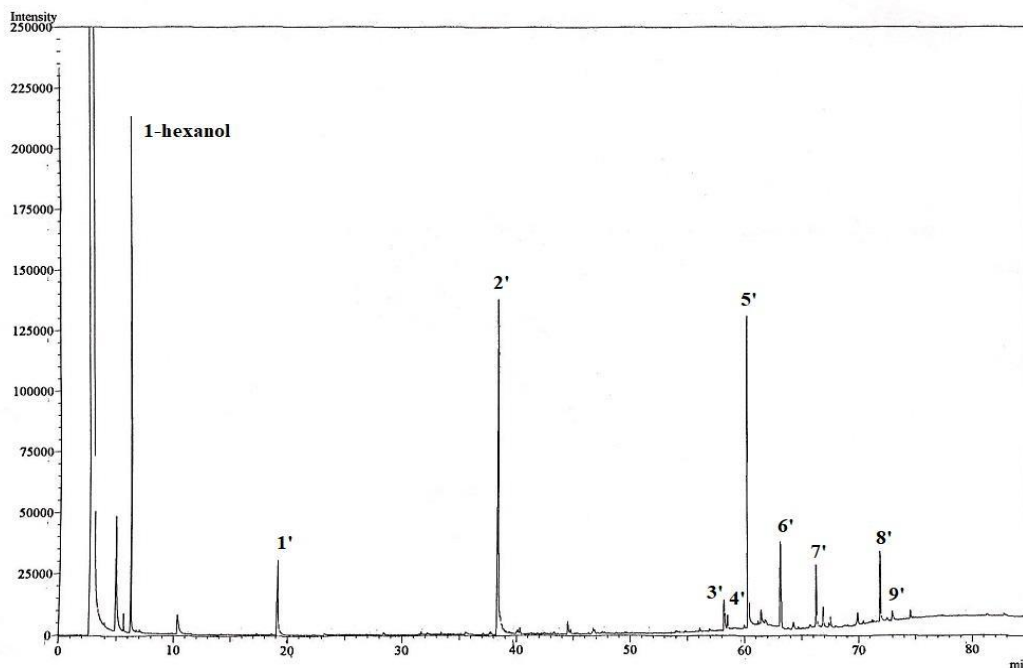

Figure 13: GC-FID of *Zingiber officinale* extracts where 1') decanal; 2') zingerone; 3') 6-isoshogaol; 4') 6-paradol; 5') 6-shogaol; 6') 6-gingerol; 7') 8-shogaol; 8') 10-shogaol; 9') 10-gingerdione and the addition of 1-hexanol.

In order to obtain gingers compound concentrations, the areas of each peak marker and the standard were integrated. The area values were added in the Equation 3 described in the item 3.4.3.2. All the compounds concentration expressed in 1-hexanol of each triplicate (A', B' and C) can be seen in the Table 18 and with the correspondent correction factors in the Table 19.

Table 18. Concentration of ginger compounds (in mg/100 mg) expressed in 1-hexanol.

Ginger compounds	Replicate A' [ ]	Replicate B' [ ]	Replicate C' [ ]	Average [ ]	RSD (%)
Decanal	0.789	0.776	0.753	0.773	2.41
Zingerone	3.217	2.948	2.975	3.046	4.87
6-isoshogaol	0.379	0.330	0.355	0.355	6.85
6-paradol	0.121	0.133	0.130	0.128	5.11
6-shogaol	2.535	2.502	2.555	2.531	1.08
6-gingerol	0.791	0.909	0.820	0.840	7.29
8-shogaol	0.467	0.475	0.468	0.470	0.93
10-shogaol	0.449	0.454	0.465	0.456	1.83
10-gingerdio-ne	0.082	0.083	0.095	0.087	8.23
Total compounds	**8.831**	**8.610**	**8.616**	**8.685**	**1.45**

[ ] = concentration.

Table 19. Concentration of ginger compounds (in mg/100 mg) using the correction factors.

Ginger compounds	Average Concentration in 1-hexanol	Correction factor (k)	Average Concentration with the correction factor
Decanal	0.773	x 1.53	1.182
Zingerone	3.046	x 1.90	5.791
6-isoshogaol	0.355	x 2.71	0.960
6-paradol	0.128	x 2.73	0.349
6-shogaol	2.531	x 2.71	6.847
6-gingerol	0.840	x 2.88	2.421
8-shogaol	0.470	x 2.98	1.401
10-shogaol	0.456	x 3.25	1.484
10-gingerdione	0.087	x 3.39	0.295
Total compounds	**8.685**		**20.730**

4.5.1.3. Caffeine quantitative determination

The purpose of this determination was to quantify caffeine by adding a known concentration of 1-hexanol internal standard and then carrying out the analysis in GC-FID. The analysis was performed in duplicate (C1 and C2) according to the item 3.4.4 of this present work and one of the resulting chromatograms can be seen in the Figure 14.
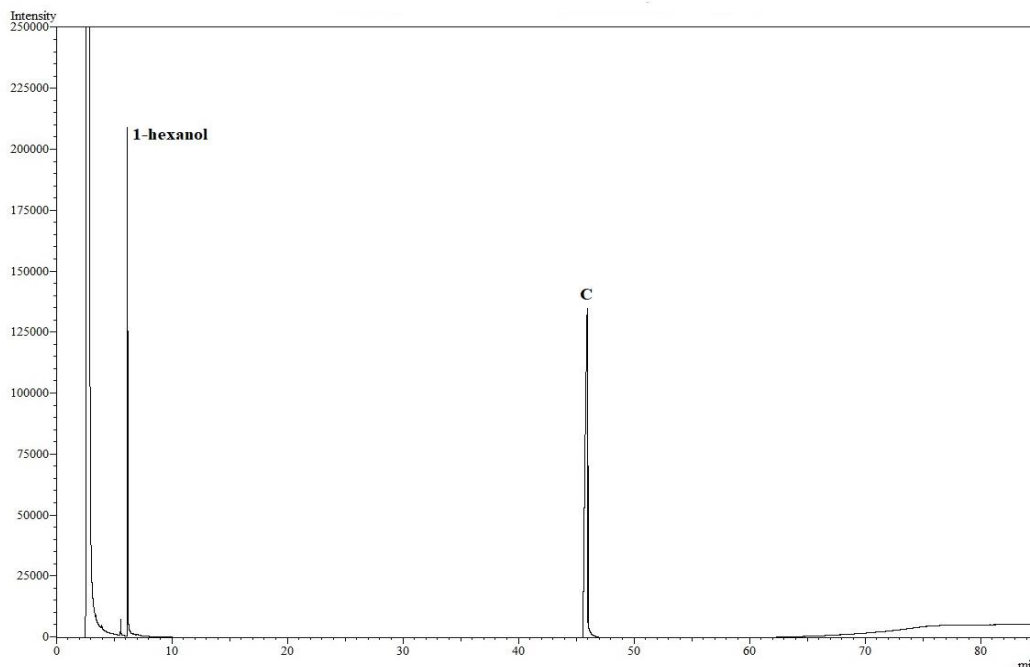
Figure 14: GC-FID of caffeine (C). Addition of 1-hexanol as the internal standard.

In order to obtain caffeine concentration the areas of its peaks and the internal standard were integrated. The area values were added in the Equation 3 described in the item 3.4.3.2. Caffeine concentration expressed in 1-hexanol can be seen in the Table 20.

Table 20. Concentration of caffeine (in mg/100 mg) expressed in 1-hexanol and using the correction factor.

Compound	Replicate C1 [ ]	Replicate C2 [ ]	Average [ ] in 1-hexanol	RSD (%)	Average [ ] with the correction factor
Caffeine	48.919	49.683	49.301	1.10	93.672

[ ] = concentration.

It can be seen in the European Pharmacopoeia and in the Brazilian Pharmacopoeia, that the caffeine dosage method is through titration in non-aqueous environment, and the content, in relation to the dried substance, should be at least 98.5% and no more than 101.5% (Brasil, 2010a; Europe, 2017). Not only the method employed was not the one recommended by the pharmacopoeia, but also the raw material was not dried. Moreover, the extraction method chosen is not the best method to extract caffeine, as previously discussed. However, a fine result was obtained by this methodology, since the concentration of caffeine was around 94%.

4.5.1.4. Nutraceutical compounds quantitative determination

All of the analyses to quantify nutraceutical compounds (those previously identified in ginger and myrrh extracts) have been carried out in duplicates of each extract prepared with the content of two capsules (randomly selected) plus the addition of a known concentration of 1-hexanol. For batch number two, three independent extractions have been prepared and analysed, and, for batch number three, five independent extractions have been performed and analysed. From the same nutraceutical extract preparation, a duplicate of each injection of each new addition of a known volume of extract and internal standard was carried out in GC-FID. These analyses were performed according to the item 3.4.4 of this present work and the resulting representative chromatograms can be seen in the Figure 15, for the batch number two, and in the Figure 16, for the batch number three. Tables 21 and 22 shows the compounds average concentrations for batch number two, and Tables 23 and 24 for batch number three.
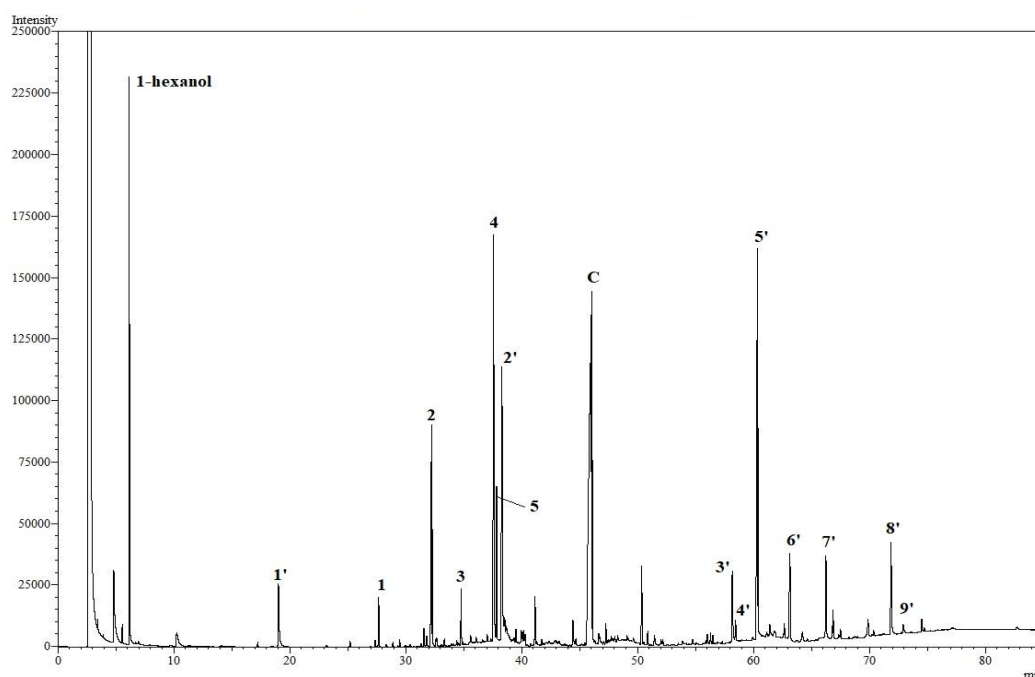
Figure 15. GC-FID of the nutraceutical batch number 2 where 1) beta-elemene; 2) curzerene; 3) gamma-elemene; 4) furanoeudesma-1,3-diene; 5) lindestrene; C) caffeine; 1') decanal; 2') zingerone; 3') 6-isoshogaol; 4') 6-paradol; 5') 6-shogaol; 6') 6-gingerol; 7') 8-shogaol; 8') 10-shogaol; 9') 10-gingerdione and the addition of 1-hexanol.

Table 21. Nutraceuticals marker compounds concentrations (in mg/100 mg) of the batch number 2 expressed in 1-hexanol.

Compounds	Replicate 1 [ ]	Replicate 2 [ ]	Replicate 3 [ ]	Average [ ]	RSD (%)
1'	0.170	0.150	0.192	0.171	12.31
1	0.067	0.068	0.071	0.069	3.032
2	0.323	0.310	0.317	0.317	2.055
3	0.079	0.079	0.078	0.079	0.734
4	0.620	0.614	0.571	0.602	4.442
5	0.215	0.223	0.207	0.215	3.721
2'	0.594	0.543	0.804	0.647	21.38
C	2.213	2.141	2.162	2.172	1.705
3'	0.162	0.173	0.185	0.173	6.637
4'	0.045	0.049	0.045	0.046	4.984
5'	0.889	0.840	0.907	0.879	3.946
6'	0.218	0.229	0.133	0.193	27.18
7'	0.177	0.158	0.160	0.165	6.327
8'	0.157	0.150	0.135	0.147	7.629
9'	0.021	0.026	0.020	0.022	14.39
Total	**5.949**	**5.753**	**5.987**	**5.896**	**2.130**

[ ] = concentration.

Table 22. Nutraceuticals marker compounds concentrations (in mg/100 mg) of the batch number 2 using the correction factors (k).

| Compounds	Average Concentration in 1-hexanol	Correction factor (k)	Average Concentration with the k
1'	0.171	x 1.53	0.261
1	0.069	x 2.00	0.137
2	0.317	x 2.12	0.671
3	0.079	x 2.00	0.157
4	0.602	x 2.20	1.262
5	0.215	x 2.10	0.451
2'	0.647	x 1.90	1.230
C	2.172	x 1.90	4.127
3'	0.173	x 2.71	2.705
4'	0.046	x 2.73	0.126
5'	0.879	x 2.71	2.377
6'	0.193	x 2.88	0.557
7'	0.165	x 2.98	0.492
8'	0.147	x 3.25	0.479
9'	0.022	x 3.39	0.076
Total	**5.896**		**15.109**
Myrrh compounds	**1.281**		**2.678**
Ginger compounds	**2.444**		**8.304**
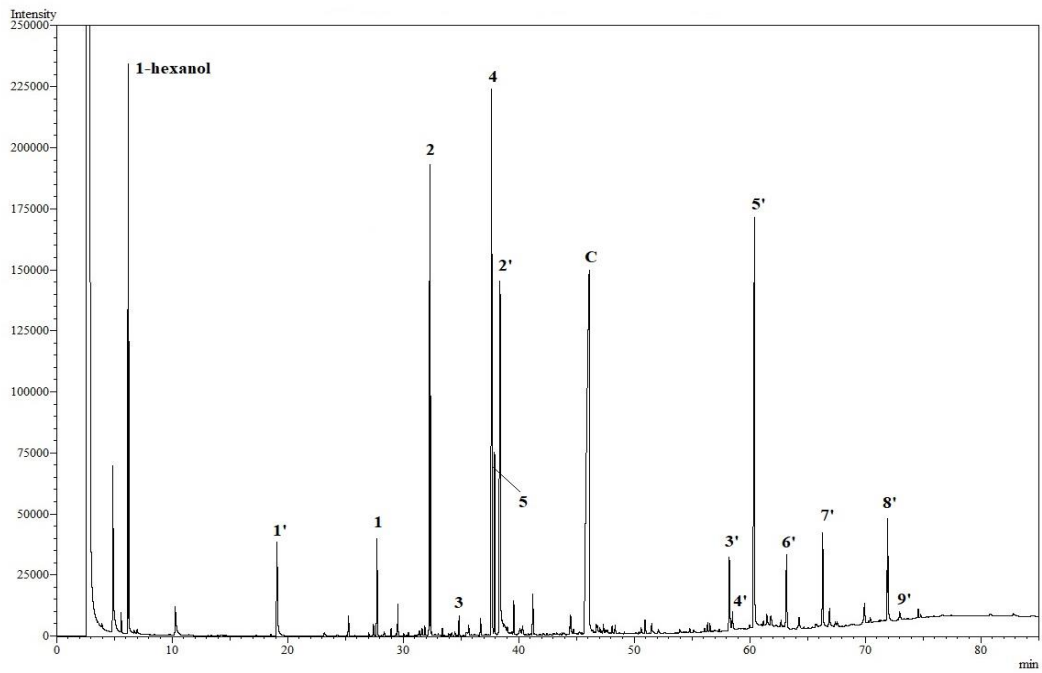
Figure 16. GC-FID of the nutraceutical batch number 3 where 1) beta-elemene; 2) curzerene; 3) gamma-elemene; 4) furanoeudesma-1,3-diene; 5) lindestrene; C) caffeine; 1') decanal; 2') zingerone; 3') 6-isoshogaol; 4') 6-paradol; 5') 6-shogaol; 6') 6-gingerol; 7') 8-shogaol; 8') 10-shogaol; 9') 10-gingerdione and the addition of 1-hexanol.

Table 23. Nutraceuticals marker compounds concentrations (in mg/100 mg) of the batch number 3 expressed in 1-hexanol.

Com-pounds	Replicate 1 [ ]	Replicate 2 [ ]	Replicate 3 [ ]	Replicate 4 [ ]	Replicate 5 [ ]	Average [ ]	RSD (%)
1'	0.269	0.249	0.219	0.216	0.241	0.239	9.205
1	0.172	0.188	0.175	0.179	0.203	0.183	6.817
2	0.912	1.031	0.967	0.959	1.103	0.994	7.446
3	0.035	0.041	0.038	0.038	0.045	0.039	9.598
4	1.140	1.309	1.233	1.217	1.430	1.266	8.664
5	0.335	0.379	0.359	0.357	0.412	0.368	7.852
2'	1.023	0.823	0.713	0.657	0.727	0.789	18.26
C	2.932	3.093	2.872	3.036	3.509	3.088	8.110
3'	0.212	0.217	0.207	0.211	0.236	0.217	5.700
4'	0.054	0.054	0.054	0.041	0.057	0.052	12.09
5'	1.199	1.271	1.202	1.259	1.387	1.264	6.036
6'	0.221	0.302	0.312	0.381	0.433	0.330	24.54
7'	0.228	0.226	0.220	0.232	0.274	0.236	9.186
8'	0.229	0.271	0.275	0.302	0.335	0.282	13.93
9'	0.025	0.032	0.038	0.042	0.047	0.037	23.33
Total	**8.985**	**9.488**	**8.885**	**9.128**	**10.438**	**9.384**	**6.730**

[ ] = concentration

Table 24. Nutraceuticals marker compounds concentrations (in mg/100 mg) of the batch number 3 using the correction factors (k).

Nutraceutical compounds	Average Concentration in 1-hexanol	Correction factor (k)	Average Concentration with the k
1'	0.239	x 1.53	0.365
1	0.183	x 2.00	0.367
2	0.994	x 2.12	2.106
3	0.039	x 2.00	0.079
4	1.266	x 2.20	2.655
5	0.368	x 2.10	0.773
2'	0.789	x 1.90	1.499
C	3.088	x 1.90	5.867
3'	0.217	x 2.71	0.586
4'	0.052	x 2.73	0.142
5'	1.264	x 2.71	3.418
6'	0.330	x 2.88	0.950
7'	0.236	x 2.98	0.703
8'	0.282	x 3.25	0.919
9'	0.037	x 3.39	0.125
Total	**9.384**		**20.56**
Myrrh compounds	**2.851**		**5.979**
Ginger compounds	**3.445**		**8.708**

A significant lack of uniformity in the quantitative composition is evident for the content of the capsules in the final products corresponding to both batches. The non-uniformity of content does not ensure constancy of effect, which could lead to a major problem. It is possible to affirm, through the analysis performed, that the qualitative composition of the nutraceutical

is in accordance with that intended, meaning, the nutraceutical is composed of myrrh, ginger and caffeine, which is what it claims. However, quantitatively there is great variation. Comparing the amount of active compounds in the standard extracts of ginger, myrrh and caffeine and the amount of active compounds in the nutraceutical product it can be observed a smaller amount of active substances in the latter. Nevertheless, there is no regulation establishing the specifications regarding the quantity of these actives in nutraceuticals worldwide, which difficult the assessment of the product suitability. Thus, it can only be concluded that the product is not suitable in terms of uniformity of content and that the quantities of active compounds presented in the final product are considerably lower than those of the standards extracts. The establishment of standards and specifications regarding the quantities of active compounds in a given nutraceutical product should be carried out by a regulatory agency. Only then will it be possible to conclude on the total suitability of the nutraceutical product.

The intake values for two capsules were calculated according to the average weight determined for batches two and three. However, it is worth remarking that batch three presented a very uneven average weight. These intake values can be seen in the Table 25.

Table 25. Intake values for two capsules of total marker compounds, ginger and myrrh compounds and caffeine for batches two and three.

	Batch n° 2	Batch n° 3
Average weight for two capsules	665.1 mg	449.5 mg
Total marker compounds	100.50 mg/ 2 capsules	92.42 mg/2 capsules
Myrrh compounds	17.82 mg/2 capsules	26.88 mg/2 capsules
Ginger compounds	55.20 mg/2 capsules	39.15 mg/2 capsules
Caffeine	27.47 mg/ 2 capsules	26.39 mg/2 capsules

The intake values for the total marker compounds varies around 8 mg in two capsules between batches two and three. However, the greatest variation between batches can be observed in myrrh compounds, in greater quantity in batch three, and in ginger compounds, in greater quantity in batch two. It is possible to observe that the presented values for caffeine are

quite close to those indicated by the supplier (30 mg of caffeine in two capsules) in both batches. It can be assumed, then, that the methodology applied is adequate.

4.5.2. Repeatability analysis

The repeatability analyses of batch number two and number three were performed according to the NMKL method (1971) and have their results shown in the Table 26 and 27, respectively.

Table 26. Raw data for the calculation of RSDr of the batch number two.

N° analysis (d)	a (mg/100 mg)	b (mg/100 mg)	Average (M)	(a-b)/M	[(a-b)/M]²
1	6.007	5.968	5.988	0.0065	0.00004
2	5.937	5.961	5.949	-0.0040	0.00002
3	5.782	5.723	5.753	0.0103	0.00011

<div align="center">Number of duplicate analyses, d = 3   Sum, Σ[(a-b)/M]² = 0.00016</div>

$$RSDr = \sqrt{\frac{\Sigma[(a-b)/M]^2}{2d}} = \sqrt{\frac{0.00016}{2\ x\ 3}} = 0.00523$$

$$U = k\ x\ RSDr\ x\ C = 2\ x\ 0.00523\ x\ 5.896 = 0.0616$$
Final concentration = 5.896 ± 0.0616 mg/100 mg

Where:

U = measurement of uncertainty

k = the coverage factor with a confidence levels of 95 %

RSDr = relative standard deviation of repeatability calculated as described before

C = concentration of the analyte

Table 27. Raw data for the calculation of RSDr of the batch number three.

N° analysis (d)	a (mg/100 mg)	b (mg/100 mg)	Media (M)	(a-b)/M	[(a-b)/M]²
1	10.445	10.430	10.438	0.0014	0.00000
2	9.510	8.745	9.128	0.0838	0.00702
3	8.885	8.884	8.885	0.0001	0.00000
4	9.762	9.213	9.488	0.0579	0.00335
5	9.013	8.957	8.985	0.0062	0.00004

Number of duplicate analyses, d = 5   Sum, Σ[(a-b)/M]² = 0,01041

$$RSDr = \sqrt{\frac{\Sigma[(a-b)/M]^2}{2d}} = \sqrt{\frac{0.01041}{2\ x\ 5}} = 0.03227$$

$$U = k\ x\ RSDr\ x\ C\ = 2\ x\ 0.03227\ x\ 9.384 = 0.6057$$
Final concentration = 9.384 ± 0.6057 mg/100 mg

Where:

U = measurement of uncertainty

k = the coverage factor with a confidence level of 95 %

RSDr = relative standard deviation of repeatability calculated as described before

C = concentration of the analyte

As a result, in both batches two and three, the analyses showed high accuracy.

4.6. Preliminary studies of products stability

Only the capsules from batches number two and three have been made available for this study. Those from batch number one were not available for the analyses because they were insufficient.

Regarding the physical characteristics of the capsules, all of them from the tested batches presented the same ones for a given time zero. These capsules were presented as a

whitish and opaque shell, upstanding, with no distinctive odour. Its content, instead, was presented as a pale-yellow dry powder with ginger and myrrh characteristic aromas.

After five days under heating conditions the capsules began to present integrity modification. They continued exhibiting the whitish and opaque shell, however, when the capsules were opened and their contents removed, they have shown to be very brittle and fragile. Changes in the capsules integrity submitted to freezing conditions also occurred after five days. The whitish and opaque shell remained, but it was very soft and weak while its content was humid and clumpy, very adherent to the walls capsule. All of these characteristics remained until the end of 30 days of analyses.

Concerning batch number two chemical characteristics, a decrease in marker compounds concentration could be observed under heating conditions at 40 °C. At time zero, the marker compounds total concentration was 5.96 mg/100 mg. After 15 days this concentration decreased to 5.52 mg/100 mg and at 30 days it was 5.12 mg/100 mg. On the other hand, when capsules were submitted to freezing conditions, they showed low or no significant decrease in their marker compounds concentration. At time zero, the marker compounds total concentration was 5.96 mg/100 mg. After 15 days this concentration decreased to 5.94 mg/100 mg and at 30 days it was 5.90 mg/100 mg. The following results can be observed in the graphic of Figure 17 and the chromatograms in Figures 18 and 19.
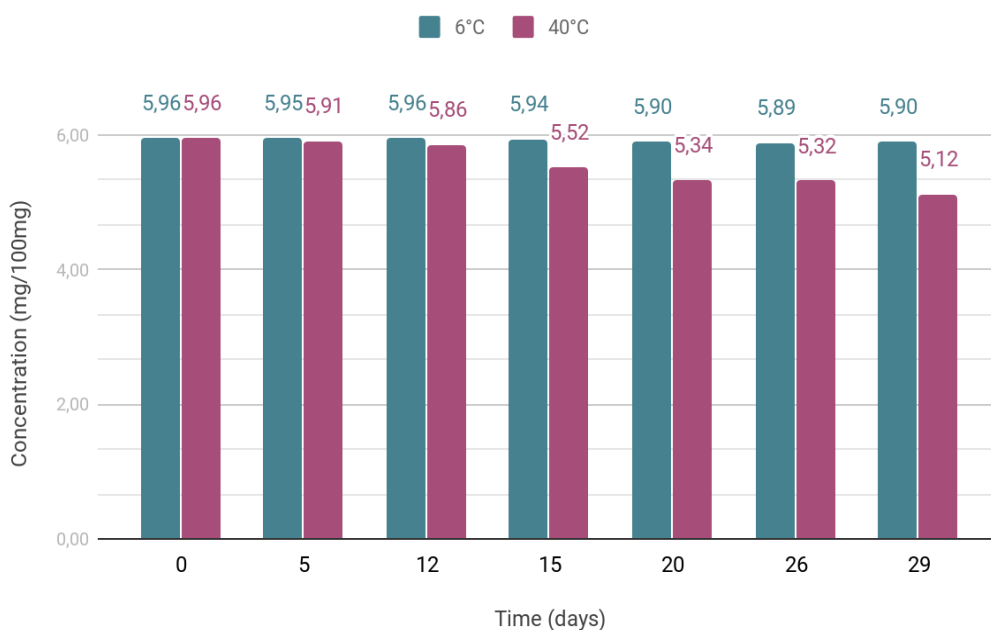

Figure 17. Graphic of batch number 2 nutraceutical marker compounds concentration (in mg/100 mg) *versus* time (in days) after the capsules exposure at 6 °C and 40 °C for 30 days.

Figure 18. GC-FID of nutraceuticals batch number 2 after 15 days of the capsules exposure at 40 °C.

Figure 19. GC-FID of nutraceuticals batch number 2 after 29 days of the capsules exposure at 40 °C.

Concerning batch number three chemical characteristics, decrease in marker compounds concentration could be observed under heating conditions at 40 °C. At time zero, the marker compounds total concentration was 9.76 mg/100 mg. After 15 days this concentration decreased to 8.59 mg/100 mg and at 30 days it was 7.83 mg/100 mg. On the other hand, when capsules

were submitted to freezing conditions, they showed a minor decrease in their marker compounds concentration. At time zero, the marker compounds total concentration of batch number three was 9.76 mg/100 mg. After 15 days this concentration decreased to 9.11 mg/100 mg and at 30 days it was 9.08 mg/100 mg. The following results can be observed in the graphic in the Figure 20 and the chromatograms in Figures 21 and 22.

Figure 20. Graphic of batch number 3 nutraceutical marker compounds concentration (in mg/100 mg) *versus* time (in days) after the capsules exposure at 6 °C and 40 °C for 30 days.
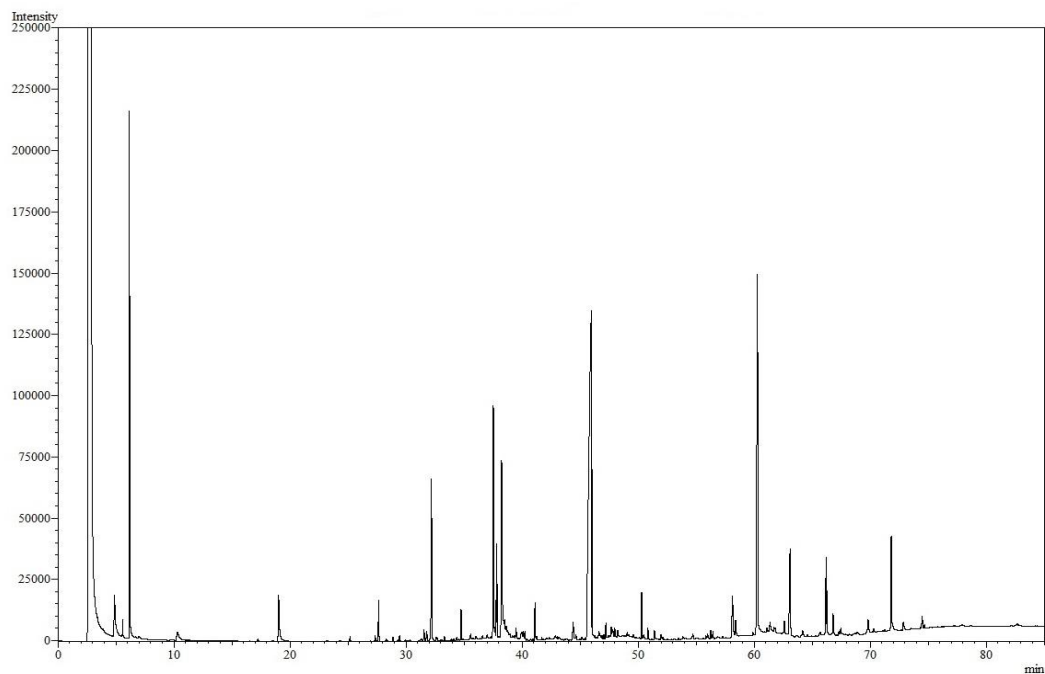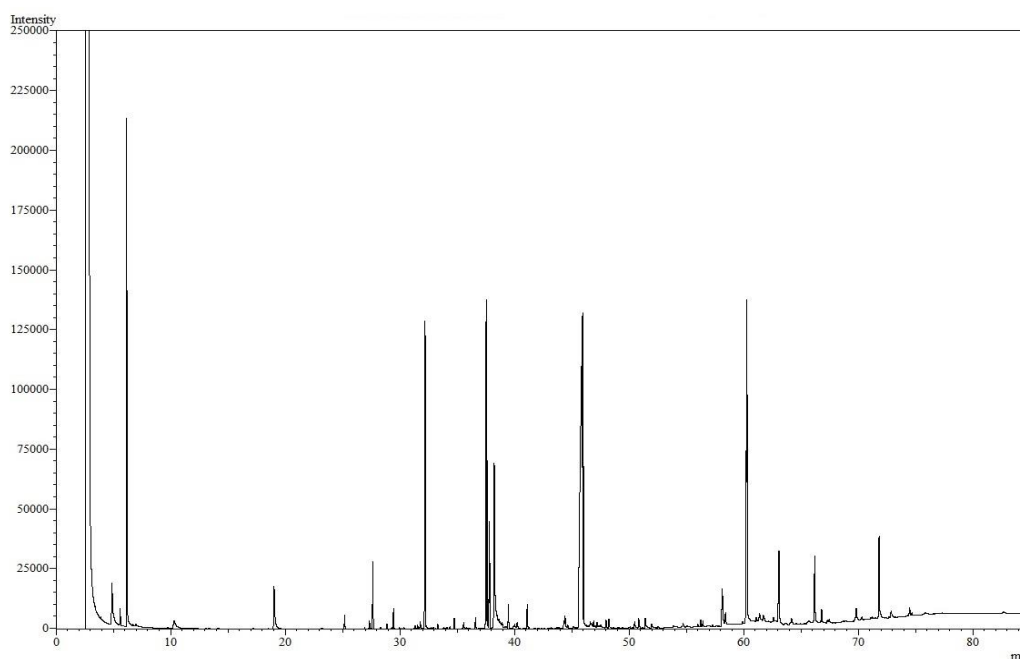

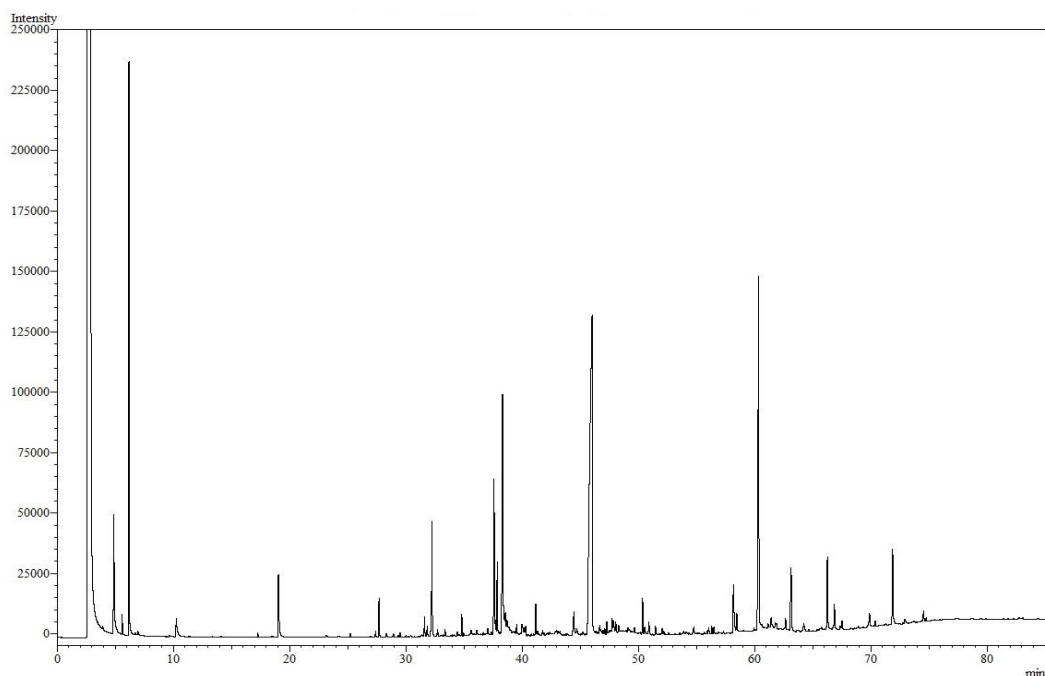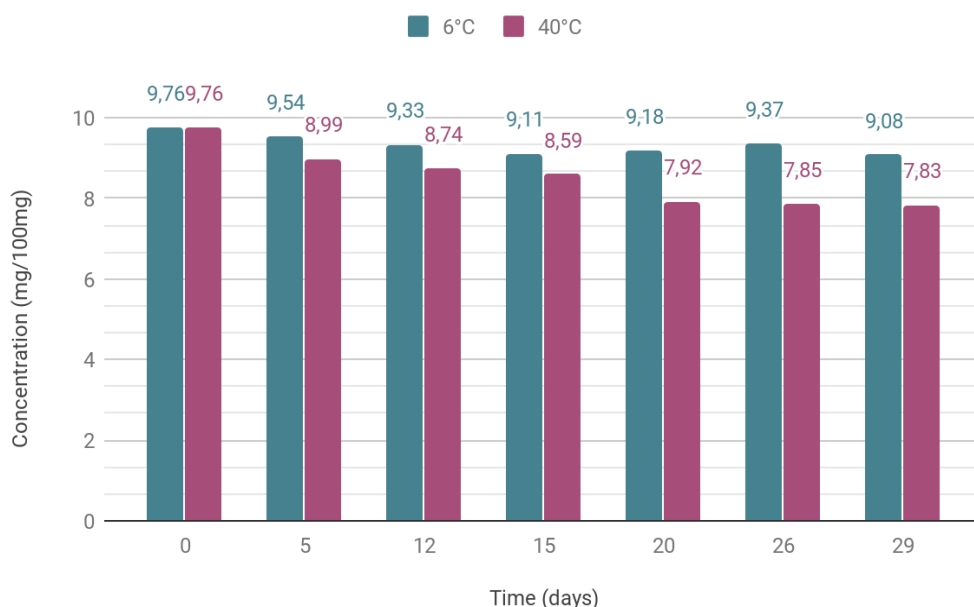Figure 21. GC-FID of nutraceuticals batch number 3 after 15 days of the capsules exposure at 40 °C.
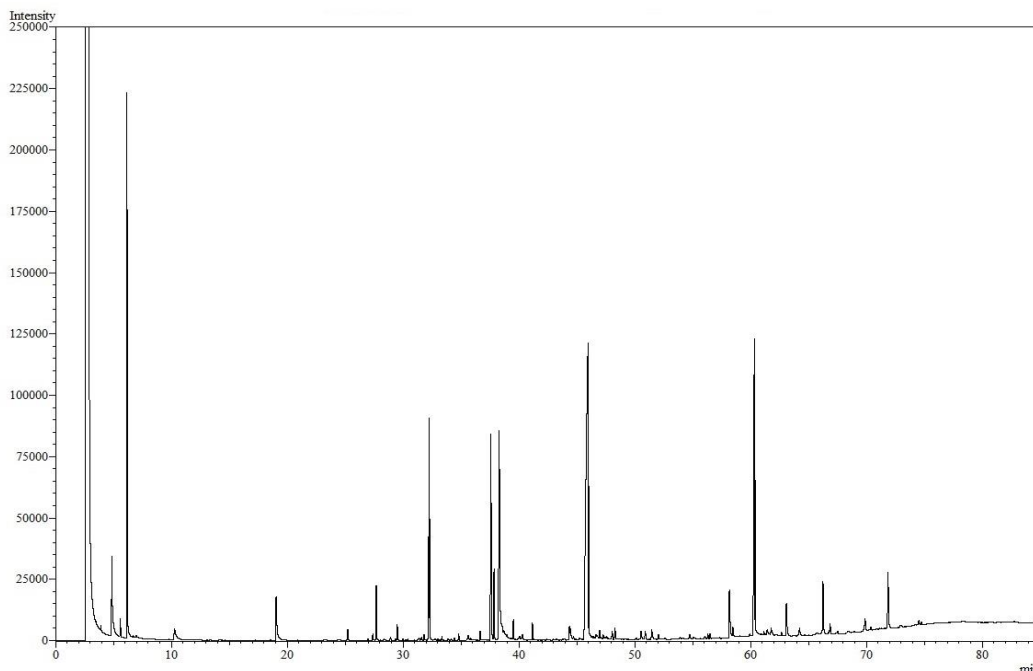
Figure 22. GC-FID of nutraceuticals batch number 3 after 29 days of the capsules exposure at 40 °C.

An interesting data observed is that compounds do not behave in the same way. For example, 6-shogaol (marker compound for ginger extract) showed minor changes in its concentration at 6 °C and 40 °C exposure temperatures over 30 days of analyses. The initial concentration was 1.28 mg/100 mg and after 29 days the concentration at 6 °C was 1.21 mg/100 mg and at 40 °C was 1.22 mg/100 mg. Meanwhile, furanoeudesma-1,3-diene (marker compound for myrrh extract) exhibited major differences in its concentration at 6 °C and 40 °C exposure temperatures over 30 days of analyses. The initial concentration was 1.36 mg/100 mg and after 29 days the concentration at 6 °C was 1.20 mg/100 mg and at 40 °C was 0.60 mg/100 mg. All data can be seen in the Figure 23.
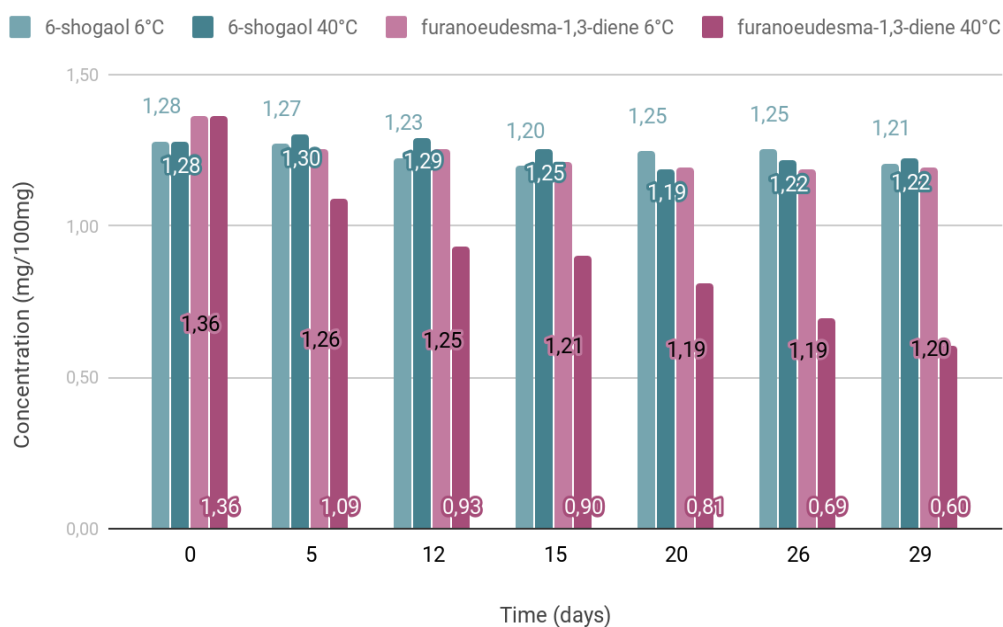
Figure 23. Graphic of batch number 3 nutraceuticals marker compounds 6-shogaol and furanoeudesma-1,3-diene concentration (in mg/100 mg) *versus* time (in days) after the capsules exposure at 6 °C and 40 °C for 30 days.

The blister-protected capsules showed no important changes in their physical structures. The capsules remained intact, both in heating and freezing conditions after 15 days of analyses. It shows that the primary packaging (i.e. the blister) protected a little the capsules from the outside exposure, in terms of humidity at least. Likewise, its chemical characteristics showed minor decrease in their marker compounds concentration at 6 °C. At time zero, the marker compounds total concentration of batch number three was 9.76 mg/100 mg. After 9 days this concentration decreased to 9.45 mg/100 mg and after 14 days it was 9.12 mg/100 mg. Meanwhile, marker compounds concentration at 40 °C exhibited more considerable differences. After 9 days this concentration decreased to 9.20 mg/100 mg and after 14 days it was 8.89 mg/100 mg. The following results can be observed in the graphic in the Figure 24.

Figure 24. Graphic of blister-protected capsules from batch number 3 nutraceutical marker compounds concentration (in mg/100 mg) *versus* time (in days) after the capsules exposure at 6°C and 40°C for 15 days.
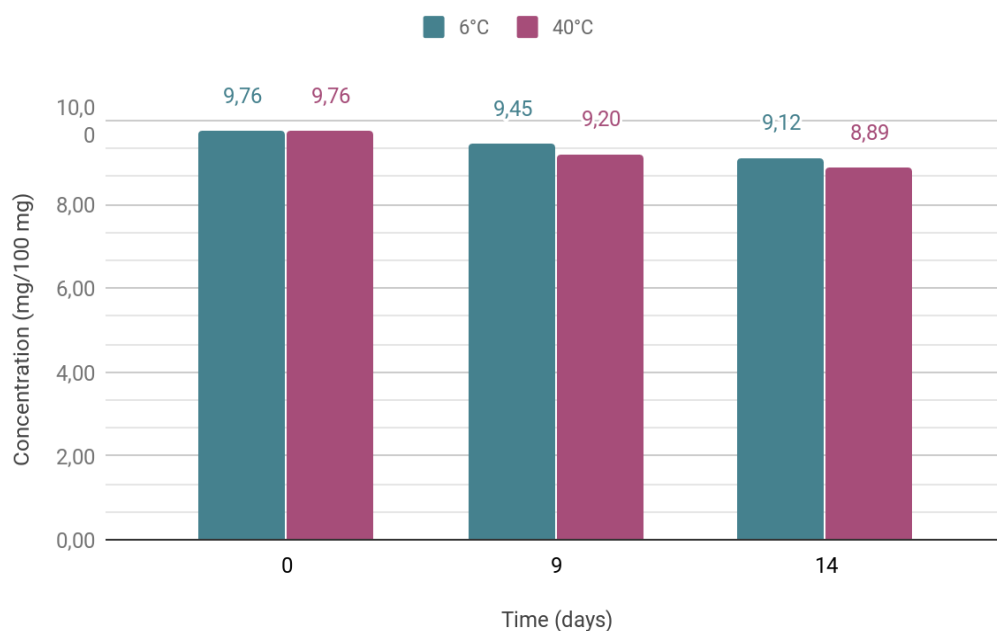
Comparing the data of the blister-protected capsules and the non-protected capsules, it is possible to see that the blister helped in slightly minimizing the effects on compounds concentration from the outside exposure, specially in higher temperatures. After 5 days of heating exposure, the non-protected capsules presented a total concentration of marker compounds of 8.99 mg/100 mg. But after 9 days of heating exposure, the blister-protected capsules showed a total concentration of marker compounds of 9.20 mg/100 mg. And after 15 days the non-protected capsules total concentration was 8.59 mg/100 mg and after 14 days the blister-protected capsules total concentration was 8.89 mg/100 mg.

Based on preliminary results obtained by the developed stability study, further analyses could be interesting. For starters, perform thermal experiments for a longer period than 14 days for the blister-protected capsules and also longer than 30 days for the non-protected capsules. In a month, it was possible to observe only a few aspects of the product and draw a trend line. With a longer evaluation time, it could be verified whether this trend would continue or not, for example. Also, perform photostability tests to evaluate how marker compounds behave when in contact with visible light and UV.

It is also important to take into consideration that the tests were carried out at the suggested temperatures according to the World Health Organization (WHO) Guidelines on Stability Testing. The climate zone II (subtropical and mediterranean climate, where Italy is

located) is where the annual average temperature is lower than in the climate zone IV-B (hot and very humid climate, where Brazil is located) (WHO, 2006). So, according to this matter, 40 °C and around 4 °C and 8 °C were the temperatures used for the preliminary studies.

5. CONCLUSION

The importance of a well-designed, established and standardised quality control for nutraceuticals is quite evident. Applying appropriated analytical methodologies on nutraceuticals raw materials could minimize mistakes with plant substitution, contamination or misidentification and ensure its quality in terms of chemicals contents. Also, on nutraceuticals final products these methodologies could guarantee the total content of the declared compounds on the label. Although adequate and simple methodologies can already provide satisfactory outcomes, it is important to validate the analytical methodology for more accurate results. The validation shall guarantee that the method satisfies the requirements of the analytical applications and is suitable for the intended purpose, ensuring the reliability of the results. Analytical parameters should be based on the intention of the method application, so the experiments can be limited for the actual needs. The analytical parameters for method validation are generally: selectivity, linearity and application range, precision, accuracy, limit of detection, limit of quantification and robustness.

In addition, the implementation of pre-clinical and clinical studies is crucial for providing the basis for health claims and consequently having an impact on consumers approval as well as on the nutraceutical companies, providing reliable products. With the increasing interest in nutraceuticals, it is essential to implement appropriate and specific legislation for these products requiring the above-mentioned studies to be carried out. The establishment of stricter rules of standards and specifications regarding the quantities of active compounds, along the lines of what is already preconized for medicines and phytomedicines, is important precisely because of the pharmacological property claim that nutraceuticals enforce.

Thus, nutraceuticals present no possibility of approval or disapproval regarding the quantity of its assets. The non-uniformity of content could signify a major problem since it cannot guarantee constancy of pharmacological effect. Through the qualitative and quantitative analyses performed, it is possible to say that the qualitative composition of the nutraceutical is in accordance with the alleged composition. Quantitatively, however, there is great variation, when comparing the amount of active compounds in the standard extracts and in the nutraceutical product, on which a smaller amount of active substances can be observed. The lack of extracts standardization used in the production of the nutraceutical product, incorrect mixing of plant extracts and caffeine with the excipients or even encapsulation defects could be hypothesized as causes for what was observed.

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