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"Eco-friendly extraction of bioactive compounds from olive (drupes and leaves), Stevia and Schizocytrium sp."

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A Matteo & Simone,

la mia gioia, la mia forza, la mia Vita

"Eco-friendly extraction of bioactive compounds from olive (drupes and leaves), Stevia and Schizochytrium sp."

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Abstract

The use of nutraceutical compounds, as well as their extraction from natural sources, is now the subject of studies in various sectors.

The research groups working in this field focus their attention on the optimization of extraction methods that combine the economic aspect with the ecological one.

High-value active principles can be recovered from wastes of agri-food farms or industries.

The purpose of this work was the evaluation of green extracting techniques and characterization of different bio-active compounds recovered from different matrices.

The main goals were:

- ✓ The development of an easy analytical approach for the identification and assay of Stevia sweeteners in commercially available soft drink based on liquid chromatography coupled to tandem mass spectrometry;
- ✓ Extraction and characterization of active compounds from Olive (drupes and leaves) wastewaters, monitored by Liquid Chromatography (LC)-Electro Spray Ionization (ESI)-tandem mass spectrometry (MS/MS)
- ✓ Extraction and characterization of lipids from alghe *Schizochytrium sp*. and identification of new adducts of fatty acids, monitored by Gas Chromatography/Liquid Chromatography (LC)-Electro Spray Ionization (ESI)-tandem mass spectrometry (MS/MS)

Introduzione

L'uso di composti nutraceutici, così come la loro estrazione da fonti naturali, è attualmente soggetto di studi in diversi settori. I ricercatori stanno focalizzando la loro attenzione sull'ottimizzazione di metodi estrattivi che combinino l'aspetto economico con quello ecologico. Principi attivi ad alto valore possono essere recuperate da scarti agricoli ed

industriali. Lo scopo di questo lavoro è stato quello di valutare tecniche estrattive green e caratterizzazione dei composti bio-attivi recuperati da diverse matrici.

Gli argomenti principali sono stati:

- ✓ Approccio analitico per l'identificazione e la caratterizzazione di dolcificanti derivanti dalla Stevia utilizzati in bevande commerciali, basato sull'utilizzo della cromatografia liquida accoppiata alla spettrometria di massa tandem;
- ✓ Estrazione e caratterizzazione di composti attivi da acque di scarto della lavorazione di Olive (Drupe e foglie), monitorati tramite cromatografia liquida accoppiata alla spettrometria di massa tandem;
- ✓ Estrazione e caratterizzazione di lipidi alge *Schizochytrium sp*. Ed identificazione di nuovi addotti di acidi grassi, monitorati tramite gas cromatografia/ cromatografia liquida accoppiata alla spettrometria di massa tandem.

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CHAPTER 1 : NUTRACEUTICAL COMPOUNDS

1.1. Introduction

The use of bio-active compounds, deriving from animals and plants, is constantly increasing. De Felice defined nutraceuticals as "*any substance that is a food or part of a food and provides medical or health benefits, including the prevention and treatment disease*". This definition is based on Hippocratic principle "*let food be thy medicine and medicine be thy food*". Nowadays the term nutraceutical is regarded as the bioactive substance and natural bioactive compounds include a broad diversity of structures and functionalities that provide an excellent pool of molecules for the production of therapeutic compounds (Ganapathy M., 2016).

Those compounds can be divided in two categories: potential nutraceuticals and established nutraceuticals. Subdivision is made on the basis of :

- the food materials and nutrients
- their effects on the body
- chemical constituents
- active ingredients

The term "nutraceuticals" can be divided in *nutrient* (nutrient food components) and *pharmacologicals* (a medical drug) (Figure 1).

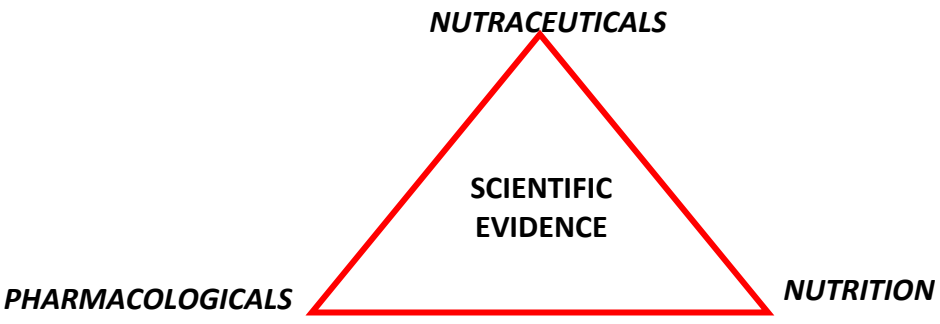

Figure 1 Representation of the nutraceutical concept

The nutraceuticals compounds can be organized into three groups:

- nutrients, compounds with specific nutritional characteristics (vitamins, amino acids, fatty acids, minerals);
- herbals, extracts or concentrates from herbs or botanicals;
- dietary supplements, integrators coming from other sources (pyruvate, chondroitin, steroid hormone precursor).

Nutrients are substances necessary for a healthy human development. The most common nutrients are vitamins and antioxidants (water and fat-soluble), known for their potential health benefits when they are added as dietary supplements.

Vitamins play an important role in the production on macrophage and T cells and have the potential to improve the antibody response to some vaccines (Villamor E., 2005). Antioxidant help in the prevention of the initiation and progression of cardiovascular disease (Penn M.S., 2003). It has been found that supplementation of the diet with various antioxidants before the development of vascular disease inhibited the atherogenic process (Prasad K., 1993) (Parker R.A., 1995).

Plant extracts have been used for the treatment of numerous diseases and such traditional medicine is still widely practiced today. Nutraceutical compounds are found in all parts of the plant (seeds, berries, leaves, roots, flowers and bark) (Jayaprakasha G.K., 2011). The relationship between dietary and prevention of diseases it is known. Epidemiological studies have shown that food has a direct impact on health. It is generally accepted that plant derived foods such as wine, fruits, nuts, vegetables, grains, legumes, spices, etc. exert some beneficial effects on human health, particularly on age-related diseases (Espìn J.C., 2007).

Nutraceuticals are sold in presentations similar to drugs: pills, extracts, tablets, etc. The Food and Drug Administration regulates dietary supplements under a different set of

regulations than those covering conventional foods and drug products. However, no specific regulation exists in Europe to control nutraceuticals (Espìn J.C., 2007).

1.2 Sources of natural Nutraceutical Compounds

Nutraceutical compounds have been studied in different natural matrices (tea, olive oil, exotic fruits, plants, algae, microalgae, bacteria, and fungi).

Natural products are the most source of nutraceutical compounds. A lot of natural-product-related drugs, that are recently approved (Newman D.J., 2007) (Butler M.S., 2008), include compounds from plants and algae.

They cover a range of therapeutic indications (anti-cancer, anti-infective, anti-diabetic, among others) (Konno K., 1999) (Swanson D., 2012) (Mazzotti F., 2013) (Albert C. M., 2002) and they show a great diversity of chemical structures. Natural products are more readily absorbed than synthetic drugs.

Plant natural compounds are divided in primary and secondary metabolites (Wu S., 2008) The primary metabolites include sugars, amino acid, nucleic acids and fatty acids, while secondary metabolites are polyketides, isoprenoids, alkaloids, phenylpropanoids, and flavonoids (Oksman-Caldentey K., 2004).

2/3 Of the world population use natural extracts for their primary pharmaceutical care (McChesney J., 2007) and most scientific evidence is needed in order to support their efficacy and to ensure their safety.

Microorganism are another important source of natural compounds, particularly because they use biological systems for the production of important molecules (Demain A., 2000) (Donnez D., 2009). Like plants, fungi synthesize bioactive compounds thanks to their ecological function. All those compounds have different functions and can be used to produce antibiotics, enzymes and organic acids (Liu J., 2004) (Silveira S., 2008).

Nutraceutical compounds produced from microorganism can be incorporated into functional food as nutritional supplements.

A large variety of bio-active compounds can be extracted from algae, each of which exhibit diverse biological activities (Wijesekara I., 2011).The compounds extracted from microalgae possesses antimicrobial, antioxidant and antiviral activities (Onofrejovà L., 2010) (Plaza M., 2010). Thanks to the extreme conditions in which they live, microalgae must adapt rapidly and efficiently in order to produce a great variety of biologically active secondary metabolites that participate in natural defense mechanisms (Rodrìguez-Meizoso I., 2010). These defense strategies can result in a high level of structural and chemical diversity of compounds, originating from different metabolic pathways (Gil-Ch´avez G.J., 2013) .

1.3 Extraction methods of nutraceutical compounds

Various techniques have been developed for the extraction of bioactive compounds from natural samples in order to shorten the extraction time, enhance the quality of extracts and increase the extraction yield.

A classical process is Soxhlet extraction and it is the main reference technique for the solid-liquid extraction. A suitable extracting solvent should be selected for the extraction of targeted nutraceuticals using soxhlet apparatus. Based on the characteristics of the solvent, different yields and composition from extracts are obtained. The most used solvent is hexane, an excellent organic solvent in terms of oil solubility and easy recovery. The use of alternative solvents has increased due to environmental, health and safety concerns (see par.2.3).

In recent years, the use of microwave-assisted extraction (MAE) is widespread. With this technique cell disruption is promoted by internal superheating, which facilitates desorption

of chemicals from the matrix, improving the recovery of nutraceuticals (Kaufmann B., 2001). Two important parameters influence the efficiency of MAE: plant particle size and size distribution (usually particle size are in the range 100μm-2mm) (Spar Eskilsson S., 2000). The solvent is chosen based on the solubility of the extracts and by the interaction between solvent and plant matrix (Csiktusnadi Kiss G. A., 2000).

One of the new extraction methods is supercritical fluid extraction. This process has several major advantages when compared with the preceeding techniques. The dissolving power of a supercritical fluid solvent depends on its density, which is highly adjustable by changing the pressure or/and temperature. Supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer (Wang L., 2006). The selection of supercritical fluids, as well as the choice of solvent in the previous methods, is critical for the development of extraction. For example, nutraceuticals like phenols are poorly soluble in carbon dioxide and hence not extractable. The solubility of polar compounds can be increased with the addition of polar co-solvent (methanol, ethanol, acetone, etc) to the supercritical CO_2.

Kaufmann reviewed developments in accelerated solvent extraction of bioactive compounds from natural matrix (Kaufmann B., 2002). The solvent is still below its critical condition during accelerated solvent extraction. This techniques is similar to soxhlet with respect to recovery, selectivity and repeatability but extraction time and solvent are reduced.

1.4 Use of Bioactive Compounds in the Food and Pharmaceutical Industries

The interest in nutraceuticals is supported by the results obtained by several epidemiological studies that show the correlation between a specific diet rich in these compounds with a lower risk of chronic disease (Biesalski H., 2009). Nutraceuticals are

usually consumed in pharmaceutical preparations such as pills, capsules, tablets, powder, and vials (Espin and others 2007). Bio-active compounds can be extracted from different matrices (plants, fruits, animals, algae) and show different biological activities.

The food products used as nutraceutical are subdivided as follows (Kokate C.K., 2002):

- Probiotic
- Prebiotic
- Dietary fiber
- Omega 3 fatty acid
- Antioxidant

For example monosaturated fatty acids (MUFA) ,commonly found in olive oil and nuts, have been shown to lower the risk of cardiovascular disease (CVD) and metabolic syndrome (MS) (Kastorini C.M., 2011) and polyunsatured fatty acids (PUFA) are involved in the prevention of Amyotrophic Lateral Sclerosis (Veldink J.H., 2007).

Probiotics are selected from strains most beneficial for the host intestinal bacteria (Eckburg P.B, 2005). The main probiotic preparations currently on the market belong to a large group of bacteria designated as lactic acid bacteria (e.g. lactobacilli, streptococci, bifidobacteria), which are important and normal constituents of the human gastrointestinal microflora (Penner R., 2005).

1.5 Characterization: the importance of mass spectrometry

Innovation in analytical technology have often played an important role in the progress of natural product chemistry. The characterization of metabolites in complex mixtures requires advanced analytical techniques that should provide high sensitivity and selectivity as well as structural information on the constituents of interest.

HPLC is used in phytochemistry to “pilot” the preparative isolation of natural products (optimization of the experimental conditions, checking of the different features throughout the separation) and to control the final purity of the isolated compounds (Tringoli C., 2001).

Hyphenated techniques are advanced analytical technologies that combine the high separation efficiency of HPLC with different detectors to provide the acquisition of on-line complementary data on LC peak of interest within a complex sample matrix.

The growing interest in nutraceutical compounds leads to the need to use rapid methods of isolation and identification of natural products. Mass spectrometry is one of the most sensitive methods of molecular analysis. Mass spectrometry allows the information on the molecular weight as well on the structure of the analytes. There are different ionization methods, but the most commonly used are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). In conjunction with these interfaces, various types of analyzers can be used (quadrupoles-Q, ion trap-IT, time of flight-TOF). An important advace in LC/MS is soft ionization. Indeed, the spectra acquired, unlike electron impact ionization (EI), display mainly the molecular ion species and a very few fragment ions. The structure information given by a single LC/MS spectrum is rather poor. It’s possible to remedy this problem with the use of tandem mass spectrometry (MS/MS). The basic principle of MS/MS is the selection of precursor ion, fragmentation of this ion, usually by collision-induced dissociation (CID), and measurement of the m/z ratio of the product ions formed (Hird S.J., 2014). Tandem mass spectrometers can be operated in a variety of modes, those with a QqQ configuration are typically operated in SRM mode (single reaction monitoring) or in multiple-reaction monitoring (MRM) mode (Hird S.J., 2014) . With an LC-MS/MS instrument in SRM mode, two SRM transitions are acquired for each compound of interest – one for quantification and one qualifier transition. For

identification, ion ratios generated from analysis of the sample and ion ratios from a reference strand and are compared (Benincasa C., 2015) (Malaj N., 2013) (Russo A., 2012).

Permitted tolerances for the relative intensities of the detected ions, set for official control purposes within the EU, specify the maximum deviation between the observed and the expected ion ratios (European Commission., 2002) (European Commission., 2007). A mass spectrometry analysis is more reproducible and the use of a database of natural products can be considered for validation. Spectral MS/MS libraries, such as MassBank or NIST, contain reference spectra for many compounds.

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CHAPTER 2: GREEN CHEMISTRY

2.1 Introduction

Many governments have focused the attention on environmental problems, so as to promote an eco-compatible development in different areas. Industry wants to achieve the desired combination of environmental, economic and social objective. Various chemical disciplines are involved to design and development these ideas.

The approach, which has come to be known as Green Chemistry, intends to eliminate intrinsic hazard itself, rather than focusing a reducing risk by minimizing exposure (WarnerJ.C, 2004).

Green Chemistry is diffusing throughout the chemical industry (SIC 28, NAICS 325) and includes use and development of new substances and processes that impact other sectors such as agriculture, healthcare, automotive, aerospace, energy, electronics, and advanced materials. Process innovation in the chemical sector is often risky, expensive, difficult, requires a broad combination of skills, and takes a long time (Freeman, 1986).

The theory and practice of Green Chemistry is associated (in concept) with a reorientation in the paradigm for conducting science-based investigations—that is 'use-inspired basic research (Stokes, 1997) the pursuit of fundamental understanding motivated by a practical problem. Green Chemistry can be viewed as ''work that locates the center of research in an area of basic scientific ignorance that lies at the heart of a social problem''.

The production of bio-products is one of the most important sector in which green chemistry is developing.

2.2 What is Green Chemistry?

The basic philosophy of Green Chemistry can be defined as:

"*the design of chemical products and processes which reduce or eliminate the use and generation of hazardous substances*" (Anastas P.T., 1998).

Green chemistry utilizes a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture, and application of chemical products (Collins T.J., 1997) Guidelines on adoption of this approach is portrayed in the "12 principles of Green Chemistry" (Anastas P.T. & Warner J.C., 2000) outlined in Table 1.

Those principles should be understood in terms similar to other criteria historically used by chemists such as yield, number of total synthetic steps, or selectivity. It may be impossible to maximize all of the factors simultaneously, so trade-offs may be necessary to optimize the various criteria for highest benefit.

Environmentally friendly technologies and the needs of the marketplace can coexist in Green Chemistry.

2.3 The Solvent in Green Chemistry

In the chemical industry, solvents are used in large quantities. In particular, in fine-chemical and pharmaceutical production, large amounts are used per mass of final products. Solvents, used in commercial manufacturing and service industries are one of the major ecological problems. Solvents are difficult to contain, recycle and so contaminate air, water and land. The idea of "green" solvents expresses the goal to minimize the environmental impact resulting from the use of solvents in chemical production.

The 12 principles of Green Chemistry
1. It is better to prevent waste than to treat or clean up waste after it is formed
2. Synthetic methods should be designed to maximize the incorporation of all materials used into the final product
3. Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment
4. Chemical products should be designed to preserve efficacy of function while reducing toxicity
5. The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary wherever possible and, innocuous when used
6. Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure
7. A raw material of feedstock should be renewable rather than depleting wherever technically and economically practicable
8. Unnecessary derivatization (blocking group, protection/deprotection, temporary modification of physical/chemical processes) should be avoided whenever possible
9. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents
10. Chemical products should be designed to preserve efficacy of function while reducing toxicity
11. Analytical methodologies need to be developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances
12. Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents, including releases, explosions and fires

Table 1. 12 principles of Green Chemistry (Anastas P.T., 2000)

One of the twelve principles of green chemistry is "use safer solvents and auxiliaries". Alternative solvents suitable for green chemistry are those that have low toxicity, are easy to recycle, are inert and do not contaminate the product. There is no perfect green solvent that can be applied to all situations and therefore decisions have to be made.

Recently, four directions towards green solvents have been developed (Capello C., 2007):

- ✓ substitution of hazardous solvents with ones that show better EHS (environmental, health and safety) properties, such as increased biodegradability or reduced ozone depletion potential;

- ✓ use of solvents produced with renewable resources (such as ethanol produced by fermentation of sugar-containing feeds);
- ✓ substitution of organic solvents either with supercritical fluids that are environmentally harmless; or
- ✓ with ionic liquids that show low vapour pressure, and thus less emission to air.

Environmental improvements are achieved with all alternatives in different ways. Therefore, the question is raised of how to measure how green a solvent is. To this end, comprehensive evaluations of the pros and cons of these alternatives and their environmental performance have to be conducted and compared (Capello C., 2007).

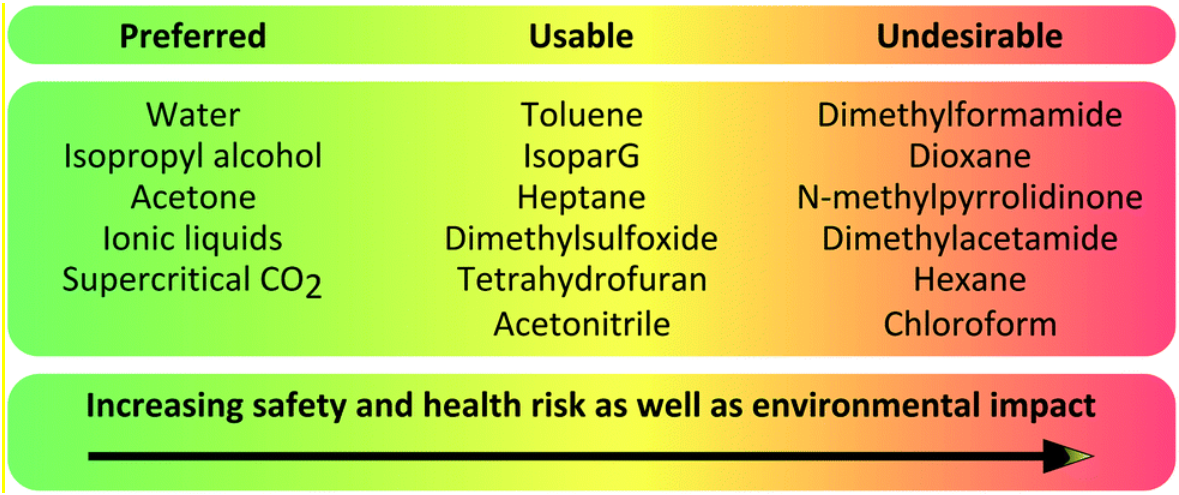

Figure 2 Greenness of solvents commonly used in analytical chemistry.

Within the framework of green chemistry, solvents occupy a strategic place and in the literature novel approaches have also been proposed addressing the use of safer alternatives with more eco-friendly characteristics for the synthetic transformation (Nardi M., 2015) (Nardi M., 2015) (Nardi M., 2017) (Nardi M., 2017).

Eco-sustainable solvents become even more important when applied to the extraction of nutraceuticals or particular molecules of pharmacological interest.

Alternative solvents can be produced from biomasses such as wood, starch, vegetable oils or fruits (Table 3) and they are used in alternative extraction techniques of nutraceutical

compounds. Often such solvents are associated with extraction techniques associated with the use of microwaves or ultrasounds.

Solvent	Extraction Technique (Application)	Health & Safety	Cost	Environmental Impact
Solvent-free	Microwave Hydrodiffusion and Gravity (antioxidants, essential oils)	xxx	x	xxx
	Pulse Electric Field (antioxidants, pigments)	xxx	x	xxx
Water	Steam distillation (essential oils)	x	xx	x
	Microwave-assisted distillation (essential oils)	x	x	xx
	Extraction by sub-critical water (Aromas)	x	x	x
CO_2	Supercritical fluid extraction (decaffeination of tea and coffee)	x	x	x
Ionic liquids	Ammonium salts (Artemisinin)	-	-	xx
Agrosolvents	Ethanol (pigments and antioxidants)	-	xx	x
	Glycerol (polyphenols)	-	x	x
	Terpenes such as *d*-limonene (fats and oils)	-	x	x
Petrochemical solvents	*n*-Hexane (fats and oils)	---	xx	--

Table 2 Alternative solvents of Green Chemistry

Ethanol is obtained by fermentation of sugars and is a solvent completely biodegradable. Bio-solvents also include *d*-limonene, a compound mainly extracted from Citrus fruits that is used for the extraction of oils. Glycerol is perhaps the most used solvent in the cosmetic sector and is obtained by trans-esterification of vegetable oils.

2.4 Food Waste Recycling

The need to both avoid waste and find renewable resources has led to a new and promising research avenue: the use of food supply chain waste (FSCW) as a renewable bio-refinery

feedstock. Food supply chain waste could be defined as “the organic material produced for human consumption discarded, lost or degraded primarily at the manufacturing and retail stages” (Lin S.K.C., 2013).

Wastes can be reused as a raw material for the production of marketable chemicals such as silica based bio-derived adhesives from wheat straw or bio-derived surfactants produced from bread waste, gradually helping our society to achieve a circular economy based on zero waste (Leunga C. C. J., 2012).

FSCW contain many functionalized molecules that can be recovered and reused (Figure 3). For example, waste processing of food products as well as gardening and orchard waste can be an important source of nutraceuticals (Figure 3).

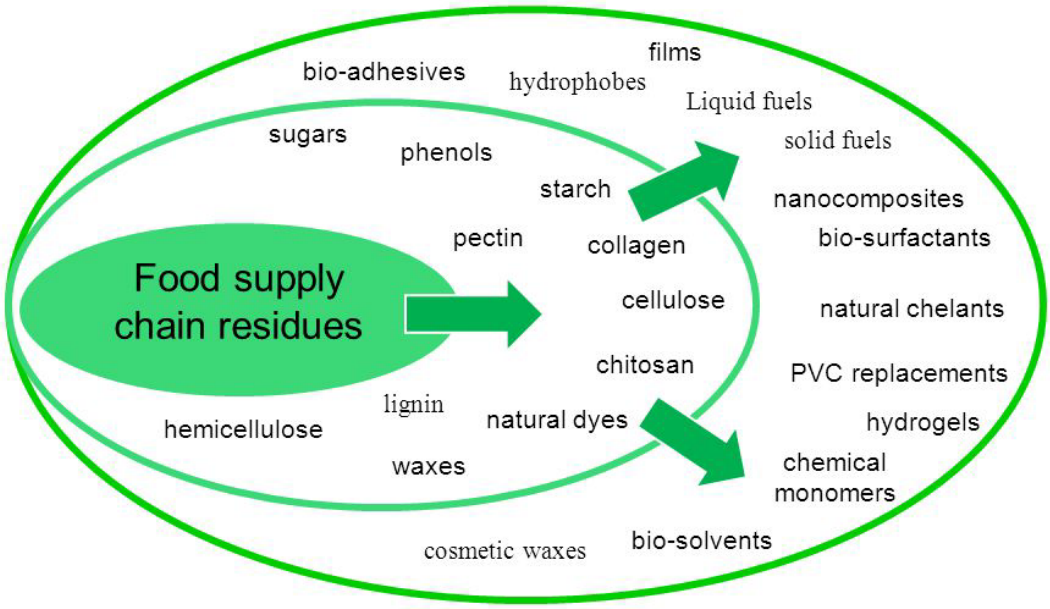

Figure 3 Components present in food chain waste and their uses in common consumer applications.

Plant-derived waste represents a higher proportion (63%) of FSCW in comparison to animal-derived waste (on a wet basis) (Pfaltzgraff L.A., 2013). For any profitable waste treatment facility, there needs to be segregated waste from a single fruit or vegetable so that it may be treated accordingly for generation of various pharmaceutical, nutraceutical or food ingredient (Rudra S.G., 2015).

The waste materials such as peels, seeds and stones produced by the fruit and vegetable processing can be successfully used as a source of phytochemicals and antioxidants. The entire tissue of fruits and vegetables is rich in bioactive compounds, such as phenolic compounds, carotenoids, vitamins and in most cases, the wasted by products can present similar or even higher contents of antioxidant and antimicrobial compounds than the final produce can (Rudra S.G., 2015). The new aspects concerning the use of these wastes as by-products for further exploitation on the production of food additives or supplements with high nutritional value have gained increasing interest because these are high value products and their recovery may be economically attractive. The by-products represent an important source of sugars, minerals, organic acid, dietary fibers and phenolics which have a wide range of action which includes anti-tumoral, antiviral, antibacterial, cardio protective and anti-mutagenic activities. Utilization of by-products is, however, limited due to the poor understanding of the nutritional and economic value (Rudra S.G., 2015).

2.5 Green Extraction of Natural Products

The design of green extraction methods of natural products is currently an active research topic in the multidisciplinary area of applied chemistry, biology and technology.

All industries (perfume, cosmetic, pharmaceutical, food, bio fuel, or fine chemicals industries) use extraction processes (maceration, steam or hydro-distillation, pressing, decoction, infusion, percolation and Soxhlet extraction) for the preparation of final products.

In the food industry, besides the well established huge extraction processes of sugar beet and sugar cane, and the preparation of decaffeinated tea and coffee, many formulations have been developed by adding plant extracts and nutraceuticals concentrates (Chemat F.,

2012). Precursor of bioactive compounds are extracted with classical methods or with modern technology.

The directive REACH (*Registration, Evaluation, Authorisation and Restriction of Chemicals*) is changing the European industry. This directive puts the spotlight on processes by defining the notion of BAT (Best Available Technology) for each professional sector. For this reason, the development of green technologies occupy a central role in environmental-friendly processes (Chemat F., 2012).

Green extraction of natural products can be defined as:

> "*discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product".*

Just as the 12 principles of green chemistry have been listed, also for the green extractions it is possible to summarize 6 similar principles:

- Innovation by selection of varieties and use of renewable plant resources;
- Use of alternative solvents and principally water or agro-solvents;
- Reduce energy consumption by energy recovery and using innovative technologies;
- Production of co-products instead of waste to include the bio- and agro-refining industry.
- Reduce unit operations and favour safe, robust and controlled processes.
- Aim for a non denatured and biodegradable extract without contaminants.

The principles have been identified and described not as rules but more as innovative examples to follow, discovered by scientists and successfully applied by industry.

In particular, the second principle (use of alternative solvents and principally water or agro-solvents) suggests a reduction in the consumption of petrochemical solvents and volatile organic compounds (VOC's) with alternative solvents (Table 2).

Extraction with supercritical CO_2 is one of the most used green extraction methods. Using this technique, pure perfumes, fragrances and active ingredients can be obtained with no traces of solvent. Supercritical CO_2 extraction is being applied in several sectors such as food, cosmetics and pharmaceutical industry. It is used for weakly polar compounds of low molecular weight such as carotenoids, triglycerides, fatty acids, aromas, *etc*. The main drawbacks remain its high initial investment and difficulties to perform continuous extractions (Herrero M., 2006).

Microwave Hydrodiffusion and Gravity (MHG) is a new microwave (mw) solvent-free extraction technique used for the extraction of essential oils. Based on a relatively simple principle, this method also involves placing the plant material in a microwave reactor, without adding any solvent or water (Chemat F., 2012). The internal heating of the *in situ* water within the plant material distends the plant cells and leads to the rupture of glands and oleiferous receptacles. The heating action of microwaves thus frees essential oil and *in situ* water which are transferred from the inside to the outside of the plant material, and drop by gravity out of the microwave reactor (Chemat F., 2012).

In recent years the use of microwaves for the extraction of nutraceuticals from food waste has been used. The MW-Assisted extraction from olive leaves of polyphenols is a valid alternative to classical extraction procedures (Procopio A., 2009).

Ultrasound assisted hydrodistillation can contribute to intensify and to improve the efficiency of essential oil extraction and considerably reduce extraction time and energy used, in particular on citrus fruit (Sawamura M., 2010). With this process extraction yield is 44% higher than the conventional method.

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CHAPTER 3: EXTRACTION AND CHARACTERIZATION OF BIO-ACTIVE COMPOUNDS

3.1 Stevia

3.1.1 Introduction

Since the introduction, 50 years ago, of aspartame, a synthetic dipeptide sweetener, many efforts have been made to introduce new highly potent sugar surrogates to alleviate the medical and nutritional concerns raised by the intake of this molecule. Stevia, a sweet plant of the family Asteraceae, which is native in South America (Paraguay and Brazil), contains a number of sweet flavored glycosides of the diterpene steviol type. It was reported that there are around 150 species within the *Stevia* family including *Stevia dianthoidea*, *Stevia Phlebophylla, Stevia anisostemma, Stevia bertholdii, Stevia crenata, Stevia enigmatica, Stevia eupatoria*, *Stevia lemmonii, Stevia micrantha*, *Stevia ovata*, *Stevia plummerae*, *S. rebaudiana*, *Stevia salicifolia*, *Stevia serrata* and *Stevia viscida* with all plants being sweet but *rebaudiana* having the highest sweetness levels.

3.1.2 Botanical description

Stevia is a perennial semi-shrub up to 65 cm in height. It's leaves are sessile, 3-4 cm long, elongate-lanceolate or spetulate shape with blunt tipped lamina, serrate margin from the middle to the up and entire below. The upper surface of the leaf is slightly glandular pubescent. The stem is weak- pubescent at bottom and woody. The rhizome has slightly branching roots. Flowers are composite surrounded by an involucres of epicalyx. The

capitula are in loose, irregular, sympodial cymes. The flowers are pentamerous, white in color with a pale purple. The fruit is a five-ribbed spindle-shaped achene (Figure 4).

The suitable natural climate is semi humid subtropical with temperatures extremes from 21 to 43°C and overage 24°C (Huxley A., 1992).

Figure 4 Plant of Stevia Rebaudiana Bertoni

3.1.3 Chemical constituents

The complete chemical composition of Stevia species is not yet available despite a variety of Stevia species having been tested. The useful part of this shrub is the leaves. The steviol glycosides which are secondary metabolites responsible for the sweet taste (Kinghorn A., 1985) were first isolated in 1931 by two French chemists (Bridel M., 1931). Eight sweet diterpene glycosides are presents in stevia leaves, including isosteviol, rebaudiosides (A, B, C, D, E, F), stevioside, dulcoside A and steviolbioside (Rajasekaran T, 2008) (Goyal S.K., 2010).

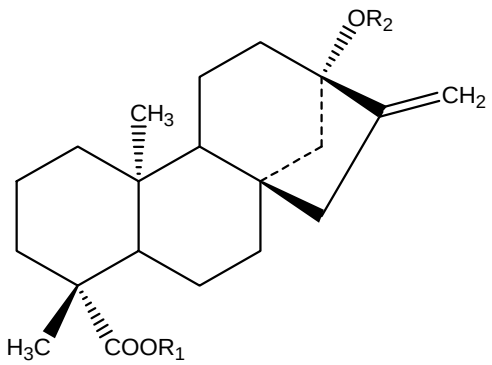
These glycosides are mainly compounds of the diterpene derivative steviol (Shibata H, 1991). Stevia rebaudiana Bertoni, the sweetest species, contains in its leaves all of the eight ent-kaurene glycosides (Kinghorn A.D., 1984). In addition, stevia rebaudiana Bertoni contains stigmasterol, b-sitosterol, and campesterol (D'Agostino M., 1984). The same species also contains steviol, a product formed by enzymatic hydroxylation within the

plant (Kim K.K, 1996) Other chemicals with no sweet taste are also found in Stevia species and some may even be bitter in taste. Stevisalioside A (from the roots of Stevia salicifolia) (Mata R., 1992), longipinane derivatives in the roots of Stevia connata (Sánchez-Arreola E., 2000), epoxylabdane diterpenes and a clerodane derivative in the leaves of Stevia subpubescens (Román L.U., 2000), flavonoids from the leaves of S. rebaudiana (Soejarto D.D., 1982), Stevia nepetifolia (Rajbhandari A., 1983), Stevia microchaeta, Stevia monardifolia, Stevia origanoides (Rajbhandari A., 1985) and Stevia procumbens (aerial parts) (Sosa V.E., 1985), and sesquiterpene lactones from the aerial parts of stevia procumbens and the leaves of stevia origanoides (Calderon J.S., 1987) are in this group.

The contents of sweet glycosides found in the leaves depends on variety (Giraldo C. E., 2005), growing conditions (Pól J., 2007) and on the adoption of modern agronomical techniques (Nepovim A., 1998).

Work to elucidate the chemical structures of stevia rebaudiana sweeteners began in the early twentieth century, but proceeded slowly. The structures of rebaudioside A and stevioside were not fully determined until 1960 (Kinghorn A.D., 1989) (Kinghora A. D., 1986) (Kinghorn A.D., 2001) and 1970 (Kinghorn A.D., 1985), respectively. During the 1970s, additional sweet components, including rebaudiosides A–E, were isolated from S. Rebaudiana leaves and characterized by Osamu Tanka and co-workers (Kohda H., 1976). However, some evidence exists that rebaudioside B and steviolbioside are not native stevia rebaudiana constituents, but are formed by partial hydrolysis during the extraction process (Wiet S. G., 1988). All Stevia compounds have the same chemical backbone structure (steviol) but differ in the residues of carbohydrate at positions C-13 and C-19. Structures of the sweet components of Stevia occurring mainly in the leaves are given in Figure 5. Stevioside is a glycoside with a glucosyl and sophorosyl residue attached to the aglycon

steviol; the latter has a cyclo-pentanoperhydrophenanthrene skeleton. The C4 and C13 of steviol are connected to the β-glucosyl and β-sophorosyl group, respectively. The structure of rebaudioside A is the same as that of stevioside except that the sophorosyl residue is replaced by a glucosyl-(1-3)-sophorosyl residue (Prakash I., 2008). The Stevia sweeteners are similar in structure, in that a steviol aglycon is connected at C4 and C13 to trisaccharides consisting of glucose and/or rhamnose residues (Kobayashi M., 1977) Stevioside is a natural sweetener extracted from leaves of Stevia (Genus J. M.C., 2003).



Compound name	R-groups in backbone figure above	
	R_1	R_2
Steviol	H-	H-
Steviolbioside	H-	β-glc-β-glc-
Stevioside	β-Glc-	β-glc-β-glc-
Rebaudioside A	β-Glc-	$(β\text{-glc})_2$-β-glc-
Rebaudioside B	H-	$(β\text{-glc})_2$-β-glc-
Rebaudioside C (Dulcoside B)	β-Glc-	(β -glc,α-rha-)-β-glc-
Rebaudioside D	β-Glc-β-Glc-	$(β\text{-glc})_2$-β-glc-
Rebaudioside E	β-Glc-β-Glc-	β-glc-β-glc-
Rebaudioside F	β-Glc-	(β-glc,β-xyl-)-β-glc-
Dulcoside A	β-Glc-	α-rha-β-glc-

Figure 5 Structures of steviol glycosides

The most abundant substances, present in Stevia, are stevioside and rebaudioside A. Of the Stevia glycosides, rebaudioside A is the sweetest and the most stable, and it is less bitter than stevioside. Rebaudioside E is as sweet as stevioside, and rebaudioside D is as sweet as

rebaudioside A, while the other glycosides are less sweet than stevioside (Cramer B., 1987). According to Pederson (Pederson P., 1987), stevioside is extracted from the leaves of the Stevia plant.

Each Stevia glycoside possesses a different sweetening power. Stevioside, a white, crystalline powder, for instance, is between 110 and 270 times sweeter than sucrose, while reabudioside A is between 150 and 320, rebaudioside C is between 40 and 60 and dulcoside is about 30 times sweeter (Phillips K. C., 1989).

The steviol aglycon in Stevia ranges between 4% and 20% of the dry weight of the leaves, depending on the cultivar and growing conditions (Gardana C., 2010).

Stevioside and Rebaudioside A are the most abundant components of Stevia and are considered safe for type 2 diabetes patients (Gregersen S., 2004).

The stevioside diterpenoid glycoside is composed by the aglycone steviol, to which it is glycosylated with three molecules of glucose. In addition to stevioside, several other sweet terpene glycosides, such as steviolbioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside E and dulcoside were isolated from Stevia leaf (Rajasekaran T, 2008). Presently, different type of foods containing Stevia glycosides are available in the market. The FDA and EFSA authorities' cautions require that any of these glycosides to be sold have to be analytically controlled, by special experts in this new important field of nutraceuticals used for human health (Santini A., 2017).

3.1.4 Beneficial properties

Several studies are developing around the beneficial properties of Stevia. Leaves of S. rebaudiana has many medical applications like antimicrobial, antiviral, antifungal, anti-hypertensive, anti-hyperglycaemic, anti-tumour, anti-inflammatory, anti-diarrhoeal,

diuretic, anti-human rota-virus activities, anti-HIV, hepatoprotective and immunomodulatory effects.

Anti-hyperglycemic activity - Diabetes mellitus is a group of disease characterized by hyperglycemic and varying degrees of an insufficient insulin effect (De Fronzo R.A., 1991). In addition to insulin abnormalities, pancreatic α-cell dysfunction and relative glucagon excess are involved (Unger J., 1997). Postprandial hyperglycemia observed in type 2 diabetes is usually due to an increase in basal hepatic glucose production and a decrease in peripheral glucose disposal. Therefore, correction of this imbalance at either the entry or the exit step of plasma glucose should help to correct this pathological condition. *Stevia* leaf extract has been used traditionally in the treatment of diabetes (Megeji N. W., 2005) (Soejarto D.D., 1982).

Stevioside, the major component of the extract, has a high sweetness with no calorie and only a small amount is needed for sweetening purposes. Thus, it should be a good alternative to sugar for diabetic patients. Suzuki et al. (Suzuki H., 1977) monitored a sample of rats treated with a diet containing 0.5 g % of stevioside and 10 g % stevia powder leaves, for 4 weeks. The result of the study was a significant reduction in blood glucose level following the 4 weeks of treatment (Suzuki H., 1977). Subsequently, the effect of aqueous extract of stevia leaves on the glucose tolerance test was investigated in humans. Aqueous extracts of 5.0 g of leaves were administered to volunteers at regular six-hour intervals for 3 days. Glucose tolerance tests were performed before and after extract administration. A second group of six healthy volunteers who ingested an aqueous arabinose solution was also studied. The extract of S. rebaudiana increased glucose tolerance. The extract significantly decreased plasma glucose levels during the test and after overnight fasting in all volunteers from a basal value of 82 mg/dl at the beginning of

the analysis to 68 mg/dl after three hours of ingestion. Nevertheless, a possible effect of this plant on insulin secretion or peripheral action should be considered.

In 2011, Mishra performed an analysis of antidiabetic activity of S. rebaudiana extract on a sample of fifteen patients, man and woman aged 25 to 60 years (Mishra N., 2011). During the experimental period, the patients did not use hypoglycemic drugs. Stevia leaf powder (0.5–1.0 g) was used in place of sugar in drink intake. the study was performed for 45 days divided into three steps of 15 days each. First 15 days patient received normal hypoglycemic drug and their postprandial glucose level (PGL) and fasting glucose level (FGL) were measured (153.54 mg/dl and 189.56 mg/dl respectively); for next 15 days; they were not given any medicine under normal diet and their PGL and FGL were measured (208.6 and 283 mg/dl,); in the last 15 days, they were given Stevia leaf three times (3.0 g each time) a day with beverages, and their glucose levels were measured (FGL levels were 195.7 and PGL were 271.3 mg/ml (means). Based on the results obtained the authors concluded that stevia rebaudiana extract decreased glucose levels, but not statistically significant. For more significant results larger sample size should be considered for use under controlled conditions and for extended periods of time.

Antimicrobial activity - There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and re-emerging infectious diseases and development of resistance to the antibiotics in current clinical use (Cowan M. M., 1999). There are many studies on the antimicrobial activity of various extracts of stevia rebaudiana (with water, acetone, chloroform, methanol, ethyl acetate or hexane as solvents). The effects of stevia on some selected microorganisms such as Salmonella typhi, Aeromonas hydrophila, Vibrio cholerae, Bacillus subtilis, Staphylococcus aureus and

others have been examined (Ghosh S., 2008); (Jayaraman A., 2008); (Seema T., 2010) (Tadhani M. B., 2006). S. rebaudiana Bertoni has the ability to inhibit the growth of certain bacteria, which helps to explain its traditional use in treating wounds, sores, and gum disease. Tomita et al. studied the bactericidal activity of a fermented hot-water extract from S. rebaudiana Bertoni towards enterohaemorrhagic Escherichia coli and other food-borne pathogenic bacteria (Tomita T., 1997). Other researcher groups evaluated the antimicrobial activity of S. rebaudiana leaf extracts on microorganisms like Salmonella typhimurium, B. subtilis, and S. aureus. Gosh et al. (Ghosh S., 2008) evaluated the antimicrobial activity of S. Rebaudiana leaf extracts (i.e., petroleum ether, cyclohexane, chloroform, water, acetone, and ethanol) against 10 selected pathogenic as well as food-spoilage fungal (Alternaria solani, Helminthosporium solani, Aspergillus niger, and Penicillium chrysogenum) and pathogenic bacterial (E. coli, B. subtilis, Enterococcus faecalis, Proteus mirabilis, P. aeruginosa, and S. aureus) isolates, using streptomycin and cotrimazole as controls. The study shows that petroleum ether extracts at 250 mg/ml (MIC) inhibit the growth of E. coli and S. aureus among bacteria and P. chrysogenum among fungi. So, use of petroleum ether for extraction exhibited the best antimicrobial potential followed by water, chloroform cyclohexane, acetone, and ethanol. The result of the study is that extracts of S. rebaudiana act on a wide spectrum of microorganisms. While most of the extracts obtained with organic solvents showed antimicrobial activity, only those that are obtained with water or ethanol are used in a large number of traditional natural products from plants applied in allopathic medicine.

The results obtained by various authors indicate that the solubility, concentration, and composition of secondary metabolites are responsible for the antimicrobial activity of the different extracts (Ruiz-Ruiz J.C., 2017) (Jayaraman A., 2008).

Anti-inflammatory activity - Inflammation is a very complex, multifactorial, and dynamic process involving many systems, which is closely associated with the process of repair. Inflammation is defined as a local response of mammalian tissue to injury due to any agent, and manifests usually in form of painful swelling associated with some changes in skin covering the site. Inflammation can be classified either as acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is due to the increased movement of plasma and leukocytes from the blood into the injured tissues. Chronic inflammation is due to a progressive shift in the type of cells that are present at the site of inflammation, which is characterized by simultaneous destruction and healing of the tissue due to the inflammatory process (Loganayaki N, 2013). Carrageenan-induced paw edema is widely used for determining the acute phase of the inflammation and is characterized by biphasic event with involvement of different inflammatory mediators. In the first phase chemical mediators such as histamine, dextran, and serotonin play a role, while in the second phase kinins and prostaglandins are involved. Arya and co-workers studied the anti-inflammatory activities of methanolic extract of callus and intact plant parts of Stevia rebaudiana Bertoni (Arya A., 2012). The studies revealed that stevia can inhibit the edema starting in the first phase of inflammation and during all the processes that probably leads to inhibition of different aspects and chemical mediators of inflammation. It is well known that in chronic and sub-acute inflammation, reactive oxygen species (ROS) are important in the modulation of the extent of the inflammatory response and consequent tissue and cell injury, and antioxidants are considered as possible protective agents that reduce oxidative damage to the human body from ROS and retard the progress of many diseases. Results from the anti-inflammatory activity testing indicated that the callus extract showed a less significant inhibition in the edema as compared to extract of intact plant. It was also revealed that there is an increase in the

accumulation of secondary metabolites in callus cultures in sixth week of incubation. The natural phenolic, alkaloids, tannins, glycosides, and flavonoid compounds function as antioxidants by different mechanisms and, according to Arya et al. (2012), the high contents of these phytochemicals in both the extracts can explain their anti-inflammatory activity. The methanolic extract of callus culture and intact plant part exhibited significant, dose dependent activity on the tested experimental animal models and produced a significant inhibition of carrageenan-induced paw edema in rat. The studies suggest that S. rebaudiana is also a potent source for phytomedicine development in the future.

Antitumor activity - Cancer is a group of diseases caused by loss of cell-cycle control and is associated with uncontrolled cell growth. Cancer can be caused by many external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). Cancer is a significant worldwide health problem because of the lack of widespread and comprehensive early detection methods, the associated poor prognosis of patients diagnosed in later stages of the disease, and its increasing incidence on a global scale. Many scientific studies focused on the use of non-nutritional compounds present in the diet, preventing the phenomenon of degenerative disease such as cancer. This heterogeneous class of molecules is generally known as phytochemicals (polyphenols, flavonoids, phytoalexins, phenolic acids, indoles, and sulfur-rich compounds) (Sporn M. B., 2002) (Surh Y.J., 2003) (Russo G. L., 2007). These compounds are present in plant-derived food, beverages and many dietary supplements (Russo G. L., 2007). Fifty pecent of the drugs in clinical trials for anticancer are isolated from natural source or related to them (Cragg G. M., 2000). A great number of in vitro and in vivo methods have been developed to measure the efficiency of natural anticancer compounds either as pure compounds or as

plant extracts (Chanda S., 2013); (Krishnamurthi K., 2007). The antitumor potential of S. rebaudiana was evaluated in vitro by (Jayaraman A., 2008). These authors obtained leaf extracts with organic solvents (i.e., ethyl acetate, acetone, and chloroform) and water. These authors performed an antitumor assay on human laryngeal epithelioma cells (HEp2). The aqueous extract of S. rebaudiana showed no pronounced antitumor activity, but the acetone and EAEs were more cytotoxic to HEp2 cells. Acetone extracts showed the highest cytotoxic activity, followed by ethyl acetate and chloroform extracts. An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to evaluate cytotoxicity based on metabolic reduction of MTT. In treatments with Vero (African green monkey kidney cells) cells, 1:2 and 1:4 dilutions of the acetone extract showed cytotoxicity, but there was no apparent cytotoxicity at 1:8 dilution. Further dilutions also had no toxic effects on Vero cells. The 1:2 and 1:4 dilutions were cytotoxic to HEp2 cells, whereas the 1:8 dilution caused more than 50% cytotoxicity and also cessation of cell growth. Further dilutions had less effect on the viability of the cancerous cells. Thus, the 1:8 dilution of the acetone extract of S. rebaudiana is nontoxic to the normal cells and also has both anti-cancer and antiproliferative activities against the cancerous cells (Ruiz-Ruiz J.C., 2017). These results points to the probable antitumor potential of some solvent extracts of S. rebaudiana leaves. There is a need for further investigation of this plant in order to identify and isolate its active anticancer principles. The results of these studies will also need to be confirmed using in vivo models and clinical trials.

Anti-hypertensive activity - The lifestyle-related diseases like hypertension, a risk factor for cardiovascular diseases, such as coronary heart disease, peripheral arterial disease, are increasing. Hypertension is defined as an increase in blood pressure above certain measured levels (systolic blood pressure of 140 mmHg and a diastolic blood pressure of 90

mmHg). High blood pressure results in pathological changes that accrue in medium-sized and small arteries that cause further increases in blood pressure. The pathology is a thickening of the walls of these blood vessels so that the diameter of the vessels is effectively diminished, which causes the heart to work harder to pump enough blood to meet the demands of all the tissues, thus increasing the risk of a heart attack or stroke (Gupta E., 2013). Studies in animals have shown that stevia reduce blood pressure. Intraperitoneal injection of stevioside (25 mg/kg) leads to antihypertensive effect (Lee C. N., 2001). Experiments on isolated aortic rings from rats have shown how stevioside relaxed the vasopressin-induced vasoconstriction in both the presence and absence of the endothelium. Chan et al. (1998) studied vasorelaxation induced by stevioside and shown the clinical efficacy of stevia leaves in reducing chronic hypertension. For the study, a sample of 106 hypertensive subjects who consumed capsules containing either stevioside (750 mg daily) or placebo was used. The study was conducted for one year and starting from the third mounth that the subjects consumed stevioside, a greater decreases in systolic and diastolic blood pressure was observed, without any adverse effects. Some other studies have reported benefits of daily consumption of stevia in the decrease of blood pressure (Hsieh M. H., 2003) (Sharma N., 2009).

Anticariogenic activity - Dental caries are considered to be a localized disease that results from the metabolic processes of the biomass in contact with the tooth surface. The diet provides the energy to proliferate to the microorganism of the oral microbiota (Vitery, 2010). Stevia has been investigated recently and was shown to be noncariogenic. Several studies have been conducted that confirmed this property. Contreras (Contreras M. S., 2013) describe the mechanism of action divided it into three groups (antibacterial effect, low acidogenic potential, antiplaque effect). On the surface of the teeth a quantity of

bacteria proliferate that makes it difficult to associate a specific group with the cause of teeth decay. It is know that Streptococcus sobrinus is involved the development of tooth decay (Okada M., 2005). Mohammadi-Sichani et al. (Mohammadi-Sichani M., 2012) studied the in vitro effect of stevia extracts in different solvents on Streptococcus mutans using tetracycline (1%) as a positive control. The effects of different concentrations of stevia extracts in different solvents (water, methanol, ethanol, hexane and ethyl acetate) were compared on strains of sterptococcus mutans and Lactobacillus acidophilus using vancomycin as a positive control. The stevia extract in hexanoic acid showed the best inhibition of the growth of both bacteria after 48 hours. Other solvents also showed activity against the studied bacteria, although higher concentrations were necessary. Oral bacteria metabolize sucrose, and the result is the release of acids that are responsible for the demineralization of dental tissues in the dynamic process of caries. An in vivo study performed by Goodson (Goodson J., 2010) evaluated the cariogenic activity of rebaudioside A, and the effect of the presence of this glycoside steviolic and sucrose on the pH. The results of this and other studies (Giacaman R. A., 2013) showed that stevia leads to a minimal loss of surface hardness compared to sucrose. The formation of dental plaque, complex structures that are associated with similar microorganisms and different bacterial species, is the first step in the development of mouth disease. De Slavutzky (de Slavutzky S.M.B., 2010) measured the accumulation of dental plaque in a period of 5 days, after the use of a solution of sucrose and one of stevia for mouth washes. The result shows that the accumulation of plaque after the mouthwashes with stevia was less than that with sucrose mouthwashes. Dental treatment is a field new for the application of stevia extracts. The right solvent has get to be found that achieves the best use of active compounds of this plant (Ruiz-Ruiz J.C., 2017).

3.1.5 Food Application

Some studies showed that the Stevia sweetener was heat-stable up to 200°C, acid-stable, and not fermentable, which makes it suitable for use in different food products (Kroyer G., 2010). The use of these sweeteners does not vary the sensory characteristics of the food to which they are added (Parpinello G.P., 2001). As such the functional food sector may be able to make use of stevioside in the preparation of dietetic and other low calorie foods (Puri M., 2011). Stevia extracts are now used by many international food companies like Coca Cola, which uses stevia for its diet coke and have field patents applications in 2007 related to extracting the tastiest parts of the Stevia plant.

Only in recent years the confectionery industry has started to use stevia to replace sugar as a sweetener. Stevia can be used in chocolates and candies, not only to meet market demand by diabetics but also to harvest the added advantage of this herb's actions against tooth decay (Lindsay R., 2007). Stevia extracts can also be used in chewing gum, mints, mouth refreshers, toothpaste, and some cosmetics. This low-calorie natural sweetener is used extensively in various food products such as biscuits, jams, chocolates, ice-creams, baked foods, soft drinks and fruit drinks (Goyal S.K., 2010) (Jayaraman A., 2008) (Tadhani M. B., 2006), sauces, sweet corn, delicacies, pickles (Koyama E., 2003), yoghurt, soju, soy sauce (Hossain M. A., 2010) candies, sea foods (Goyal S.K., 2010) (Koyama E., 2003), and the common beverages like diptea, coffee, and herbal tea.

Studies on the stability of steviol sweeteners have been performed in different food matrices. The stability of stevioside, for example, is maintained up to 120 ° C, consequently, the application of stevioside as sweetening agent might not be suitable and recommended for baking processes or processes requiring high temperatures (Kroyer G., 2010).

In the presence of other low calorie sweeteners (saccharin, cyclamate, aspartame, acesulfame and neohesperidin dihydrochalcone), Stevioside shows excellent stability. No interaction between the individual sweeteners were found in the course of thermal treatment at 80°C up to 4 h as well as of 4 months incubation at room temperature indicating that there are chemically no objections to the simultaneous application of Stevioside with other low calorie sweeteners (Kroyer G., 2010). Stevia extracts are commonly used as sweeteners in beverages like tea or coffee.

In soft drinks the concentration of Stevioside and rebaudioside A decreases over time. After 24 h, the stevioside concentration decreased in the caffeinated soft drink by nearly 32%. The highest degradation of 71% was observed in the caffeinated lemonade after 72 h, whereas the energy drink was decomposed to only 27%. . The highest degradation of rebaudioside A, nearly 54%, was observed in the caffeinated soft drink after 72 h, and the lowest one again in the energy drink. It is obvious that their stability mainly depends on pH and increases with rising pH values (Wölwer-Rieck U., 2010).

3.1.6 Health and Safety

Currently, the number of countries accepting the use of Stevia is increasing.

Stevia has been confirmed as a safe for use as a general purpose sweetener by the majority food-standard regulating agencies globally. For instance, in 2008 the Food and Agriculture Organization/World Health Organization's Joint Expert Committee on Food Additives (JECFA), a gobal panel of food ingredient safety experts, and in 2009 the United States Food and Drug Administration (FDA) stated that the use of pure steviol glycosides (95 %) is safe for human consumption as a nonmedical ingredient up to 4 mg/kg of body weight/day. The European Food Safety Authority (EFSA) have expressed in April 2010 a positive scientific opinion on the use of steviol glycosides (Efsa A.N.S., 2013); even some

concern was raised on the uncertainty of the safety of the steviol aglycon produced in the metabolic pathways. Some studies have been reported that Steviol was found in the blood of rodents after feeding stevioside; no steviol was detected in the blood of chickens and pigs (Geuns J. M., 2003). In uptake studies, it was shown that stevioside and rebaudioside A are not absorbed by the intestine, whereas steviol was easily absorbed (Geuns J. M., 2003) (Koyama E., 2003). The free steviol concentration in the plasma was estimated to be around 102 μg/mL in pigs as well as in human volunteers concentrations were never detected in the plasma after administration of 68 mg stevioside/kg BW (pigs) or 11 mg/kg BW (humans), indicating that stevioside can be used as a sweetener (Genus J. M.C., 2003).

3.1.7 Genetic toxicology testing

A report from JECFA confirm that steviol glycosides have not shown evidence of genotoxicity in vitro or in vivo (JECFA, 2005). The report also confirm that steviol shows signs of genotoxicity activity in vitro but not in vivo up to doses greater than 2000 mg/Kg bw/day. However, since all steviol glycosides are metabolized to steviol many genetic tests were performed with stevia extracts in order to evaluate genetic toxicology. Stevioside was reported to be non-mutagenic in Ames strains of S. typhimurium, and in strains of E. coli and B. subtilis (Tama, 1981). These studies included rat liver S9 supernatant. Stevioside was evaluated in the Ames strains TA98 and TA100 using a pre-incubation exposure technique (Suttajit M., 1993).

The major constituents of stevia extract (stevioside and rebaudioside A) are relatively un-reactive compounds without clear evidence of DNA binding or mutagenicit. Rebaudioside A was evaluated for genotoxicity with a set of in vitro and in vivo assays covering mutation, chromosome damage and DNA strand breakage with consistent and uniformly negative results (Pezzuto J. M., 1985) (Nakajima M., 2000). High concentrations of

stevioside failed to either induce mutations in bacteria or in cultured mammalian cells or chromosome breakage in vitro and in vivo.

The toxicology and safety of stevioside used as a sweetener were recently reviewed (Geuns J.M., 2002) (Huxtable, 2002). An acceptable daily intake (ADI) of 7.9 mg stevioside/kg BW was calculated (Xili L., 1992).

3.1.8 Extraction and characterization of glycosides

Different method for glycosides extraction from stevia have been developed, those based on solvent extraction (Bondarev N., 2001) (Pól J., 2007), ion exchange (Fuh W. S., 1990) (Payzant J.D., 1999), supercritical fluids (Choi Y. H., 2002), chromatographic adsorption, selective precipitation (Kumar S., 1986) and membrane processes (Zhang S. Q., 2000). Hot water extraction was very effective for extracting glycosides at selected pH and temperatures (Rai C., 2012) since the better-tasting rebaudioside A was more soluble than stevioside in water. Ray and collaborators have optimized the parameters for stevioside extraction for human consumption.

Some studies claimed the advantage in the use of solvents (methanol, chloroform, ethanol, glycerin, sorbitol) to extract stevioside from dried leaves (Liu J. O. C., 1997).

One of the most used methods is CO_2-supercritical fluid extraction. Pol et al. shows the benefits from the physico-chemical properties of this extraction which possess a higher diffusivity and lower viscosity than conventional liquid solvents (Pól J., 2007). A negative aspect is that pure CO_2 does not have sufficient solvation power for polar stevioside and therefore a polar co-solvent (methanol, water, ethanol) must be added.

Different protocols have been devised for the characterization of Stevia active principles. High-performance liquid chromatography (HPLC) is one of the most commonly used methods for the determination of sweet tasting stevioside (STS) in plant material and food samples (Wölwer-Rieck U., 2010). The determination of the diterpene glycosides can also

be carried on by desorption electrospray ionization mass spectrometry (Jackson A. U., 2009), near infrared reflectance spectroscopy (Hearn L.K., 2009) and capillary electrophoresis (Liu J. L. S., 1995, Wölwer-Rieck U., 2010) have developed an HPLC method for the quantification of steviol glycosides after water leaching and SPE clean up with C18 cartridges. HPLC can be performed on a Hydrophilic Interaction Liquid Chromatography (HILIC) column with nearly 100% recoveries. Bergs et al. (Bergs D., 2012) have introduced an HPLC method for the quantification of steviol glycosides in Stevia leaves based on the application of an extraction mode performer, in a five-step crossflow, each step for 3 h in an overhead shaker. Hutapea et al. (Hutapea A. M., 1999) have presented an HPLC method for identification of the metabolites of SV found in blood, feces and urine of hamsters after ingestion of SV. The separation was carried out on a reversed phase C18 column with gradient elution of acetonitrile/water mixture, whereas T. Rajasekaran et al. (Rajasekaran T, 2008) have developed a liquid chromatograpy–M/S-ESI method for the determination of eight components of Stevia. Mass spectrometry is the analytical technique of choice for the identification and assay of target species in complex mixtures. Recently, mass spectrometry has been widely applied to discern origin, quality and safety of foods (Sindona G., 2014) (Benincasa C., 2015) (Di Donna L., 2015) (Di Donna L., 2007) (Taverna D., 2016).

3.1.9 Characterization by LC-MS/MS

LC-ESI-MS and low-energy collision induced dissociation CID-MS/MS by multiple reaction monitoring (MRM) scans, is applied in the assay of Steviol glycosides in food beverages. Brutieridin, a natural molecule present in bergamot fruits only, was used as internal standard. This approach should allow an easy and scientifically based evaluation of the healthiness of commercially available Stevia sweetened food bevarages.

The identification and assay of the active principles present in soft drink sweetened with Stevia glycosides has a twofold purpose. The first one is directly linked to the daily doses as suggested by the international EFSA and FDA organizations and is related also to food quality principles, whereas the second is linked to safety, thus preventing fraudulently addition of other sugar surrogates. The proposed method has been exploited in food chemistry with excellent outcomes.

The internal standard Brutieridin, a natural compound chemically very close to steviol glycosides, was selected either for its flavonoid glycoside structure, and because it is a natural molecule present in bergamot citrus fruit only, hence it cannot interfere with the analytical evaluation of Stevia containing drinks. The procedure is, therefore, independent from the variety of analyzed beverages edulcorated with Stevia, and was checked on very popular drinks available in the local market.

The accuracy of the method was determined from samples prepared by adding known quantities of the analytes to samples of soft drinks. In the two examined cases (Table 3), representative of low and a highly value of calibration curve, the accuracy was higher than 95%.

Spiked sample amount	Calculated amount mg/L	RSD %	Accuracy %
0.08 mg/L			
Sb	76.31 ± 7.21	9.45	95.39
SV	78.45 ± 6.45	8.34	98.06
Rc	81.22 ± 5.32	6.55	101.53
Ra	79.52 ± 8.22	10.34	99.4
0.350 mg/L			
Sb	343.45 ± 25.34	7.38	98.13
SV	351.54 ± 22.58	6.42	100.44
Rc	348.91 ± 28.89	8.28	99.69
Ra	345.16 ± 24.45	7.08	98.62

Table 3 Analytical parameters of Accuracy

The RSD value was in all cases under 13% thus showing good repeatability of the measurements. The proposed approach has been applied to real samples obtained by local market (Table 4).

Analyte	cola soft drink (mg/L)	RSD %	Pineapple and Litchi juice (mg/L)	RSD %	Pineapple and Lime juice (mg/L)	RSD %	Sweetener (%)
Sb	0.8 ± 0.08	10	0.71 ± 0.02	2.82	0.56 ± 0.006	1.07	0.07
SV	3.9 ± 0.42	10.77	22.04 ± 2.48	11.25	4.28 ± 0.55	12.85	0.055
Ra	83.11 ± 8.55	10.29	95.81 ± 12.04	12.56	134.97 ± 10.79	7.99	3.6
Rc	1.89 ± 0.17	8.99	3.34 ± 0.26	7.78	1.68 ± 0.024	1.43	0.17

Table 4 Amount of steviol glycosides found in all analyzed samples

The data reported in table 4 clearly show that the examined commercial products fits well FDA and EFSA requirements, moreover the *quality* of the analytical protocol guarantees its use as a scientific approach to appropriately label commercial products based on the use of Stevia edulcorator.

Quality and safety of Stevia based functional foods can be properly controlled by the application of the trustworthy MS protocols here proposed. This approach is ready to be transferred to Food Manufactures to favor the introduction in the market of scientifically checked Stevia based functional foods.

3.2 Olive drupes and olive leaves: biological properties

3.2.1 Introduction

The olive fruit, its oil, and the leaves of the olive tree have a rich history of nutritional, medicinal, and ceremonial uses. The introduction of olive cultivation coincided with the expansion of the Mediterranean civilizations, and the olive has been used widely in traditional remedies in European Mediterranean, islands and countries such as Spain, Italy, France, Greece, Israel, Morocco, Tunisia, and Turkey. In Mediterranean countries several olive cultivars are processed as table olives, probably the most popular fermented vegetables.

Fruits are harvested at different stages of ripening depending on the cultivar and processing method, the most common being the Spanish-style (for green olives which ferment after a treatment with NaOH to hydrolyze the bitter constituent oleuropein, followed by washing step to remove the excess of alkali), both relying on a fermentation process which involves, in different ways, mixed populations of microorganisms, mainly represented by lactic acid bacteria and yeasts.

The study of the fermentation process and physical, chemical and microbiological control is the fundamental basis for understanding and improving the preparation, storage and safety of the final product. This has been the subject of serious and continuous research during the past 60 years.

A characteristic common to almost all olive varieties is their extreme bitterness when tasted fresh. The glucoside oleuropein is responsible for this, and the different processing methods are aimed at removing this compound in order to obtain more-palatable fruits products.

Oil and table olives are not the only products deriving from the olive tree. Olive-leaf tea is one of the most common traditional herbal tea used among Mediterranean people to cure

certain diseases. For this reason, interest in the potential health benefits of olive leaves has increased among scientists in various fields. Recently, antioxidant, hypoglycemic, antihypertensive, antimicrobial, and anti-atherosclerotic effects of olive leaves have been reported in various studies (Somova L. I., 2003) (Ribeiro R. D. A., 1986) (Alarcon-Aguilara F. J., 1998) (Panizzi L., 1960).

These properties are attributed also to the phenolic compounds of olive leaves.

The phenolic composition of olive leaves varies depending on several conditions such as origin, proportion of branches on the tree, storage conditions, climatic conditions, moisture content, and degree of contamination with soil and oils (Briante R. L., 2001) (Briante R. P., 2002) (Morello J. R., 2004a) (Morello J. R., 2004b). In addition, the structural carbohydrates and nitrogen content in olive leaves depends on factors such as the variety of the olive tree, climatic conditions, age, proportion of wood, etc. (Molina-Alcaide E., 2008) (Delgado-Pertíñez M., 2000) (Martin-García A. I., 2003) (Martin-Garcia A.I., 2008). The phenolic compounds content is an important parameter in the evaluation of virgin olive oil quality given that phenols contribute to oil flavor and aroma and protect it from oxidation through their free radical scavenging and metal chelating properties.

There are five groups of phenolic compounds principally present in olive leaves: oleuropeosides (oleuropein and verbascoside); flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7- glucoside, luteolin, and diosmetin); flavonols (rutin); flavan-3-ols (catechin), and substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid).

Oleuropein is the compound most studied that is present in the olive plant and the most important of the glucosidic fraction of the Olea europaea. Oleuropein was first named and studied by Bourquelot and Vintilesco (Bourquelot E., 1908) and investigated in humans by

Panizzi et al. (Panizzi L., 1960) who reported on the hypotensive properties of this complex phenol.

3.2.2 Structure, occurrence and biosynthesis of phenolic compounds

Oleuropein belong to a very specific group of coumarin-like compounds, called secoiridoids, which are aboundant in Oleaceae, Gentianales, Cornales and many other plants. Iridoids and secoiridoids are compounds, usually, glycosidically bond, produced from the secondary metabolism of terpene as precursor of various indole alkaloids.

Oleuropein (Figure 6) is a glucoside that undergoes hydrolysis and yields several simpler molecules (simple phenols) that build up the well-known olive oil complex taste during olive maturation. Moreover, oxidative modifications of phenolic compounds are sometimes advantageous, as they enhance the aroma and flavor of foods, including olive oils. Two of its by-products are also present in the olive plant together with the oleuropein: demethyl-oleuropein and glucoside of the elenolic acid (Figure 7). This acid compounds are two indicators of maturation of the olives. Their relative quantity increase as soon as the maturation of the olives. This datum is relative to the increase of the activity o the hydrolytic enzymes with the progress of maturation. Oleuropein amounts to up to 14% of the dry weight in unripe olives but, during maturation, undergoes hydrolysis and yields several simpler molecules (simple phenols) that build up the well-known olive oil complex taste (Amiot M. J., 1989). It is noteworthy that olives contain complex phenols as glycosides, that is, they are present in a rather polar and hydrophylic form, whereas the oil contains their aglyconic form, that is the more lipid-soluble residue of their molecule. The phenolic constituents confer a bitter and pungent taste to the oil. The effect of bitterness and pungency is the result of complex interactions between the ‘‘minor constituents’’ and the taste buds, including inactivation of ptyalin. In particular, phenolic acids such as phenol

and cinnamic acid are responsible for the bitter sensation that can be felt on the lateral and posterior areas of the tongue, while secoiridoids confer the peculiar pungency (Visioli F. P. A., 2002). Any alteration in the concentration of the various chemicals changes olive oil's particular taste. Phenolic compounds, and in particular oleuropein, give the oil a bitter taste (Visioli & Galli, 2001). This is confirmed by panel test in which oils produced from greener olives usually obtain higher scores because of their ''fruity'' and complex aroma, provided by their high phenols content (Visioli F. G. S., 2006). High phenol levels in virgin olive oils are very likely to exhibit a high stability and a strong, fruity flavor, indicating a high, but not necessarily the most preferred, organoleptic quality of the oil (Visioli F. G. S., 2006).

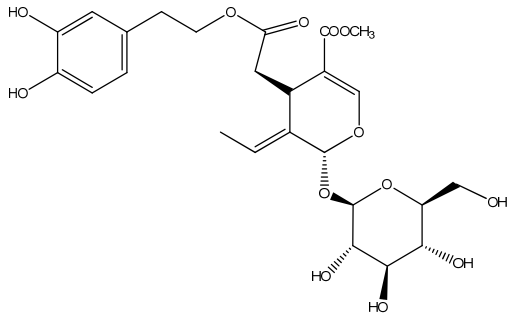


Figure 6 Structure of Oleuropein

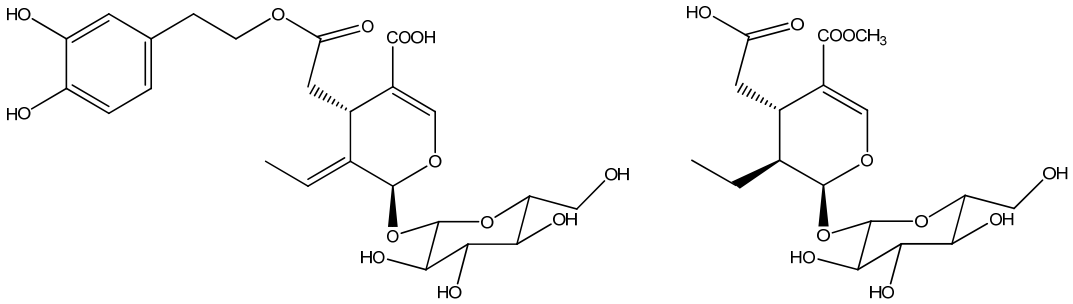


Figure 7 Structures of demethyl-oleuropein and glucoside of the elenolic acid

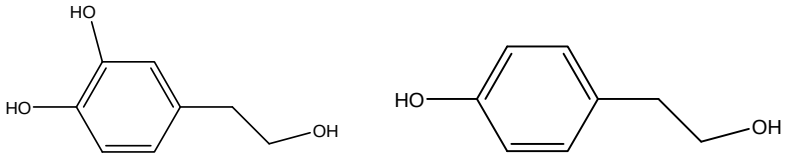


Figure 9 Structures of Hydroxytyrosol and Tyrosol

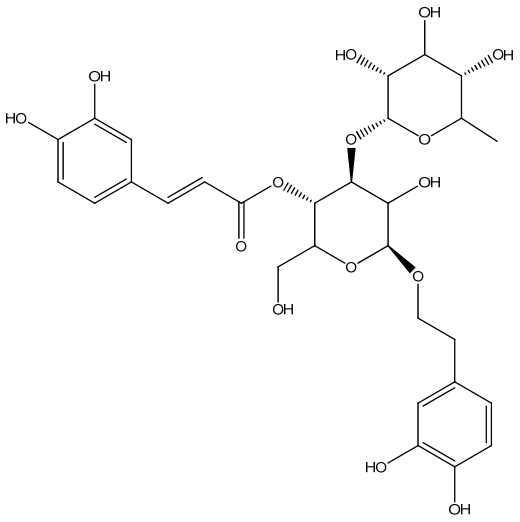
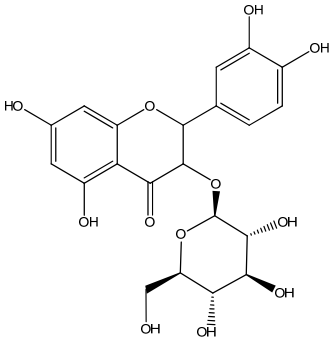
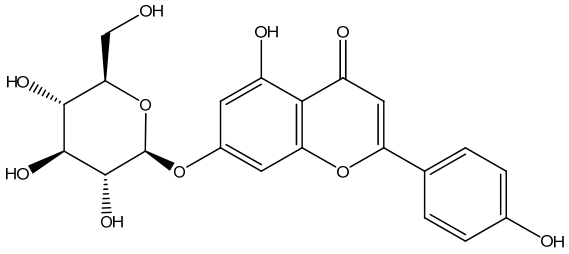


Figure 8 Structure of Verbascoside

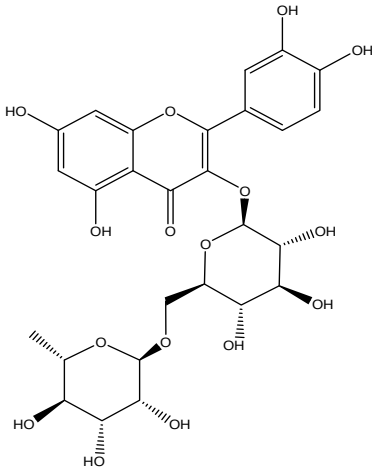
Two important phenolic alcohols of olives are hydroxytyrosol and *p*-hydroxyphenylethanol (tyrosol) (Figure 8). Contrary to oleuropein, Tyrosol ad Hydroxytyrosol increases during repining of olives. Hydroxytyrosol can be present as a simple or esterified phenol with elenoic acid, forming oleuropein and its aglycone, or as part of the molecule of verbascoside (Amiot M. J., 1989) (Figure 9); it can also be present in several glycosidic forms, depending on the hydroxyl group to which the glucoside is bound. Oleuropein, demethyoleuropein and verbascoside are present in all the constituent parts of the fruit, but more abundantly in the pulp (Tripoli E., 2005). Other flavonoid compounds present in olive are flavonol glycosides such as luteolin 7-O-glucoside, rutin, apigenin 7-O-glucoside, anthocyanins, cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside (Amiot M. J., 1989) (Romani A., 1999) (Figure 10).



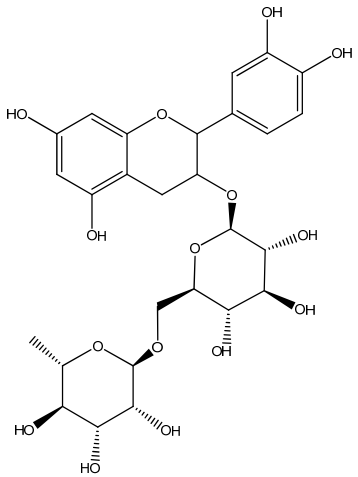
luteolin 7-O-glucoside



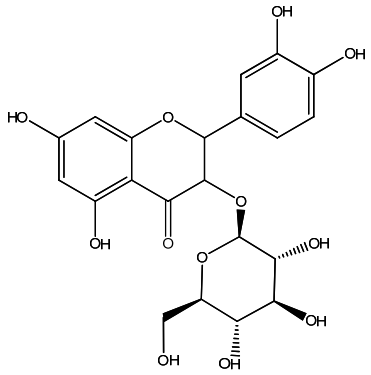
apigenin 7-O-glucoside



Rutin



cyanidin 3-O-rutinoside



cyanidin 3-O-rutinoside

Figure 10 Structures of flavonol glycoside in olive

Small-fruit cultivars are characterized by high oleuropein and low verbascoside contents, while large-fruit cultivars are characterized by low oleuropein and high verbascoside contents.

The influence of the elaboration process on the phenolic content is yet to be fully elucidated. It appears, for instance, that oils that have been obtained by centrifugation have a lower phenols content, possibly because this process involves the use of large quantities of warm water, with which the olive paste is continuously hosed during the milling. This is named ‘‘waste water’’ and is produced in extremely large quantities (800,000 tons/year in Italy). Despite the fact that a considerable amount of phenols, according to their partition coefficient, end up in the waste water, the latter is currently disposed of. A series of experiments performed by Visioli et al. (Visioli F. G. C., Oleuropein protects low density lipoprotein from oxidation, 1994) demonstrated that waste water extracts have powerful (in the ppm range) antioxidant activity and might therefore be recovered and employed in preservative chemistry as a cheap, as yet unused, source of natural antioxidants.

3.2.3 Pharmacological activities

A lot of studies have reported the amazing physiological and pharmacological properties of oleuropein. Accordingly, in vitro and in vivo studies have documented the antioxidant, antimicrobial, antifungal, anti-tumoral, hypolipidaemic, and especially hypotensive, anticancer and cardioprotective properties of oleuropein (Carrera-González M. P., 2013) (Rubió L., 2014) (Sepporta M. V., 2014).

Antioxidant activity **-** One of the more prominent properties of oleuropein is its strong antioxidant activity, particularly as a free radical scavenger (Cicerale S., 2012). The proof of such feature has been established by using in vitro and in vivo tests. In these assays, the ability of oleuropein and oleuropein-rich extracts to scavenge the 2,2-diphenyl-1-picryhydrazyl (DPPH) radical was found to be higher than the synthetic antioxidant BHT (butylated hydroxytoluene) (Hassen I., 2015). Such effect was primarily attributed to their

donating electron ability (Lee O. H., 2009). The scavenging activity of oleuropein was extensively investigated and the structure–activity relationship has been studied by different experimental models namely Trolox-equivalent antioxidant capacity (TEAC), superoxide and hydrogen peroxide produced from linoleic acid in β-carotene bleaching assay superoxide dismutase (SOD)-like activity, peroxide value (POV), conjugated diene values (CDV), xanthine oxidase, and also by metal-related methods namely ferric-reducing ability power (FRAP) assay (Hassen I., 2015). The potent antioxidant activity of oleuropein is mainly due to the presence of hydroxyl groups (particularly the 1,2-dihydroxybenzene moiety) in its chemical structure. These hydroxyl groups could donate hydrogen to prevent oxidation.

Anti-inflammatory activity - Oleuropein increases nitric oxide (NO) production in macrophages challenged with lipopolysaccharide through induction of the inducible form of the enzyme nitric oxide synthase, thus increasing the functional activity of these immunocompetent cells. It is well known that oleuropein elicits anti-inflammatory effects by inhibiting lypoxygenase activity and the production of leukotriene B4 (Omar S.H., 2010).

Anti-atherogenic activity - *T*he anti-atherogenic activity of Oleuropein was showed in many research (Visioli F. G. C., 2001) (Carluccio M.A., 2003). Oleuropein reduces monocytoid cell adhesion to stimulated endothelium as well as vascular cell adhesion molecule-1 (VCAM-1) mRNA and protein. Reflow in ischemic hearts was accompanied by a prompt release of oxidized glutathione; in ischemic hearts pretreated with oleuropein, this release was significantly reduced and was accompanied by prevention of membrane

lipid peroxidation, which is considered a key factor in the pathogenesis of atherosclerosis (Manna C., 2004).

Anti-cancer activity - Epidemiological studies suggest that olive oil intake is associated with a reduced risk of cancer. Recently, the chemopreventive activity of olive oil has been attributed to its unique phenolic compounds represented by phenolic alcohols, hydroxytyrosol (3,4-dihydroxyphenylethanol: 3,4-DHPEA) and tyrosol (*p*-hydroxyphenylethanol: *p*-HPEA), and their secoiridoid derivatives 3,4-DHPEA-EA (oleuropein aglycon), *p*-HPEA-EA (ligstroside aglycon), 3,4-DHPEA-EDA, *p*-HPEA-EDA (oleocanthal), and oleuropein. Several studies have demonstrated that these compounds are able to inhibit proliferation and induce apoptosis in different tumor cell lines (Fabiania R., 2016).

Anti-microbial activity - Sudjana et al. (Sudjana A. N., 2009) showed the antimicrobial activity of commercial *Oleaeuropaea* (olive) leaf extracts against *Campylobacter jejuni, Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA). The authors also demonstrated these extracts play a role in regulating the composition of the gastric flora by selectively reducing levels of *H. pylori* and *C. jejuni*. Many research confirm the anti-microbial activity of phenolic compounds in olive. Oleuropein and hydrolysis products are able to inhibit the development and production of enterotoxin B by *Staphylococcus aureus*, the development of *Salmonella enteritidis* and the germination and consequent development of spores of *Bacillus cereus* (Bisignano G., 1999) (Aziz N.H., 1998) (Fleming H.P., 1973) (Tassou C.C., 1991) (Tranter H.S., 1993) . Oleuropein and other phenolic compounds (p-hydroxybenzoic, vanillic and p-coumaric acids) completely

inhibit the development of *Klebsiella pneumoniae, Escherichia coli* and *B. cereus* (Aziz N.H., 1998).

Anti-viral activity - Fernández-Bolaños et al. (2012) have identified Hydroxytyrosol and oleuropein as HIV-1 inhibitors that prevent HIV from entering into the host cell and binding the catalytic site of the HIV-1 integrase. Hydroxytyrosol was also found to be useful as a microbicide for preventing HIV infection. Because of this property, it has recently been patented as a product for topical use (Gómez-Acebo et al., 2011).

In a U.S. patent (no 6,117,844) is claimed that oleuropein has potent antiviral activities against herpes mononucleosis, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus, and feline leukemia virus (Fredrickson W.R., 2000). Studies have also shown that oleuropein exhibits a significant antiviral activity against respiratory syncytial virus and para-influenza type 3 virus (He Z.D., 2001).

3.2.4 Characterization by LC-MS/MS

Developed and developing countries are often characterized by the presence in their environment of traditional plants, which are considered as a new source of medical supply. The beneficial effects of such compounds depend on the amount consumed and their bioavailability. Leaves are potential source of diverse nature of phenolic compounds: flavones (luteolin), flavonols (rutin), substituted phenols (tyrosol, hydroxytyrosol) and oleuropein, typical of the Oleaceae family.

The Mediterranean diet, rich in fresh fruit and vegetables, is an important source of phenols. In fact, the main lipid component is the constituent of olive oil that is unique for its particular composition of unsaponifiables (Benincasa C.., 2015) (Di Donna L., 2011). Olive leaves have been the subjects of many epidemiological studies as they showed

greater beneficial effects on health such as the reduction of cardiac disorders (Sindona G., 2010) (Soler-Rivas, 2000) (Visioli F.G.C., 1994). Moreover, hydroxytyrosol and tyrosol are two of the main compounds that contribute to the oxidation resistance in olives (Konno K., 1999). All the known methods for extracting phenols from leaves are based on the use solvents, supercritical fluids and classical analytical techniques. The results presented here are, instead, based on the improvement of green extraction techniques and on the exploitation of mass spectrometric methodologies to fully characterize phenolic compounds from different food matrices (Sindona G., 2010) (McDonald S., 2001). Optimizing a "green" mining method for the recovery of such important functional compounds from the residues of olive cultivation would be very beneficial.

Olive drupes and leaves were left in maceration for 10 days and monitored every 24 hours. LC-ESI-MS/MS analysis was performed on aliquots of each sample collected. The results obtained and a discussion for each compound analysed (below) are also presented in Figure 12 and Figure 13. The MS instrument operating parameters were optimized for each analyte of interest by direct injection of the analyzed solution. The CID-MS/MS spectra of the deprotonated molecular ion $[M-H]^{-1}$ for several analytes are shown in Figure 11.

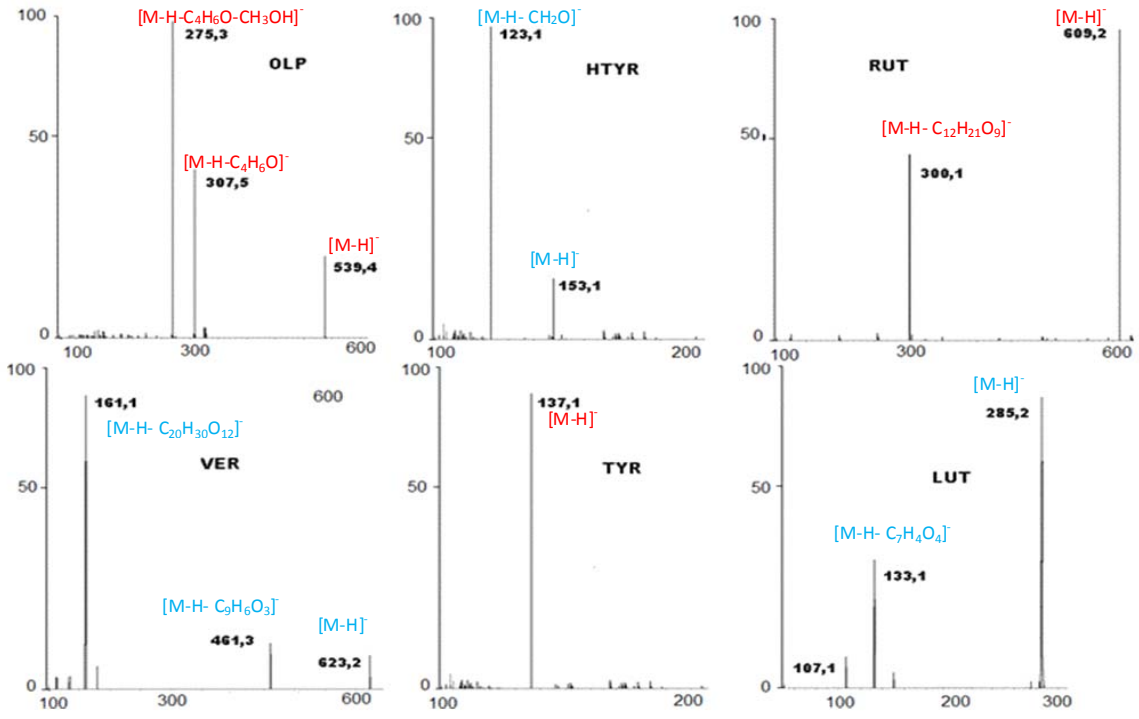


Figure 11 ESI-MS/MS spectra of oleuropein, hydroxytyrosol, tyrosol, verbascoside, luteolin and rutin showing the deprotonated molecular ion $[M-H]^-$ and the major fragments utilised to set up the MRM method.

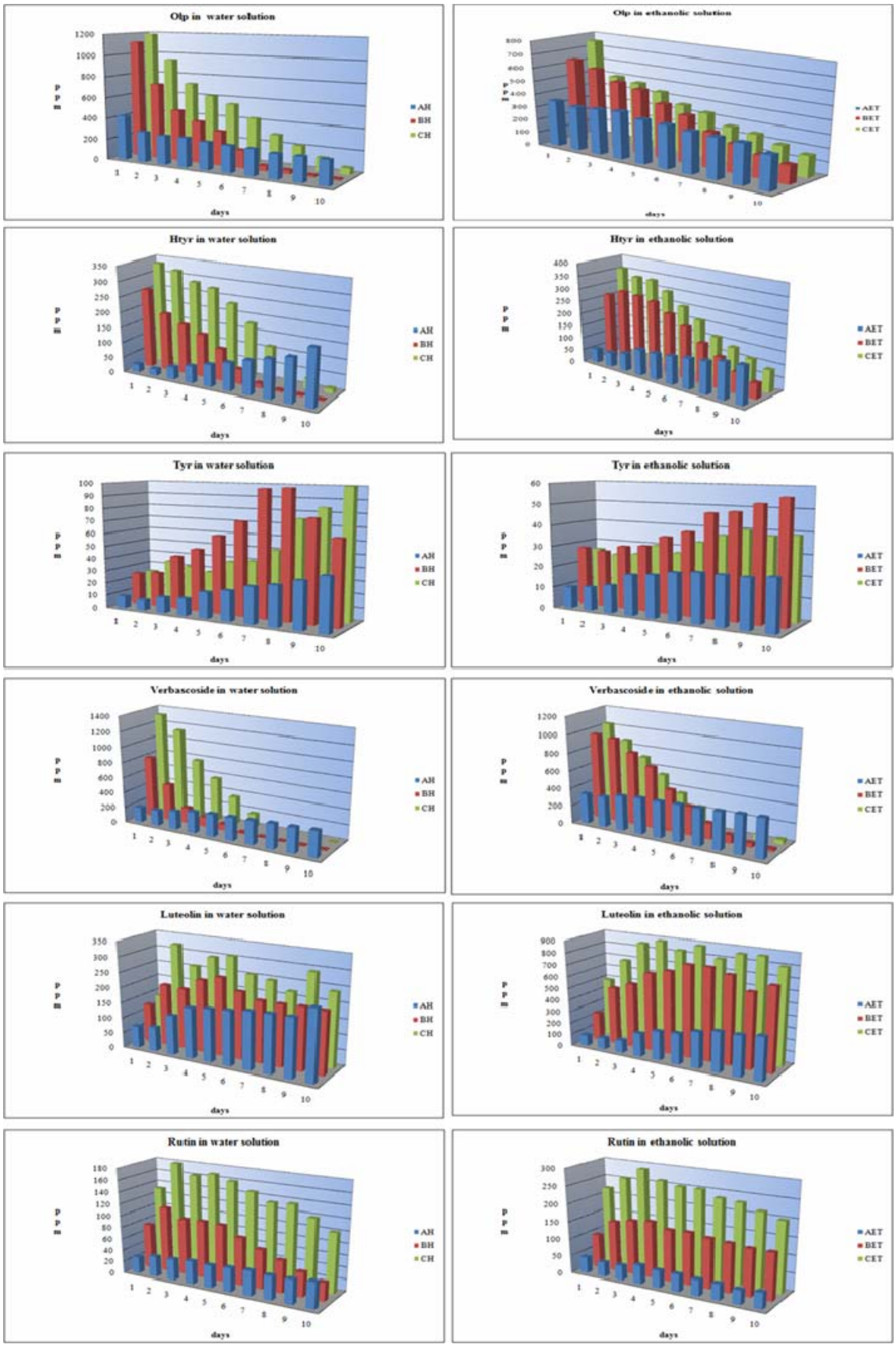


Figure 12 Trend of the debittering process in whole olives, crushed olives and pitted olives. The letters A, B and C are for whole olives, crushed olives and pitted olives respectively. H and ET are for water solution and 20% ethanolic solution respectively.

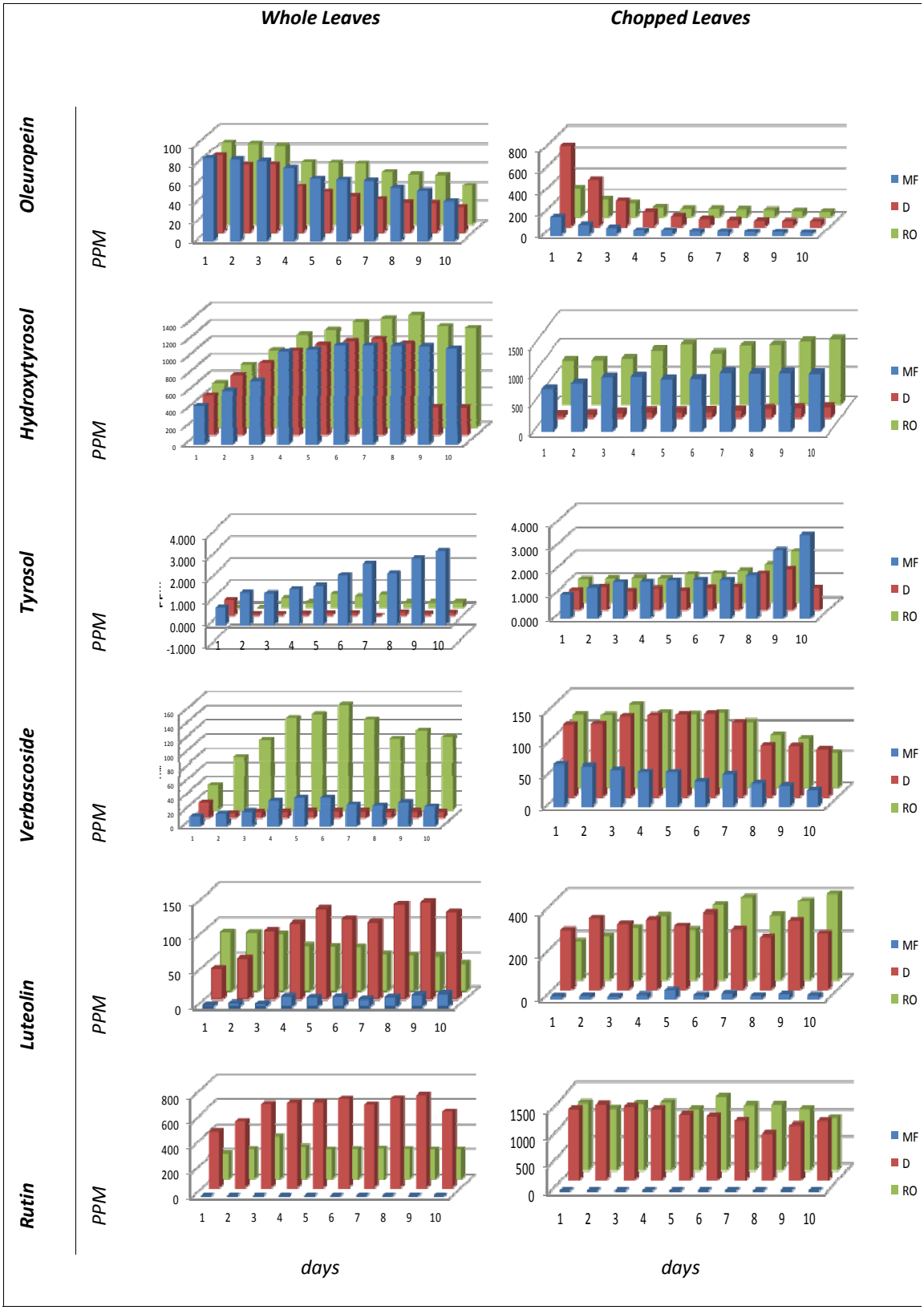


Figure 13 Trend of the variation in composition of phenolic compounds in whole and chopped leaves. Bar charts show the decay of active antioxidant compounds in the three thesis. The letters MF, D and RO are for microfiltered waters, distilled water and osmotized water respectively.

3.2.4.1 Oleuropein (Olp)

According to Figure 11, ESI mass spectrum of the negative (-) ions shows the deprotonated molecule at m/z 539 together with the known diagnostic product ions at m/z 275 and m/z 307 (Ryan D., 1999) (De la Torre-Carbot K., 2005) (Figure 11). Oleuropein, as expected, was one of the major components of wastes.

Drupes: The maximum concentration of this secoiridoid (409 mg/kg), reached after the first day when plain water is used, slowly decreases to half (228 mg/kg) in the subsequent days (Figure 9). A similar trend was observed when water/ethanol (80:20) was used although the maximum concentration observed after one day (369 mg/ kg) reaches the mean value 264 mg/kg.

A marked difference was observed when crushed and pitted olives were debittered (Figure 12). The maximum concentration achieved after one day was 1187 mg/kg and 1118 mg/kg for pitted and crushed drupes, respectively. This value decreases according to the enzymatic kinetics to reach a value of 8 mg/kg on the last day. The solution containing water/ ethanol (80:20) since day one gave a lower content of oleuropein (665 mg/ kg for crushed olives and 754 mg/kg for pitted olives). These values decrease during the days until they reached the mean values 139 mg/ kg and 162 mg/kg for crushed and pitted olives, respectively (Figure 12).

Leaves: The variation trend of the concentration of oleuropein in whole and chopped leaves varies considerably depending on the days of infusion of the leaves. Oleuropein reaches its maximum concentration in the first day of infusion in chopped leaves (753 mg/kg) (Figure 13). After 4 days this quantity is halved to became almost null at the end of the sampling. This value decreases according to the enzymatic kinetics. In whole leaves, the amount of Oleuropein extracted does not depend on the type of water used for the

infusion. However, water seems to be incisive for chopped leaves. The extracting power of water D is quite strong comparing to RO water. In fact, oleuropein concentration by RO water is one third of that of D water. The extraction power of MF water is very weak instead.

3.2.4.2 Hydroxytyrosol (HTyr)

According to Figure 11, the mass spectrum for this compound in negative ion mode gave the deprotonated molecule at m/z 153 and the fragment at m/z 123 due to the loss of the CH_2OH group (Figure 11). HTyr is frequently detected in olive pulp and has been widely studied, in particular its anti-oxidant activity and its health-beneficial properties.

Drupes: The concentration of HTyr in the solution containing pure water and whole olives on the first day was 21 mg/kg. This content increases during the next nine days of experimentation to reach the mean value of 171 mg/kg. The solution containing 20% of ethanol gave an initial value of HTyr double than the solution of pure water (50 mg/ kg). Moreover, the concentration of the compound at the end of the experimentation was found to be less than in the previous one (149 mg/kg). The solutions containing pure water and either crushed and pitted olives showed a high content of HTyr already in the first day of the experimentation (261 and 334 mg/kg, respectively). These values decrease over time to almost zero (2 and 14 mg/kg, respectively). It is to be noted that by using 20% of ethanol the content of HTyr is preserved over time and, in fact, the concentration of the compound found at the end of the experimentation was 60 and 87 mg/kg in the solution containing crushed and pitted olives, respectively. The patterns observed in the three cases (whole, pitted and crushed olives) in the two solutions (pure water and water/ethanol (80:20)) are quite different. In the case of the solution containing whole olives the concentration of the compound increases over time and this increment is due both to its initial content and both

to the degradation of secoiridoid compounds. This low accumulation observed could be attributed to the fact that in whole olives the membrane is not damaged and the release of the active compound is slowed down. In the case of the solution containing crushed and pitted olives the concentration of HTyr decreases over the time. The presence of the stone seems to favour a faster degradation of the compound under investigation (Figure 12).

Leaves: The fragmentation pattern for this compound, in negative ion mode, shows the presence of the molecular ion at m/z 153 and the loss of CH_2OH at m/z 123. The concentration of HTyr, in whole leaves, increases during the first four days of infusion to stabilize afterwards. The quantity registered varies from 441 mg/kg to 1077 mg/kg. Waters do not seem to vary the quantity of HTyr extracted for whole leaves. Important differences can be highlighted in samples of chopped leaves instead. In this case the amount of nutraceutical extracted is very low compared to the other extractions (from 56 mg/Kg to 189 mg/Kg) (Figure 13).

3.2.4.3 Tyrosol (Tyr)

In all cases analysed the content of Tyr increases. This increase can be attributed both to its initial content and to the degradation processes of higher molecular weight molecules.

Drupes: In the solutions containing whole olives the concentrations of Tyr on the first day was found to be 8 mg/kg in pure water and 9 mg/kg in water/ ethanol (80:20). At the end of the debittering process these contents were 39 and 31 mg/kg, respectively. The concentration of Tyr in crushed olives on the first day of the debittering process was 25 mg/ kg in pure water and 27 mg/kg water/ethanol (80:20). At the end of the experimentation these contents were 65 and 56 mg/kg, respectively. The solvent in these two cases does not affect the Tyr content. The situation appears different in the solutions

containing pitted olives. In fact, the Tyr content varies from 24 to 100 mg/kg in pure water and from 25 to 40 mg/kg in water/ethanol (80:20)(Figure 12).

Leaves: Quantities of tyrosol close to zero have been extracted both in whole and chopped leaves, regardless of the type of water used (Figure 13).

3.2.2.4 Verbascoside (Ver)

According to Figure 11, the ESI mass spectrum in negative ion mode was represented by the deprotonated ion at m/z 623 with few other fragments of lower intensity (Figure 11). Particularly, two ions characteristic of verbascoside at m/z 461 and m/z 161 were observed. The loss of caffeic acid produced an ion at m/z 461 and a ketene as a neutral fragment, while the peak at m/z 161 resulted from a proton transfer and production of an anionic ketene (Ryan D., 1999).

Drupes: The trend observed for all the solutions was similar to the one observed for HTyr: the verbascoside tends to increase during the process of debittering. In the solutions containing whole olives the content of the active compound varies from 196 to 365 mg/kg on the first and last day, respectively. In water/ethanol (80:20) its content varies from 338 to 441 mg/kg on the first and last day, respectively.

The solution containing crushed and pitted olives in pure water showed a high content of verbascoside already on the first day of the experimentation (802 and 1319 mg/kg for crushed and pitted olives respectively). These values decrease over the time to almost disappear after 10 days (1 and 4 mg/kg, respectively) (Figure 12).

Leaves: The trend of infusions of whole leaves was found to be completely different from all those observed so far. MF and distilled water are not very efficient for verbascoside extraction,resulting in 40 mg/kg in the first case and almost nothing in the second one.

Infusion of whole leaves using RO water gives, in five days, a concentrations of verbascoside of 148 mg/kg. After this period the value decreases to 103 mg/kg (Figure 13).

3.2.2.5 Luteolin

According to Figure 11, the ESI mass spectrum was characterized by an intense ion at m/z 285 and a fragment of m/z 133 of lower intensity (Figure 11).

In several studies this flavonoid has been detected in olive fruits of different cultivars and has proved to possess important biological properties, such as anti-oxidant, anti-inflammatory, anti-microbial and cardio-tonic activity, ability to scavenge free radicals and to inhibit low-density lipoprotein oxidation . Luteolin is responsible, along with other carotenoid compounds, for the colour of the drupes. Fermentation and debittering processes promote its release into solution that is pronounced if olives are damaged. Moreover, in the presence of solvents such as ethanol, this release is marked. Also, the absence of light preserves the colour of the final solutions.

Drupes: During the ten days of experimentation, depending on the olives utilized, different gradients of colour were obtained. Whole olives gave, at the end of the experimentation, a dark green colour, whereas crushed and pitted ones gave a browny green colour that was more evident in the ethanolic solutions. All solutions analysed showed an increase of the active compound. In particular, in aqueous solution containing whole olives the luteolin content varies from 66 to 218 mg/kg during the experimental period, whereas in the ethanol solution the result was from 83 to 287 mg/kg. The solutions containing crushed and pitted olives in pure water showed a higher content of luteolin during the first days of the experimentation; these values did not vary significantly over the period of the debittering process. In fact, the content of luteolin for crushed olives varies from 126 to 202 mg/kg whereas for pitted olives from 110 to 190 mg/kg. The ethanol solutions have a

different pattern with respect to the previous ones examined. In fact, from day one the solutions are very rich in luteolin and this increases during all the debittering process. At the end of the experimentation almost 700 mg/ kg were found in the solution containing crushed olives and almost 800 mg/kg in the solution containing crushed ones (Figure 12).

Leaves: Sample preparation and infusion water play an important role for the extraction of luteolin. In whole leaves, Lut concentration varies from 45 mg/kg to 127 mg/kg if distilled water is used, while it decreases with the use of RO water. In chopped leaves, the amount of luteolin registered is 300 mg/kg, on average, if distilled water is used and does not vary very much over the experimental time. When chopped leaves are left soaked in RO water, this amount varies from 178 mg/Kg on the first day to 400 mg/Kg on the tenth day (Figure 13).

3.2.2.6 Rutin

According to Figure 11, the ESI mass spectrum was characterized by an intense ion at m/z 285 and a fragment of m/z 133 of lower intensity (Figure 11).

ESI mass spectrum was characterized by an intense deprotonated molecular ion at m/z 609 and a fragment at m/z 301 which is diagnostic of quercetin derivatives (Figure 11). Many biological effects have been attributed to this flavonoid, which shows anti-oxidant, anti-inflammatory, anti-thrombotic, cytoprotective, vasoprotective and anti-microbial activity . This glycoside flavone is stable in aqueous solution at neutral or alkaline pH; in an acid or by the action of enzymes specific hydrolase, the glycosidic bond is broken with the formation of anomeric hemiacetals.

Drupes: In the aqueous solutions the concentration of rutin, from the first to the last day of experimentation, does not significant change and remains constant when whole olives are used (from 40 to 44 mg/kg). A decrease in the concentration can be observed for crushed

olives (from 110 to 31 mg/ kg) and for pitted olives (from 179-101 mg/kg). The difference that can be noticed when ethanol is added is that the content of rutin released is far greater, but constant throughout the experimentation period. For whole olive the content of rutin is the same as in the aqueous solution (from 41 to 49 mg/kg). The solutions containing pitted olives showed an increased content of the compound than those containing crushed olives (from 144 to 132 mg/kg and from 221 to 200 mg/kg, respectively) (Figure 12).

Leaves: In whole leaves infuses, during all the experimental trial, the concentration of rutin varies from 454 mg/kg to 610 mg/kg reaching the value of 713 mg/kg in the fifth day of experimentation. The lower amount of extracted rutin is when RO water is used, average value recorded of 230 mg/kg; this quantity remains constant over the time. Small differences can be noted for chopped leaves using distilled and RO water. However, the quantity of rutin extracted is greater than that of whole leaves reaching the value of 1331 mg/kg on the first day of sampling to decrease, over time, up to 1099 mg/kg. No extraction of rutin is obtained from the infusion using MF water, in both whole and chopped leaves (Figure 13).

The results discussed above show, from a chemical point of view that, as expected, the glycosidic antioxidants are less stable in water than the other detected active principles, even in the absence of chemical catalysts that could affect the process. The observed behaviour is, therefore, probably driven by those deglycosylation enzymes that are extracted from the drupes together with the active species listed (Table 11). This observation allows the choice of the experimental conditions to be applied to favour the recovery of a particular family of nutraceuticals from olive drupes.

In conclusion, the results presented above provide evidences on the possibility of devising new strategies in the recovering of active principles from olives. The results previously discussed clearly show that eco-friendly chemical procedures can be applied to isolate the

drugs from olive waste and that the use of modern analytical methods of analysis, such as those based on MS/MS procedures, provide unique information on the identification assay and isolation of the active principles.

3.3 Algal Oil

3.3.1 Introduction

The development of products rich in omega-3/omega-6 fatty acids is one of the main topics on which the pharmaceutical and food industries have focused in recent years. The importance of these compounds is that PUFAs are precursors of hormone like compounds known as eicosanoids, which are involved in many important biological processes in the human body (Braden L.M., 1986).

3.3.2 Biochemistry of long-chain PUFA

Fatty acids are almost entirely straight chain aliphatic carboxylic acids. Fatty acid chain lengths vary from two to more than thirty carbon atoms, with C18 most common. They can be saturated, monounsaturated and polyunsaturated. There are two kinds of nomenclature to identify fatty acids. In the systematic nomenclature, the position of double bond is given by numbering from the carboxyl group. The second nomenclature numbers the chain from the terminal methyl group. Two numbers separated by a colon give the chain length and number of double bonds: octadecenoic acid with 18 carbons and 1 double bond is therefore 18:1. The position of double bonds is indicated in a number of ways: explicitly, defining the position and configuration; or locating double bonds relative to the methyl or carboxyl ends of the chain. Double-bond position relative to the methyl end is shown as *n-x* or *ox*, where *x* is the number of carbons from the methyl end (Shahidi, 2005).

The principals fatty acids are subdivided in two groups: omega-3 and omega-6 fatty acids. This notation is the generally used because the action of fatty acids in the human body depend on whether they are members of one or the other group. The human body can introduce additional double bonds but cannot interconvert omega-3 and omega-6 fatty acids, while plant enzymes normally introduce a new double bond between an existing double bond and the terminal methyl group.

Two families with opposing effects which are referred to as essential fatty acids. The omega-6 (arachidonic acid) and the omega-3 (eicosapentaenoic acid) are the precursors of prostaglandine, thromboxanes and leukotrienes.

Figure 14 shows the most significant steps transforming linoleic acid and α-linoleic acid to their higher unsaturated derivatives (arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid) by the activities of consecutives desaturation and elongation reactions. The insertion of these unsaturations occurs in the presence of plant enzymes. Since the human organism is devoid of desaturases Δ12 and Δ15 that are necessary for the synthesis of linoleic acid and α-linolenic acid, it needs to integrate these compounds through the diet.

The two fatty acids being considered may undergo further desaturation and elongation: LA can be converted to g-linolenic acid (18:3 omega-6), which can be elongated to dihomo-gamma-linolenic acid (20:3, omega-6), and further desaturated to yield arachidonic acid (20:4, omega-6). Using the same series of enzymes involved in the metabolism of n-6 PUFA, ALA can be converted to EPA (20:5, omega-3). Further conversion of EPA to DHA (22 : 6n-3) involves chain elongation to yield docosapentaenoic acid (22 : 5n-3), then 24:5 omega-3, followed by desaturation at the D6 position to form 24:6 omega-3. Then, 24:6 omega-3 is translocated from the endoplasmic reticulum to peroxisomes where two carbons are removed by limited b-oxidation to yield DHA. EPA and docosapentaenoic acid

can also be synthesised from DHA by retroconversion due to limited peroxisomal b-oxidation. In humans, approximately 1% of DHA may be retroconverted to EPA (Ruxton C.H.S., 2005).

3.3.3 Protective effects of ω-3 PUFA and eicosanoids

ω-3 Fatty acids play important roles in human body for the normal composition of sperm, retina and brain lipids (Neuringer M., 1988) and the development of the fetus and the infant (Kang J.X., 1994) (Harris W.S., 1997) (Hoffman D.R., 1993). Studies have demonstrated that DHA deficiency is associated with abnormalities in brain function. omega-3 Fatty acids stimulate growth and exert a protective effect on the development of cardiovascular (Kromhout D., 1985), atopic dermatitis and psoriasis (Bittiner S.B., 1988), inflammatory symptoms (rheumatoid arthritis, and ulcerative colitis) (Lorenz R., 1989) (Hawthorne A.B., 1992), and malignant diseases (Anti M., 1992).

In the body, AA and EPA are converted to prostaglandines and to LT by COXs and lipoxygenases (5-lipoxygenase: 5-LO), respectively. The omega-6 derived eicosanoids have general proinflammatory effects whereas omega-3 derived eicosanoids have anti-inflammatory effects. Omega 3 fatty acids reduce the production of B4 leukotriene, of platelet activating factors released by cytokines and reduce the cytokine-induced synthesis of PG E2 and thromboxane B2 in the colonic mucosa (Endres S., 1989) (Lowry S.F., 1994).

It is important to determine the ratio between omega-3 and omega-6 fatty acids because they act in opposing ways in the modulation of inflammation. There are, for example, two main disorders of inflammatory bowel disease: Crohn's disease and ulcerative colitis. A diet rich in omega-6 fatty acids contributes to the development of ulcerative colitis. The presence of omega-3 fatty acids improves intestinal damage (Shoda R., 1996).

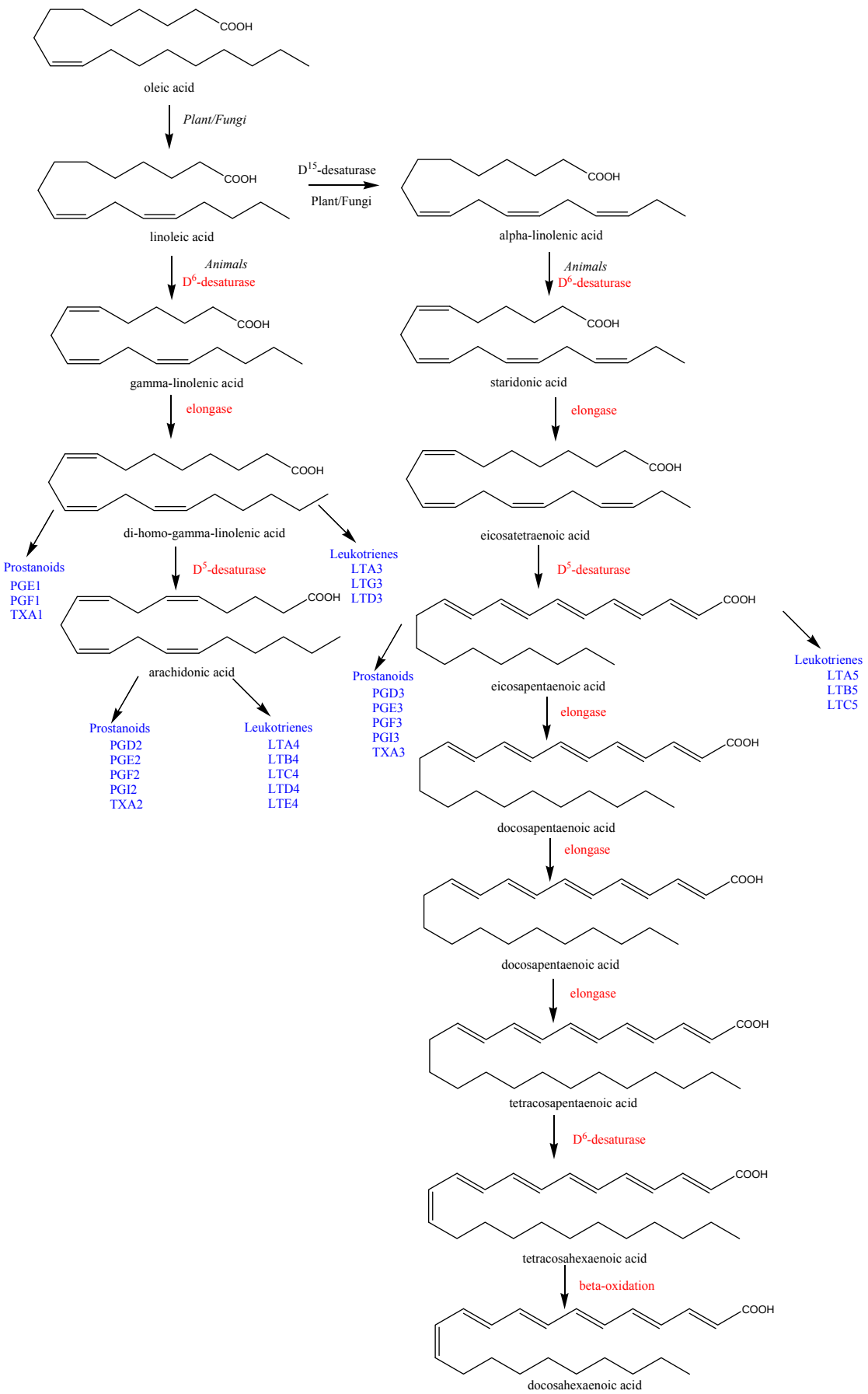


Figure 14 Desaturation and elongation of ω-3 and ω-6 fatty acids.

Arrington and co-workers showed that The inflammation experienced in rheumatoid arthritis is due, in part, to hyperproliferation of T cells and the subsequent production of proinflammatory cytokines. Recently, it was shown that consumption of a diet enriched in n-3 PUFA results in down-regulation of IL-2 production in purified T cells stimulated with α-CD3/α-CD28, and in Jurkatt cells stimulated with PMA/ionomycin/α-CD28 (Arrington J.L., 2001). The production of interleukin (IL-1) and tumour necrosis factor (αTNF) are reduced by the consumption of EPA in response to an endotoxin stimulus.

In recent years several studies have been published on the positive effects of a diet rich in omega-3 and omega-6 fatty acids on cardiovascular disorders (Simopoulos A.P., 2011) (Hu F.B., 2002) (Harris W.S., 2009).The benefits deriving from the consumption of fish depend on the type and preparation of the fish consumed. In particular, DHA and EPA fatty acids have been reported to lower blood pressure and prevent the development of hypertension (Woodman R.J., 2003), one of the most critical factors involved in cardiovascular pathologies such as atherosclerosis or stroke.

The mechanisms of action are still under study, but it is hypothesized that desaturases Δ5 and Δ6 are involved. These enzymes convert fatty acid 20:3(n-6) to 20:4(n-6) and 18:2(n-6) to 18:3(n-6), respectively. The conversion of 18:2(n-6) to 18:3(n-6) is a rate-limiting step in the biosynthesis of 20:3(n-6) from 18:2(n-6). The inhibition of Δ5 desaturase activity by EPA and DHA will reduce the amount of 20:4(n-6) precursor of TxA2 (proaggregator) synthesis and increase the amount of 20:3(n-6), precursor of PG E1 (vasodilator) [3]. It is possible to by-pass the enzymatic step by increasing the dietary γ-linolenic acid (18:3 n-6) which is rapidly converted to 20:3(n-6), precursor of PG E1, anti-aggregator and vasodilator (Tapiero H., 2002). Nieuwenhuys and Hornstra have compared the effects on arterial thrombus formation of dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in vivo using a well validated rat model. Platelet aggregation

(triggered by collagen or adenosine diphosphate in whole hirudinized blood), thromboxane formation (TxB2) and platelet phospholipid fatty acid composition were measured also. The study showed that EPA and DHA ethyl esters affected thrombosis tendency, platelet aggregation and TxB2 formation to a similar extent.

In addition, both polyenes increased the apparent thromboxane A_2-sensitivity of platelets, which appeared negatively related to arterial thrombosis tendency. We conclude that EPA and DHA have similar reducing effects on arterial thrombogenesis in vivo in rats and have comparable effects on the selected platelet functions in vitro (Nieuwenhuys C.M., 1998).

3.3.4 Characterization

The alga oil, thanks to its many characteristics, can be used in many sectors, including the production of biofuels, in nutraceutical as nutritional supplements and in cosmetics. Microalgae are a potential sources of a wide range of high value products for different biotechnological uses (Table 5).

Type of Nutraceutical	High Value Product	Applications	Ref.
PUFA's	EPA	Nutritional supplement and aquaculture feed constituent	(Devaraj S., 2006) (Le T.M., 2014; Omega-3 Fatty Acids EPA and DHA: HealthBenefits Throughout Life, 2012)
	DHA	Nutritional supplement and constituent of infant and aquaculture feeds	
	GLA, AA	Nutritional supplement	
Phycobiliproteins	Phyocyanin	Natural colorant for food and cosmetological products, anti-oxidant	(Glazer A.N., 1994)
	Phycoerythrin	Diagnostic fluorescence agent	
Carotenoids	β-Carotene	Natural food colorant, antioxidant, anti-cancer properties	(Li Y., 2008)
	Astaxantin	Pigment and antioxidant	
	Echinenone, Zeaxanthin Lutein, Phycocyanobilin	Food colorant	
Aminoacids	MAA (mycosporine-like amino acids)	Sunscreen agent	(Brown M.R., 1991; Fleurence J.I., 1999)
Polysaccharides	Carragenan/aliginate	Viscosifier, lubricant, flocculant, antiviral agent	(Wijesekara I., 2011)
Phycotoxins	Okadaic acid, Gonyautoxins, yessotoxins	Diagnostic agent for neurodegenerative diseases	(Rossini G.P., 2010)
Lipids	triglycerides and hydrocarbons	Biofuels	(Brennan L., 2010; Reddy B.V.S., 2008)

Table 5 Principal nutraceuticals from microalgae and their Biotechnological applications. modified from (Chu W.L., 2012)

According to the previous studies based on the use of non-toxic solvent (Nardi M., 2017) we have focused our attention on the development of selective extractive processes rich in PUFA rather than saturated fatty acids and vice versa. For this reason, it was deemed

necessary to characterize the various lipid components within each fraction. Oil extraction yield has been reported in Figure 15

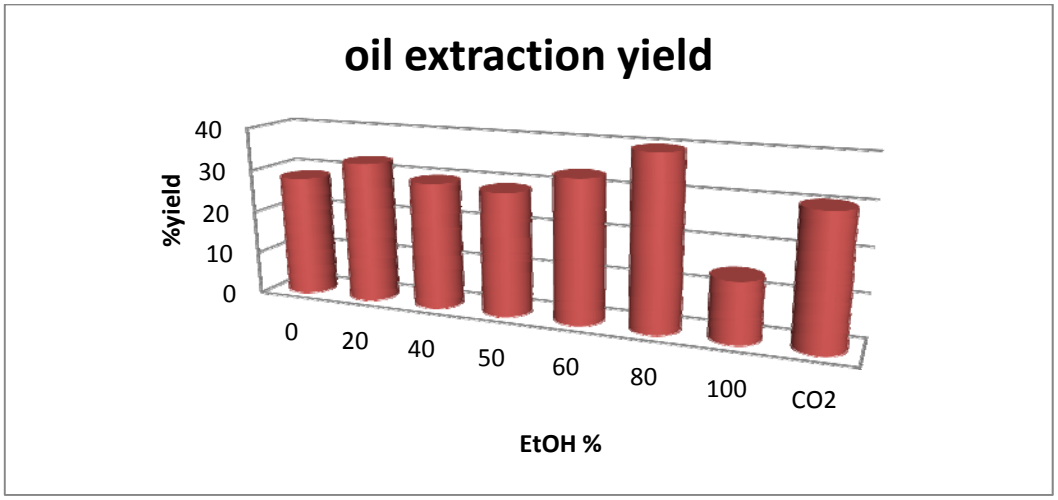


Figure 15 Oil extraction yield in the variation of the extraction solvent

The identification of the individual fatty acids was performed by studying all the masses of mass obtained from the GC / MS analysis, shown below (Figure 16).

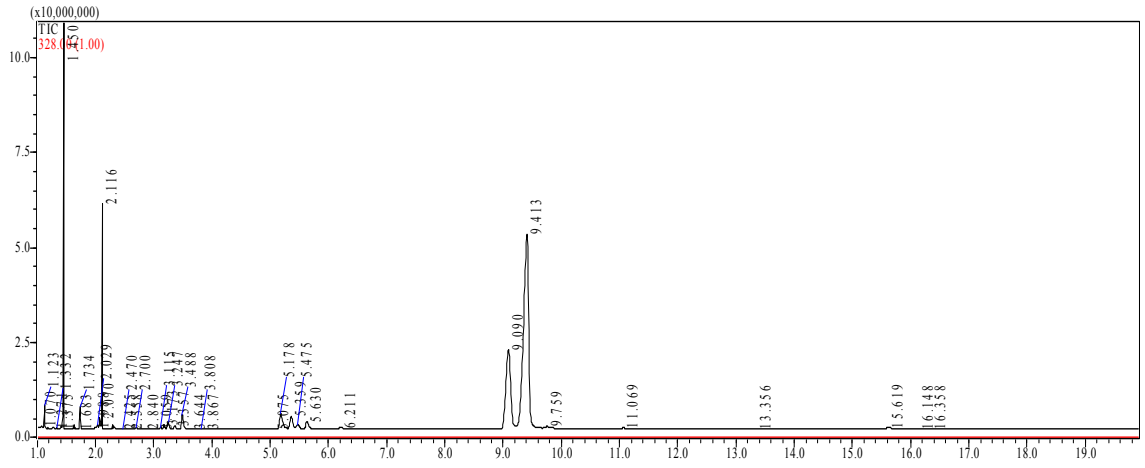


Figure 16 GC/MS Chromatogram of algal oil extracted

The peak at retention time 13.4 is referred to the internal standard (Figure 17).

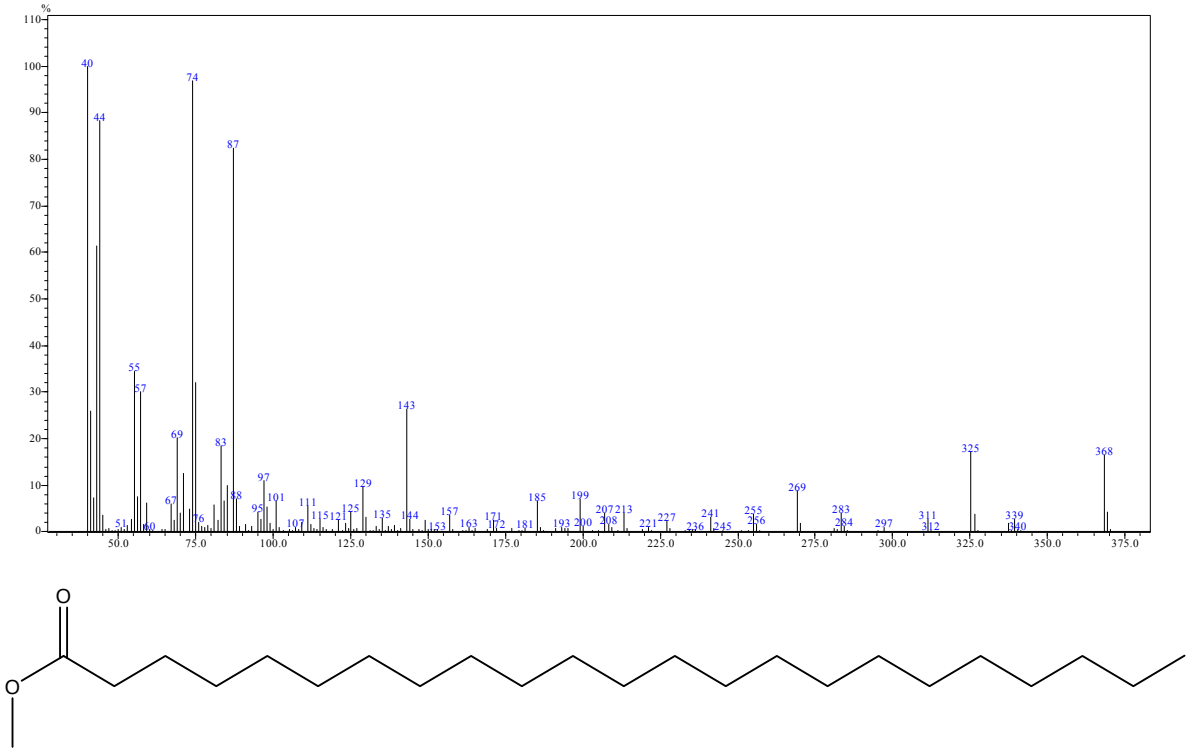


Figure 17 EI-MS spectrum (retention time 13.4 min) and structure of methyl tricosanoate.

The EI-MS spectrum at retention time 1.5 min was characterized by a clear molecular ion [M+] at m/z = 242 (Figure 18). The ion at m/z = 211 represents a loss of 31 mass units as shown, typical of the presence of a methyl ester. Satured fatty acids generally show the McLafferty ion (m/z 74) as the most intense ion and m/z 87 that is formed via β-cleavage as the second most abundant fragment ion. The ion at m/z 227 showns the loss of the methyl fragment. The trend of the peaks with consecutive losses of 14 amu is characteristic of saturated fatty acids (m/z = 87, 101, 115, 129, 143, 157, 199, etc. of general formula $[CH_3OCO(CH_2)_n]^+$). This spectrum is consistent with the structure of myristic acid (14:0).

The EI-MS spectrum at retention time 1.7 min was characterized by a clear molecular ion $[M^+]$ at m/z = 256 (Figure 19). This mass spectrum, like the previous one, can be associated with the presence of a saturated fatty acid thanks to the characteristic trend of

peaks. The loss of the methyl moiety from the molecular indicates back to the presence of pentadecanoic acid (15:0).

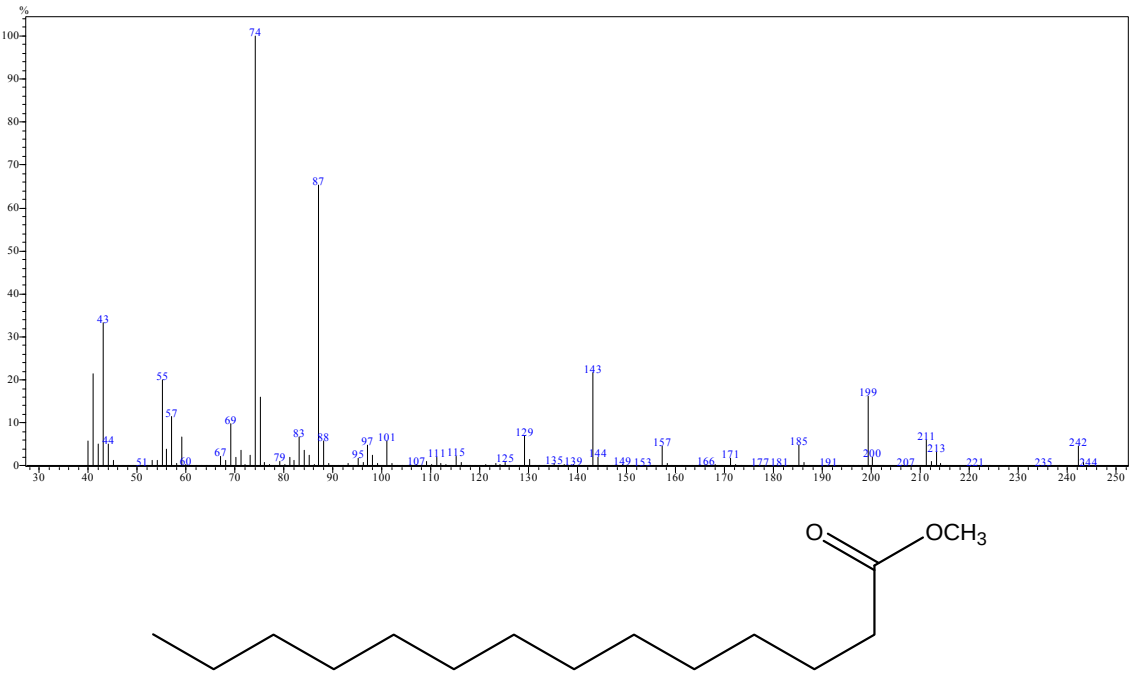


Figure 18 EI-MS spectrum (retention time 1.5 min) and structure of methyl myristate.

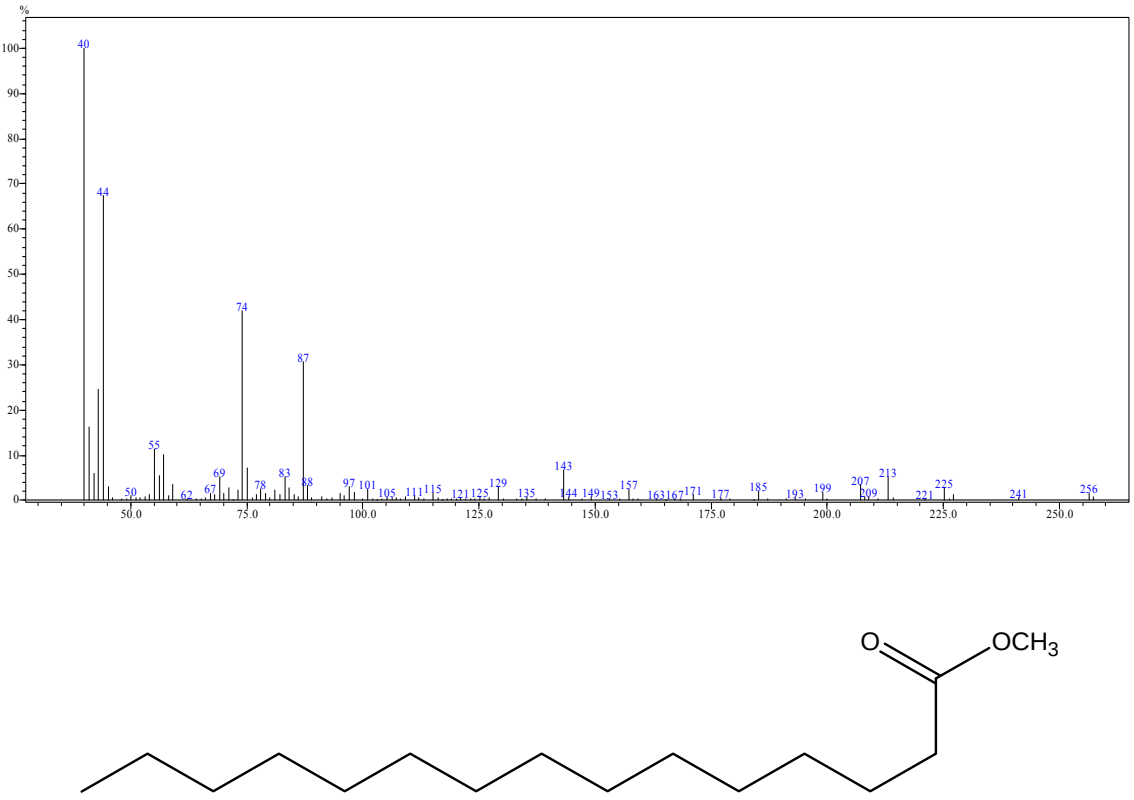


Figure 19 EI-MS spectrum (retention time 1.7 min) and structure of methyl pentadecanoate.

The EI-MS spectrum at retention time 2.1 min was characterized by a clear molecular ion [M^+] at m/z = 270 (Figure 20). This mass spectrum can be associated with the presence of a saturated fatty acid thanks to the characteristic trend of peaks. All fragmentations along the chain are easily identifiable due to losses of 14 amu (m/z: 255; 241; 227; 213; 199; 185; 171; 157; 143; 129; 115; 101; 87). The loss of methyl from the molecular ion indicate the presence of palmitic acid (16:0).

The EI-MS spectrum at retention time 2.7 min was characterized by a clear molecular ion [M^+] at m/z = 284 (Figure 21). This mass spectrum, like the previous one, can be associated with the presence of a saturated fatty acid thanks to the characteristic trend of peaks. The loss of methyl from the molecular indicates to the presence of heptadecanoic acid (17:0).

Figure 20 EI-MS spectrum (retention time 2.1 min) and structure of methyl palmitate.

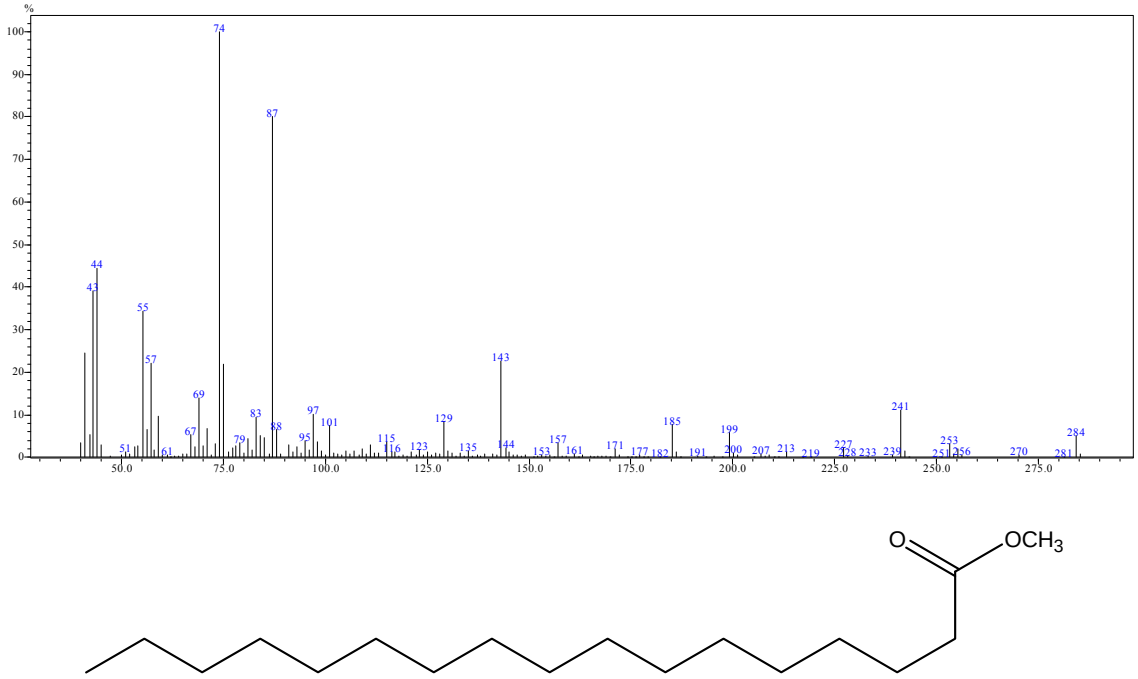


Figure 21 EI-MS spectrum (retention time 2.7 min) and structure of methyl heptadecanoate.

The EI-MS spectrum at retention time 3.1 min was characterized by a low intensity molecular ion [M^+] at m/z = 290 (Figure 22). The two characteristic fragment ion peaks (m/z 74 and m/z 87) in this mass spectrum indicates the presence of at least one unsaturation. The study of the spectrum shows consecutive fragmentation of the hydrocarbon chain indicating to the presence of 8,11,14,17- octadecatetranoic acid (18:4).

The EI-MS spectrum at retention time 3.2 min was characterized by a low intensity molecular ion [M+] at m/z = 292 (Figure 23). It is important to note the presence of the peak at m/z 150, characteristic for methyl esters of polyunsaturated fatty acids with an *n*-6 terminal double bond. The peak at m/z 194 suggests a cleavage at the carboxyl end of the molecule giving a fragment containing the first two double bonds and the second methylene group (minus a proton) that could be termed the '*alpha*' (α) ion. From these considerations, one can infer it's possible to hypothesize the presence of 6,9,12-octadecatrienoic acid (γ-linolenic acid).

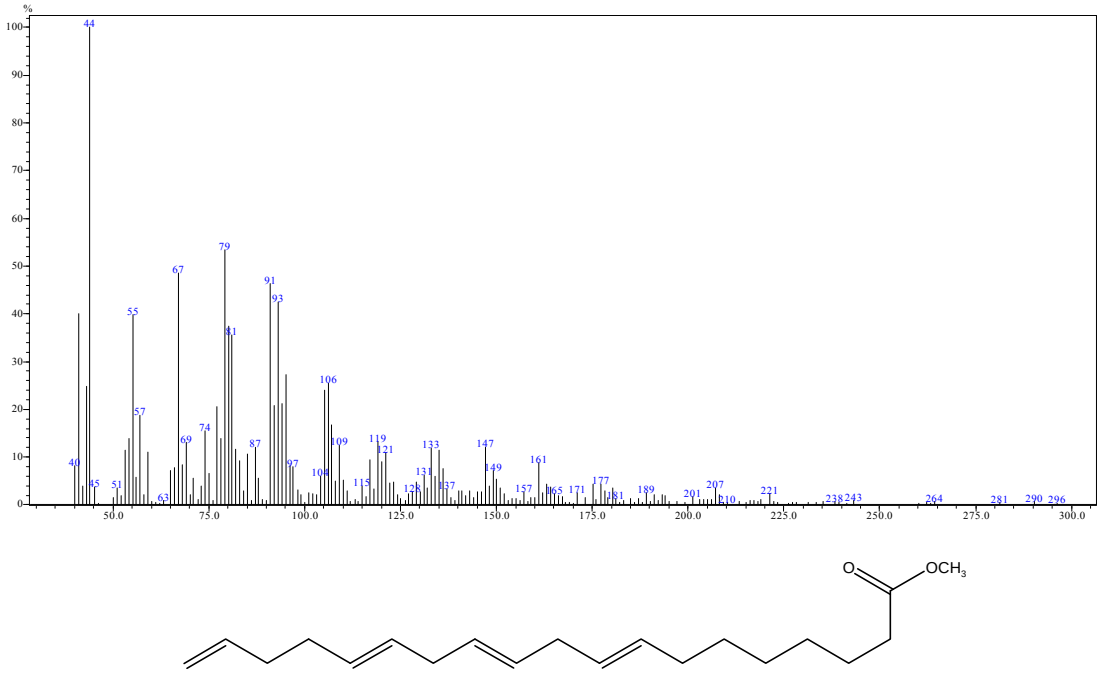


Figure 22 EI-MS spectrum (retention time 3.1 min) and structure of methyl 8,11,14,17- octadecatetraenoate.

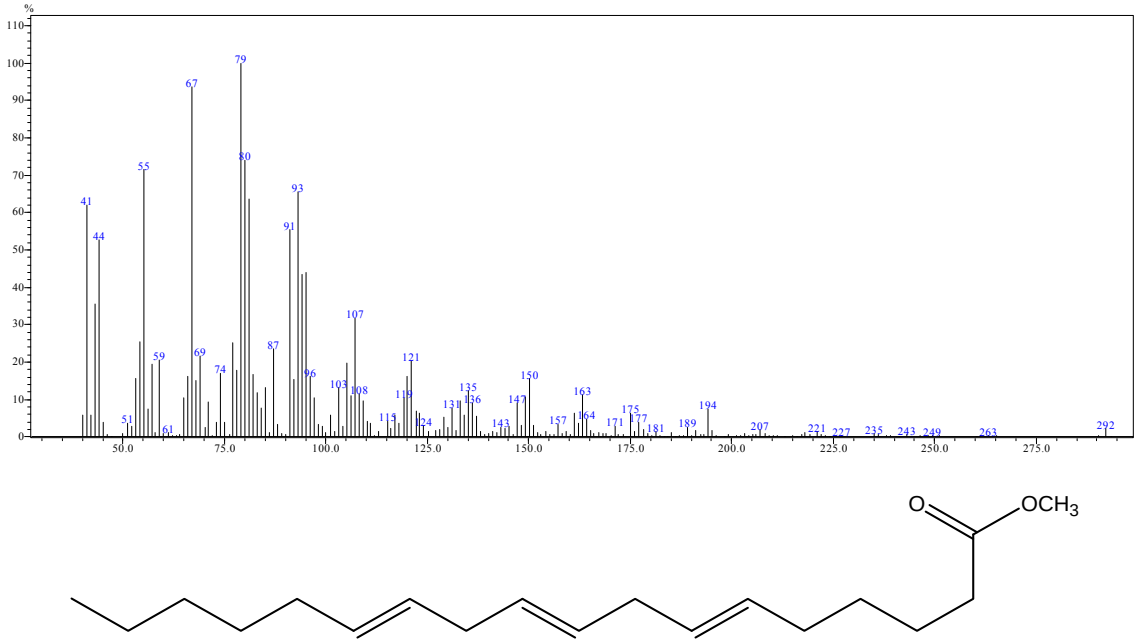


Figure 23 EI-MS spectrum (retention time 3.2 min) and structure of methyl 6,9,12-octadecatrienoate.

The EI-MS spectrum at retention time 3.4 min was characterized by a low molecular ion [M+] at m/z = 296 (Figure 24). The abundant ion at m/z 264 is produced by loss of methanol from the molecular ion, while the loss of the McLafferty rearrangement ion is

evident in the presence of ion at m/z 222. The base peak at m/z 55 usually indicates the presence of a single double bond. This spectrum indicates the presence of oleic acid.

The EI-MS spectrum at retention time 3.5 min was characterized by a clear molecular ion [M+] at m/z = 298 (Figure 25). The distribution of the peaks indicates that it is a saturated fatty acid. The analysis of the individual peaks of the fragments and the loss of methyl from the molecular ion indicates to the presence of octadecanoic acid (18:0).

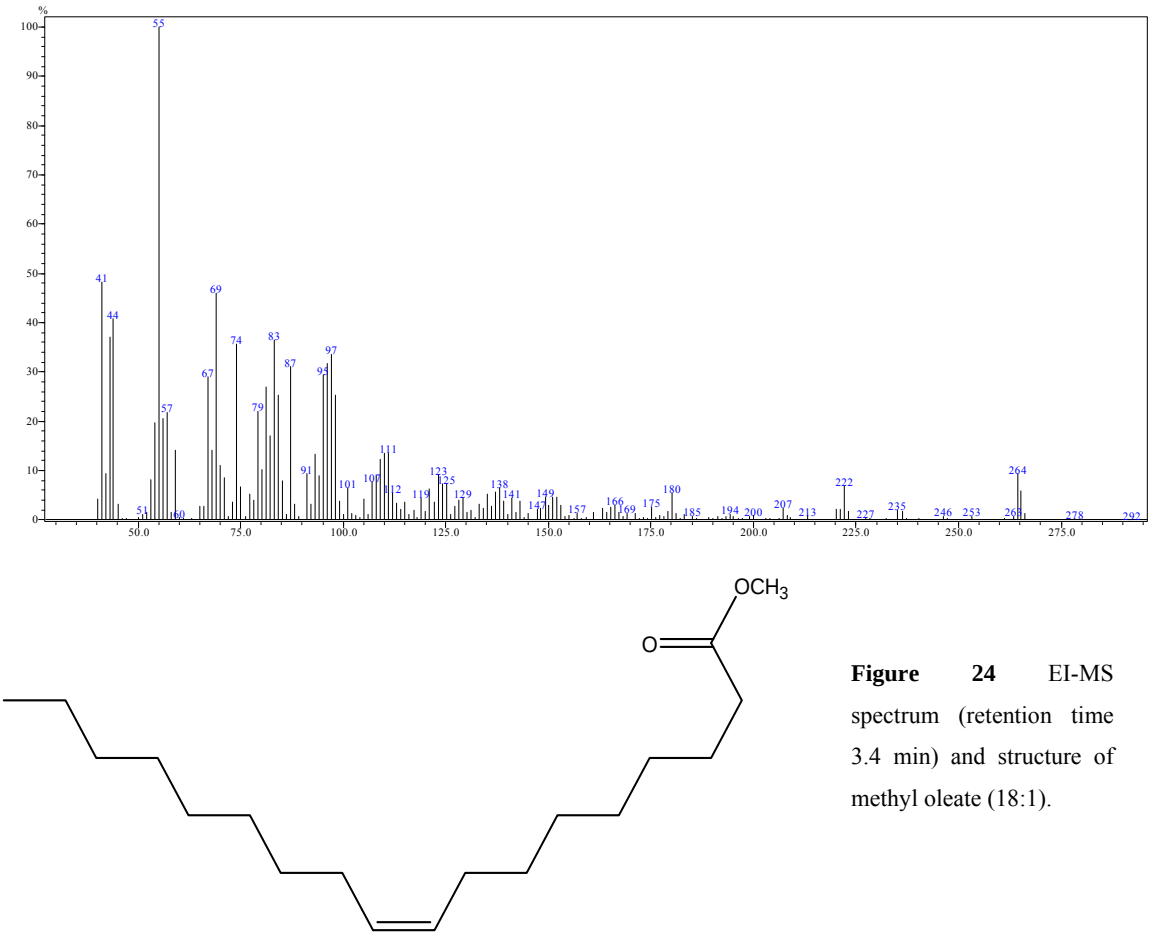


Figure 24 EI-MS spectrum (retention time 3.4 min) and structure of methyl oleate (18:1).

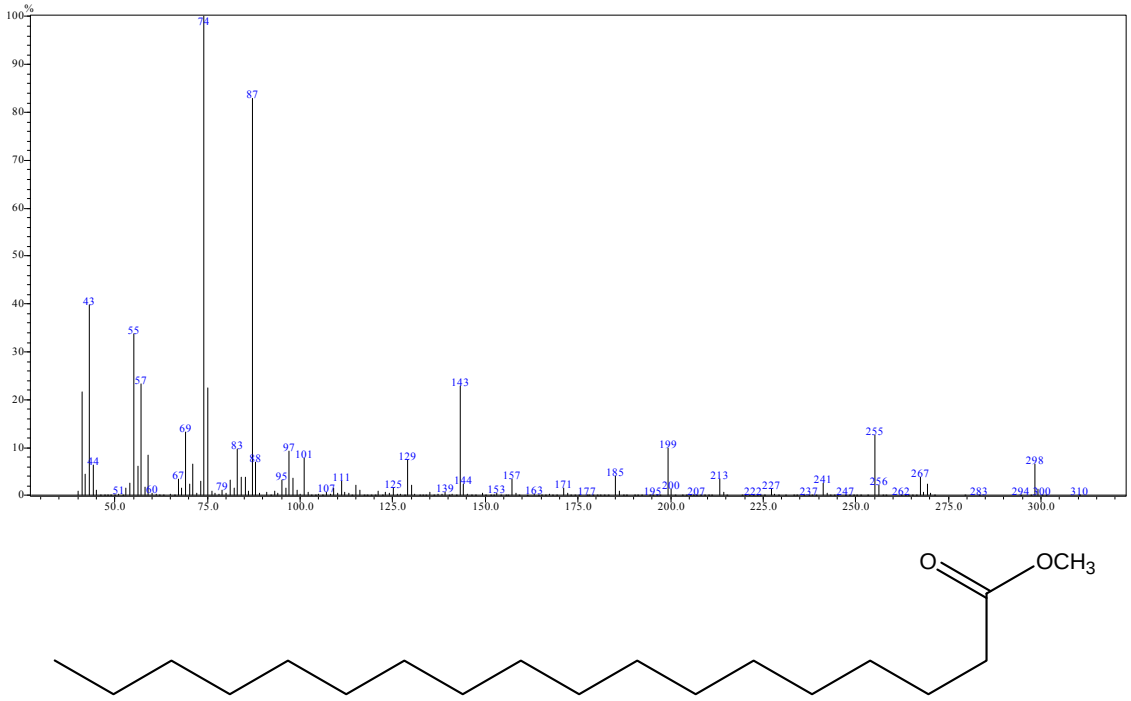


Figure 25 EI-MS spectrum (retention time 3.5 min) and structure of methyl octadecanoate(18:0).

The EI-MS spectrum at retention time 5.4 min shows a Gaussian pattern of the distribution of the peaks, characteristic of polyunsaturated fatty acids. The 'diagnostic' omega ion at m/z = 108 for an n-3 double bond is observe (Figure 26). The molecular ion peak is not evident, but from the data obtained from the spectrum, it goes back to the presence of EPA acid is indicated.

The EI-MS spectrum at retention time 5.5 min shows a Gaussian pattern of the distribution of the peaks similar to the previous one and is characterized by a clear molecular ion at m/z 320 (Figure 27). The 'diagnostic' omega ion at m/z = 150 for an n-6 double bond is present. As seen previously, the peak at m / z 222 indicates the loss of McLafferty rearrangement ion. The study of the fragmentation pattern indicates the presence of 8,11,14-eicosatrienoic acid.

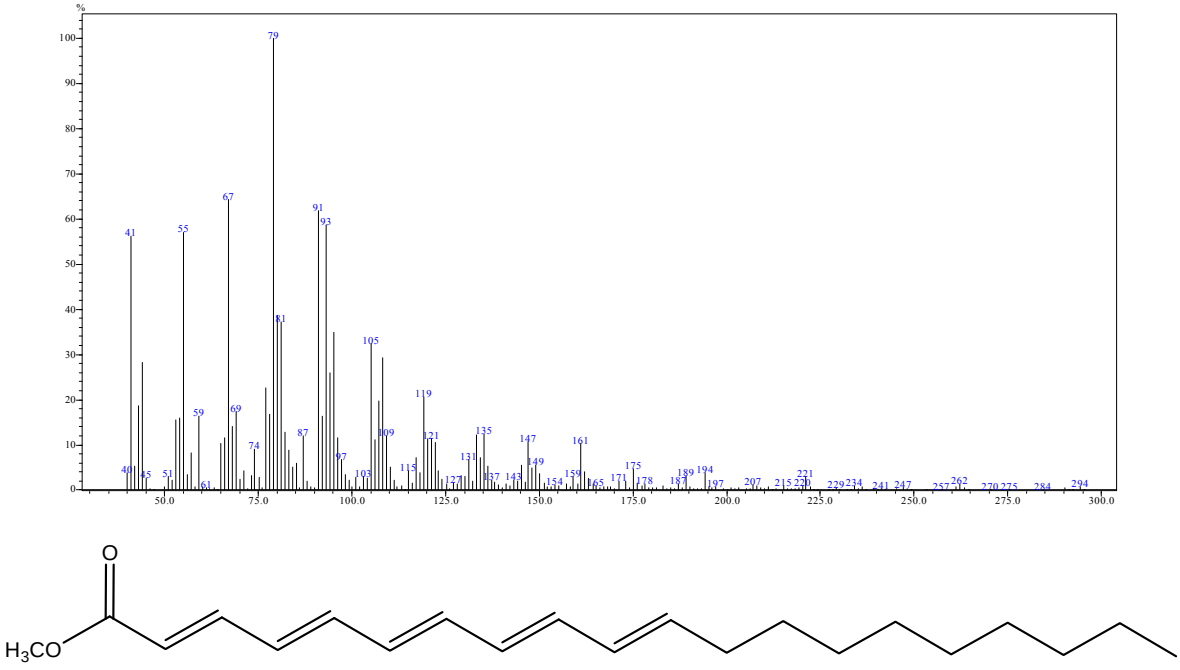


Figure 26 EI-MS spectrum (retention time 5.4 min) and structure of methyl eicosapentaenoate (20:5 ω-3).

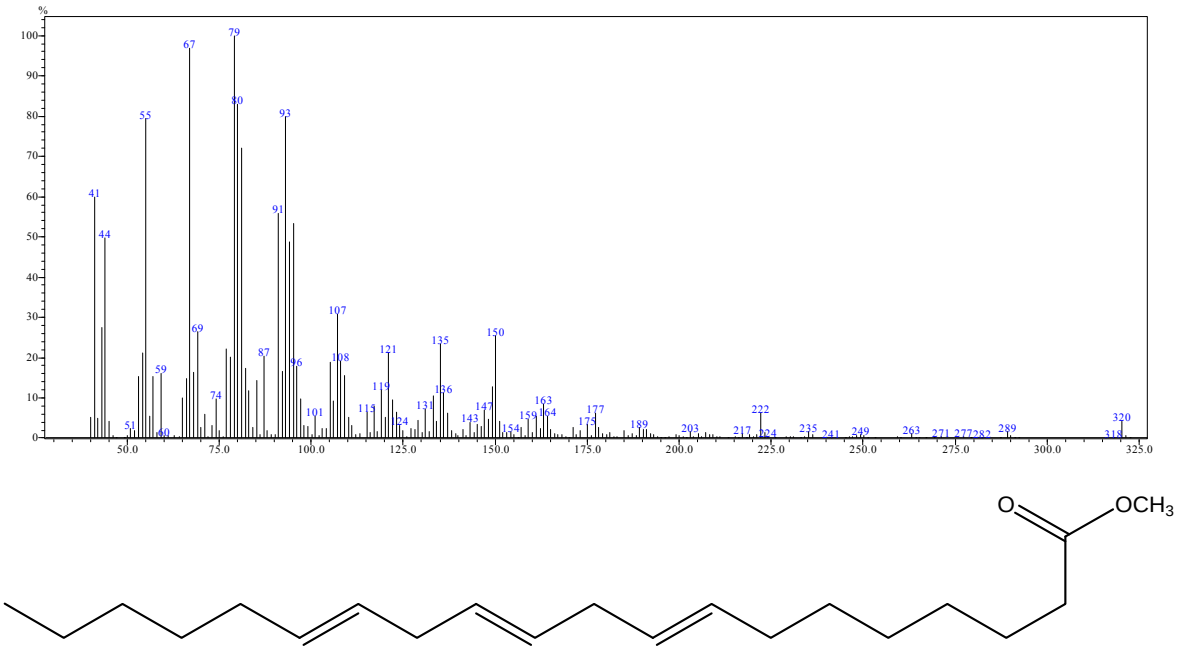
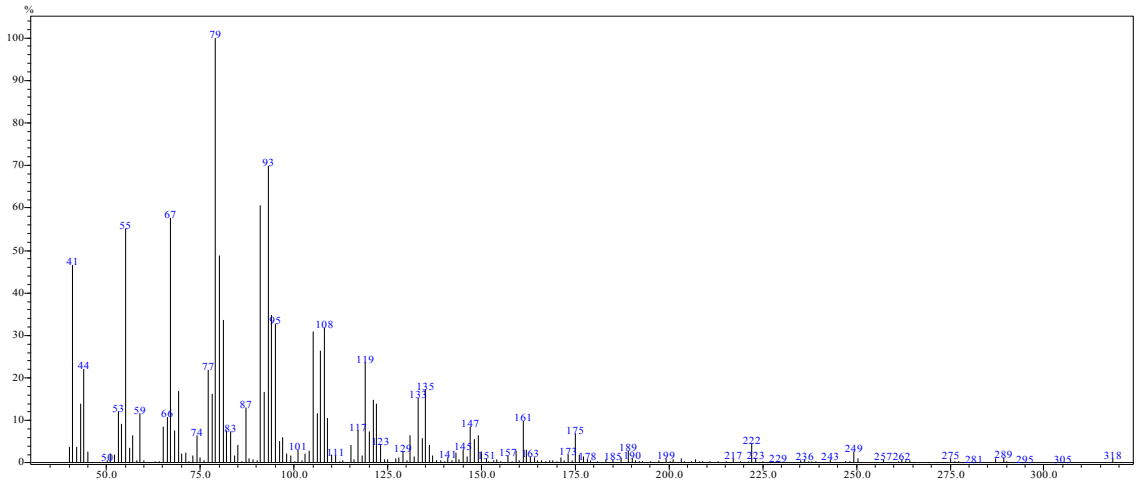


Figure 27 EI-MS spectrum (retention time 5.5 min) and structure of methyl 8,11,14-eicosatrienoate (20:3 ω-6).

The EI-MS spectrum at retention time 5.6 min is similar to the EPA spectrum and is characterized by a low intensity molecular ion at m/z 318 (Figure 28). The diagnostic omega ion at m/z 108 for the n-3 family of fatty acids is present. The alpha ion at m/z 222 suggests the presence of an Δ8, 11 double bond. The loss of a methyl group from the molecular leads indicated the presence of 8,11,14,17-eicosatetraenoic acid (20:4).

The EI-MS spectrum at retention time 6.2 min characterized by a clear molecular ion at m/z 326 (Figure 29). The intence molecular peak and the trend of the distribution of the other peaks suggest that it is a saturated fatty acid. The loss of 31 amu from the molecular ion indicates to the presence of eicosanoic acid (20:0).



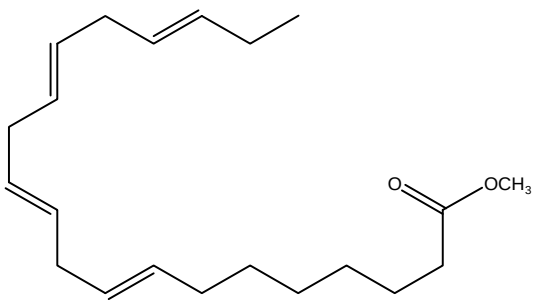


Figure 28 EI-MS spectrum (retention time 5.6 min) and structure of methyl 8,11,14,17-eicosatetraenoate (20:4).

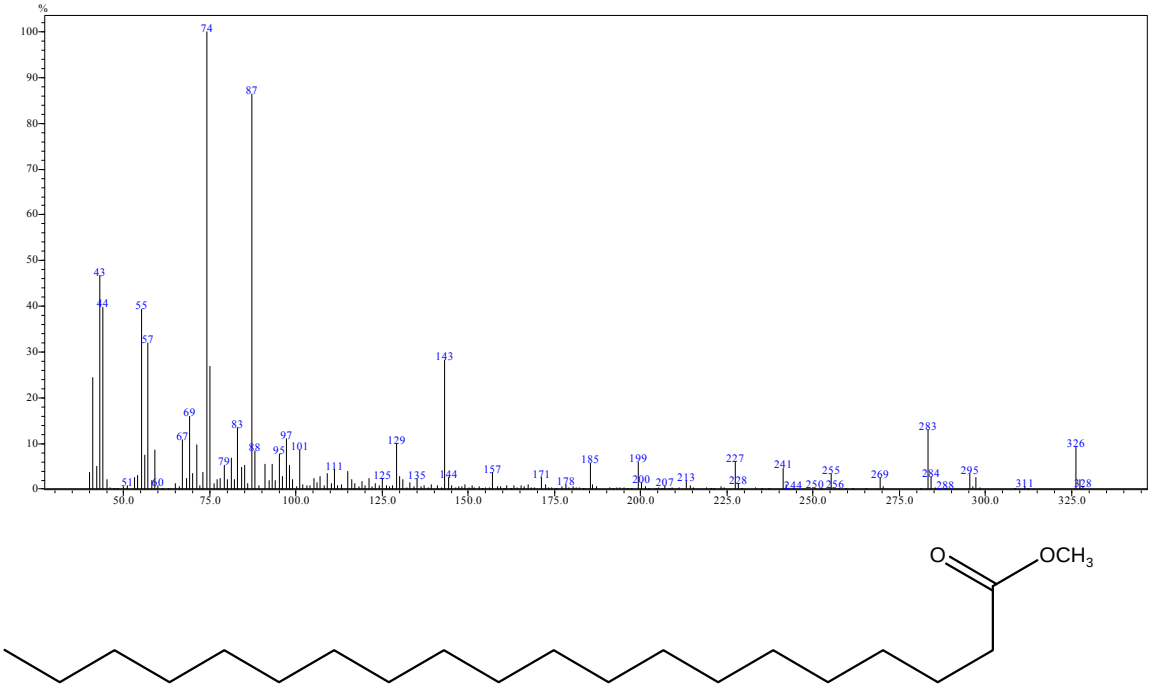


Figure 29 EI-MS spectrum (retention time 6.2 min) and structure of methyl eicosanoate (20:4).

The EI-MS spectra at retention time 9.1 (Figure 30) and 9.4 (Figure 31) are similar and are characterized by a low intensity molecular ion and by a Gaussian pattern of the distribution of the peaks. Those information suggest the presence of two PUFA. In the first one there is the diagnostic omega ion at m/z 150 for the n-6 family of fatty acids. In the second one there is the diagnostic omega ion at m/z 108 for the n-3 family of fatty acids. In both spectra the alpha ion at m/z 166 is present. The difference of 2 amu in the peaks from m/z 217 in the first spectrum and 215 m/z in the second one suggests that the two molecules differ in an unsaturation. The loss of a methyl group from the molecular ion indicates the presence of 4,7,10,13,16-docosapentaenoic acid (22:5) (DPA) at retention time 9.1 min, and docosahexaenoic acid (DHA) at retention time 9.4 min.

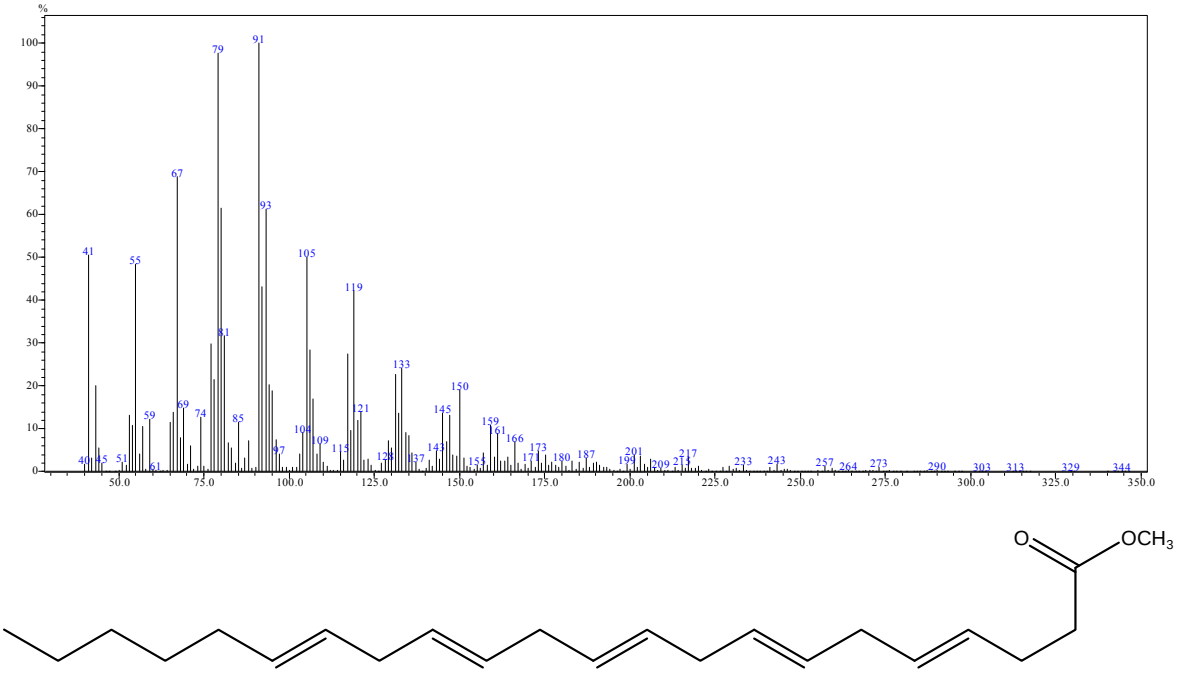


Figure 30 EI-MS spectrum (retention time 9.1 min) and structure of methyl 4,7,10,13,16-docosapentaenoate (22:5).

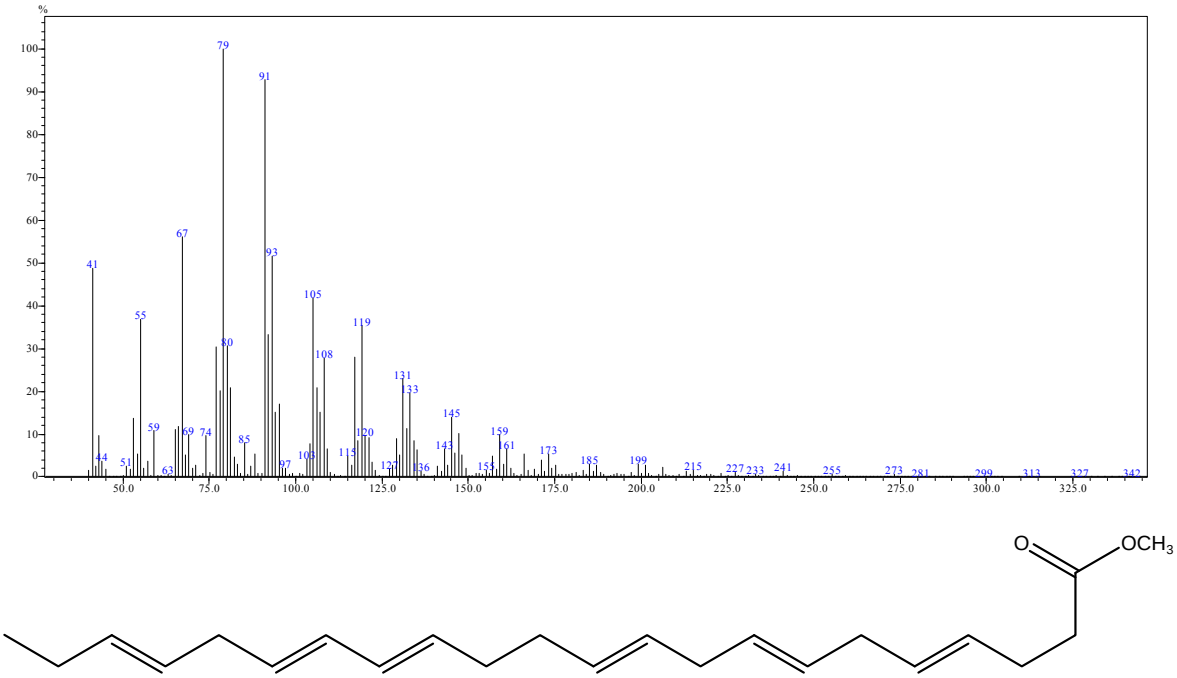
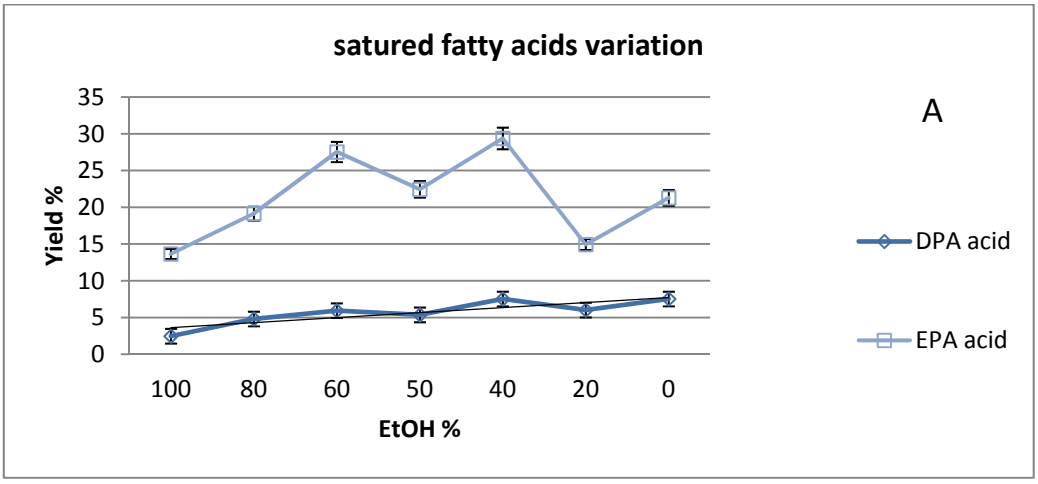


Figure 31 EI-MS spectrum (retention time 9.4 min) and structure of methyl docosahexaenoate (22:6).

A quantitative analysis was performed to evaluate the composition in terms of fatty acids in the extracted samples. Two saturated fatty acids, myristic and palmitic, and two polyunsaturated fatty acids, DPA and DHA, were considered to evaluate the performance according to solvent/solvent mixture used for extraction. It is shown in Figure 3 that the evolution of saturated fatty acid concentrations is opposite to that of PUFA (Figure 32). According to this result it is possible to choose the type of solvent/mixture of solvents to be used depending on the sector in which the algal oil is utilized.



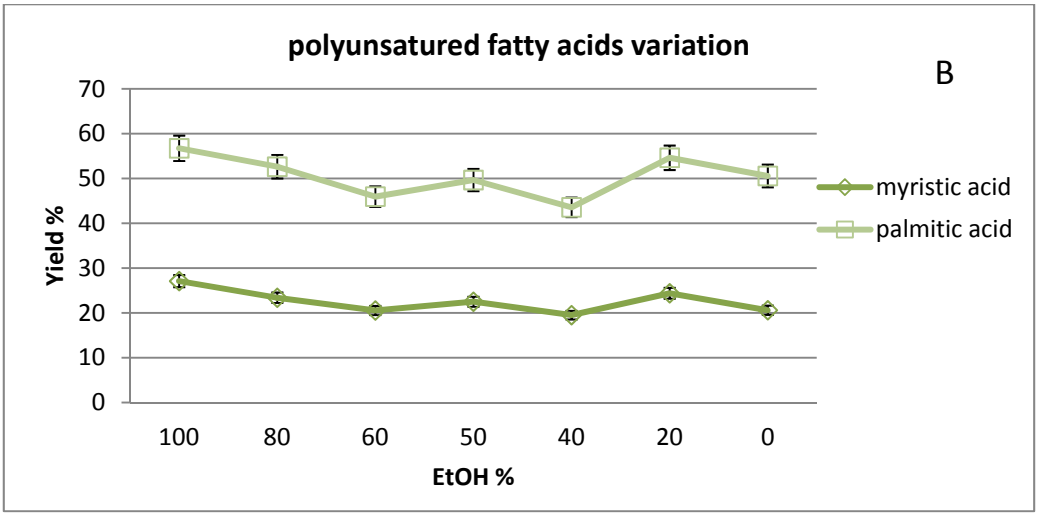


Figure 32 PUFA (A) and satured fatty acids (B) variation as a function of solvent/solvent mixture used.

To further characterize the extracts of algal oil ESI-MS analysis were performed by flow injection analysis (FIA) in both negative and positive ion modes. In general, the intensity

of ions observed as positive ions was lower than those observed as negative ions. Therefore, the experiments were conducted using the negative mode. The most important point of this experiment is to confirm that ions generated from target compounds are observed, rather than that merely any sort of ions are observed. It is necessary to attribute observed ions to specific compounds. Fundamentally, in the attribution process, assuming that positive ions are protonated molecules $[M+H]^+$ and negative ions are deprotonated molecules $[M-H]^-$, it is verified whether they are consistent with the molecular mass of target compounds. Structural assignments were, therefore, based on the accurate mass of the pseudo-molecular ion $[M-H]^-$, present in the negative ESI-MS spectrum, and on the corresponding fragments ions detected by collision-induced dissociation (CID) under nitrogen (25% normalized collision energy) in the ion trap.

The mass range scanned was between m/z 50 and m/z 1200 (Figure 33).

The initial complexity of the mass spectrum was reduced when the product ion scan and a precursor ion scan were performed. ESI-MS/MS analysis were performed for all the ions present in the full scan chromatogram for each algae extract (APPENDIX A).

Table 6 lists the deprotonated molecules $[M-H]^-$ with their corresponding fragments and precursor ions presents in an algae extract monitored. Ions included in the range of m/z 455-656 are not associated with free fatty acids, or diglycerides, or triglycerides.

For each analyte considered, ions with highest intensity are given in the Table 6. From Table 7, it is clear that the algal extract contains adducts between two identical and different fatty acid molecules.

The structure of the two components of each precursor $[M-H]^-$ species has been proved by MS/MS, in the same conditions, performed on each product ion listed in Table 7.

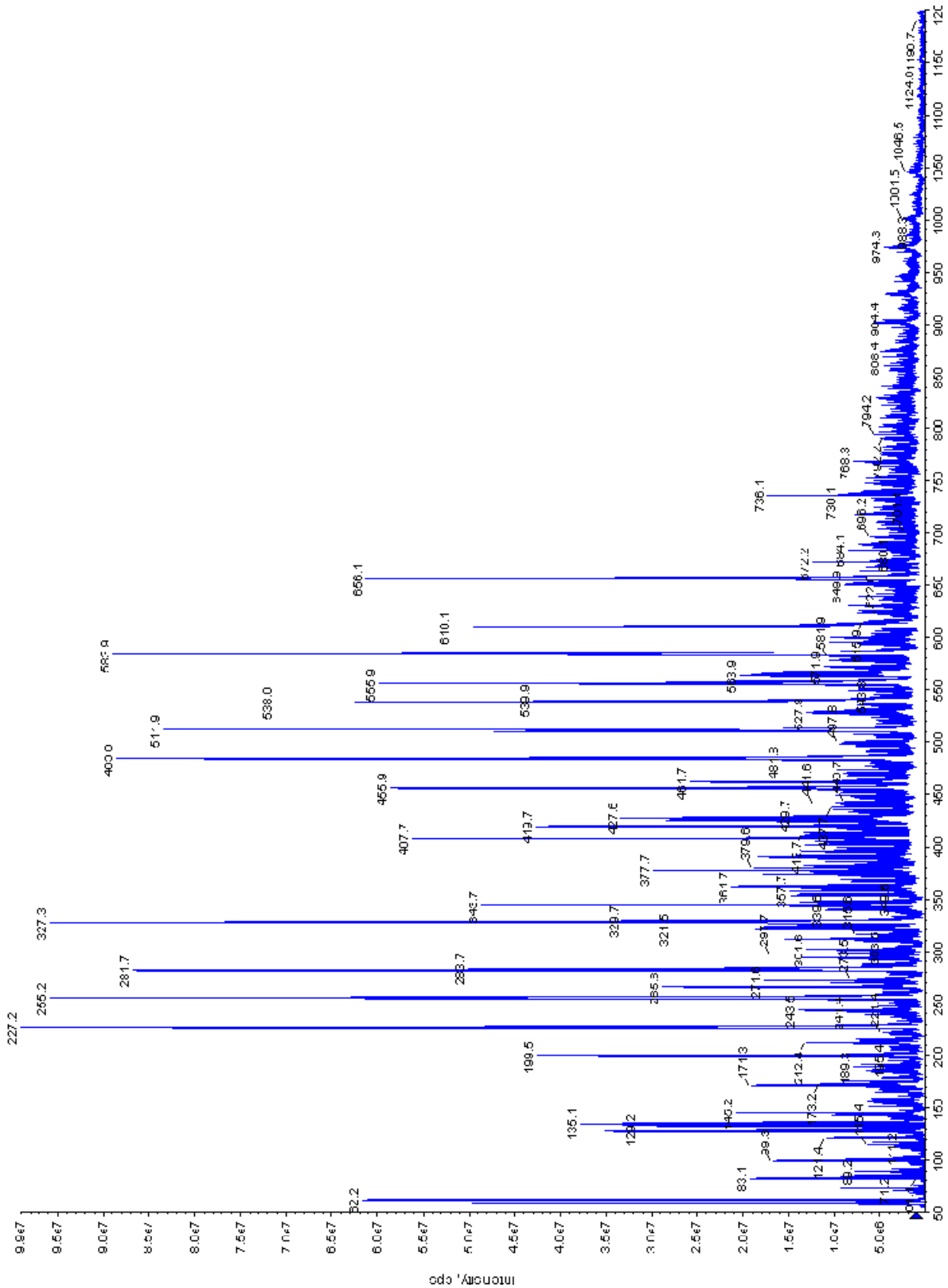


Figure 33 Negative ESI full scan mass spectrum of algal oil sample.

ANALYTE	*[M-H]⁻*	*Product ions*	*Precursor ions*
Lauric acid	199.4	181.6; 155.0	399.7; 455.9
Myristic acid	227.4	209.5; 183.3	455.7; 483.8; 509.8
Myristoleic acid	225.2	207.2; 181.5	482
Pentadecylic acid	241.4	223.2; 197.2	483.8
Palmitic acid	255.5	237.6; 211.6	511.8; 537.9; 583.9
γ-linolenic acid	277.4	259.4; 233.4	555.8
Oleic acid	281.6	263.4; 237.4	509.8; 537.9; 563.8
Stearic acid	283.5	265.4; 239.2	540.5; 568
Eicosapentanoic acid	301.6	283.6; 257.6	602.2
Docosaexaenoic acid	327.5	283.6; 309.2; 229.6	583.9; 610.0; 656.0; 658.0
Docosapentaenoic acid	329.7	311.7; 285.6	658
Behenic acid	339.6	321.3; 294.9	596.5; 569.2

Table 6 List of deprotonated molecules identified in full scan MS spectra of algal oil sample, fragment ion identified in MS/MS spectra and precursor ion identified in precursor ion spectra.

Negative ion	Fatty acid composition
455.8	2*myristic
483.9	2* Pentadecylic
511.9	2*palmitic
555.8	2*γ-linolenic
563.8	2*oleic
583.9	DHA+palmitic
610.0	DHA+oleic
656.0	2*DHA
658.0	DHA+DPA

Table 7 Molecular composition of adduct with negative molecular ion between m/z 455 and m/z 656.

For a better understanding of the type of interaction occurring between the fatty acids present into the algae extract, standard solutions were analyzed: a long chain polyunsaturated fatty acid (DHA) solution, a long chain saturated fatty acid (stearic acid) solution, a short chain fatty acid (butyric acid) solution and a solution containing all of them. The full scan ESI-MS (negative ion mode) of the DHA standard solution shows the deprotonated molecular ion at m/z 327.5 and ions at m/z 655.8 and 984.3, adducts of two and three DHA molecules, respectively (Figure 34). The stearic acid solution full scan spectrum shows the deprotonated molecular ion at m/z 283.3, and ions at m/z 567.7 and 852.2, referring to two and three interacting acid molecules. The analysis of the solution containing the three fatty acids (DHA acid, stearic acid and butyric acid) leads to a spectrum in which there are the deprotonated ions of the three acids and the ions formed by the reaction between DHA and stearic acids. Butiric acid does not form an adduct with the other two.

To better understand what the reaction site was in the saturated and unsaturated fatty acid adducts, a mixture containing oleic acid and palmitic acid was analysed. From the full scan ESI-MS (- ion mode) of this solution, it was evident that we obtained an acid-alcohol adduct confirmed by the ion at 524.2. Furthermore, the ion at m/z 255.2 represented the deprotonated molecule of palmitic acid; the ion at m/z 268.0 (Figure 35).

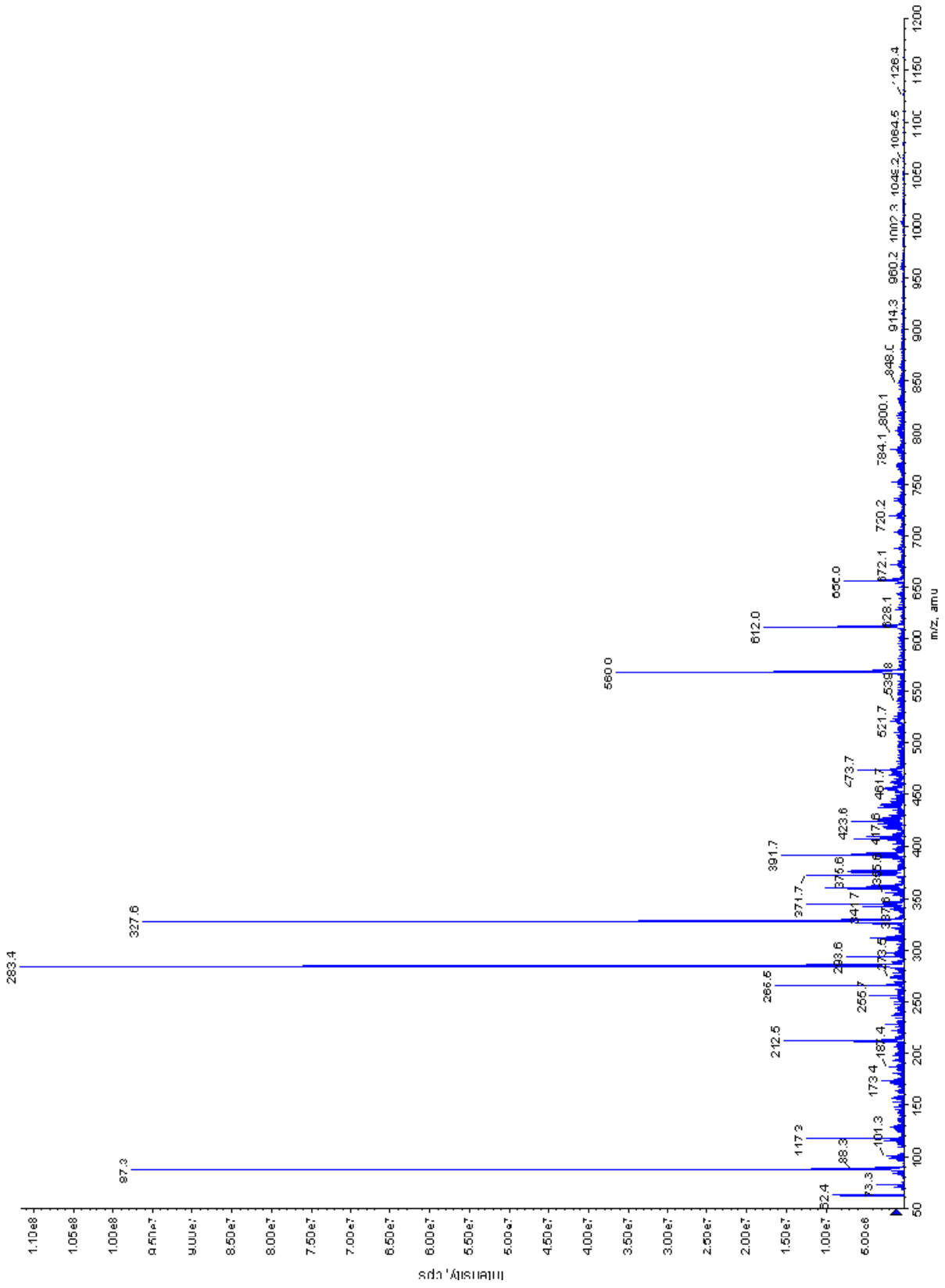


Figure 34 Negative ESI full scan mass spectrum of standard solution of DHA, Stearic acid and butyric acid.

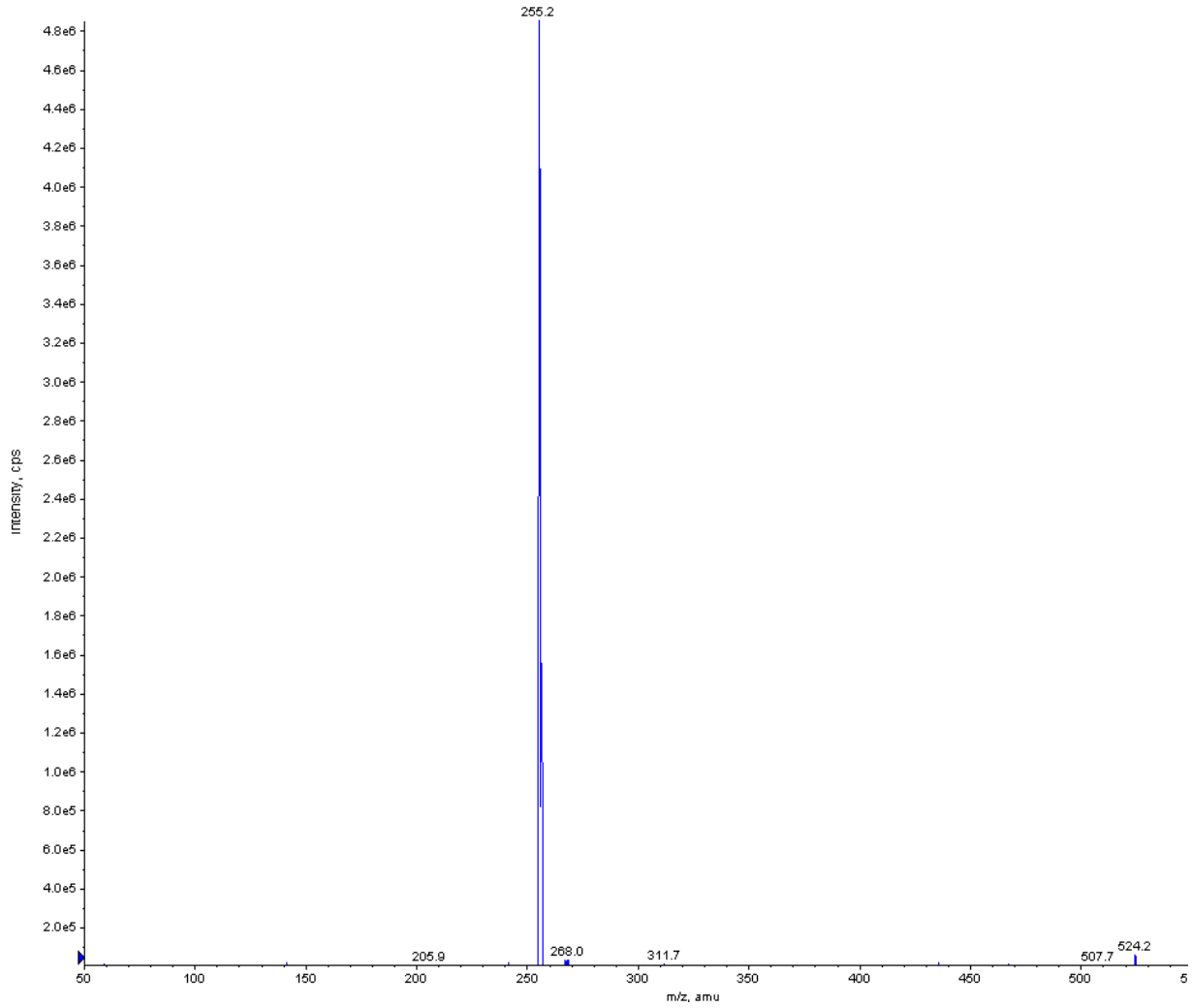


Figure 35 Negative ESI full scan mass spectrum of standard solution of oleic alcohol and palmitic acid.

New adducts of satured and unsatured fatty acids were identified in algal oil by mass spectrometry. The peak at m/z value 327.5, the most abundant in the full scan spectra, is related to the molecular ion of DHA fatty acid. The presence of the FA adducts is given indicated by the presence of the dimer and trimer ions at m/z 655.9 (related to two molecules of DHA) and m/z 984.4 (related to three molecules of DHA). From the precursor ion scan (PREC) of fatty acids, it is possible to trace the adducts that form. From the product ion scan of the ions related to the adducts indicates the fatty acids that compose them. The ion with m/z value 556.5, derived from the PREC of the ion 327.5 in full scan,

is given by the interaction between a molecule of DHA acid and a myristic acid. To understand the type of interaction that is being formed, a standard solution of DHA and Stearic acids is added to Butyric acid. It is noted that there is no adduct between the butyric acid and the two long chain acids. To define the interaction that takes place on the carboxylic part or on the hydrocarburic chain of fatty acids, a solution of Palmitic acid and Oleyl alcohol was analyzed The full scan spectrum shows that there is an interaction between the fatty acid and the alcohol. Therefore, it is reasonable to conclude that the interaction takes place on the hydrocarbon fraction of fatty acids.

3.4 References

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CHAPTER 4: EXPERIMENTAL DATA

4.1 Stevia

4.1.1 Introduction

Food safety and security has undoubtedly become a critical factor developing and developed countries. FDA food facility registration is required for all companies that manufacture, process, pack, or store food, beverages, or dietary supplements that may be consumed in the United States. Similarly, The European agency EFSA takes care of existing and emerging risks associated with the food chain. Moreover, food safety and security has become important in the NATO Science for Peace and Security Programme (Sindona G., 2014). A particular attention of researchers managing sophisticated analytical methodology, such as mass spectrometry, would provide unique contributions to the security and safety of foods.

4.1.2 Materials and methods

Solvents and reagents were commercially available (Sigma–Aldrich, St. Louis, MO). Steviol glycosides standards were purchased from Extrasynthese (Genay Cedex, France). Brutieridin internal standard was prepared in the laboratory with our in-house methods.

Three beverages (two fruits juice and a cola soft drink used as blank matrix) and a sweetener were purchased in local stores.

Two milliliters of each beverage were centrifuged at 6000 rpm for 5 min. The supernatant was quickly degassed by a nitrogen flow, when needed. All the solutions were filtered through a 0.45 μm PTFE filter. One milligrams of sweetener was diluted in 1ml of water. A 100 μl volume of each beverages solution was added to 167 μl of a 1.2 mg/l of Brutieridin internal standard, diluted to 1 ml of ACN/H_2O (50/50) and stirred for 30 s to

allow homogeneous distribution of the standards. 100 µl of a solution of 1 mg/l sweetener Stevia was added to 167 µl of a 1.2 mg/l of Brutieridin internal standard and diluted to 1 ml of ACN/H_2O (50/50). These solutions were used to quantify Sb, SV and Ra. Solutions of each sample of beverage with a dilution 1 to 20 and a solution of sweetener with a dilution 1 to 200 were prepared to quantify Rc.

The calibration curves for each of the steviol glycosides were obtained sampling five solutions containing the analytes at concentration ranging from 50 to 400 µg/l, and the internal standard at the fixed concentration of 200µg /l. The HPLC chromatogram of the STS standard is presented in Figure 37.

Analyte	Equations	R^2
Sb	Y=93.442x-12.75	0.998
SV	Y=70.561x-10.878	0.997
Ra	Y=53.072x-18.478	0.929
Rc	Y=49.261x-9.270	0.995

Table 8 Equation and the correlation coefficient found for each STS.

Table 8 shows the equation and the correlation coefficient found for each analyte in the range of concentrations employed. In all cases, an excellent linearity has been achieved. Before applying the methodology to real cases, some experiments have been planned to obtain the typical analytical parameters.

Each analysis was repeated three times and the mean value was used.

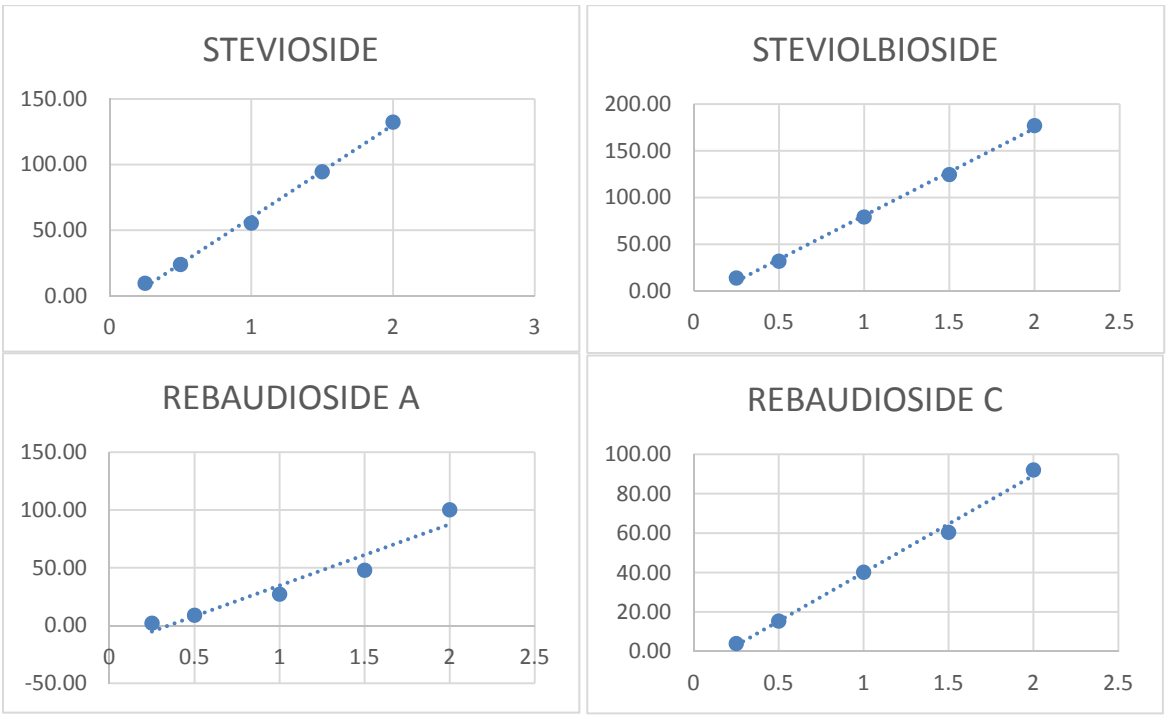

Figure 36 External calibration curve of STS obtained injecting different concentrations of the mix standard solution.

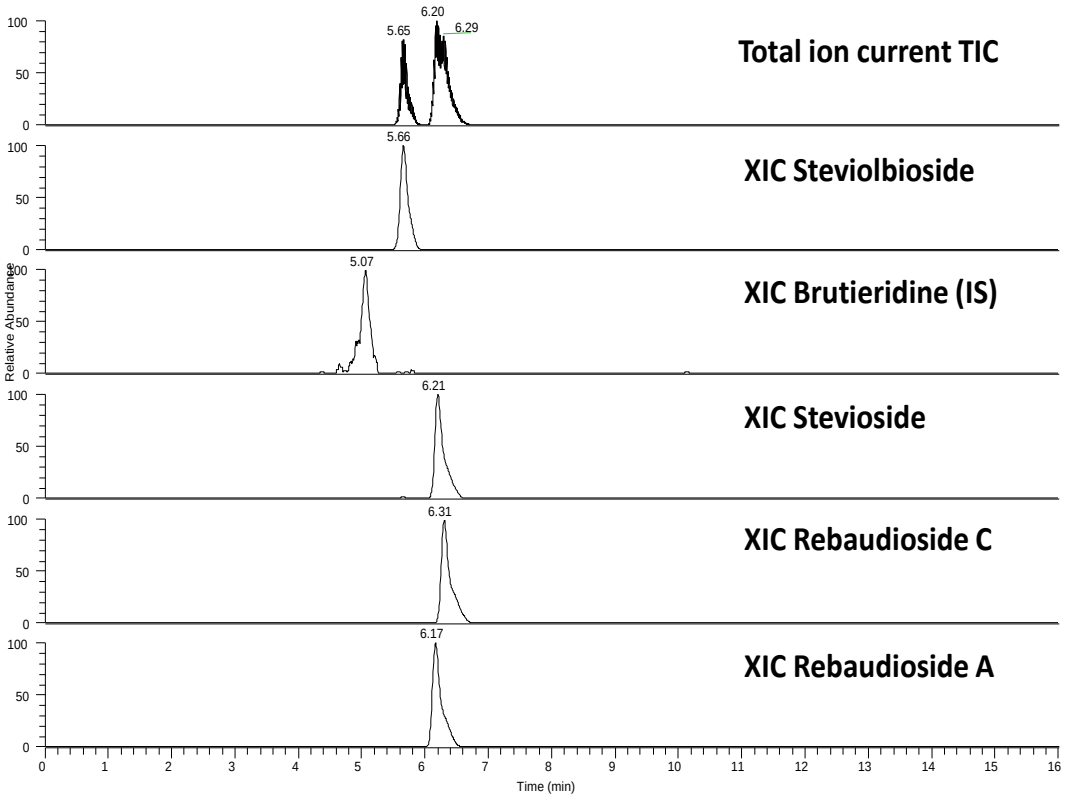

Figure 37 LC-MS/MS under MRM condition of analytes and internal standard

4.1.3 Characterization

LC–MS/MS and low-energy CID-MS/MS analysis was carried out using a Thermo Scientific UHPLC instrument coupled to a TSQ Quantum Vantage (Thermo Fischer Scientific, San José, CA) triple-stage quadrupole mass spectrometer. The chromatographic separation was achieved using a_C18 reversed-phase analytical column, Kinetex PFP (2.1_50 mm, 2.6 lm particle size, 100 Å, Phenomenex, Torrance, CA). The elution gradient consisted of mobile phase (A) ACN and (B) H_2O (0.1% $HCOONH_4^+$). The linear gradient used was the following: at t = 0.0 min, 10% A and 90% B; at t = 2.0 min, 10% A and 90% B; at t = 5.00 min, 50% A and 50% B; at t = 8.00 min, 50% A and 50% B; at t = 10.00 min, 98% A and 98% B; at t = 13.00 min, 98% A and 98% B; at t = 14.00 min, 10% A and 90% B; at t = 16 min, 10% A and 90% B. The flow rate was set at 0.3 ml/min, and the sample injection volume was 10 μL. A further switching valve located on the mass spectrometer was used to divert the LC flow to waste for the initial 1 min as well as the final 3 min of each injection to allow the protection of the MS source from contamination. All valve positions and the instrument parameters were controlled by Xcalibur software, version 2.0.0 (Thermo Fisher Scientific).

Mass spectrometric analysis was performed using a heated electrospray ionization (HESI II) source operating in positive ion mode. The following working conditions spray voltage, 3 kV; vaporizer and capillary temperatures, 280 and 270 °C, sheath and auxiliary gas at 58 and 40 arbitrary units (au), respectively. For the CID-MS/MS analysis , the collision gas was argon used at a pressure of 1.5 collision cell (Q2), and the mass resolution at the first (Q1) and third (Q3) set at 0.7 Da at full width at half-maximum (FWHM). The S-lens rf amplitude as the collision energy (CE) were both optimized individually per compound; S-ranging from 79 to 114 V, whilst CE values ranging 13–27 eV. The multiple

monitoring (MRM) mode, used to identify and assay the analytes, was following two transitions per compound, the first one for quantitative analysis second for structure validation. The formation of ammoniated ions, selected to absolute intensity of the ionized analytes in the MS spectrum represent the choice when glycosides have to be analyzed by mass spectrometry (Hogg A.M., 1972).

In Table 9 reports the parent ammoniated molecular ions of each analyte and the two main fragments obtained by CID on the given precursor species. The CE values reported for each fragment are automatically optimized by the instrument to provide the formation of the most abundant fragment peaks. The considered fragments are typical of the gas phase chemistry of glycosides (Zaia J., 2004).

Analyte	Parent Ion $[M+NH_4]^+$	Product ions quantitative (eV)	Product ions diagnostic (eV)
Sb	m/z 660	m/z 319 (18)	m/z 325 (13)
SV	m/z 822	m/z 319 (23)	m/z 325 (17)
Ra	m/z 984	m/z 319 (27)	m/z 325 (25)
Rc	m/z 968	m/z 309 (23)	m/z 471 (15)

Table 9 Monitored ions and optimized instrumental parameters. Collision energy is reported in elettronvolt (eV).

Instrument control and data processing were carried out by means of Xcalibur software. The total LC–MS/MS method run was 16 min. LC–MS/UV chromatograms were obtained using a Fractionlynx system from Waters (Milford, MA) set-up at analytical mode and equipped with a ZMD mass spectrometer and a 486 UV detector.

The accuracy of the method was determined from samples prepared by adding known quantities of the analytes to samples of soft drinks.

4.2 Olive drupes and leaves

4.2.1 Introduction

This work aims at identifying and assessing active compounds, such as oleuropein, hydroxytyrosol, tyrosol, verbascoside, luteolin and rutin, accumulating in wastes during the simplest debittering processing of olives with plain water. Solutions containing whole, pitted and crushed olives were prepared and target phenols assayed using tandem mass spectrometry. Tandem Mass spectrometry has been extensively used in the field of structure evaluation of natural products.

4.2.2 Materials and methods

Pure samples of rutin, luteolin and verbascoside were purchased from Extrasynthese (Nord B.P 62 69726 Genay Cedex France) and tyrosol, 3-hydroxytyrosol and oleuropein from Sigma–Aldrich (Riedel-de Haën, Laborchemikalien, Seelze). Methanol, ethanol, formic acid were LC/MS grade and purchased from VWR International; aqueous solutions were prepared using ultrapure water, with a resistivity of 18.2 MΩ cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA). microfiltered and osmotized water were obtained from tap water. Microfiltration (MF) was performed by using a bench laboratory plant consisting of a stainless steel feed tank, a magnetic drive gear pump and a stainless-steel cell able to accommodate a flat-sheet polymeric membrane with a surface area of 38.46 cm2. Transmembrane pressure (TMP) was measured by two manometers allocated before and after the membrane cell and regulated by a pressure control valve on the concentrate outlet. Crossflow velocity (CFV) was controlled by a digital flowmeter. Temperature was controlled by using a cooling system fed with tap water and monitored by a digital thermometer inserted in the feed tank. Tap water was microfiltered at a TMP of 0.5 bar and an operating temperature of 25±2 °C.

Reverse osmosis (RO) was performed by using a RO laboratory bench plant consisting of a control panel, a cylindrical jacketed feed tank (with a capacity of 5L) constructed from stainless steel (SS 316), a feed plunger pump with belt drive (Cat Pumps, Milano-Italy, Model 3CP1221), two pressure gauges (Wika, USA) (max pressure 100 bar, absolute error 1 bar), a digital flow meter (SM6000, ifm electronic gmbh, Germany), a thermometer placed inside the feed tank and a cylindrical housing able to accommodate a 11.74x1.75 inches spiral-wound membrane module. The adjustment of operating pressure and feed flow rate was done by simultaneously pump rotation control through a frequency inverter and a needle valve. The operating temperature was controlled by circulating a coolant (cold water) through the tank jacket. The system was operated at a TMP of 6 bar and an operating temperature of 25±2 °C. Characteristics of MF and RO membranes are reported in Table 10.

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Type	MV020 T	SC1812C-34D
Manufacturer	Microdyn-Nadir	GE Water & Process Technologies
Configuration	Flat-sheet	Spiral-wound
Membrane material	Polyvinylidene fluoride (PVDF)	Triple layer thin film of polyamide
Max. operating pressure (bar)	-	41.37
Max. operating temperature (°C)	95	50
Operating pH	2-11	2-10
NaCl rejection (%)	-	99.5
Nominal pore size (□m)	0.2	-
Membrane surface (m^2)	0.003846	0.32
Water flux	>500 L/m^2h	5 L/m^2h

Table 10 Characteristics of MF and RO membranes

4.2.3 Olive drupes and leaves sampling and maceration process

Hand-picked olives of Coratina cultivar, allegedly very rich in antioxidant compounds**,** were collected during the crop year 2013 from an olive grove located in the Basilicata region (Italy) and stored immediately at -25°C. Three different forms of olives, whole, pitted and crushed, were considered for the debittering process. Ultrapure water and ultrapure water-ethanol 80/20 (v/v) were used in two distinct protocols. In a typical experiment, 12 g of olives were placed in stoppered flask containing 100 ml of debittering solution.

Olive leaves from Coratina cultivar were collected in April during the crop year 2016 from the olive grove of the Research Centre for Olive, Citrus and Tree Fruit (CREA-OFA) located in Rende (CS) and immediately processed for the experimental study. Two different forms of olive tissues were prepared: whole and chopped leaves. In a typical experiment, 6 g of olive tissue were placed in stoppered flask containing 50 ml of water.

The maceration process was carried out in the dark at 4°C, except for the first day when the olives were subjected to two thermal shocks caused at first by passing from -25°C to room temperature in the aqueous solutions and then being frozen again. This was to promote the disintegration of the cell membranes and the release of any active compounds. After which, every day for 10 days, aliquots of supernatant were collected. The samples thus prepared were submitted to LC-MS/MS analysis.

Standard stock solutions were prepared by dissolving reference compounds in ethanol. Aliquots of these solutions were further diluted with water/0.1% formic acid to obtain calibration standards at concentrations between 1-200 μg/ml for oleuropein and hydroxytyrosol, 1-100 μg/ml for tyrosol, luteolin and rutin, and 1-150 μg/ml for verbascoside.

4.2.4 Characterization by High performance liquid chromatography (HPLC)

HPLC was performed using an Agilent Technologies 1200 series liquid chromatography system equipped with G1379B degasser, G1312A pump, and G1329A autosampler. The analytes were separated on an Eclipse XDB-C8-A HPLC column [5 μm particle size, 150 mm length and 4.6 mm i.d. (Agilent Technologies, Santa Clara, California)] at a flow rate of 350 μl/min and an injection volume of 10 μl. A binary mobile phase made up of 0.1% aqueous formic acid (A) and methanol (B) was programmed to increase B from 10% to 100% B in 10 minutes, hold for two minutes and ramp down to original composition (90% A and 10% B) in eight minutes. The total elution time was 20 min per injection.

The ESI-MS/MS analyses were performed using a MSD Sciex Applied Biosystem API 4000 Q-Trap mass spectrometer in negative ion mode using multiple reactions monitoring (MRM). The experimental conditions were set-up as follow: ionspray voltage (IS) -4500 V; curtain gas 20 psi; temperature 400°C; ion source gas (1) 35 psi; ion source gas (2) 45 psi; collision gas thickness (CAD) medium. Entrance potential (EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were optimized for each transition monitored.

Calibration curves reported Figure 38. were constructed using a least-squares linear regression analysis. For each analyte the calibration curves were linear in the concentration range 0-150 μg/mL with correlation coefficients ranging between 0.9990 and 0.9997.

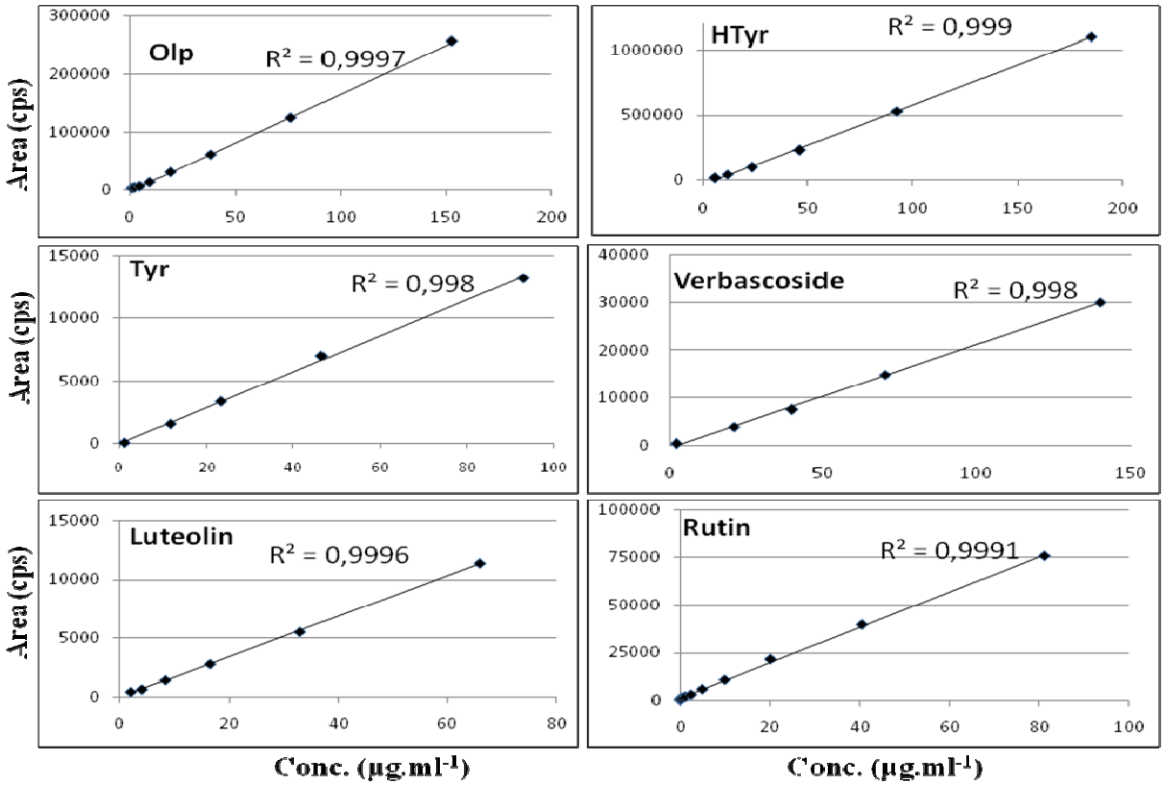

Figure 38 External calibration curve of oleuropein, tyrosol, hydroxytyrosol, rutin, luteolin and verbascoside obtained injecting different concentrations of the mix standard solution.

The MS instrument operating parameters were optimized for each analyte of interest by direct injection of the analyzed solution. The CID-MS/MS spectra of the deprotonated molecular ion $[M-H]^{-1}$ are shown in Figure 39. MRM analyses were carried out by monitoring the transition reported in Table 11.

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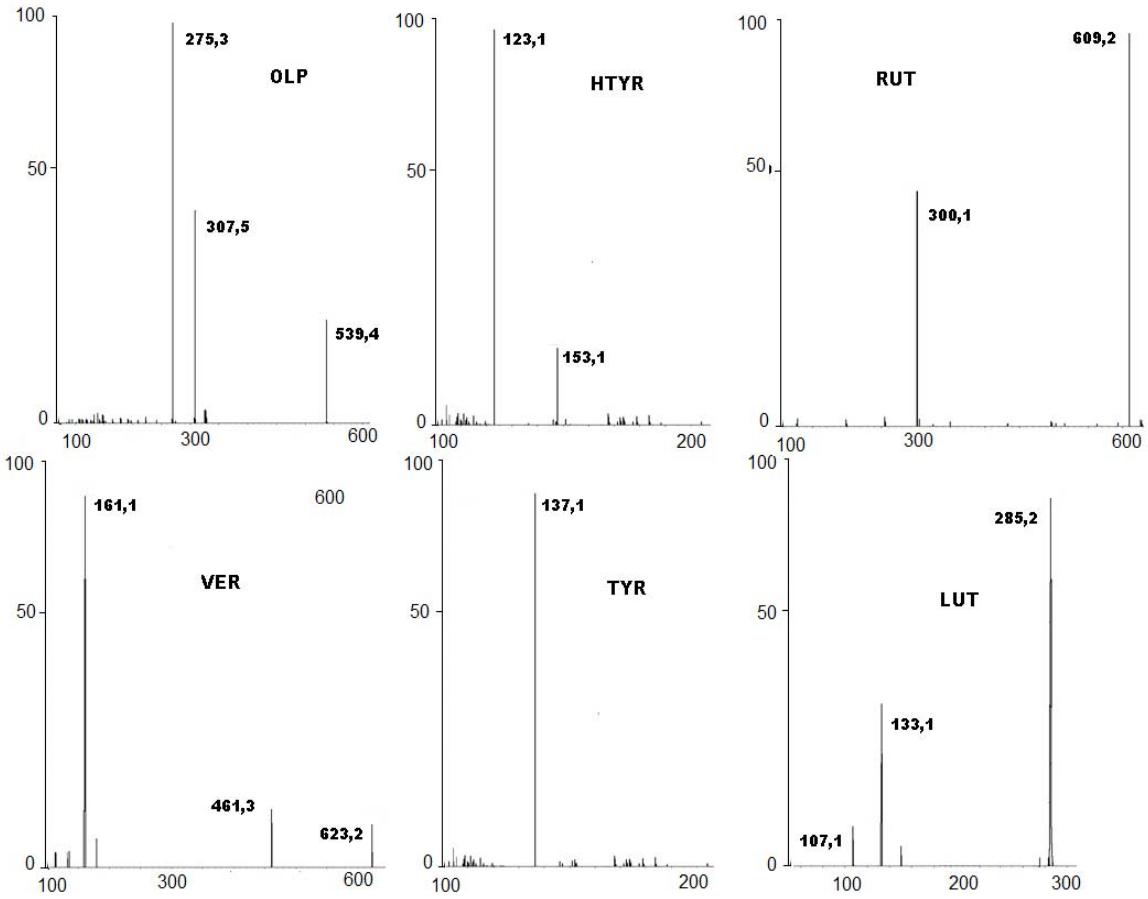

Figure 39 ESI-MS/MS spectra of oleuropein, hydroxytyrosol, tyrosol, verbascoside, luteolin and rutin showing the deprotonated molecular ion $[M-H]^-$ and the major fragments utilised to set up the MRM method.

A SIM-like analysis was considered for tyrosol (F) since its product ion spectrum under the experimental conditions adopted for the other analytes did not produce any important fragments. The MRM measurements were, therefore, carried out by modulating the first quadrupole scanning for the transmission of the (M-H)- value of the six analytes and scanning the third analyzer according to Table 11. A MRM chromatogram obtained by injecting 10 µl of a standard mixture of the compounds under investigation at 25 µg/ml is shown in Figure 40.

Analyte	1st quadrupole $(M-H)^-$	2nd quadrupole	EP	DP	CE	CXP
(A) Hydroxytyrosol	*m/z* 153	*m/z* 123	-55	-7	-25	-5
(B) Luteolin	*m/z 285*	*m/z* 133	-70	-8	-35	-8
(C) Oleuropein	*m/z* 539	*m/z* 307 *m/z* 275	-65	-10	-30	-5
(D) Rutin	*m/z* 609	*m/z* 301	-65	-8	-45	-10
(E) Verbascoside	*m/z* 623	*m/z* 161 *m/z* 461	-70	-8	-35	-8
(F) Tyrosol	*m/z* 137	No scan	-45	-8	-25	-5

Table 11 Data set for the assay of active principles A-F. Optimized entrance potential(EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP)

Recovery tests were carried out injecting three times two standard solutions at different concentration of the six active molecules. Results from recovery experiments at levels of 25 and 50 μg/ml gave mean recoveries ranging from 91–116% with satisfactory precision (relative standard deviation (RSD) from 0.1-0.5%). Limits of quantitation (LOQs) from 1.258-2.847 μg/ml (Table 12).

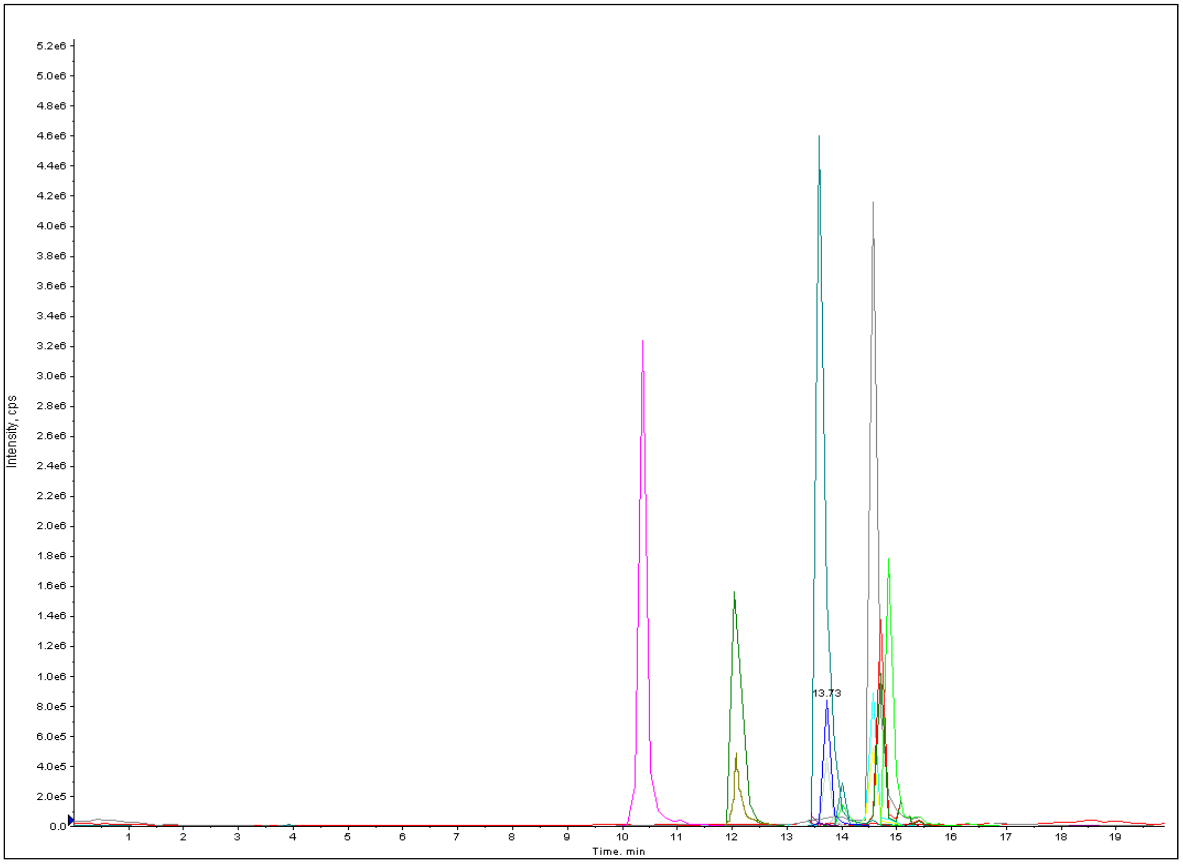

Figure 40 MRM chromatogram obtained injecting 10 µl of a standard mixture of the analytes of interest at 25 µg/ml.

			Spiked solutions		
Active compound	LOD (µg/ml)	(a) Found mean ± RSD[a]	(a) Recovery (%)	(b) Found mean ± RSDa	(b) Recovery (%)
Oleuropein	1.258	58.0 ± 0.1	116	25.3 ± 0.2	101
Hydroxytyrosol	1.479	52.5 ± 0.1	105	24.5 ± 0.3	98
Tyrosol	2.418	49.5 ± 0.2	99	26.0 ± 0.3	104
Verbascoside	1.215	45.5 ± 0.5	91	27.3 ± 0.1	109
Luteolin	1.658	50.5 ± 0.3	101	24.8 ± 0.2	99
Rutin	2.847	51.5 ± 0.1	103	22.5 ± 0.1	90

[a] Each value is the mean of three determinations, RSD = relative standard deviation.
Spiked solutions (a) 50 µg/ml; (b) 25 µg/ml.

Table 12 LOD, recoveries and precision (RSD) of the active compounds analysed by LC-ESI-MS/MS.

4.3 Algal Oil

4.3.1 Introduction

The information on complete lipid characterization of extracts is essential for selection of the successful extraction process most useful for production of biofuels and for develop of potentials nutraceutical.

4.3.2 Materials and methods

Solvents, reagents and thimbles were purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO), while Microalgae from Aquafauna Biomarine inc. (P.O. Box 5, Hawthorne, California, 90250 USA).

Five g of dried algae were placed in a thimble of the Soxhlet apparatus (Figure 41) The thimble consists of a thick filter paper, which is loaded into the main chamber of the Soxhlet extractor, placed on a balloon containing extraction solvent. The Soxhlet is equipped with a condenser and the solvent is heated to reflux. The solvent forms vapors, which travels up a distillation arm, and floods into the chamber housing the thimble containing the sample. A condenser ensures that any solvent vapor that cools drips down into the chamber that slowly fills with warm solvent. When the Soxhlet chamber is almost full, is automatically emptied by the siphon side arm, with solvent running back to the distillation flask. This cycle was repeated several times to ensure an adequate production of oil into the distillation flask. After solvent removal, the oil obtained was treated for fatty acid analysis. The insoluble portion of the algae remains in the thimble. The same process was repeated with different solvent mixtures; hexane 100%, hexane/ethanol (80:20, 60:40, 50:50, 40:60, 20:80) and ethanol 100%.

A supercritical CO_2 extraction was performed to compare with sohlet extraction method. 5 g of dried algae were extracted with a flow of 2 g / min for 1 hour. The oven temperature used is 50 ° C and the vessel temperature is 120 ° C. The vessel used measures 25.4 cm x 1.27 cm.

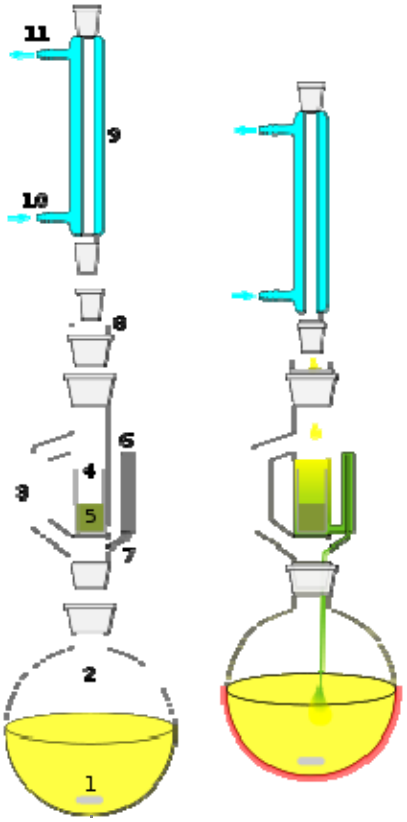

Figure 41 Soxhlet apparatus: 1. Stirred bar; 2. Distillation flask; 3. Distillation arm; 4. Thimble; 5. Dried algae; 6. Siphon top; 7. Siphon exit; 8. Expansion adapter; 9. Condenser; 10. Cooling water in; 11. Cooling water out.

Fatty acids compositions were determined by their conversion to methyl esters. Fifteen mg of each oil was added to the internal standard (250 ng/ 100 µl chloroform, methyl tricosanoate, 23:0). The transmethylation reagent used was prepared with 6 ml of concentrated sulfuric acid, 100 mL of methanol and 15 mg of hydroquinone (as antioxidant). The mixed was incubated for 12 h at 60°C and subsequently cooled. 1 mL of distilled water was added to each vial and extracted 3 times with 1.5 mL of hexane. Some crystals of hydroquinone was added prior the extraction with hexane. The fraction extracted with hexane were collected and washed 2 times with distilled water. In the first

wash, the aqueous layer was removed and in the second wash the hexane layer was separated and evaporated under a stream of nitrogen.

The GC-MS analysis for FAME's was performed on Shimadzu GC/MS-QP 2010 gas chromatography instrument with autosampler AOC-20i (Shimadzu). A capillary column DB- 5ms with dimension of 30m*0.25mm i.d. 0.25 μm film thickness was used for the separation of fatty acids methyl esters. The temperature program was as follows: from 70 to 135 °C with 2°C min−1, hold for 10 min, from 135 to 220 °C with 4 °C min−1, hold for 10 min, from 220 to 270 °C with 3.5 °C min−1and then hold for 20 min. The injector and detector temperature were 280°C and 290°C respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range 50-500 m/z. Peak identification of the fatty acids in the analyzed microalgae oil samples was carried out by the comparison with retention time and mass spectra of know standard. Samples were analyzed in triplicate.

4.3.3 Flow injection analysis/mass spectrometry (FIA/MS)

The mass spectrometer system used for the qualitative analysis of the algal oil extracts was a Q-Trap API 4000 (MSD Sciex Applied Biosystem). The instrument parameters were set as follow: ionspray voltage (IS) -4600 V; curtain gas 10 psi; ion source gas 12 psi; collision gas thickness medium; entrance potential 10 eV, declustering potential 70 eV, collision energy (CE) between 15 and 30 eV and collision exit potential (CXP) between 5 and 9 eV.

Table 13 lists the deprotonated molecules $[M-H]^-$ with their corresponding fragments and parent ions presents in an algae extract monitored. Ions included in the range of m/z 455-

556 are not associated with free fatty acids, or diglycerides, or triglycerides.

ANALYTE	$[M-H]^-$	PIS	PREC
Lauric acid	199.4	181.6; 155.0	399.7; 455.9
Myristic acid	227.4	209.5; 183.3	455.7; 483.8; 509.8
Myristoleic acid	225.2	207.2; 181.5	482
Pentadecylic acid	241.4	223.2; 197.2	483.8
Palmitic acid	255.5	237.6; 211.6	511.8; 537.9; 583.9
γ-linolenic acid	277.4	259.4; 233.4	555.8
Oleic acid	281.6	263.4; 237.4	509.8; 537.9; 563.8
Stearic acid	283.5	265.4; 239.2	540.5; 568
Eicosapentanoic acid	301.6	283.6; 257.6	602.2
Docosaexaenoic acid	327.5	283.6; 309.2; 229.6	583.9; 610.0; 656.0; 658.0
Docosapentaenoic acid	329.7	311.7; 285.6	658
Behenic acid	339.6	321.3; 294.9	596.5; 569.2

Table 13 List of deprotonated molecules identified in full scan MS spectra of algal oil sample, fragment ion identified in MS/MS spectra and precursor ion identified in precursor ion spectra.

For each analyte considered, ions with highest intensity are given in the Table 14. From Table 14, it is clear that the algal extract contains adducts between two identical and different fatty acid molecules.

Negative ion	Fatty acid composition
455.8	2*myristic
483.9	2* Pentadecylic
511.9	2*palmitic
555.8	2*γ-linolenic
563.8	2*oleic
583.9	DHA+palmitic
610.0	DHA+oleic
656.0	2*DHA
658.0	DHA+DPA

Table 14 Molecular composition of adduct with negative molecular ion between m/z 455 and m/z 656.

A solution containing DHA, stearic acid and butyric acid was analyzed using the same method. All the spectra are shown in Appendix A.

To better understand what the reaction site was in the saturated and unsaturated fatty acid adducts, a mixture containing oleic acid and palmitic acid was analysed using the method describe before. All the spectra are shown in Appendix A.

4.4 References

Hogg A.M., Nagabhushan T.L.. "Chemical ionization mass spectra of sugars" *Tetrahedron Letters 13*.47 (1972): 4827-4830.

Sindona G.. "Identification of fraudolently modified foods" *In Detection of Chemical, Biological, Radiological and Nuclear Agents for the Prevention of Terrorism* (2014) (p. 207-222). Dordrecht: Springer.

Zaia J. "Mass spectrometry of oligosaccharides" *Mass spectrometry reviews 23*.3 (2004): 161-227.

Conclusions

This thesis proposes new, simple and sustainable methods for the extraction of bioactive compounds from food waste. Green extraction techniques are proposed considering the 12 principles of Green Chemistry.

The main objectives achieved are:

1. Quality and safety of Stevia-based functional foods can be properly controlled by the application of validated MS protocols proposed here.
2. Identification and characterization of new adducts of satured and unsatured fatty acids present in algal oil by the use mass spectrometry.
3. Application of eco-friendly chemical procedures to isolate the drugs from olive waste. The use of modern analytical methods of analysis, such as those based on MS/MS procedures, is important in providing information on the identification assay and isolation of the active principles.

Annessex A: Mass Spectra of algal oil

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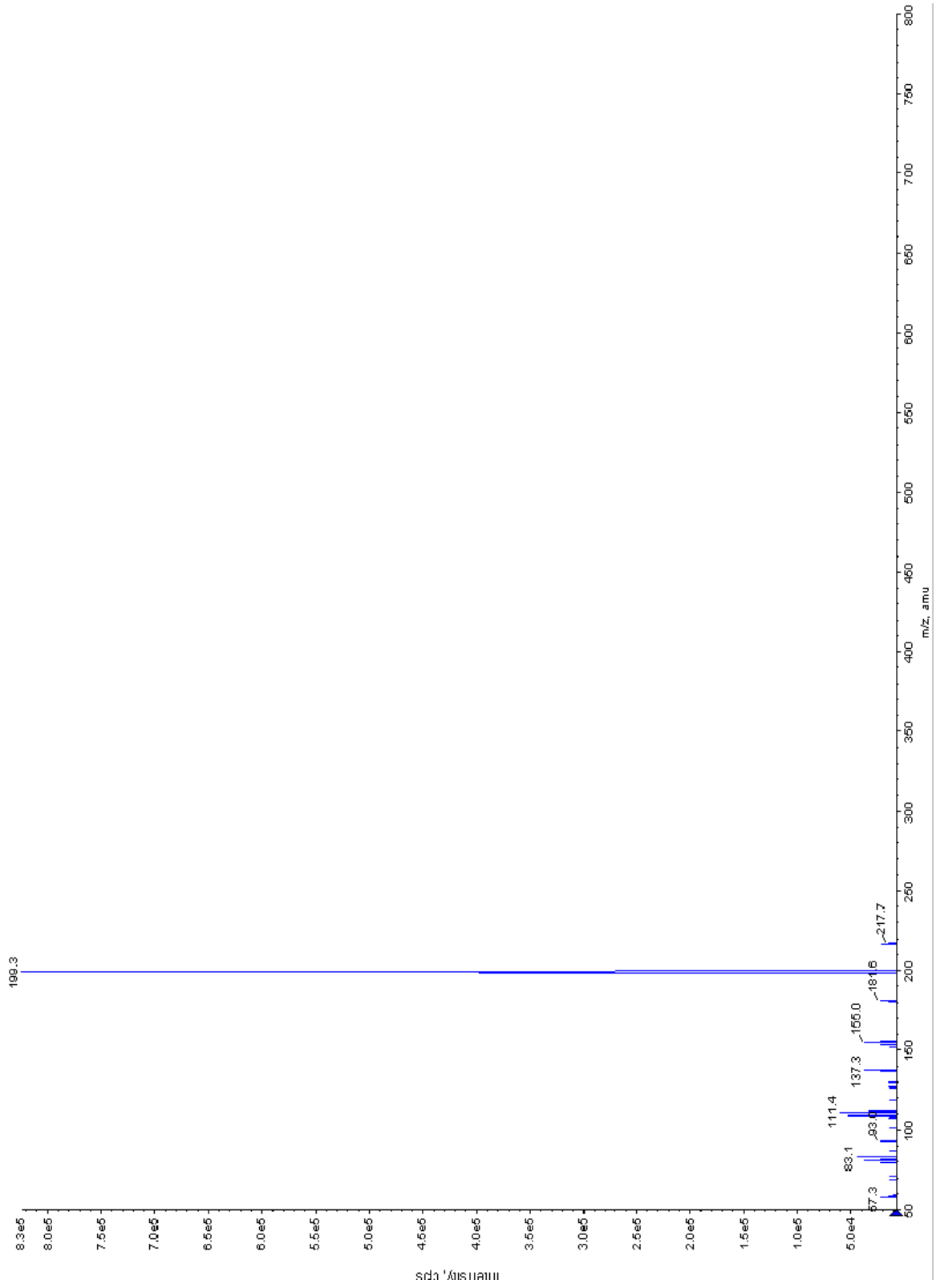

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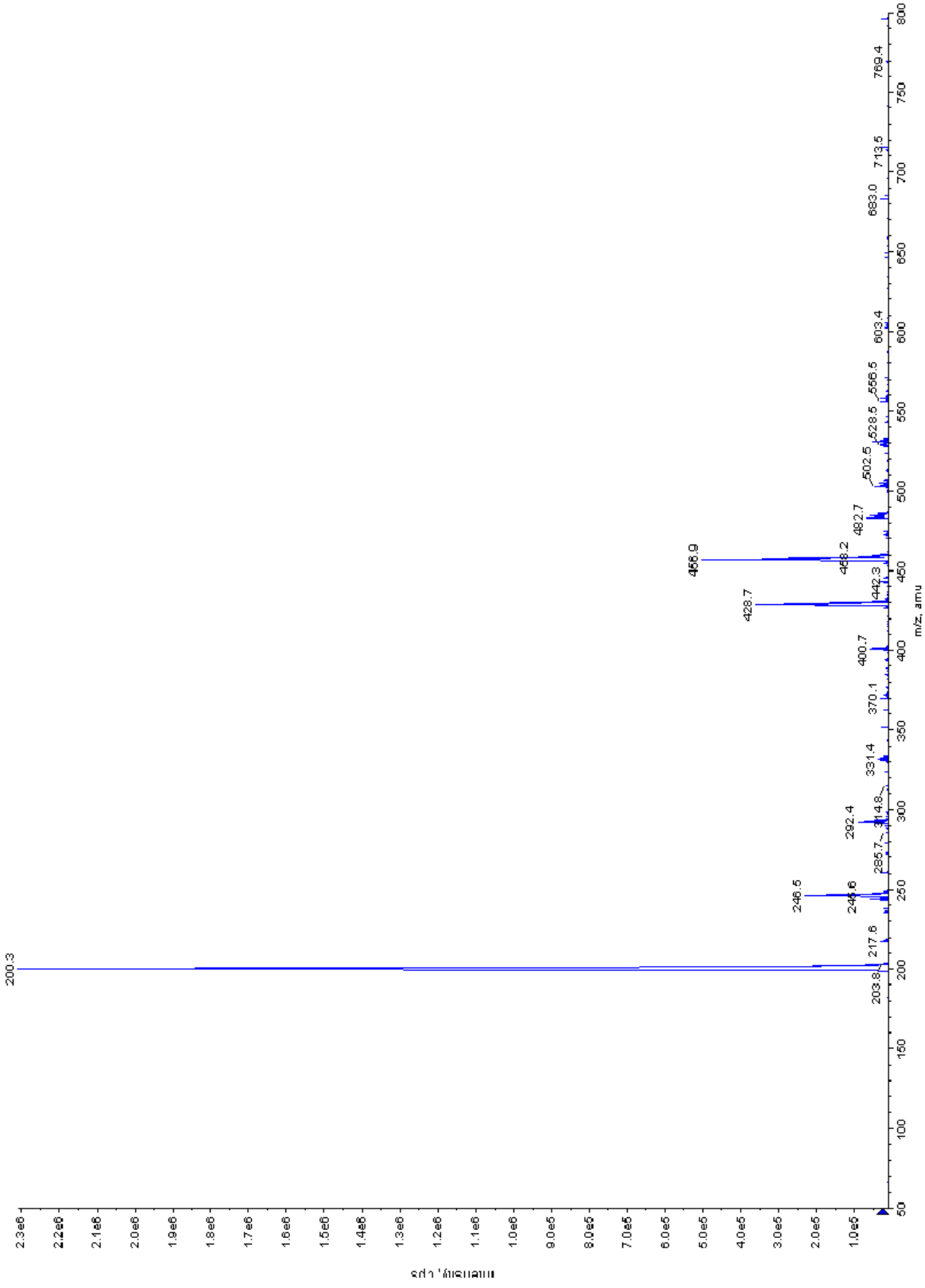

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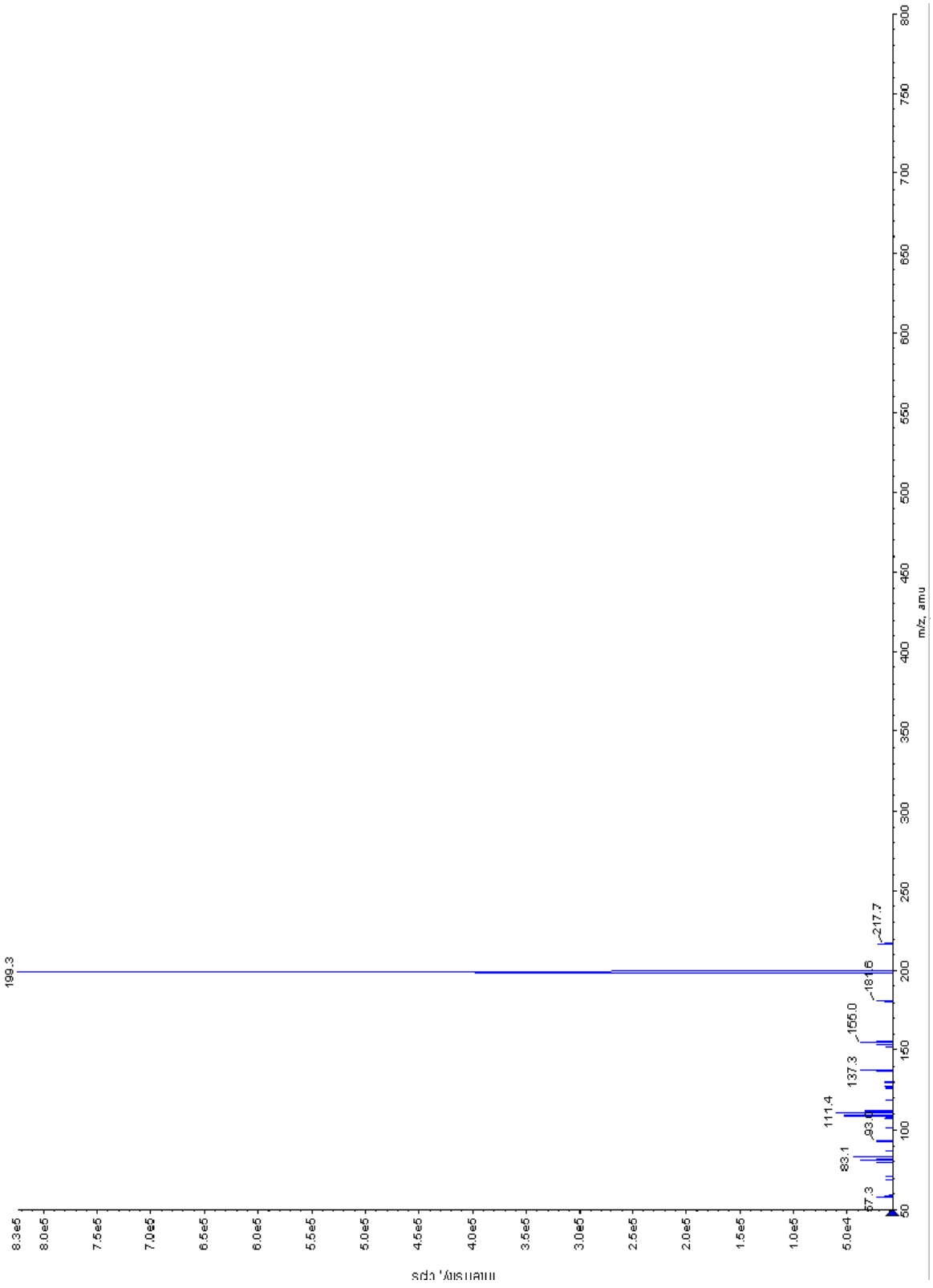

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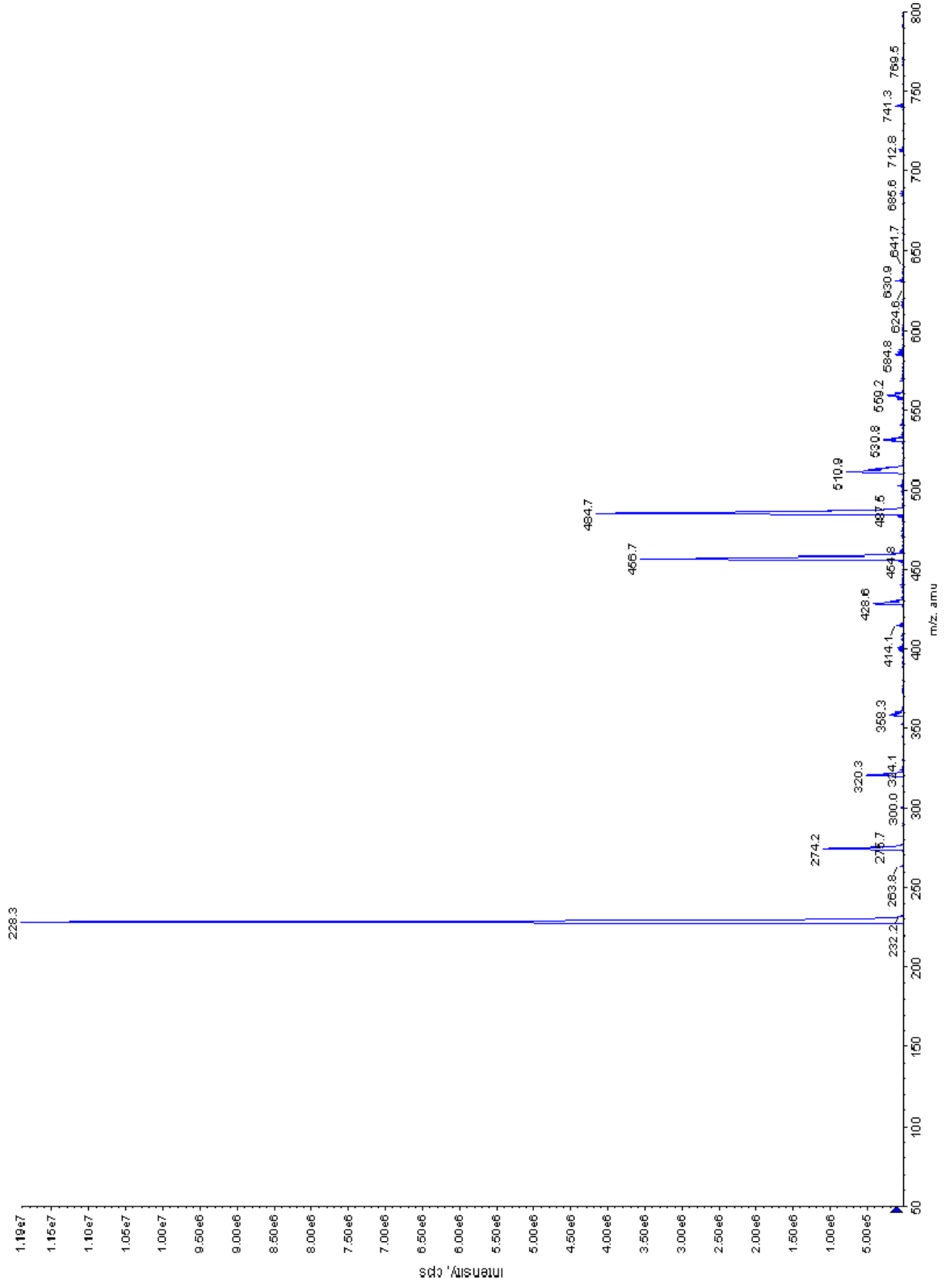

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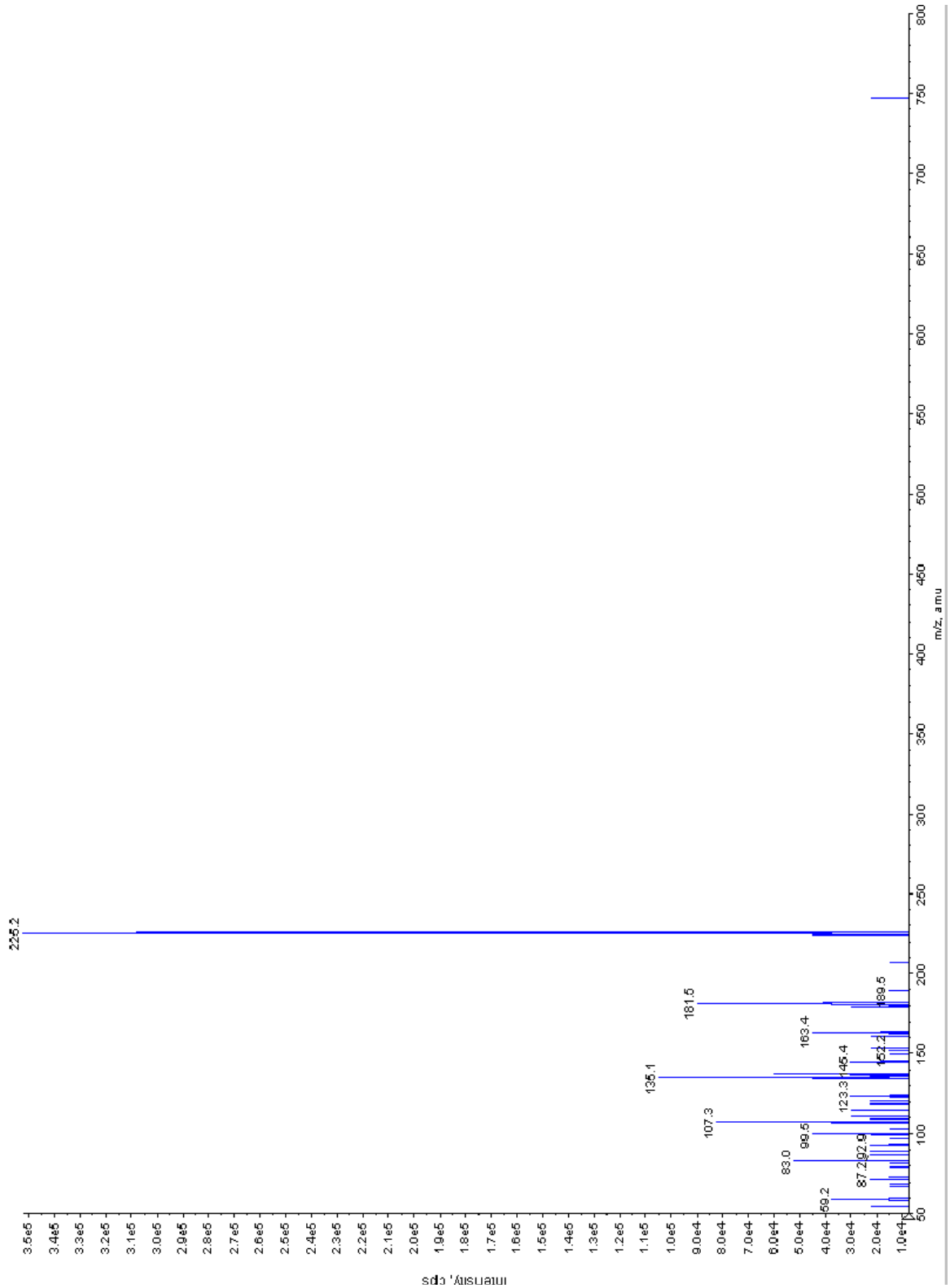

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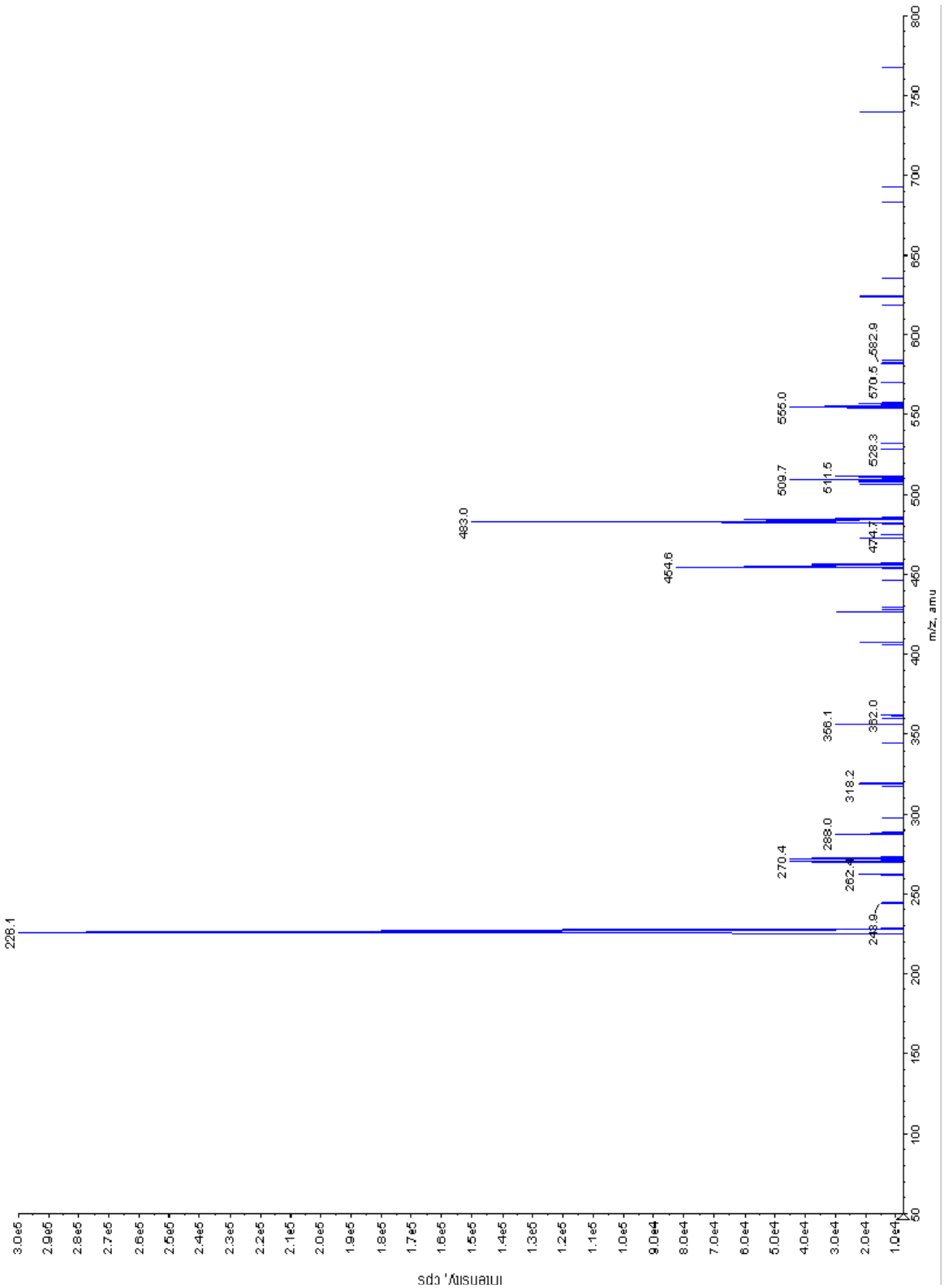

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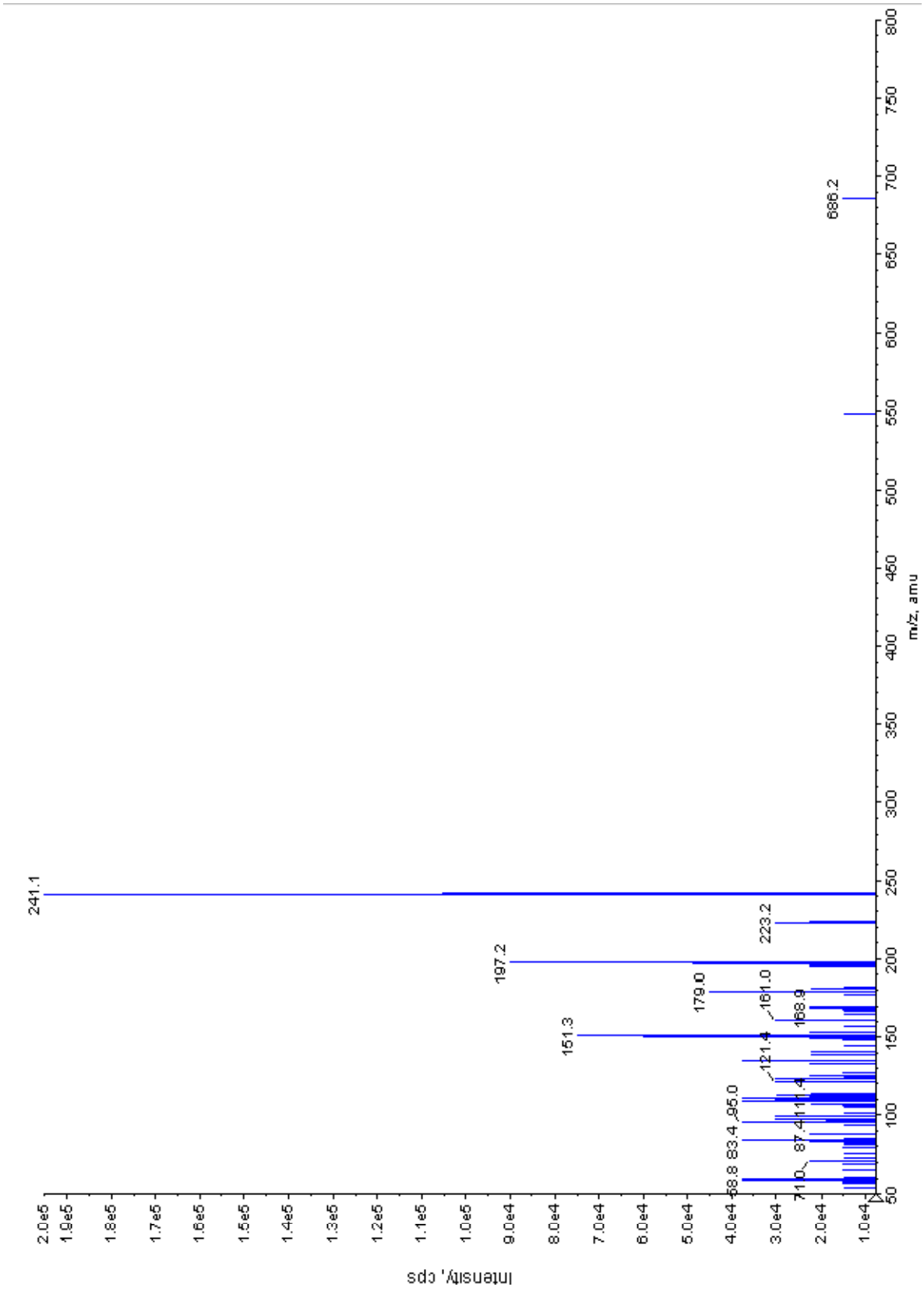

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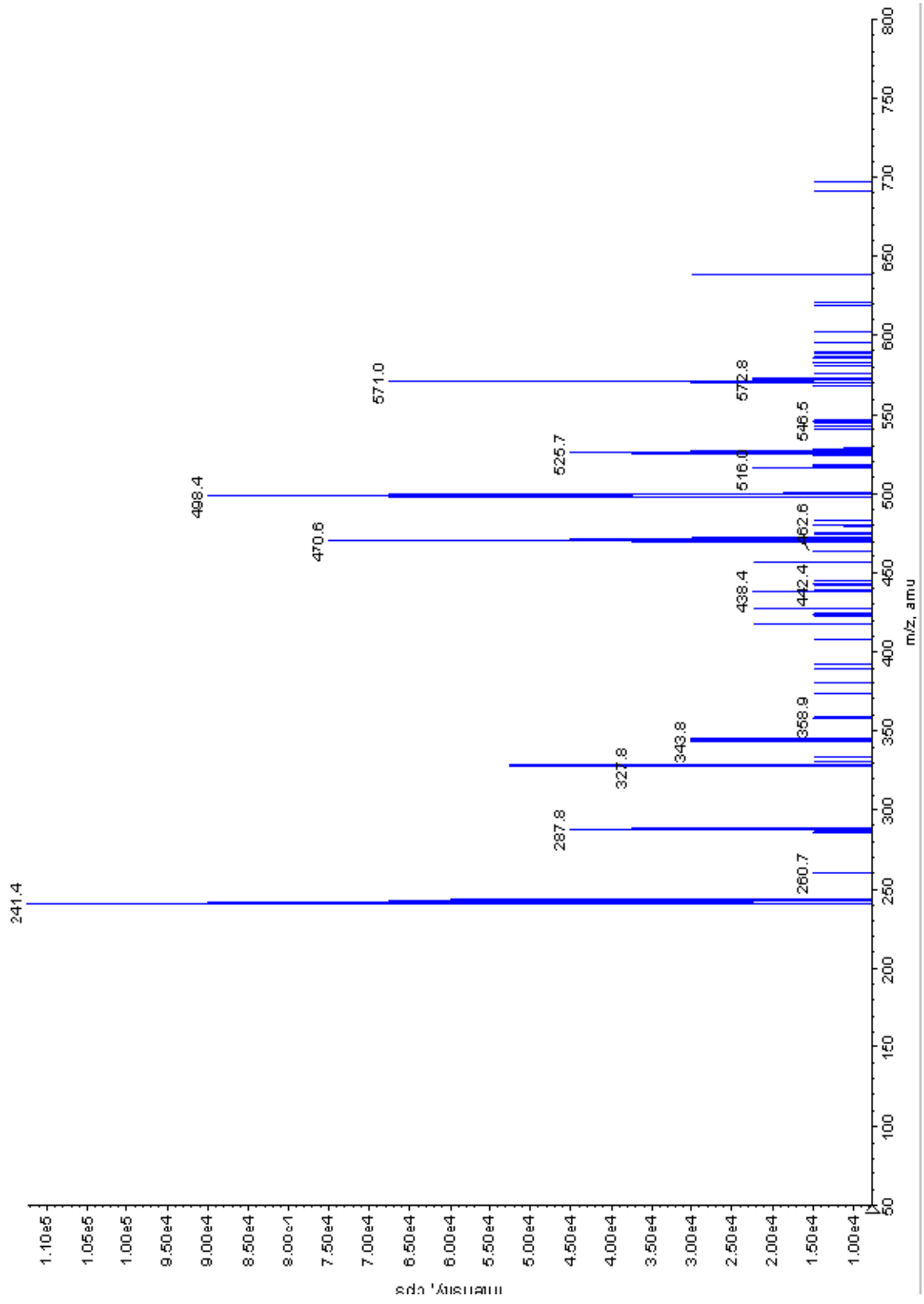

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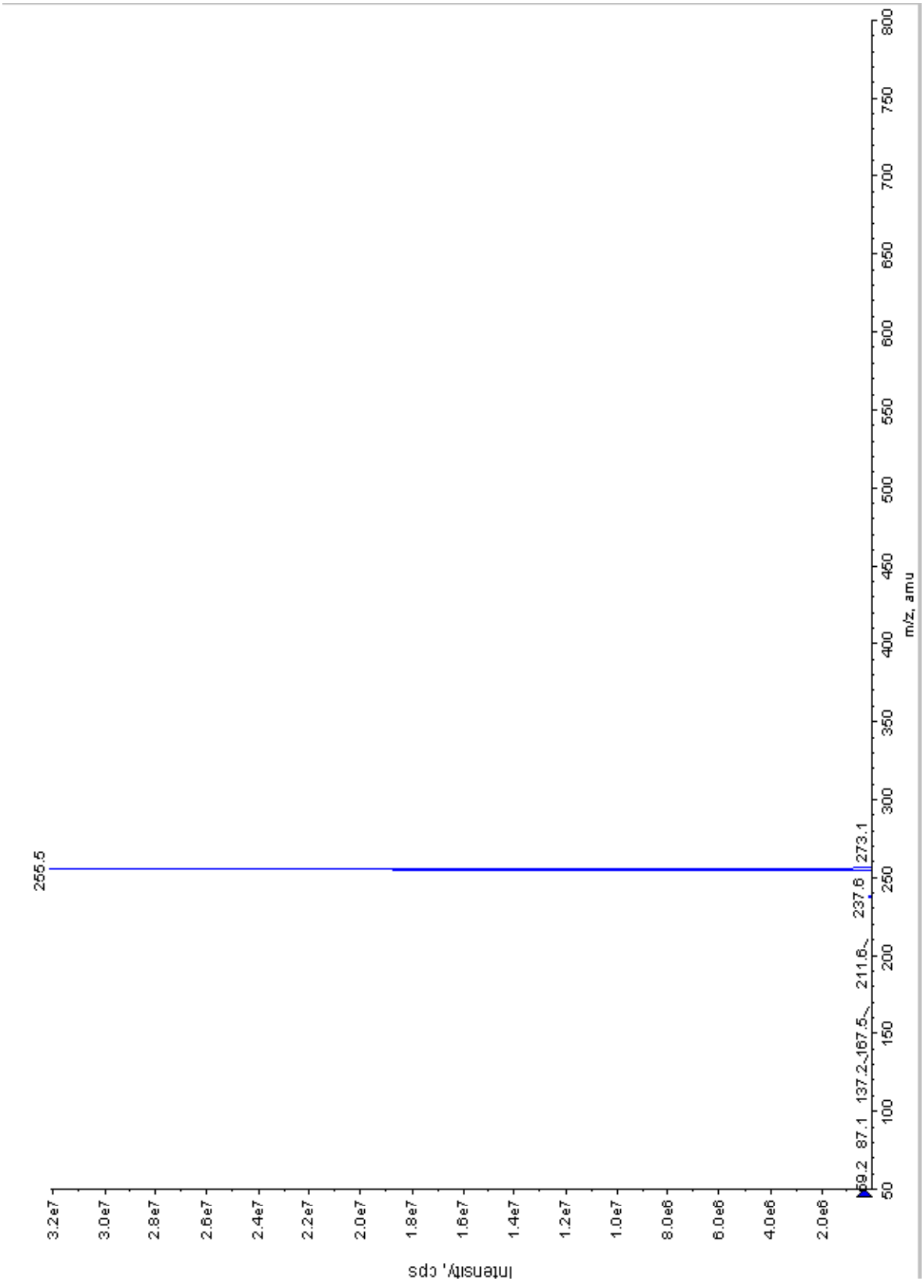

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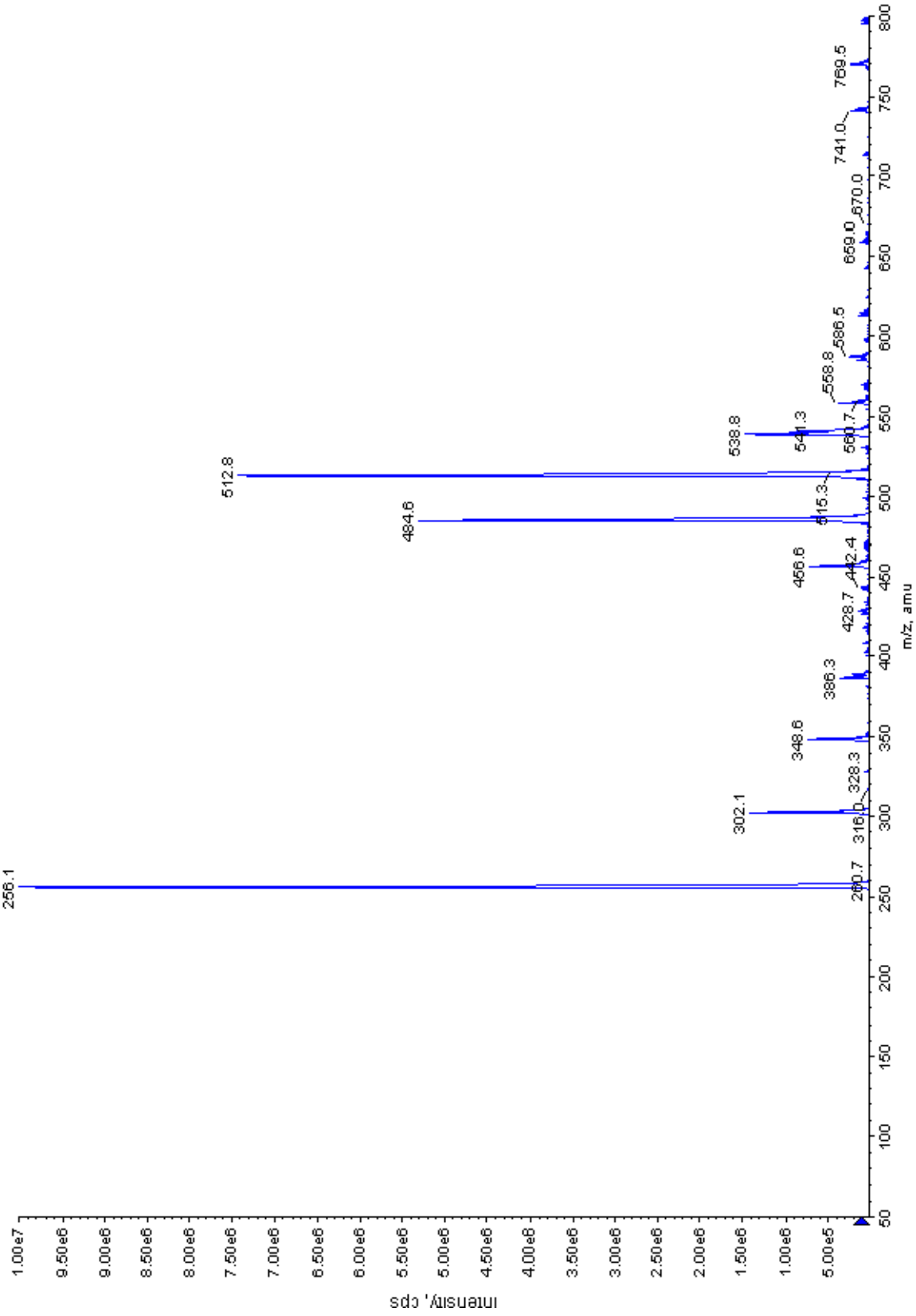

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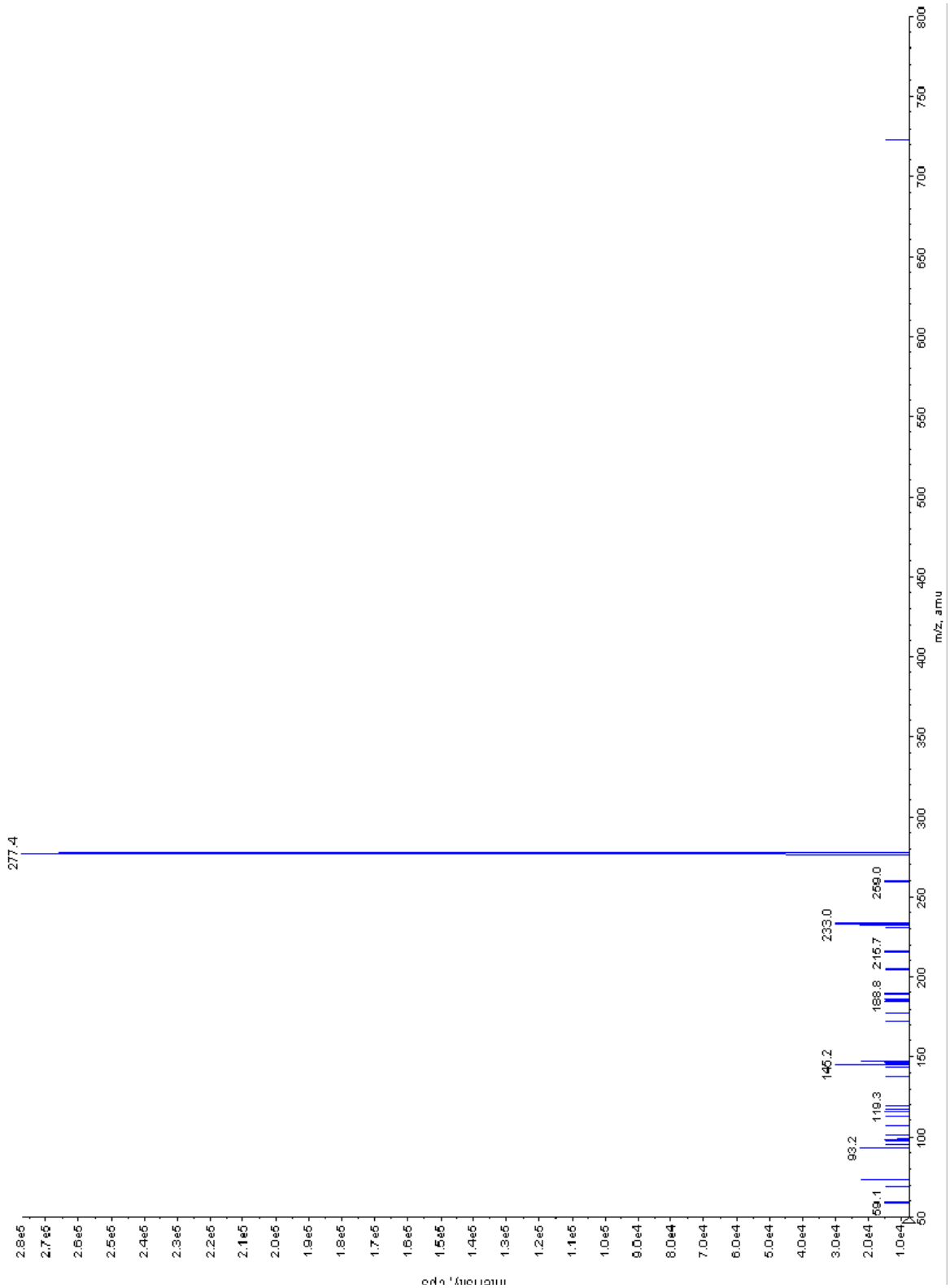

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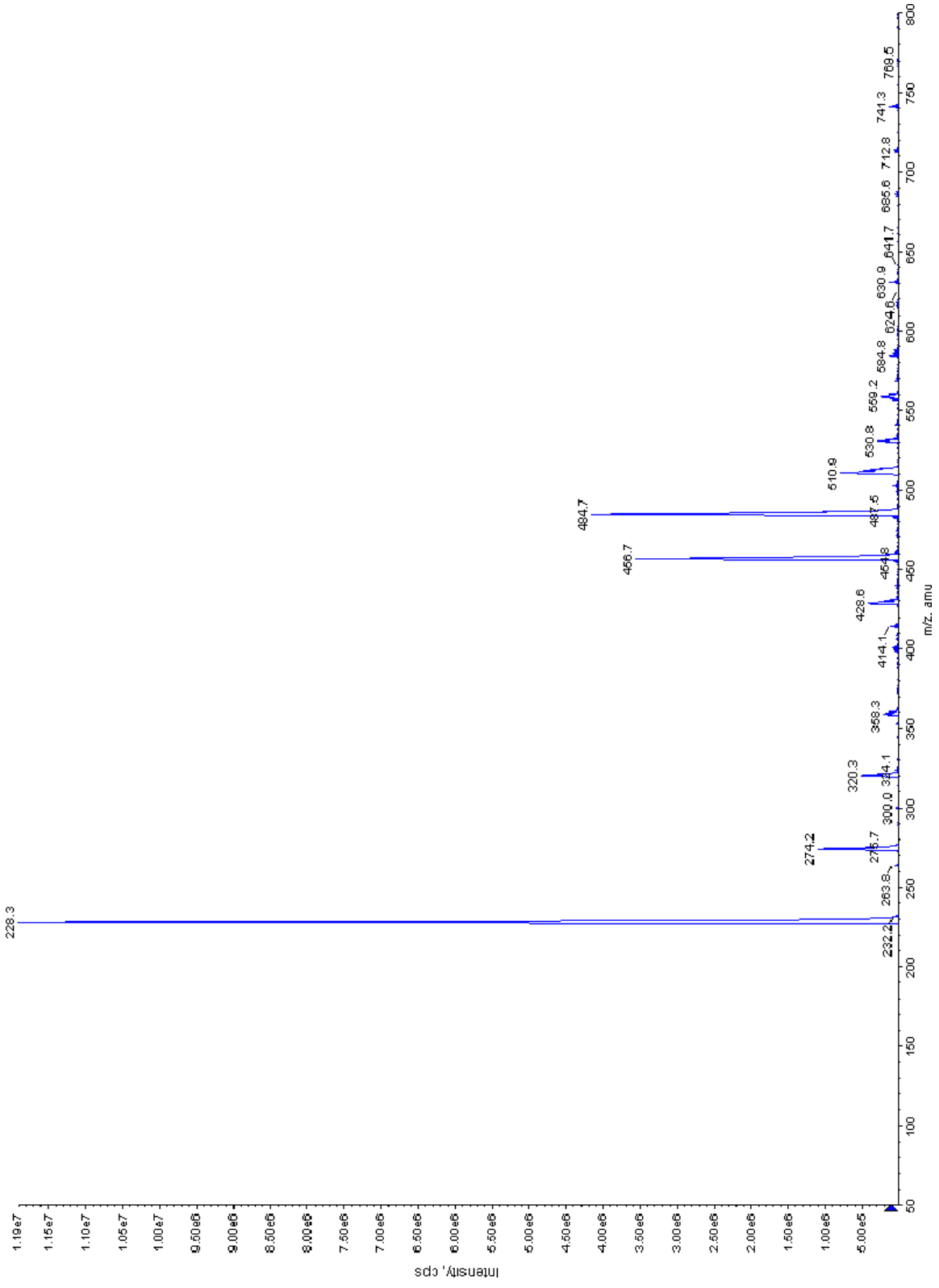

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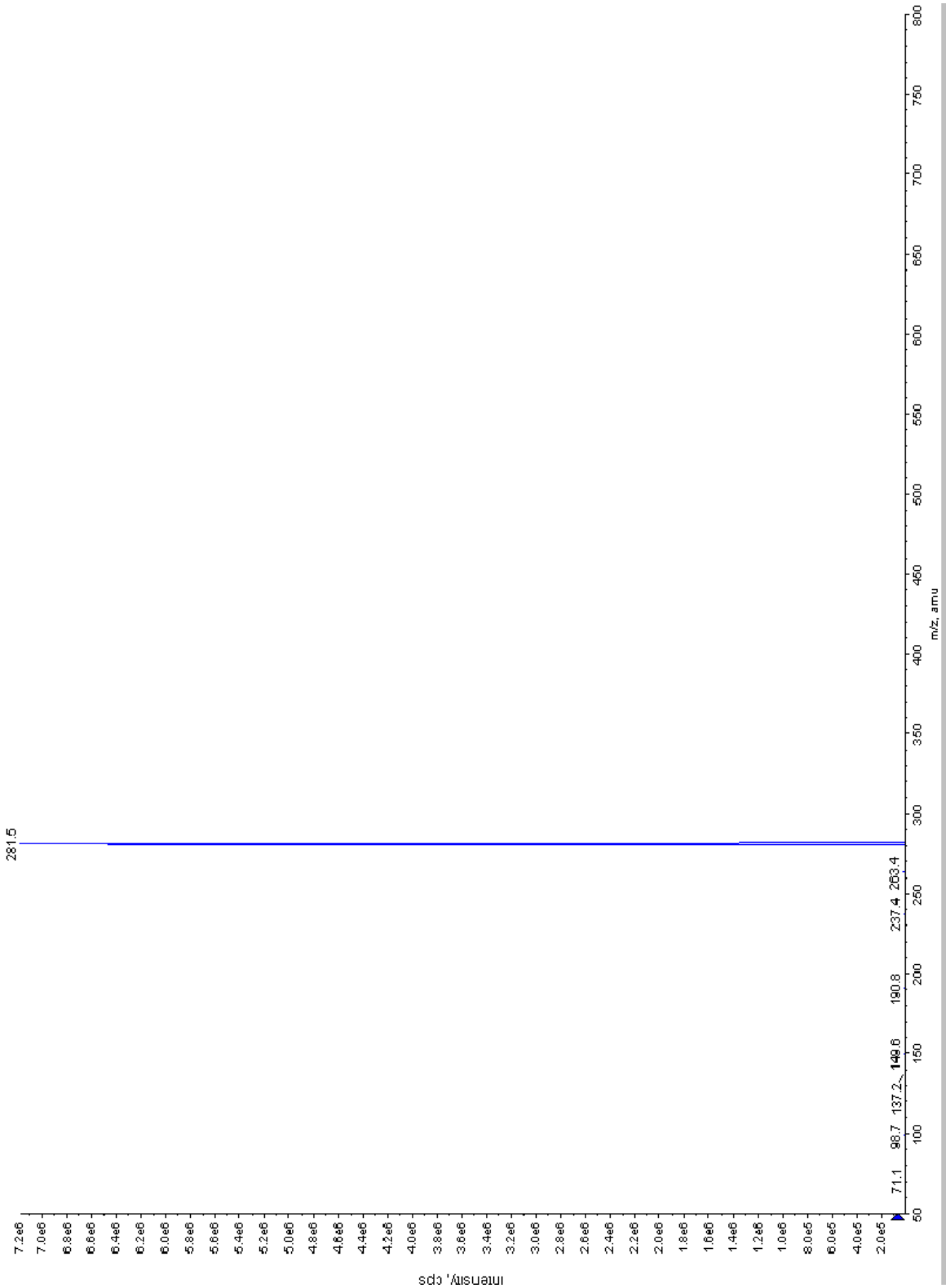

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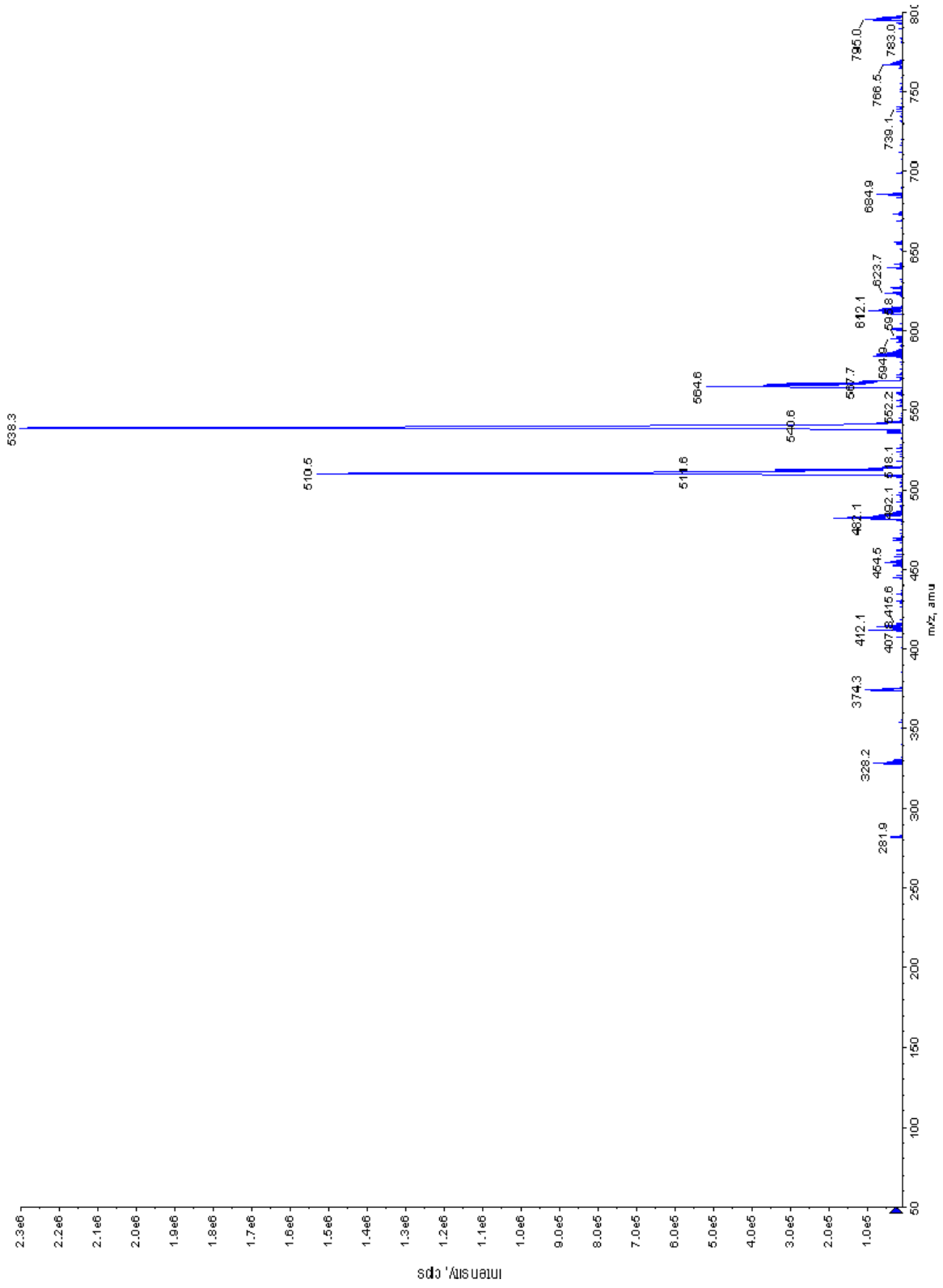

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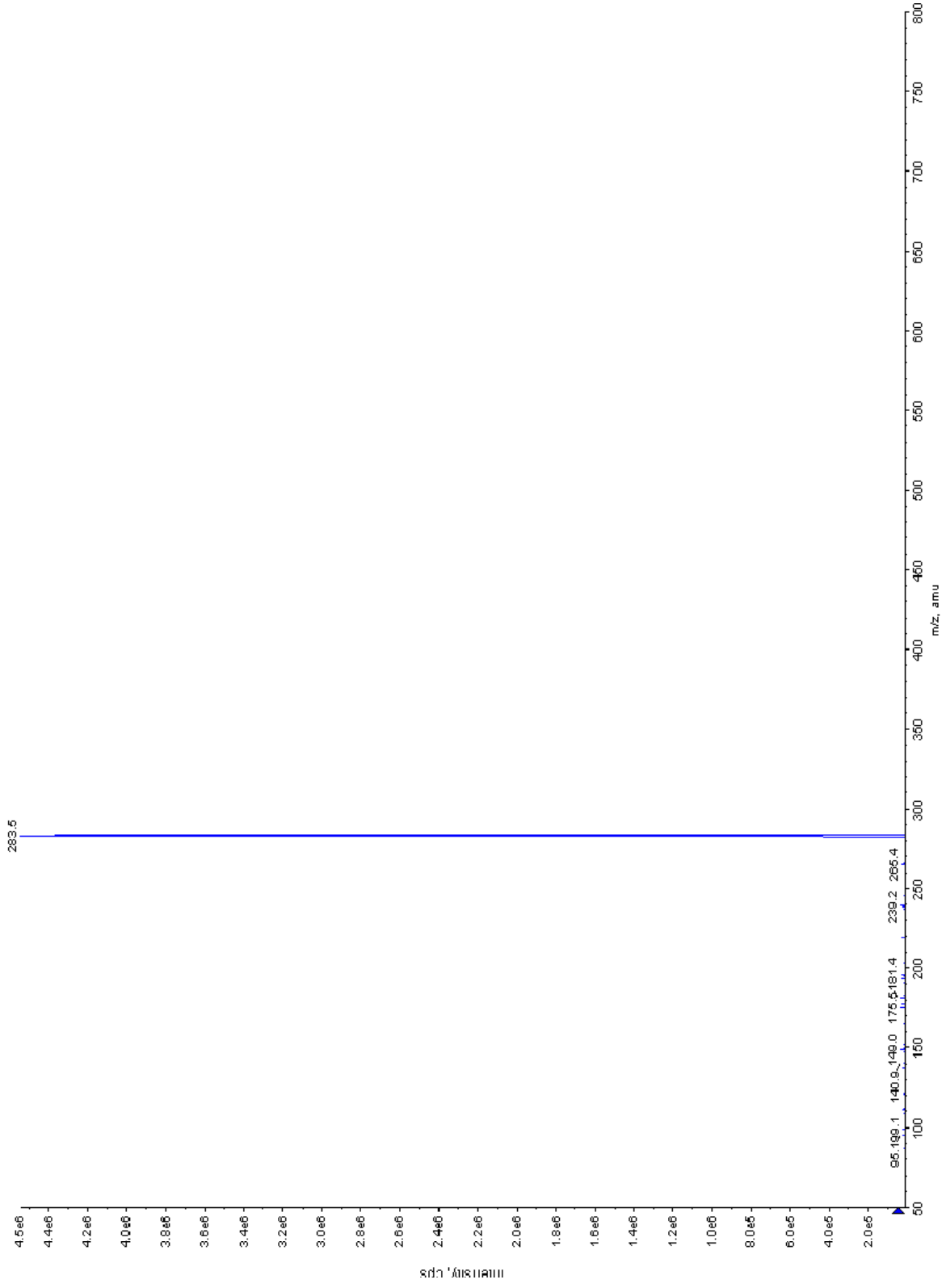

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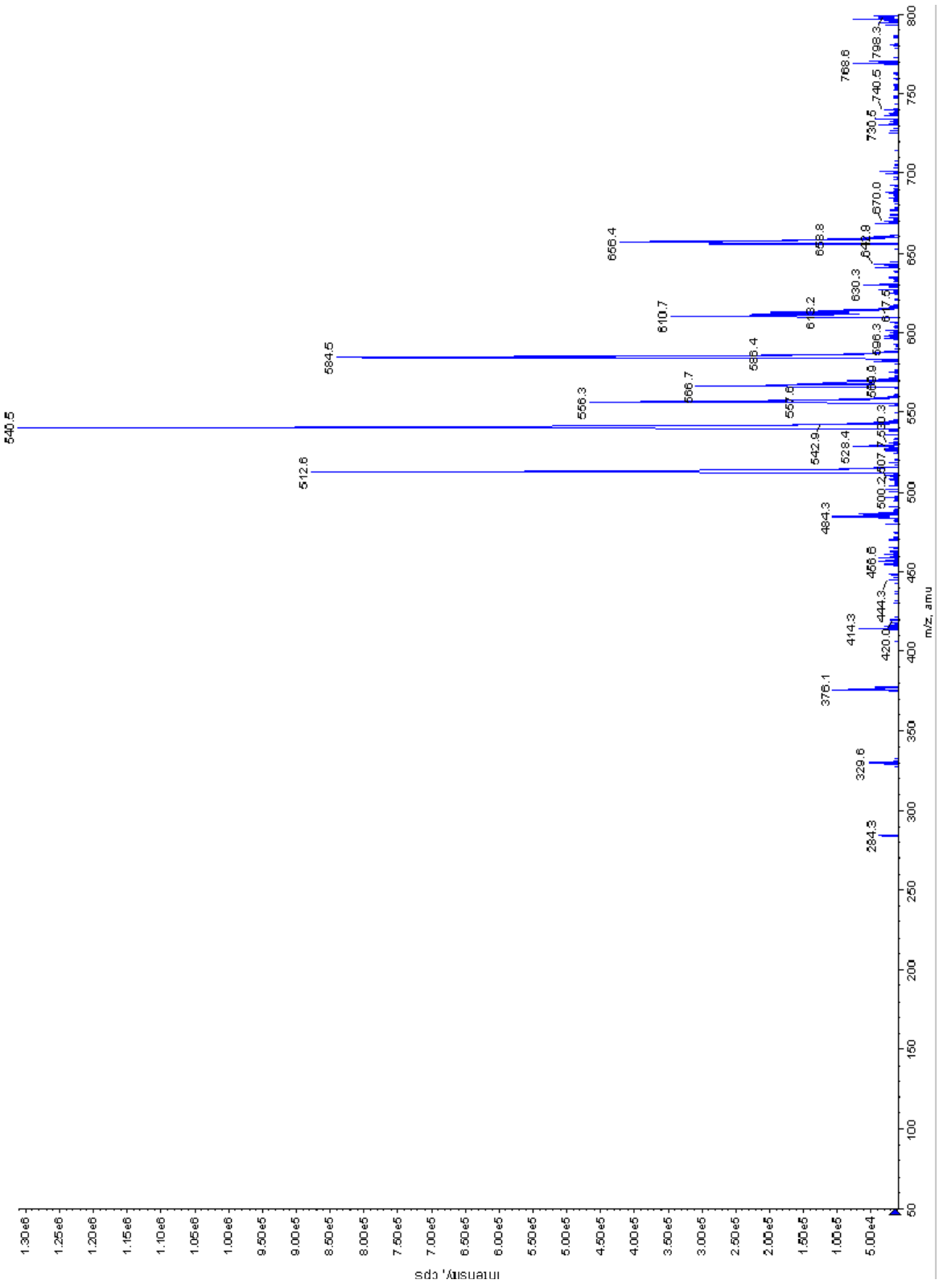

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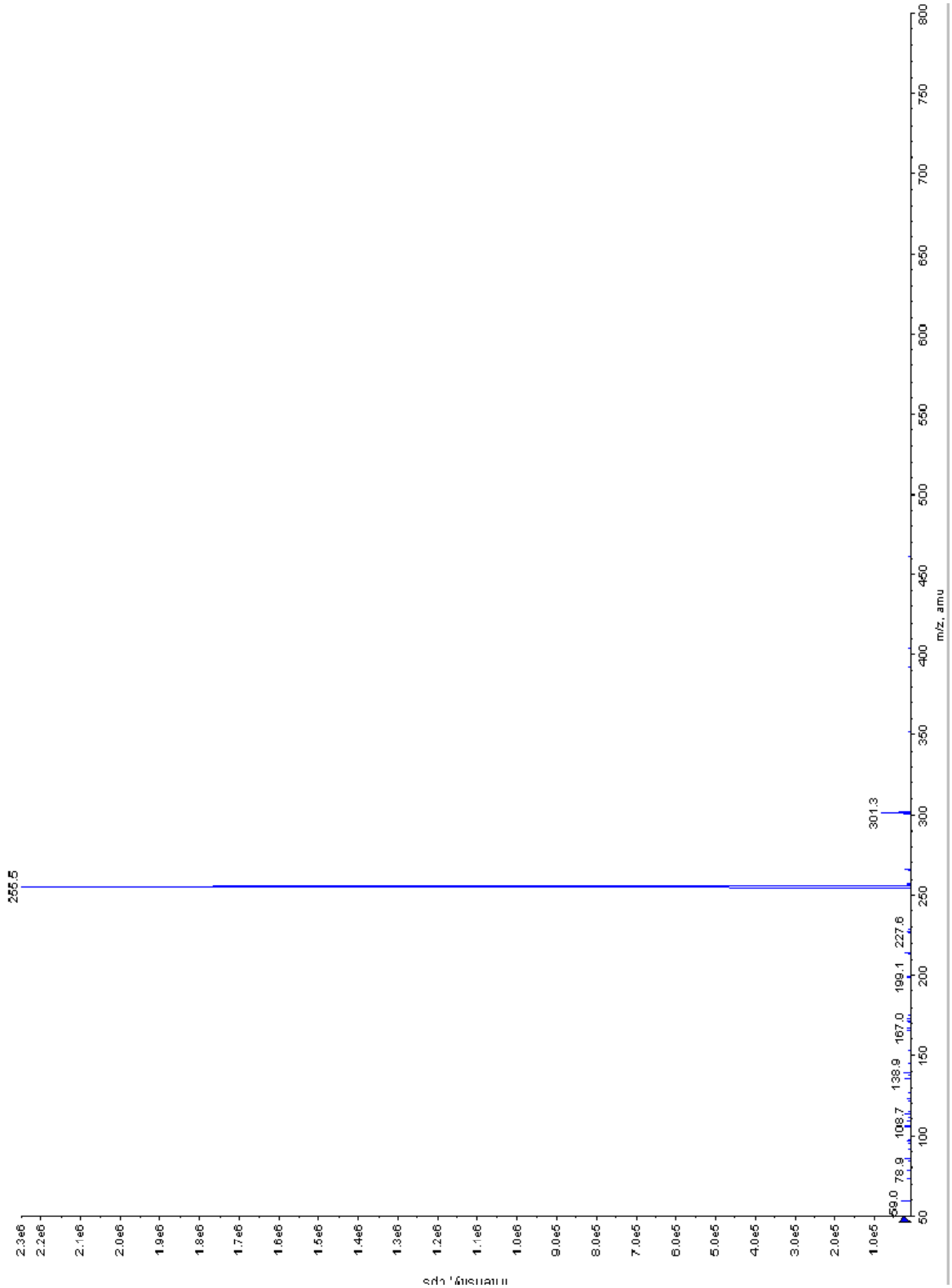

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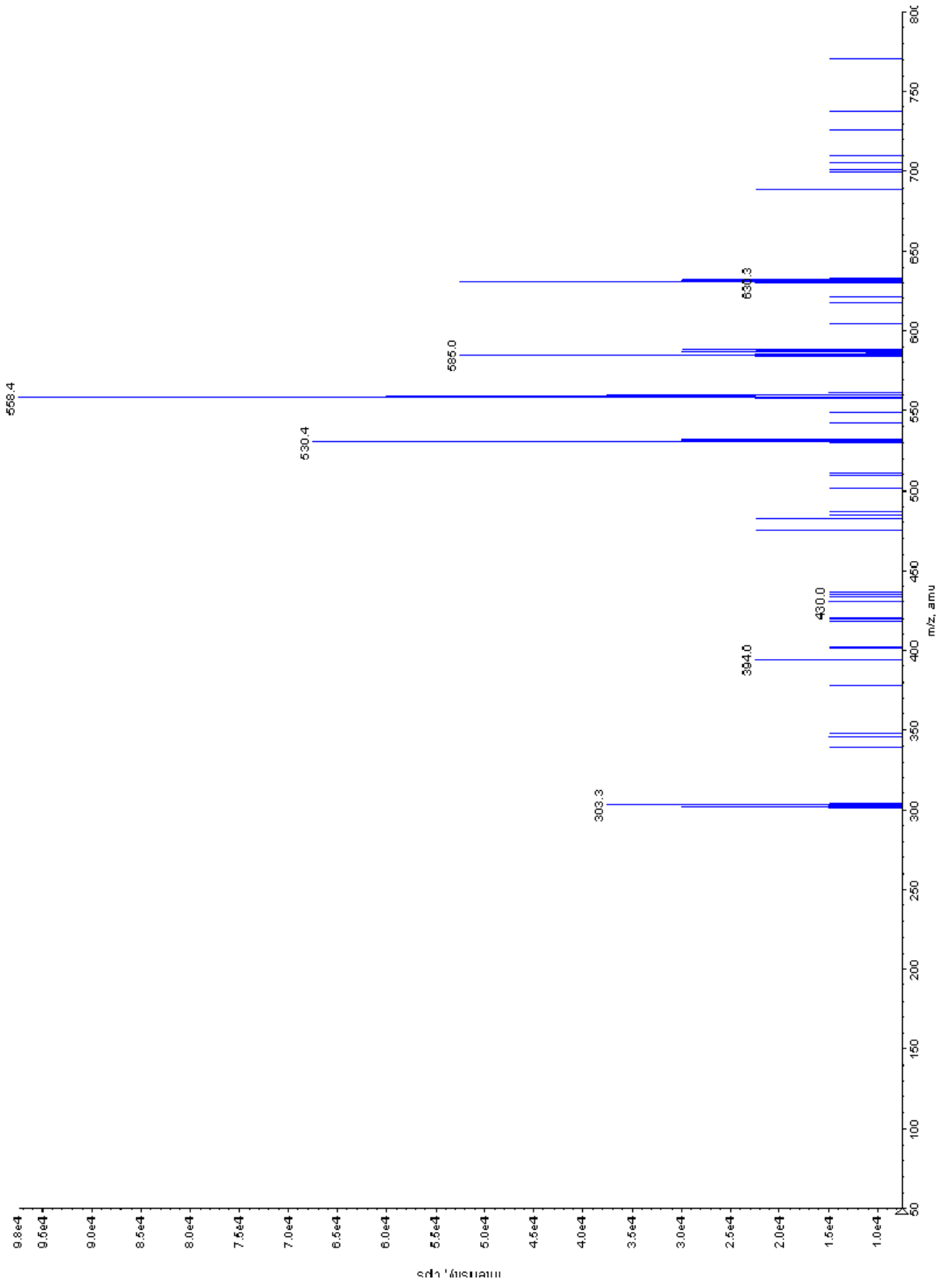

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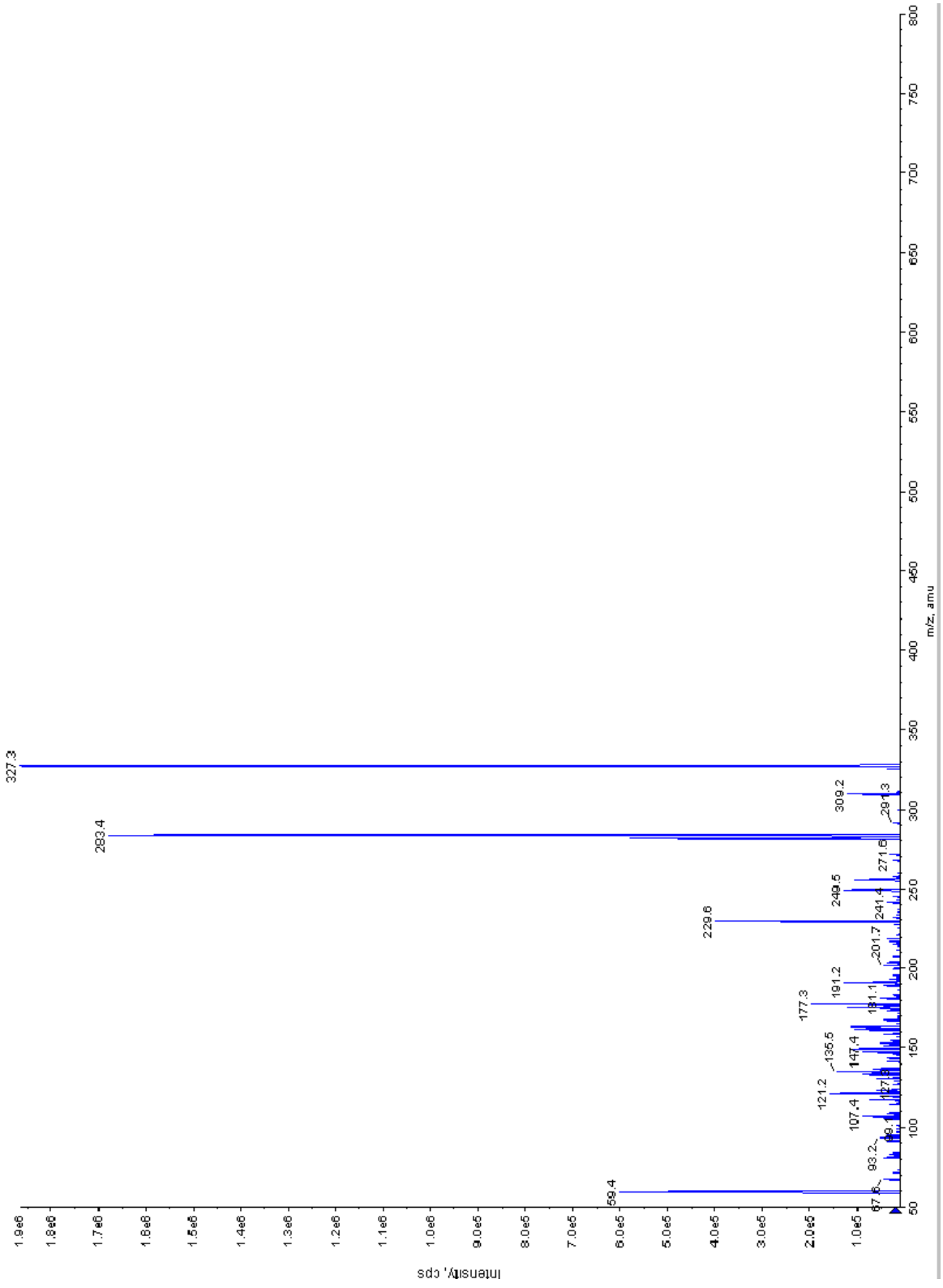

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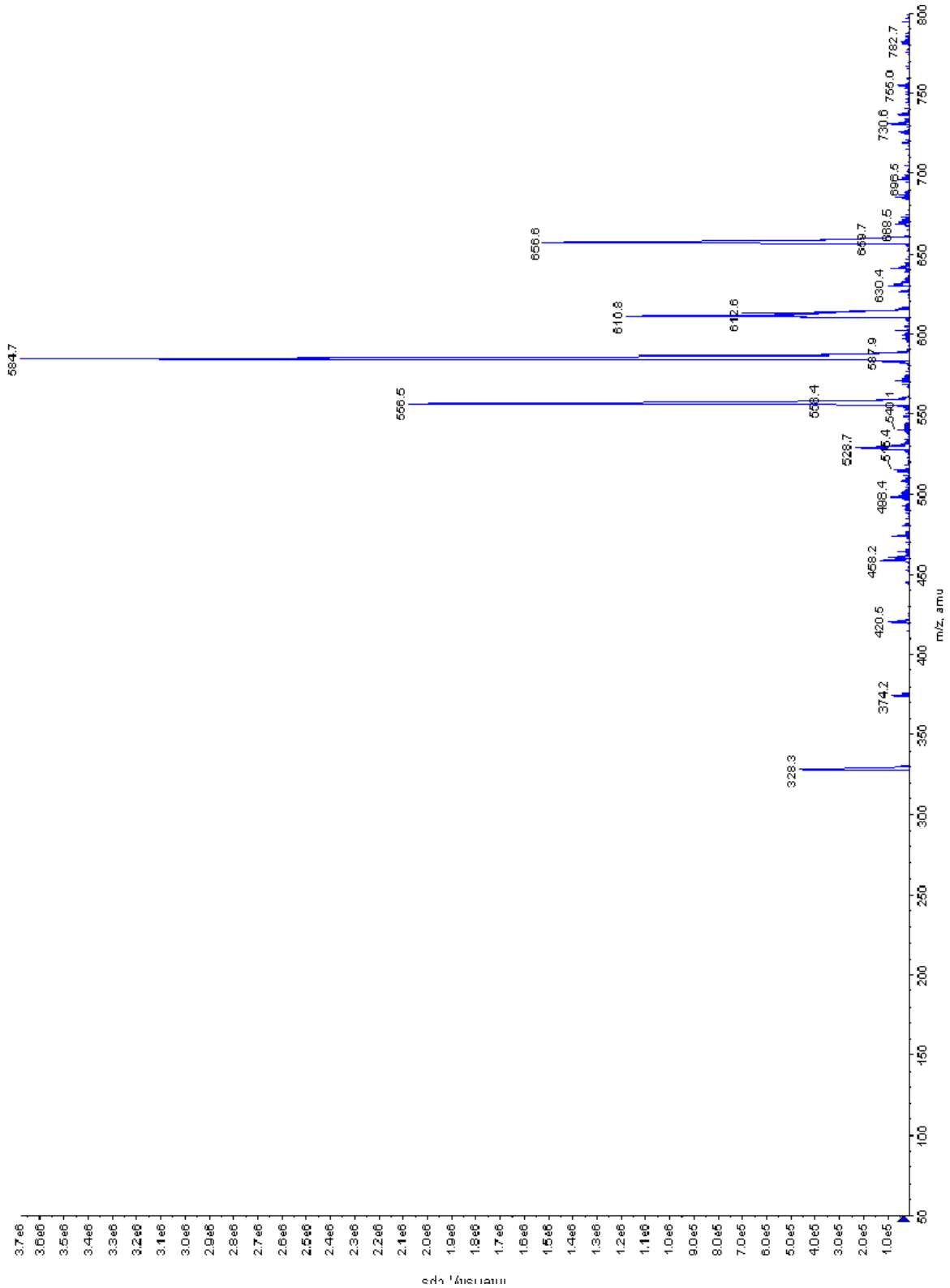

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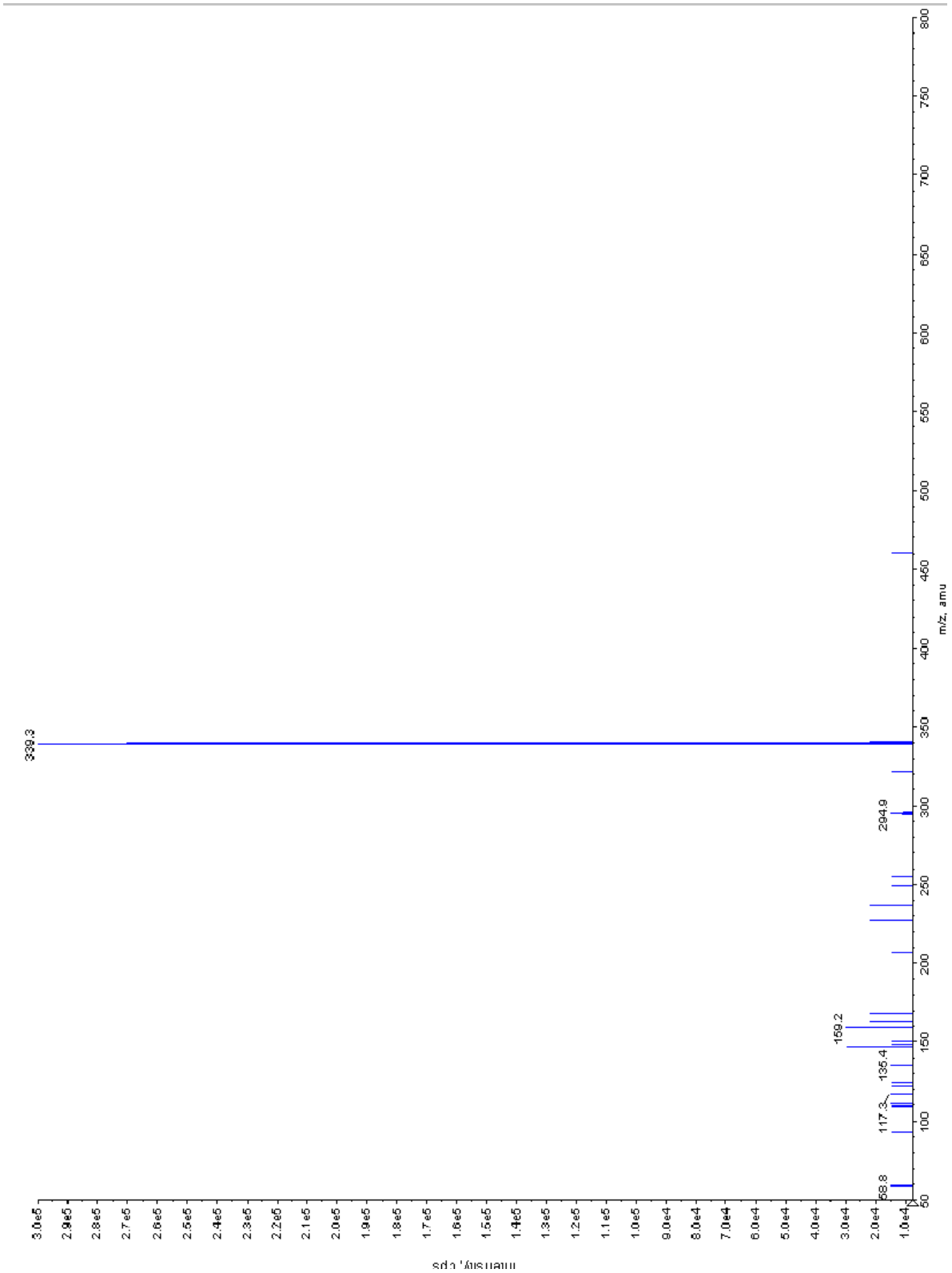

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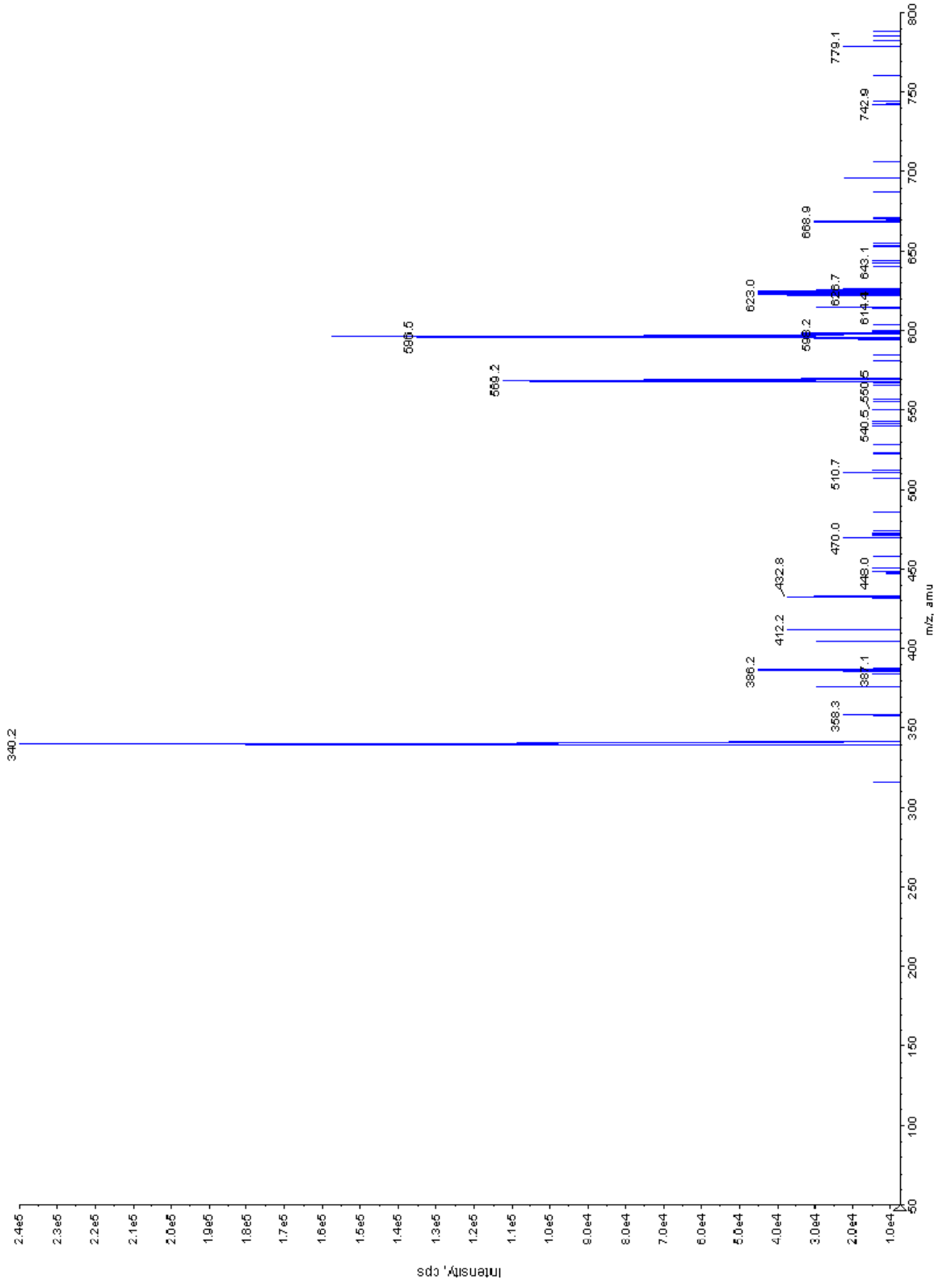

Annessex : Scientific Publications

The scientific publications produced during the doctoral work are:

- Di Donna, L., Mazzotti, F., **Santoro, I.,** & Sindona, G. (2017). Tandem mass spectrometry: a convenient approach in the dosage of steviol glycosides in Stevia sweetened commercial food beverages. Journal of Mass Spectrometry, 52(5), 290-295.
- Leggio, A., Leotta, V., Belsito, E. L., Di Gioia, M. L., Romio, E**., Santoro, I**., ... & Liguori, A. (2017). Aromatherapy: composition of the gaseous phase at equilibrium with liquid bergamot essential oil. Chemistry Central Journal, 11(1), 111.
- Benincasa, C., Perri, E., Romano, E., **Santoro, I.,** & Sindona, G. (2015). Nutraceuticals from Olives Plain Water Extraction, Identification and Assay by LC-ESI-MS/MS. Journal of Analytical & Bioanalytical Techniques, 6(6), 1.

Manuscripts submitted:

- **Santoro I.**, Nardi M., Perri E., Russo A., Sindona G..
  "Lipid *peroxidation in algae oil: Antagonist effects of natural antioxidants*"
  Submitted to *Food Chemistry*
- **Santoro I**., Benincasa C., Nardi M., Sindona G.
  "*Eco-friendly Production of Phenolic nutraceuticals from Olive Leaves*
  The Nagoya Protocol"
  Submitted to *Food and Function*
- **Santoro I.,** Benincasa C., Nardi M., Sindona G.,
  "Improvements of extraction and identification methodologies of PUFA from algae"
  Submitted to *Journal of Mass Spectrometry*

Research article

Journal of MASS SPECTROMETRY

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(wileyonlinelibrary.com) DOI 10.1002/jms.3925

Tandem mass spectrometry: a convenient approach in the dosage of steviol glycosides in Stevia sweetened commercial food beverages

L. Di Donna, F. Mazzotti, I. Santoro and G. Sindona*

The use of sweeteners extracted from leaves of the plant species *Stevia rebaudiana* is increasing worldwide. They are recognized as generally recognized as safe by the US-FDA and approved by EU-European Food Safety Authority, with some recommendation on the daily dosage that should not interfere with glucose metabolism. The results presented here introduce an easy analytical approach for the identification and assay of Stevia sweeteners in commercially available soft drink, based on liquid chromatography coupled to tandem mass spectrometry, using a natural statin-like molecule, Brutieridin, as internal standard.

Keywords: *Stevia rebaudiana*; steviol glycosides; tandem mass spectrometry; multiple reaction monitoring; stevioside food labeling

Introduction

Since the introduction, 50 years ago, of aspartame, a synthetic dipeptide sweetener, many efforts have been made to introduce new highly potent sugar surrogates to alleviate the medical and nutritional concerns raised by the intake of this molecule. Stevia, a sweet plant of the family *Asteraceae*, which is native to South America (Paraguay and Brazil), contains a number of sweet flavored glycosides of the diterpene steviol type; these are up to 300 times sweeter than sucrose.[1]

Each Stevia glycoside possesses a different sweetening power. Stevioside (SV), for instance, is between 110 and 270 times sweeter than sucrose, while rebaudioside A(Ra) is between 150 and 320, rebaudioside C (Rc) is between 40 and 60 and dulcoside (Du) is about 30 times sweeter.[2] In December 2008, the Food and Drugs Administration (FDA) recognized Stevia as generally recognized as safe and allowed its use in mainstream US food production. The structures of these diterpene steviol series are indicated in Fig. 1.

The European Food Safety Authority (EFSA) have expressed in April 2010 a positive scientific opinion on the use of steviol glycosides[3]; even some concern was raised on the uncertainty of the safety of the steviol aglycon produced in the metabolic pathways. The steviol aglycon in Stevia ranges between 4% and 20% of the dry weight of the leaves, depending on the cultivar and growing conditions.[4] SV and Ra are the most abundant components of Stevia and are considered safe for type 2 diabetes patients.[5] The SV diterpenoid glycoside is composed by the aglycone steviol, to which it is glycosylated with three molecules of glucose. In addition to SV, several other sweet terpene glycosides, such as steviolbioside (Sb), Ra, rebaudioside B (Rb), Rc, rebaudioside E (Re) and Du were isolated from Stevia leaf.[6] The major glycosides of Stevia plant were identified as SV (4–13% *w*/w), Ra (2–4% *w*/w) and Rc (1–2% *w*/w).[7] Presently, different type of foods containing Stevia glycosides are available in the market. The FDA and EFSA authorities' cautions require that any of these sold glycosides have to be analytically controlled, by special experts in this new important field of nutraceuticals used for human health.[8]

All Stevia compounds have the same chemical backbone structure (steviol) but differ in the residues of carbohydrate at positions C-13 and C-19. Structures of the sweet components of Stevia occurring mainly in the leaves are given in Fig. 1.

Different protocols have been devised for the characterization of Stevia active principles. High-performance liquid chromatography (HPLC) is one of the most commonly used methods for the determination of sweet tasting stevioside (STS) in plant material and food samples.[9] The determination of the diterpene glycosides can be also carried on by desorption electrospray ionization mass spectrometry,[10] near infrared reflectance spectroscopy[11] and capillary electrophoresis.[12]

Woelwer-Rieck *et al.*[9] have developed an HPLC method for the quantification of steviol glycosides after water leaching and SPE clean up with C18 cartridges. HPLC can be performed on a Hydrophilic Interaction Liquid Chromatography (HILIC) column with nearly 100% recoveries.

Bergs *et al.*[13] have introduced an HPLC method for the quantification of steviol glycosides in Stevia leaves based on the application of an extraction mode performer, in a five-step crossflow, each step for 3 h in an overhead shaker.

Hutapea *et al.*[14] have presented an HPLC method for identification of the metabolites of SV found in blood, feces and urine of hamsters after ingestion of SV. The separation was carried out on a reversed phase C18 column with gradient elution of acetonitrile/water mixture, whereas T. Rajasekaran *et al.*[6] have

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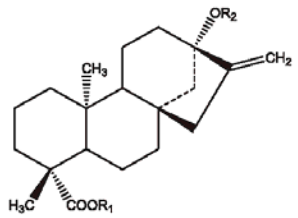

R-groups in backbone figure above

Compound name	R_1	R_2
Steviol	H-	H-
Steviolbioside	H-	β-glc-β-glc-
Stevioside	β-Glc-	β-glc-β-glc-
Rebaudioside A	β-Glc-	(β-glc)$_2$-β-glc-
Rebaudioside B	H-	(β-glc)$_2$-β-glc-
Rebaudioside C	β-Glc-	(β -glc,α-rha-)-β-glc-
Rebaudioside D	β-Glc-β-Glc-	(β-glc)$_2$-β-glc-
Rebaudioside E	β-Glc-β-Glc-	β-glc-β-glc-
Rebaudioside F	β-Glc-	(β-glc,β-xyl-)-β-glc-
Dulcoside A	β-Glc-	α-rha-β-glc-

Figure 1. Structures of steviol glycosides. Glc, glucose; Rha, rhamnose; Xyl, xylose.

developed a liquid chromatograpy–M/S-ESI method for the determination of eight components of Stevia.

Mass spectrometry is the analytical technique of choice for the identification and assay of target species in complex mixtures.

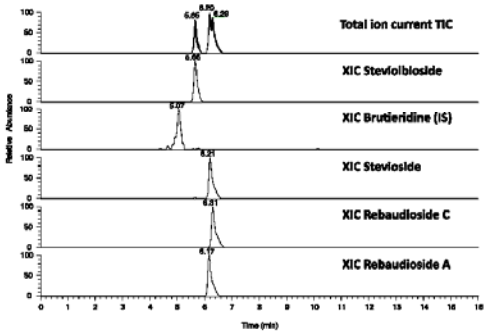

Figure 2. LC–MS/MS under multiple reaction monitoring condition of analytes and internal standard. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. Equation and the correlation coefficient found for each STS.

Analyte	Equations	R^2
Sb	Y = 93.442x−12.75	0.998
SV	Y = 70.561x−10.878	0.997
Ra	Y = 53.072x−18.478	0.929
Rc	Y = 49.261x−9.270	0.995

STS, sweet tasting stevioside; Sb, steviolbioside; SV, stevioside; Ra, rebaudioside A; Rc, rebaudioside C.

Table 2. Monitored ions and optimized instrumental parameters. Collision energy is reported in electronvolt (eV).

Analyte	Parent ion $[M + NH_4]^+$	Product ions quantitative (eV)	Product ions diagnostic (eV)
Sb	*m/z* 660	*m/z* 319 (18)	*m/z* 325 (13)
SV	*m/z* 822	*m/z* 319 (23)	*m/z* 325 (17)
Ra	*m/z* 984	*m/z* 319 (27)	*m/z* 325 (25)
Rc	*m/z* 968	*m/z* 309 (23)	*m/z* 471 (15)

Sb, steviolbioside; SV, stevioside; Ra, rebaudioside A; Rc, rebaudioside C.

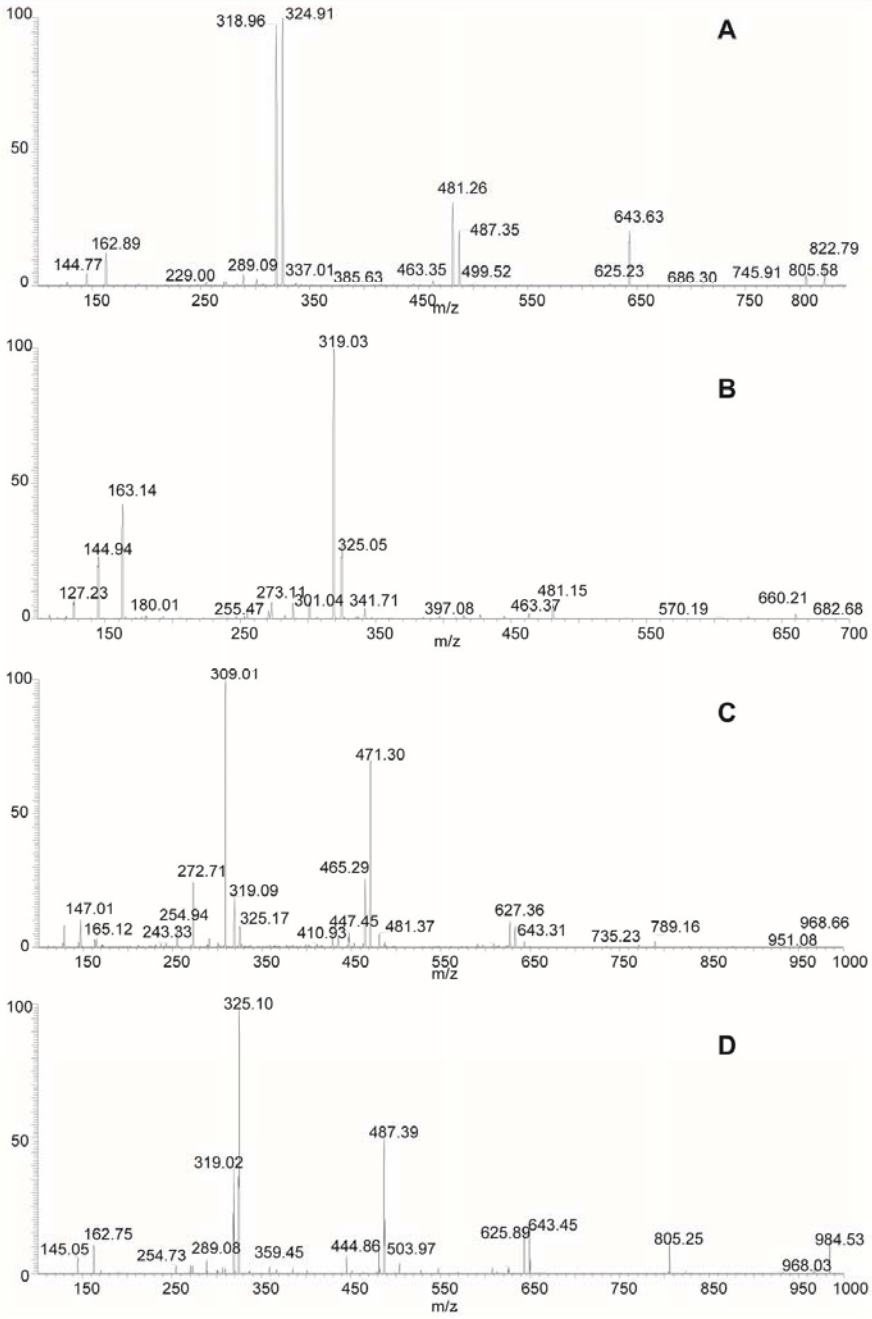

Figure 3. MS/MS spectra of (a) rebaudioside A, (b) steviolbioside, (c) stevioside and (d) rebaudioside C.

Journal of MASS SPECTROMETRY

Recently, mass spectrometry has been widely applied to discern origin, quality and safety of foods.[15–22] We now present the results obtained by LC-ESI-MS and low-energy collision induced dissociation (CID-MS/MS) using the multiple reaction monitoring (MRM) scans, applied in the assay of steviol glycosides in food beverages. Brutieridin, a natural molecule present in bergamot fruits only,[23] was used as internal standard. This approach should allow an easy and scientifically based evaluation of the healthiness of commercially available Stevia sweetened food beverages.

Materials and methods

Chemicals

Solvents and reagents were commercially available (Sigma-Aldrich, St. Louis, MO, USA). Steviol glycosides standards were purchased from Extrasynthese (Genay Cedex, France). Brutieridin internal standard was prepared in the laboratory with our in-house methods.

Samples

Three beverages (two fruits juice and a cola soft drink used as blank matrix) and a sweetener were purchased in local stores.

Sample preparation

Two milliliter of each beverage was centrifuged at 6000 rpm for 5 min. The supernatant was quickly degassed by a nitrogen flow, when needed. All the solutions were filtered through a 0.45-µm polytetrafluoroethylene (PTFE) filter. One milligrams of sweetener was diluted in 1 ml of water. A 100-µl volume of each beverages solution was added to 167 µl of a 1.2 mg/l of Brutieridin internal standard, diluted to 1 ml of ACN/H_2O (50/50) and stirred for 30 s to allow homogeneous distribution of the standards. One-hundred microliter of a solution of 1 mg/l sweetener Stevia was added to 167 µl of a 1.2 mg/l of Brutieridin internal standard and diluted to 1 ml of ACN/H_2O (50/50). These solutions were used to quantify Sb, SV and Ra. Solutions of each sample of beverage with a dilution 1 to 20 and a solution of sweetener with a dilution 1 to 200 were prepared to quantify Rc.

Calibration curves

The calibration curves for each steviol glycosides were obtained, sampling five solutions containing the analytes at concentration ranging from 50 to 400 µg/l and the internal standard at the fixed concentration of 200 µg/l. The HPLC chromatogram of the STS standard is presented in Fig. 2.

Table 1 shows the equation and the correlation coefficient found for each analyte in the range of concentrations employed. In all cases, an excellent linearity has been achieved. Before applying the methodology to real cases, some experiments have been planned to obtain the typical analytical parameters.

Each analysis was repeated three times, and the mean value was used.

Mass spectrometry

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) and low-energy CID-MS/MS analysis were carried out using a Thermo Scientific UHPLC instrument coupled to a TSQ Quantum Vantage (Thermo Fischer Scientific, San José, CA, USA) triple-stage quadrupole mass spectrometer. The chromatographic separation was achieved using a C18 reversed-phase analytical column, Kinetex PFP (2.150 mm, 2.6 lm particle size, 100 Å, Phenomenex, Torrance, CA, USA). The elution gradient consisted of mobile phase (A) ACN and (B) H_2O (0.1% $HCOONH_4^+$). The linear gradient used was the following: at t = 0.0 min, 10% A and 90% B; at t = 2.0 min, 10% A and 90% B; at t = 5.00 min, 50% A and 50% B; at t = 8.00 min, 50% A and 50% B; at t = 10.00 min, 98% A and 98% B; at t = 13.00 min, 98% A and 98% B; at t = 14.00 min, 10% A and 90% B; and at t = 16 min, 10% A and 90% B. The flow rate was set at 0.3 ml/min, and the sample injection volume was 10 µl. A further switching valve located on the mass spectrometer was used to divert the LC flow to waste for the initial 1 min as well as the final 3 min of each injection to allow the protection of the MS source from contamination. All valve positions and the instrument parameters were controlled by Xcalibur software, version 2.0.0 (Thermo Fisher Scientific).

Mass spectrometric analysis was performed using a heated electrospray ionization source operating in positive ion mode. The following working conditions were applied: spray voltage, 3 kV; vaporizer and capillary temperatures, 280 and 270 °C, respectively; and sheath and auxiliary gas at 58 and 40 arbitrary units (au), respectively. For the low-energy CID-MS/MS analysis, the collision gas was argon used at a pressure of 1.5 mTorr in the collision cell (Q2), and the mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum. The S-lens rf amplitude as well as the collision energy (CE) were both optimized individually per compound; S-lens values ranging from 79 to 114 V while CE values ranging 13–27 eV. The MRM mode, used to identify and assay the analytes, was performed following two transitions per compound, the first one for quantitative analysis and the second for structure validation. The formation of ammoniated ions, selected to enhance the absolute intensity of the ionized analytes in the MS spectrum represent the procedure of choice when glycosides have to be analyzed by mass spectrometry.[24] Table 2 reported the parent ammoniated molecular ions of each analyte and the two main fragments obtained by CID on the given precursor species. The CE values reported for each fragment are automatically optimized by the instrument software that provide the formation of the most abundant fragment peaks. The MS/MS spectra of Ra, Sb, SV and RC are reported in Fig. 3. The MS/MS fragmentation pattern and the observed product ions are typical of the gas phase chemistry of glycosides.[25]

Table 3. Analytical parameters of accuracy.

Spiked sample amount	Calculated amount (mg/l)	RSD %	Accuracy %
0.08 mg/l			
Sb	76.31 ± 7.21	9.45	95.39
SV	78.45 ± 6.45	8.34	98.06
Rc	81.22 ± 5.32	6.55	101.53
Ra	79.52 ± 8.22	10.34	99.4
0.350 mg/l			
Sb	343.45 ± 25.34	7.38	98.13
SV	351.54 ± 22.58	6.42	100.44
Rc	348.91 ± 28.89	8.28	99.69
Ra	345.16 ± 24.45	7.08	98.62

Sb, steviolbioside; SV, stevioside; Ra, rebaudioside A; Rc, rebaudioside C.

Table 4. Amount of steviol glycosides found in all analyzed samples.

Analyte	Cola soft drink (mg/l)	RSD %	Pineapple and litchi juice (mg/l)	RSD %	Pineapple and lime juice (mg/l)	RSD %	Sweetener (%)
Sb	0.8 ± 0.08	10	0.71 ± 0.02	2.82	0.56 ± 0.006	1.07	0.07
SV	3.9 ± 0.42	10.77	22.04 ± 2.48	11.25	4.28 ± 0.55	12.85	0.055
Ra	83.11 ± 8.55	10.29	95.81 ± 12.04	12.56	134.97 ± 10.79	7.99	3.6
Rc	1.89 ± 0.17	8.99	3.34 ± 0.26	7.78	1.68 ± 0.024	1.43	0.17

Sb, steviolbioside; SV, stevioside; Ra, rebaudioside A; Rc, rebaudioside C.

Instrument control and data processing were carried out by means of Xcalibur software. The total LC MS/MS method run was 16 min. LC-MS/UV chromatograms were obtained using a Fractionlynx system from Waters (Milford, MA, USA) set-up at analytical mode and equipped with a ZMD mass spectrometer and a 486 UV detector.

Result and discussion

Food safety and security has become undoubtedly a must of developing and developed countries. FDA food facility registration is required for all companies that manufacture, process, pack or store food, beverages or dietary supplements that may be consumed in the United States. Similarly, The European agency EFSA takes care of existing and emerging risks associated with the food chain. Moreover, food safety and security has become a target of the NATO Science for Peace and Security Program.[15] A particular attention of researchers managing sophisticated analytical methodologies, such as mass spectrometry, would provide unique contributions to the security and safety of foods.

The identification and assay of the active principles present in soft drink sweetened with Stevia glycosides has a twofold purpose. The first one is directly linked to the daily doses as requested by the international EFSA and FDA organizations and is related also to food quality principles. The second is associated to the safety of the aliment, thus preventing fraudulently addition of other sugar surrogates. The proposed method has been exploited in food chemistry with excellent outcomes.

The internal standard Brutieridin, a natural compound chemically very close to steviol glycosides, was selected either for its flavonoid glycoside structure, and because it is a natural molecule present in bergamot citrus fruit only, hence it cannot suffer by any interferences with the analytical evaluation of Stevia-containing drinks. The procedure is, therefore, independent from the variety of analyzed beverages edulcorated with Stevia and was checked on very popular drinks available in the local market.

The accuracy of the method was determined from samples prepared by adding known quantities of the analytes to samples of soft drinks. In the two examined cases (Table 3), representative of low and a highly value of calibration curve, the accuracy was higher than 95%.

The Relative Standard Deviation (RSD) value was in all cases under 13% thus showing a good repeatability of the measurements. The proposed approach has been applied to real samples obtained by local market (Table 4).

The data reported in Table 4 clearly show that the examined commercial products fits well FDA and EFSA requirements; moreover, the *quality* of the analytical protocol guarantees its use as a scientific approach to appropriately label commercial products based on the use of Stevia edulcorator.

Conclusions

Quality and safety of Stevia-based functional foods can be properly controlled by the application of the trustworthy MS protocols here proposed. This approach is ready to be transferred to Food Manufactures to favor the introduction into the market of scientifically checked Stevia-based functional foods.

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RESEARCH ARTICLE Open Access

Aromatherapy: composition of the gaseous phase at equilibrium with liquid bergamot essential oil

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Abstract

This work compares the composition at different temperatures of gaseous phase of bergamot essential oil at equilibrium with the liquid phase. A new GC–MS methodology to determine quantitatively the volatile aroma compounds was developed. The adopted methodology involved the direct injection of headspace gas into injection port of GC–MS system and of known amounts of the corresponding authentic volatile compounds. The methodology was validated. This study showed that gaseous phase composition is different from that of the liquid phase at equilibrium with it.

Keywords: Bergamot, Essential oil, Volatile compounds, Gaseous phase, Gas chromatography–mass spectrometry, Aromatherapy

Introduction

Phytotherapy employs fully characterized active ingredients extracted from plants for the treatment and prevention of many diseases.

Essential oils and their components exhibit various biological activities and are also used for human disease prevention and treatment. They exert antiviral, antidiabetic, antimicrobial and cancer suppressive activities [1, 2], furthermore they play a key role in cardiovascular diseases prevention including atherosclerosis and thrombosis [3, 4].

Today aromatherapy, a branch of phytotherapy, is gaining momentum as complementary therapy to the traditional medicine [5]. Aromatherapy uses essential oils via inhalation or massage as the main therapeutic agents to treat several diseases. The inhalation of volatile aromatic substances extracted from plants can affect the mood and state of health of the person by inducing psychological and physical effects [6–10]. The transdermal and transmucosal application of essential oils also concerns the phytotherapy [11].

Recently, some papers [12, 13] have tried to give scientific value to the aromatherapy, traditionally based on empirical observations and evaluations also poorly stringent, by establishing criteria similar to those that support the rigorous scientific research [14]. It has been verified in fact, which among hundreds of papers related to aromatherapy inhalation only a few are scientifically significant [15].

In order to use the essential oil appropriately it is important knowing its chemical compositions and characteristics. It seems clear, however, that if the essential oils are delivered by inhalation, the determination of gas phase (or headspace) composition above the liquid essential oil sample becomes critical [16, 17].

The migration of volatile molecules into the headspace phase does not just depend on their volatility but also on their affinity for the liquid phase sample; volatile compounds relative concentrations between the two phases will reach an equilibrium value. At equilibrium, the partial pressure of each volatile component in the headspace vapor will be equivalent to the vapor pressure that is directly proportional to its mole fraction in the liquid

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phase. In essence, the concentration of a compound in the headspace is proportional to its concentration in the liquid phase and can be affected by temperature, respective volumes of the sample and the headspace, and other factors [18]. Thus, headspace phase composition can be very different from that of the liquid phase.

Over the years specific studies designed to identify an analysis procedure for the determination of headspace gas at equilibrium with liquid essential oil have been reported [19–21]. These works are mostly based on the use of solid-phase microextraction (SPME) by which the headspace gas is extracted by a fused silica fiber coated with a suitable stationary phase (HS-SPME) [19, 20]. The volatile compounds adsorbed on the fiber are then thermally desorbed in the GC injector port of a GC–MS instrument to perform the qualitative analysis and GC–FID for the quantitative determination [22].

However, the composition of volatile compounds adsorbed on the fiber is different from that of headspace gas in equilibrium with the essential oil since the adsorption on the fiber depends on the fiber characteristics and extraction conditions used for the analysis. Therefore, this procedure is not sufficient to define the actual composition of the vapor phase in equilibrium with the essential oil, and hence poorly applicable to the study of aromatherapy.

Bergamot (*Citrus bergamia*) is an endemic plant of the Calabria region in the south of Italy and its fruit is used for the extraction of bergamot essential oil (BEO). Bergamot essential oil is the basic component of perfumes and is also used in the formulation of cosmetic products, food and confections as a flavouring.

The therapeutical applications of Bergamot essential oil are related to its antiseptic, antibacterial and anti-inflammatory properties. Of particular interest is also the composition of bergamot juice and albedo because of the presence of molecules with important biological and pharmacological activities [23–26]. Furthermore, it is employed in aromatherapy as an antidepressant to reduce anxiety and stress by improving mood and facilitating sleep induction [27–33].

The determination of headspace composition in bergamot essential oil is extremely useful in aromatherapy. Nevertheless, greater efforts are still needed to develop a simple and objective methodology.

In the present work, we studied the composition of the gaseous phase at equilibrium with the liquid phase of bergamot essential oil by developing a gas chromatography–mass spectrometry (GC–MS) method useful for the determination of the volatile aroma components.

Experimental

Materials

Bergamot essential oil (*Citrus bergamia* Risso et Poiteau) was supplied by the "Consorzio del Bergamotto di Reggio Calabria" (Southern Italy).

Chemicals and reagents

α-Pinene, α-fellandrene, α-terpinene, linalyl acetate, neral, geranial were purchased from Sigma-Aldrich Co. (Italy). β-Pinene, *p*-cimene, γ-terpinene, terpinolene, linalool, α-terpineol were purchased from Fluka. Mircene, ocimene, neryl acetate, octyl acetate, β-caryophyllene and limonene were purchased from Merck KGaA. Anisole was purchased from Sigma-Aldrich Co (Italy) and used as internal standard.

GC–MS analysis

GC–MS analyses were performed using a 6890N Network GC System (Agilent Technologies Inc., Palo Alto, CA) equipped with a HP-35MS (35% diphenylsiloxane; l = 20 m, d = 0.25 mm 0.25 μm) capillary column and with a mass spectrometer 5973 Network MSD operated in electron impact ionization mode (70 eV). GC–MS analyses were carried out in split mode, using helium as the carrier gas (1 mL/min flow rate). The column was maintained at an initial temperature of 40 °C for 0 min, then ramped to 250 °C at 3 °C/min, to 280 °C at 5 °C/min, where it was maintained for 15 min. Quantitative GC–MS analysis was carried out in splitless mode (splitless time, 1 min), by using anisole as the internal standard. The identification of the compounds was based on comparison of their retention times with those of authentic samples, and on comparison of their EI-mass spectra with the NIST/NBS, Wiley library spectra and literature [26].

GC–FID analysis

GC–MS analyses were performed using a HP6890 A series 2 GC System (Agilent Technologies Inc., Palo Alto, CA) equipped with a HP-35MS (35% diphenylsiloxane; l = 20 m, d = 0.25 mm 0.25 μm). The column temperature was programmed at 40 °C for 0 min, to 250 °C at 3 °C/min, to 280 °C at 5 °C/min, where it was maintained for 15 min. The injector and detector temperatures were programmed at 230 and 300 °C, respectively. Helium was used as the carrier gas at a flow rate 1 mL/min.

Table 1 Stock solutions for the quantitative analysis at 0 °C

Stock solutions F α-Pinene; *p*-cimene; mircene; linalool	Concentration for each analyte (mg/mL)
Solution 1	0.015
Solution 2	0.025
Solution 3	0.050
Solution 4	0.065
Solution 5	0.075
Solution 6	0.085
Stock solutions H Limonene; β-pinene	**Concentration for each analyte (mg/mL)**
Solution 1	0.225
Solution 2	0.30
Solution 3	0.375
Solution 4	0.450
Solution 5	0.525
Solution 6	0.60

Quantitative analysis of bergamot essential oil

Sample preparation

Three aliquots of the essential oil bergamot (55, 95 and 147 mg), containing anisole (0.1 mL) as internal standard, were diluted to 5 mL with diethyl ether and then subjected to the quantitative analysis. Quantitative data were obtained by comparing the analyte/anisole area ratios in the standard solutions with the corresponding ratios in the oil samples solutions.

Internal standard solution

40 mg of anisole were diluted to 100 mL with diethyl ether.

Preparation of stock solutions A–D

For the quantitative analysis of β-pinene limonene, γ-terpinene, linalool, linalyl acetate, five stock solutions A were prepared using 150 mg of each analytes and dissolving them in 5 mL of diethyl ether. Solutions A were further used to prepare diluted working solutions B. In particular, 0.1, 0.2, 0.5, 1, 1.3 and 1.5 mL of each stock solution A, after adding 0.1 mL of the internal standard solution, was made up to 5 mL volume with diethyl ether. The final concentrations of each analyte in working solutions B were 0.6, 1.2, 3, 6, 7.8, 9.6 mg/mL respectively.

For the quantitative analysis of α-pinene, α-phellandrene, α-terpinene, p-cimene, terpinolene, myrcene, ocimene, neral, geranial, neryl acetate, α-terpineol, octyl acetate, caryophyllene, thirteen stock solutions C were prepared as follows: 50 mg of each analyte was diluted to 100 mL with diethyl ether. Aliquots of these solutions C were then used to prepare diluted working solutions D. In particular, 0.2, 0.5, 1, 1.3, 1.7 and 2.5 mL of each analyte, after adding 0.1 mL of the internal standard solution, was made up to 5 mL volume with diethyl ether. The final concentrations of each analyte in working solutions D were 0.02, 0.05, 0.10, 0.13, 0.17, 0.21 mg/mL.

Quantitative analysis of the gaseous phase of bergamot essential oil

Sample preparation

Three samples of the gaseous phase of the bergamot essential oil were prepared as follows: 100 mg of bergamot essential oil and 7 mg of anisole used as the internal standard, were transferred to three 10 mL vials that were sealed and then maintained at 0, 22 and 40 °C respectively.

The temperature of 0 °C was obtained using an ice bath in which liquid phase and solid phase coexist. The temperature of 22 °C was that measured in a conditioned environment at 22 °C. 40 °C was obtained by means of a thermostated oil bath with a digital vertex thermometer.

After 30 min, a gastight syringe was used to weigh out the gaseous phase (0.4 mL) and then subjected to the quantitative analysis by both GC–MS and GC–FID. Quantitative data were obtained by comparing the analyte/anisole area ratios in the standard solutions with the corresponding ratios in the essential oil samples solutions.

Internal standard solution

20 mg of anisole was diluted to 500 mL with diethyl ether.

Preparation of stock solutions for the quantitative analysis at 0 °C (Table 1)

Preparation of stock solutions E–H

For the quantitative analysis of α-pinene, p-cimene, mircene, linalool, linalyl acetate at 0 °C, five stock solutions E were prepared using 50 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions E were further used to prepare diluted working solutions F. In particular, 0.3, 0.5, 1, 1.3, 1.5, 1.7 mL of each stock solution E, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentrations of each analyte in working solutions F were 0.015, 0.025, 0.050, 0.065, 0.075 and 0.085 mg/mL respectively.

For the quantitative analysis of limonene and β-pinene at 0 °C, two stock solutions G were prepared using 150 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions G were further used to prepare diluted working solutions H. In particular, 1.5,

Table 2 Stock solutions for the quantitative analysis at 22 and 40 °C

Quantitative analysis at 22 °C		Quantitative analysis at 40 °C	
Stock solutions J α-Phellandrene; α-terpinene; *p*-cimene; mircene; linalyl acetate	**Concentration or each analyte (mg/mL)**	**Stock solutions P α-Terpinene; *p*-cimene; mircene;**	**Concentration for each analyte (mg/mL)**
Solution 1	0.002	Solution 1	0.001
Solution 2	0.004	Solution 2	0.002
Solution 3	0.006	Solution 3	0.004
Solution 4	0.008	Solution 4	0.006
Solution 5	0.010	Solution 5	0.008
Solution 6	0.015	Solution 6	0.010

Quantitative analysis at 22 °C		Quantitative analysis at 40 °C	
Stock solutions L α-Pinene; γ-terpinene; linalool	**Concentration for each analyte (mg/mL)**	**Stock solutions R Octyl acetate; α-phellandrene; α-pinene**	**Concentration for each analyte (mg/mL)**
Solution 1	0.050	Solution 1	0.010
Solution 2	0.065	Solution 2	0.015
Solution 3	0.085	Solution 3	0.020
Solution 4	0.10	Solution 4	0.025
Solution 5	0.120	Solution 5	0.030
Solution 6	0.140	Solution 6	0.035

Quantitative analysis at 22 °C		Quantitative analysis at 40 °C	
Stock solutions N Limonene; β-pinene	**Concentration for each analyte (mg/mL)**	**Stock solutions T Limonene; β-pinene; linalyl acetate; γ-terpinene; linalool**	**Concentration for each analyte (mg/mL)**
Solution 1	0.10	Solution 1	0.070
Solution 2	0.20	Solution 2	0.150
Solution 3	0.30	Solution 3	0.20
Solution 4	0.40	Solution 4	0.250
Solution 5	0.50	Solution 5	0.30
Solution 6	0.60	Solution 6	0.350

2, 2.5, 3, 3.5 and 4 mL of each stock solution G, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentration of each analyte in working solutions H were 0.225, 0.30, 0.375, 0.450, 0.525 and 0.60 mg/mL respectively.

Preparation of stock solutions for the quantitative analysis at 22 °C (Table 2)

Preparation of stock solutions I–N

For the quantitative analysis of α-phellandrene, α-terpinene, *p*-cimene, mircene, linalyl acetate at 22 °C, five stock solutions I were prepared using 10 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions I were further used to prepare diluted working solutions J. In particular, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 mL of each stock solution I, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentrations of each analyte in working solutions J were 0.002, 0.004, 0.006, 0.008, 0.010 and 0.015 mg/mL respectively.

For the quantitative analysis of α-pinene, γ-terpinene and linalool at 22 °C, three stock solutions K were prepared using 50 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions K were further used to prepare diluted working solutions L. In particular, 1.0,

1.3, 1.7, 2.0, 2.4, 2.8 mL of each stock solution K, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentrations of each analyte in working solutions L were 0.05, 0.065, 0.085, 0.10, 0.12, 0.14 mg/mL respectively. For the quantitative analysis of limonene and β-pinene at 22 °C, two stock solutions M were prepared using 100 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions M were further used to prepare diluted working solutions N. In particular, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mL of each stock solution M, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentrations of each analyte in working solutions N were 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 mg/mL respectively.

Preparation of stock solutions for the quantitative analysis at 40 °C (Table 2)

Preparation of stock solutions O–T

For the quantitative analysis of α-terpinene, *p*-cimene and mircene, at 40 °C, three stock solutions O were prepared using 10 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions O were further used to prepare diluted working solutions P. In particular, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mL of each stock solution O, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentrations of each analyte in working solutions P were 0.001, 0.002, 0.004, 0.006, 0.008 and 0.01 mg/mL respectively. For the quantitative analysis of α-pinene, α-phellandrene and octylacetate, at 40 °C, two stock solutions Q were prepared using 10 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions Q were further used to prepare diluted working solutions R. In particular, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mL of each stock solution Q, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentrations of each analyte in working solutions R were 0.010, 0.015, 0.020, 0.025, 0.030, 0.035 mg/mL respectively. For the quantitative analysis of limonene, β-pinene, linalyl acetate, γ-terpinene and linalool at 40 °C, five stock solutions S were prepared using 100 mg of each analytes and dissolving them in 100 mL of diethyl ether. Solutions S were further used to prepare diluted working solutions T. In particular, 0.7, 1.5, 2.0, 2.5, 3.0 and 3.5 mL of each stock solution S, after adding 0.1 mL of the internal standard solution, was made up to 10 mL volume with diethyl ether. The final concentrations of each analyte in working solutions T were 0.07, 0.15, 0.20, 0.25, 0.30 and 0.35 mg/mL respectively.

Statistical analysis

Statistical analyses were carried out with the SPSS Statistics 23.0 (SPSS Inc., Chicago, IL, USA). For each compound, six solutions were prepared and analyzed by GC–MS. The statistical analysis was obtained comparing the analyte/anisole area ratios in the solutions with the corresponding concentrations. A value of P correspondent to 0.011 was considered significant.

Results and discussion

The distilled bergamot essential oil used was preliminarily analyzed to define its composition. The individual analytes present in the oil were identified by GC–MS

Table 3 Composition of BEO and gaseous phase in equilibrium with the liquid at 0 °C

Entry	Compound	Essential oil composition[a]		Gaseous phase composition at 0 °C		
		t_R (GC/MS) (min)	GC–MS (w/w% ± SD)	GC–MS (w/w% ± SD)	GC–FID (w/w% ± SD)	t_R (GC/FID) (min)
Cyclic hydrocarbon monoterpenes						
1	α-Pinene	6.14	1.03 ± 0.10	6.90 ± 0.10	7.06 ± 0.25	6.14
2	β-Pinene	8.19	6.56 ± 0.14	25.90 ± 0.40	26.68 ± 0.15	8.31
3	α-Phellandrene	9.48	0.04 ± 0.01	–	–	–
4	α-Terpinene	9.94	0.16 ± 0.02	–	–	–
5	Limonene	10.60	30.20 ± 0.77	58.07 ± 0.38	57.12 ± 0.35	10.48
6	*p*-Cimene	11.19	0.18 ± 0.01	6.36 ± 0.04	6.02 ± 0.08	11.61
7	γ-Terpinene	12.15	11.95 ± 0.32	–	–	–
8	Terpinolene	13.36	0.27 ± 0.03	–	–	–
Acyclic hydrocarbon monoterpenes						
9	Mircene	8.72	0.82 ± 0.02	2.19 ± 0.22	2.16 ± 0.15	8.49
10	Ocimene	11.03	0.08 ± 0.01	–	–	–
Acyclic oxygenated hydrocarbon monoterpenes						
11	Linalool	14.58	21.82 ± 0.87	3.04 ± 0.54	2.96 ± 0.33	14.55
12	Linalyl acetate	21.42	16.21 ± 0.84	–	–	–
13	Neral	22.94	0.21 ± 0.01	–	–	–
14	Geranial	24.46	0.11 ± 0.01	–	–	–
15	Neryl acetate	28.14	0.28 ± 0.02	–	–	–
Cyclic oxygenated hydrocarbon monoterpenes						
16	α-Terpineol	20.01	0.87 ± 0.08	–	–	–
Esters						
17	Octyl acetate	19.63	0.10 ± 0.01	–	–	–
Sesquiterpenes						
18	β-Caryophyllene	27.85	0.14 ± 0.02	–	–	–

SD standard deviations

[a] The w/w percentages were determined by the internal standard method and referred to the amount of each component contained in 100 g of essential oil

methodology by comparing the corresponding retention times and mass spectra with those of authentic sample (Table 3).

Anisole was chosen as internal standard for the quantitative measurement of the individual analytes.

For the quantitative analysis, six standard stock solution (Stock B and Stock C) containing different concentration levels of each identified analyte and the same amount of internal standard were prepared.

Each solution was injected in triplicate in the GC–MS system under optimized conditions. For each measurement, the concentration and the peak area of the analytes were compared with those of the internal standard.

Table 1 reports the quantitative results only for the identified analytes.

High contents of limonene, linalool, linalyl acetate, and α-terpinene are observed in analogy with the data reported in literature [18, 33, 34].

The determination of gas phase composition above the liquid oil has preliminarily required controlled temperature and pre-established equilibrium conditions.

To this aim, a weighed amount of essential oil was placed in a headspace vial, after adding a given amount of anisole the vial was sealed and then allowed to stand for 30 min at 0 °C to establish the equilibrium at that temperature. Once the volatile compounds have equilibrated, an aliquot of the headspace gas was withdrawn using a gas tight syringe, injected into the gas chromatograph injection port and analyzed by GC–MS. The individual analytes present in the headspace gas were identified through comparison of retention times and mass spectral data with those of authentic standards (Fig. 1).

Additional experiments using equilibration times longer than 30 min were also carried out. After 60 min equilibration time the relative ratios between the

different volatile components did not change significantly compared to those obtained after 30 min.

For the quantitative analysis, seven stock solutions containing the reference analytes at known concentrations and a given amount of anisole as internal standard were used. An aliquot (1 µL) of each of these stock solutions (Stock F and Stock H) was injected into the GC–MS injection port where it was completely turned to gas and analyzed. All the analyses were performed in splitless conditions in triplicate.

The determination of each analyte concentration level in the headspace gas of essential oil sample was performed by comparing the peak area of each individual headspace analyte with the corresponding peak area in the reference solutions, the peak area of the analytes are always compared with those of the internal standard.

The adopted methodology assumes that the total sample amount introduced into the injection port is vaporized and that all the produced gas reaches the ion source (splitless conditions).

The quantitative results are listed in Table 3.

In this study, the headspace gas in equilibrium with the bergamot oil sample at 0 °C has been also investigated by means of GC–FID in order to validate the proposed methodology (Fig. 2).

The results of GC–FID analysis are comparable to those obtained by GC–MS (Table 3). It can be observed that the gaseous phase composition is quite different from that of the liquid phase at equilibrium with it a 0 °C.

The comparison between the bergamot essential oil composition (Table 3) and that of headspace gas at equilibrium shows how the linalool and the linalyl acetate amounts decrease dramatically in the gas phase on the contrary the concentration of limonene is almost double (approximately 60%).

Furthermore, the β-pinene content, that is very low in the liquid oil, is particularly high in gaseous phase.

The composition of the gaseous phase at 22 °C (room temperature) and 40 °C was determined by using the stock solutions *I*–*N* and *O*–*T* respectively as described in "Experimental" section. The quantitative results are listed in Table 4.

At 22 °C the gas phase in equilibrium with liquid phase is enriched in some components with respect to the composition determined at 0 °C. In fact, α-phellandrene, α-terpinene, γ-terpinene, linalool and linalyl acetate, which were not detected in the gaseous phase at 0 °C, were identified and determined in the gaseous phase at 22 °C. In particular, at 22 °C γ-terpinene and linalyl acetate got to 13.13 and 0.66% respectively and linalool grew from 3 to 9% (Table 2). At both temperature, the main components were limonene (58.07% at 0 °C and 47.27% at 22 °C) and β-pinene (25.90% at 0 °C and 19.69% at 22 °C).

The composition of the headspace vapor generated at 40 °C was characterized by the presence of octyl acetate,

Table 4 Composition of BEO and gaseous phase in equilibrium with the liquid at 0, 22 and 40 °C

Entry	Compound	Essential oil composition[a] GC–MS (w/w% ± SD)	Gaseous phase composition at 0 °C GC–MS (w/w% ± SD)	Gaseous phase composition at 22 °C GC–MS (w/w% ± SD)	Gaseous phase composition at 40 °C GC–MS (w/w% ± SD)	Biological activity
Cyclic hydrocarbon monoterpenes						
1	α-Pinene	1.03 ± 0.10	6.90 ± 0.10	5.38 ± 0.10	1.29 ± 0.03	Anticancer [35] Anti-inflammatory [36]
2	β-Pinene	6.56 ± 0.14	25.90 ± 0.40	19.69 ± 0.31	7.10 ± 0.05	Anti-depressant [37] Antibacterial [38]
3	α-Phellandrene	0.04 ± 0.01	–	0.27 ± 0.02	0.39 ± 0.02	Anti-proliferative [39] Anti-inflammatory [40]
4	α-Terpinene	0.16 ± 0.02	–	0.27 ± 0.01	0.18 ± 0.02	Antioxidant [41]
5	Limonene	30.20 ± 0.77	58.07 ± 0.38	47.27 ± 0.28	37.15 ± 0.29	Anti-inflammatory [42, 43] Anxiolytic [44] Anti-proliferative [45, 46]
6	*p*-Cimene	0.18 ± 0.01	6.36 ± 0.04	0.62 ± 0.03	0.49 ± 0.01	Anti-inflammatory [47] Antifungal [48]
7	γ-Terpinene	11.95 ± 0.32	–	13.13 ± 0.29	12.22 ± 0.1	Antibacterial [49] Antioxidant [49]
8	Terpinolene	0.27 ± 0.03	–	–	–	
Acyclic hydrocarbon monoterpenes						
9	Mircene	0.82 ± 0.02	2.19 ± 0.22	1.42 ± 0.036	0.84 ± 0.02	Analgesic [50] Anxiolytic [51, 52]
10	Ocimene	0.08 ± 0.01	–	–	–	
Acyclic oxygenated hydrocarbon monoterpenes						
11	Linalool	21.82 ± 0.87	3.04 ± 0.54	9.71 ± 0.18	27.52 ± 0.24	Anti-inflammatory [53, 54] Anti-epileptic [55] Anxiolytic [56]
12	Linalyl acetate	16.21 ± 0.84	–	0.66 ± 0.03	10.40 ± 0.08	Anti-inflammatory [57] Analgesic [57] Antibacterial [58]
13	Neral	0.21 ± 0.01	–	–	–	
14	Geranial	0.11 ± 0.01	–	–	–	
15	Neryl acetate	0.28 ± 0.02	–	–	–	
Cyclic oxygenated hydrocarbon monoterpenes						
16	α-Terpineol	0.87 ± 0.08	–	–	–	
Esters						
17	Octyl acetate	0,10 ± 0.01	–	–	1.91 ± 0.04	Anti-inflammatory [59] Analgesic [59]
Sesquiterpenes						
18	β-Caryophyllene	0.14 ± 0.02	–	–	–	

SD standard deviations

[a] The w/w percentages were determined by the internal standard method and referred to the amount of each component contained in 100 g of essential oil

not detected at 22 °C, and the significant decrease of limonene, and α and β-pinene. On the contrary, linalool and linalyl acetate were appreciably increased contributing to the composition of the gaseous phase of BEO at 40 °C with 27.52 and 10.40%, respectively (Table 4).

By comparing the composition of bergamot essential oil with those of the gaseous phase in equilibrium with the liquid phase of BEO at 0, 22 and 40 °C, we observed that seven components of bergamot essential oil (terpinolene, ocimene, neral, gerianal, neryl acetate, α-terpineol, β-cariofyllene) were totally absent in the compositions of all analyzed gaseous phases.

Additionally both in the essential oil and gaseous phase at 40 °C the major components, albeit with different percentages, are limonene, linalool, γ-terpinene and linalyl acetate (Table 4).

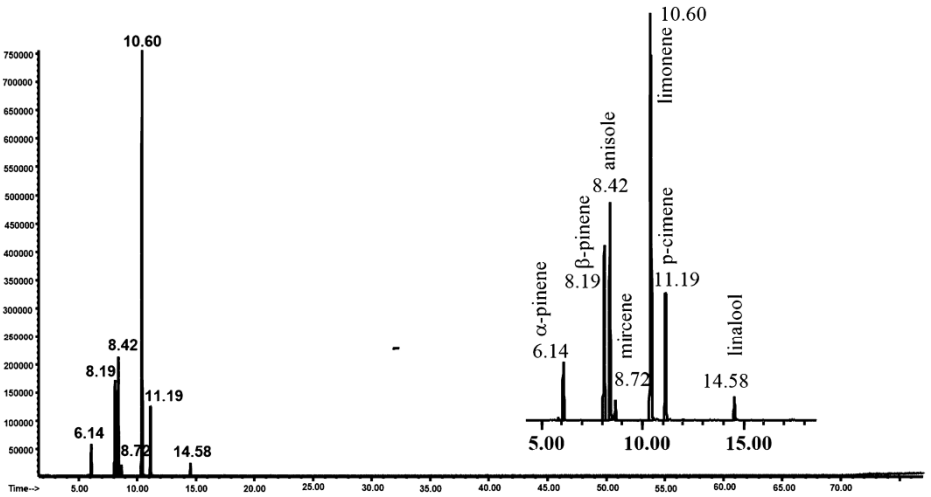

Fig. 1 GC–MS analysis of the gaseous phase of bergamot essential oil at 0 °C (α-pinene $t_R = 6.14$ min; β-pinene $t_R = 8.19$ min; anisole $t_R = 8.42$ min; mircene $t_R = 8.72$ min; limonene $t_R = 10.60$ min; *p*-cimene $t_R = 11.19$ min; linalool $t_R = 14.58$ min)

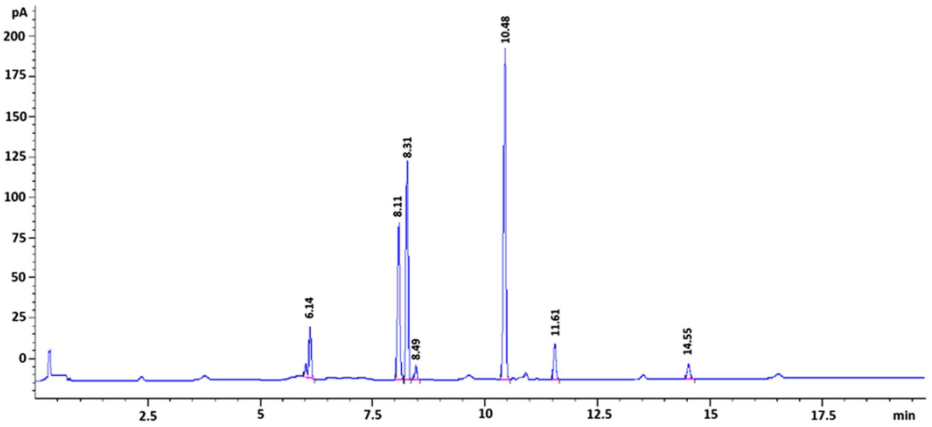

Fig. 2 GC–FID analysis of the gaseous phase of bergamot essential oil at 0 °C (α-pinene $t_R = 6.14$ min; β-pinene $t_R = 8.11$ min; anisole $t_R = 8.31$ min; mircene $t_R = 8.49$ min; limonene $t_R = 10.48$ min; *p*-cimene $t_R = 11.61$ min; linalool $t_R = 14.55$ min)

All these results showed that the compositions of the gaseous phases of BEO generated at various temperatures (0, 22 and 40 °C) are different and change also respect to the composition of the essential oil. Many of the components present in the essential oil are totally absent in the gas phase even at 40 °C while others, present in small portion in the essential oil, are concentrated in the gaseous phase.

The model we studied represents a closed system that, with some limits, mimics the open system in which

aromatherapy is usually performed where the gas composition should change until the equilibrium is achieved in the room environment.

Therefore our system could approximate the conditions under which aromatherapy is practiced.

Conclusion

These results suggest that the determination of the gaseous phase composition in equilibrium with the liquid essential oil is critical for establishing the correlation between the volatile components and their activity.

This study showed that for employing bergamot essential oil in aromatherapy it is not enough to know the essential oil composition but is extremely important to know the volatile fraction composition in equilibrium with it.

This paper reports a GC–MS methodology for the direct analysis of volatile compounds of bergamot essential oil.

The method can also be applied to environments of greater volume provided that the parameters relating to temperature are maintained and that there exist conditions whereby the vapor phase is in equilibrium with the essential oil.

The developed method is quite general and can be applied to other vegetable matrices.

Authors' contributions

AL performed research and drafted the manuscript, VL performed the research, ELB, IS and DT analyzed the data results, MLDG and ER participated in writing and editing results, GS and ALiguori proposed the subject and designed the research. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Research Article Open Access

Nutraceuticals from Olives Plain Water Extraction, Identification and Assay by LC-ESI-MS/MS

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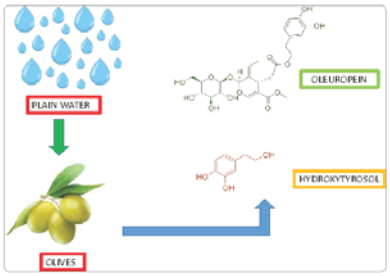
Abstract

Ancient olive trees are widespread in many countries of the Mediterranean area, including the Calabria region located in southern Italy. They have adapted to environments, climates and soil conditions and represent an ecosystem heritage. The use of their drupes for manufacturing both table olive and oil is not often economically and/or commercially attractive. The future to preserve the environment and to develop new jobs in this area is the evaluation of alternative strategies to foster the establishment of small medium enterprises (SMEs) involved in the production of high value added products which might be obtained from these now endemic trees, in an environmental safe way.

High-value active principles can be recovered from wastes of agri-food farms. Olive manufacturers are spread throughout the Mediterranean area, where incomes from this particular food industry represent an important fraction of the gross domestic product (GDP). A typical business of existing SMEs is represented by the commercialization of table olives; the necessary debittering process, traditionally performed by plain water is usually carried in alkaline conditions. The bitterness of freshly harvested olives is due to the presence of antioxidants and anti inflammatory active principles, which belong to the family of the most popular nutraceuticals.

The development of a production line whereby olive wastewaters could be used to produce end products suitable for food, pharmaceutical and cosmetic industries might induce the establishment of new SMEs. Accordingly, waste products of plain water processing of table olives were monitored by Liquid Chromatography (LC)-Electro Spray Ionization (ESI)-tandem mass spectrometry (MS/MS) over a 10-day period to assess the content of the expected phenolic compounds responsible for bitterness and generally recognized for their healthy properties.



Keywords: Table olives; Wastewater recycling; Nutraceuticals; Tandem mass spectrometry

Introduction

Old and environmentally friendly procedures might be reconsidered in olive debittering [1,2] as a *green* strategy in the production of goods in the interest of nutritional, pharmaceutical and cosmetic industries from wastes. The recovering of bergamot albedo from wastes of cosmetic industries has been recently proposed as a source of statin-like active principles [3]. Table olives are the most popular fermented agri-food in Mediterranean countries. Their composition depends on several factors such as genetics, ripening, processing technologies and storage. Standard processing technologies affect the physicochemical features of olive fruits such as color, texture, volatile compounds and phenol contents and, consequently, the organoleptic and nutritional properties of the final product. Any processing method aims, in general, at removing the natural bitterness of the fruit, caused by phenols, especially by the glucosyde known as oleuropein [4]. Oleuropein and its metabolites have powerful antioxidant activity both *in vivo* and *in vitro* making virgin olive oil a functional food. This secoiridoid is able to confer resistance to diseases and insect infestation [5], and offers antioxidant protection due to the catechol moiety [6]. It is also

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accumulated as a defense mechanism against phytophatogens [7,8]. In particular, when olive tissues are injured by diseases or by mechanical damage, a specific enzyme producing highly reactive molecules [7] hydrolyzes oleuropein. The antioxidant and antimicrobial activities of oleuropein derivative molecules against herbivores and insect attacks have been also demonstrated either in plants as well as *in vitro* against bacterial strains [9].

β-glucosidase (E.C. 3.21.1.21) [9,10] is involved during fruit ripening in the progressive degradation of oleuropein with concomitant releasing of glucose and the aglycones with a concomitant reduction in bitterness of the fruit [11,12]. The detected changes in β-glucosidase activity, thus in the products of enzymatic hydrolysis at different stages of fruit ripening [10] are strictly associated with product quality; in fact, good-tasting table olives and olive oil are greatly influenced by phenolic compound content [13-16]. Thus, debittering of green olives is a major challenge in the industrial processing of fruit. The most commonly employed methods involve dilute NaOH and/or NaCl at low concentrations. The alkaline treatment modifies the ester moieties of phenolic compounds present such as oleuropein and its derivatives. The waste produced cannot be safely and easily eliminated [17,18] while the quality of the recovered food is deeply affected. The recovering of antioxidants from olive wastes is an important issue in the Mediterranean area [19].

The alternative approaches providing both debittering and fermentation [16] through the action of bacterial β-glucosidase, interferes, unfortunately, with the antioxidant properties of the products.

A great number of studies describe the phenolic composition of olives [20-24]; their recovering by low cost innovative technologies in concentrated solutions might be of commercial interest in view of the accepted use of these natural compounds as nutraceuticals [25-30]. Nutraceuticals possess the special role of preventing or even supporting medical therapies [31].

This work aims at identifying and assessing active compounds, such as oleuropein, hydroxytyrosol, tyrosol, verbascoside, luteolin and rutin, accumulating in wastes during the simplest debittering processing of olives with plain water. Solutions containing whole, pitted and crushed olives were prepared and target phenols assayed using tandem mass spectrometry. Tandem Mass spectrometry has been extensively used in the field of structure evaluation of natural products [32].

Experimental

Chemicals

Pure samples of rutin, luteolin and verbascoside were purchased from Extrasynthese (Nord B.P 62 69726 Genay Cedex France) and tyrosol, 3-hydroxytyrosol and oleuropein from Sigma–Aldrich (Riedel-de Haën, Laborchemikalien, Seelze). Methanol, ethanol, formic acid were LC/MS grade and purchased from VWR International; aqueous solutions were prepared using ultrapure water, with a resistivity of 18.2 MΩ cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA).

Olives sampling and maceration process

Hand-picked olives of Coratina cultivar, allegedly very rich in antioxidant compounds [33], were collected during the crop year 2013 from an olive grove located in the Basilicata region (Italy) and stored immediately at -25°C. Three different forms of olives, whole, pitted and crushed, were considered for the debittering process. Ultrapure water and ultrapure water-ethanol 80/20 (v/v) were used in two distinct protocols. In a typical experiment, 12 g of olives were placed in stoppered flask containing 100 ml of debittering solution; the maceration process was carried out in the dark at 4°C, except for the first day when the olives were subjected to two thermal shocks caused at first by passing from -25°C to room temperature in the aqueous solutions and then being frozen again. This was to promote the disintegration of the cell membranes and the release of any active compounds. After which, every day for 10 days, aliquots of supernatant were collected and the solution pH was checked. The samples thus prepared were submitted to LC-MS/MS analysis.

Preparation of standard solutions

Standard stock solutions were prepared by dissolving reference compounds in ethanol. Aliquots of these solutions were further diluted with water/0.1% formic acid to obtain calibration standards at concentrations between 1-200 μg/ml for oleuropein and hydroxytyrosol, 1-100 μg/ml for tyrosol, luteolin and rutin, and 1-150 μg/ml for verbascoside.

High performance liquid chromatography (HPLC)

HPLC was performed using an Agilent Technologies 1200 series liquid chromatography system equipped with G1379B degasser, G1312A pump, and G1329A autosampler. The analytes were separated on an Eclipse XDB-C8-A HPLC column [5 μm particle size, 150 mm length and 4.6 mm i.d. (Agilent Technologies, Santa Clara, California)] at a flow rate of 350 μl/min and an injection volume of 10 μl. A binary mobile phase made up of 0.1% aqueous formic acid (A) and methanol (B) was programmed to increase B from 10% to 100% B in 10 minutes, hold for two minutes and ramp down to original composition (90% A and 10% B) in eight minutes. The total elution time was 20 min per injection.

Mass spectrometry

The ESI-MS/MS analyses were performed using a MSD Sciex Applied Biosystem API 4000 Q-Trap mass spectrometer in negative ion mode using multiple reactions monitoring (MRM). The experimental conditions were set-up as follow: ionspray voltage (IS) -4500 V; curtain gas 20 psi; temperature 400°C; ion source gas (1) 35 psi; ion source gas (2) 45 psi; collision gas thickness (CAD) medium. Entrance potential (EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were optimized for each transition monitored.

Results and Discussion

Method validation

Calibration curves reported in Figure 1 were constructed using a least squares linear regression analysis. For each analyte the calibration curves were linear in the concentration range 0-150 μg/mL with correlation coefficients ranging between 0.9990 and 0.9997. Recovery tests were carried out injecting three times two standard solutions at different concentration of the six active molecules. Results from recovery experiments at levels of 25 and 50 μg/ml gave mean recoveries ranging from 91–116% with satisfactory precision (relative standard deviation (RSD) from 0.1-0.5%). Limits of quantitation (LOQs) from 1.258-2.847 μg/ml (Table 1).

The MS instrument operating parameters were optimized for each analyte of interest by direct injection of the analyzed solution. The CID-MS/MS spectra of the deprotonated molecular ion $[M-H]^{-1}$ are shown in Figure 2. MRM analyses were carried out by monitoring the

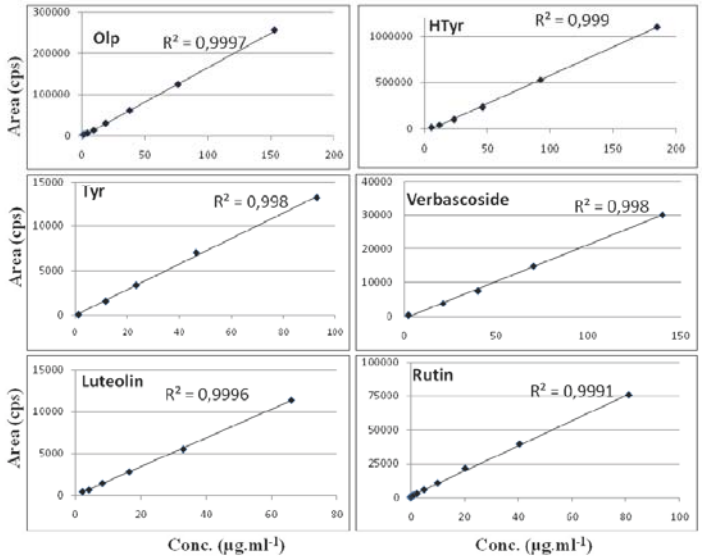

Figure 1: External calibration curve of oleuropein, tyrosol, hydroxytyrosol, rutin, luteolin and verbascoside obtained injecting different concentrations of the mix standard solution.

	Spiked solutions				
			(a)		(b)
Active compound	LOD	Found	Recovery	Found	Recovery
	(µg/ml)	mean ± RSD*	(%)	mean ± RSD*	(%)
Oleuropein	1.258	58.0 ± 0.1	116	25.3 ± 0.2	101
Hydroxytyrosol	1.479	52.5 ± 0.1	105	24.5 ± 0.3	98
Tyrosol	2.418	49.5 ± 0.2	99	26.0 ± 0.3	104
Verbascoside	1.215	45.5 ± 0.5	91	27.3 ± 0.1	109
Luteolin	1.658	50.5 ± 0.3	101	24.8 ± 0.2	99
Rutin	2.847	51.5 ± 0.1	103	22.5 ± 0.1	90

Table 1: LOD, recoveries and precision (RSD) of the active compounds analysed by LC-ESI-MS/MS. *Each value is the mean of three determinations; RSD=relative standard deviation; Spiked solutions (a) 50 µg/ml; and (b) 25 µg/ml.

transition reported in Table 2.

A SIM-like analysis was considered for tyrosol (F) since its product ion spectrum under the experimental conditions adopted for the other analytes did not produce any important fragments. The MRM measurements were, therefore, carried out by modulating the first quadrupole scanning for the transmission of the $(M-H)^-$ value of the six analytes (Table 2) and scanning the third analyzer according to Table 2. A MRM chromatogram obtained by injecting 10 µl of a standard mixture of the compounds under investigation at 25 µg/ml is shown in Figure 3.

LC-ESI-MS/MS analysis was performed on aliquots of each sample collected. Aqueous solutions containing whole and crushed olives gave a pH value of 6 regardless of whether water/ethanol (80:20) or pure water was used. However, the aqueous solutions containing pitted olives was pH 5 suggesting the presence of the stone was involved in this variation. The results obtained and a discussion for each compound analysed (below) are also presented in Figure 4.

Oleuropein (Olp)

The ESI mass spectrum of the negative (-) ions shows the $(M-H)^-$ species together with the known diagnostic fragments at m/z 275 and m/z 307 [34,35] (Figure 2). Olp, as expected, was one of the major components of wastes. The maximum concentration of this secoiridoid (409 mg/kg), reached after the first day when plain water is used, slowly decreases to half (228 mg/kg) in the subsequent days (Figure 4). A similar trend was observed when water/ethanol (80:20) was used although the maximum concentration observed after one day (369 mg/kg) reaches the mean value 264 mg/kg.

A marked difference was observed when crushed and pitted olives were debittered (Figure 4). The maximum concentration achieved after one day was 1187 mg/kg and 1118 mg/kg for pitted and crushed drupes, respectively. This value decreases according to enzymatic kinetics to reach a value of 8 mg/kg on the last day. The solution containing water/ethanol (80:20) since day one gave a lower content of olp (665 mg/kg for crushed olives and 754 mg/kg for pitted olives). These values decrease during the days until they reached the mean values 139 mg/kg and 162 mg/kg for crushed and pitted olives, respectively (Figure 4).

Hydroxytyrosol (HTyr)

The mass spectrum for this compound in negative ion mode gave the deprotonated molecule at m/z 153 and the fragment at m/z 123 due to the loss of the CH_2OH group (Figure 2).

HTyr is frequently detected in olive pulp and has been widely studied, in particular its anti-oxidant activity and its health-beneficial properties [36]. The concentration of HTyr in the solution containing

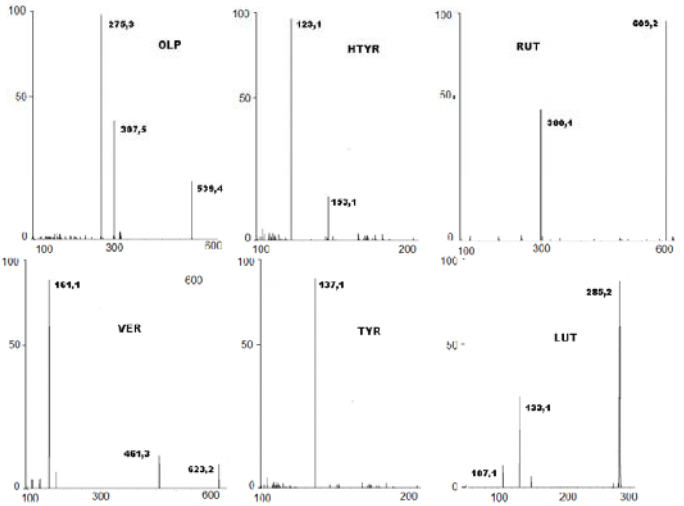

Figure 2: ESI-MS/MS spectra of oleuropein, hydroxytyrosol, tyrosol, verbascoside, luteolin and rutin showing the deprotonated molecular ion $[M-H]^-$ and the major fragments utilised to set up the MRM method.

Analyte	1st quadrupole $(M-H)^-$	2nd quadrupole	EP	DP	CE	CXP
(A) Hydroxytyrosol	*m/z* 153	*m/z* 123	-55	-7	-25	-5
(B) Luteolin	*m/z* 285	*m/z* 133	-70	-8	-35	-8
(C) Oleuropein	*m/z* 539	*m/z* 307 *m/z* 275	-65	-10	-30	-5
(D) Rutin	*m/z* 609	*m/z* 301	-65	-8	-45	-10
(E) Verbascoside	*m/z* 623	*m/z* 161 *m/z* 461	-70	-8	-35	-8
(F) Tyrosol	*m/z* 137	No scan	-45	-8	-25	-5

Table 2: Data set for the assay of active principles A-F. Optimized entrance potential (EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP).

pure water and whole olives on the first day was 21 mg/kg. This content increases during the next nine days of experimentation to reach the mean value of 171 mg/kg. The solution containing 20% of ethanol gave an initial value of HTyr double than the solution of pure water (50 mg/kg). Moreover, the concentration of the compound at the end of the experimentation was found to be less than in the previous one (149 mg/kg). The solutions containing pure water and either crushed and pitted olives showed a high content of HTyr already in the first day of the experimentation (261 and 334 mg/kg, respectively). These values decrease over time to almost zero (2 and 14 mg/kg, respectively). It is to be noted that by using 20% of ethanol the content of HTyr is preserved over time and, in fact, the concentration of the compound found at the end of the experimentation was 60 and 87 mg/kg in the solution containing crushed and pitted olives, respectively. The patterns observed in the three cases (whole, pitted and crushed olives) in the two solutions (pure water and water/ethanol (80:20)) are quite different. In the case of the solution containing whole olives the concentration of the compound increases over time and this increment is due both to its initial content and both to the degradation of secoiridoid compounds. This low accumulation observed could be attributed to the fact that in whole olives the membrane is not damaged and the release of the active compound is slowed down. In the case of the solution containing crushed and pitted olives the concentration of HTyr decreases over the time. The presence of the stone seems to favour a faster degradation of the compound under investigation (Figure 4).

Tyrosol (Tyr)

In all cases analysed the content of Tyr increases (Figure 4). This increase can be attributed both to its initial content and to the degradation processes of higher molecular weight molecules. In the solutions containing whole olives the concentrations of Tyr on the first day was found to be 8 mg/kg in pure water and 9 mg/kg in water/ethanol (80:20). At the end of the debittering process these contents were 39 and 31 mg/kg, respectively. The concentration of Tyr in crushed olives on the first day of the debittering process was 25 mg/kg in pure water and 27 mg/kg water/ethanol (80:20). At the end of the experimentation these contents were 65 and 56 mg/kg respectively. The solvent in these two cases does not affect the Tyr content. The situation appears different in the solutions containing pitted olives. In fact, the Tyr content varies from 24 to 100 mg/kg in pure water and from 25 to 40 mg/kg in water/ethanol (80:20).

Verbascoside

The ESI mass spectrum in negative ion mode was represented by

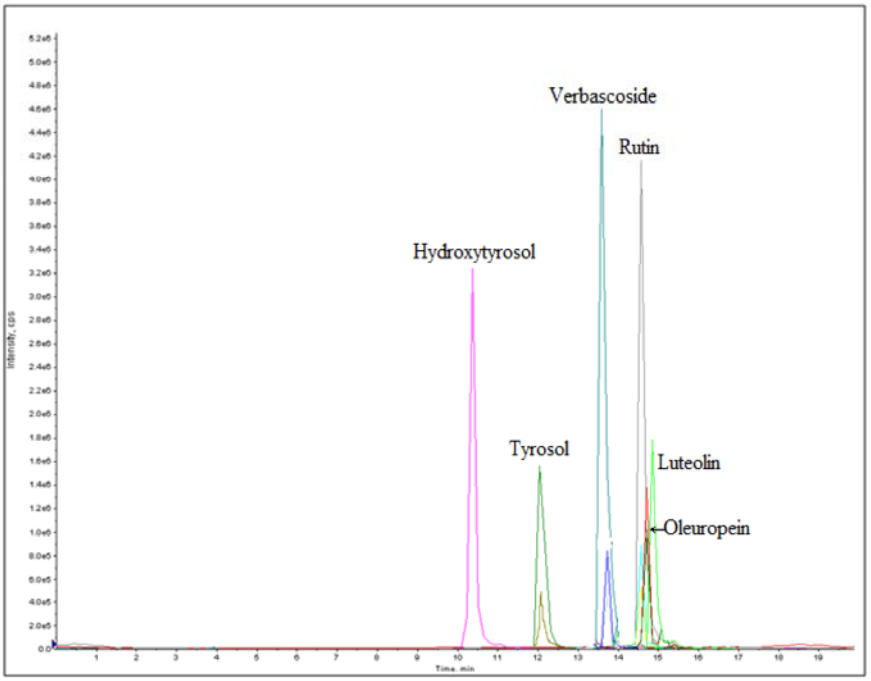

Figure 3: MRM chromatogram obtained injecting 10 µl of a standard mixture of the analytes of interest at 25 µg/ml.

the deprotonated ion at m/z 623 with few other fragments of lower intensity (Figure 2). Particularly, two ions characteristic of verbascoside at m/z 461 and m/z 161 were observed. The loss of caffeic acid produced an ion at m/z 461 and a ketene as a neutral fragment, while the peak at m/z 161 resulted from a proton transfer and production of an anionic ketene [35].

The trend observed for all the solutions was similar to the one observed for HTyr: the verbascoside tends to increase during the process of debittering. In the solutions containing whole olives the content of the active compound varies from 196 to 365 mg/kg on the first and last day, respectively. In water/ethanol (80:20) its content varies from 338 to 441 mg/kg on the first and last day, respectively.

The solution containing crushed and pitted olives in pure water showed a high content of verbascoside already on the first day of the experimentation (802 and 1319 mg/kg for crushed and pitted olives respectively). These values decrease over the time to almost disappear (1 and 4 mg/kg respectively) (Figure 4).

Luteolin

The ESI mass spectrum was characterized by an intense ion at m/z 285 and a fragment of m/z 133 of lower intensity (Figure 2).

In several studies this flavonoid has been detected in olive fruits of different cultivars and has proved to possess important biological properties, such as anti-oxidant, anti-inflammatory, anti-microbial and cardio-tonic activity, ability to scavenge free radicals and to inhibit low-density lipoprotein oxidation [34]. Luteolin is responsible, along with other carotenoid compounds, for the colour of the drupes. Fermentation and debittering processes promote its release into solution that is pronounced if olives are damaged. Moreover, in the presence of solvents such as ethanol, this release is marked. Also, the absence of light preserves the colour of the final solutions.

During the ten days of experimentation, depending on the olives utilized, different gradients of colour were obtained. Whole olives gave, at the end of the experimentation, a dark green colour, whereas crushed and pitted ones gave a browny green colour that was more evident in the ethanolic solutions. All solutions analysed showed an increase of the active compound. In particular, in aqueous solution containing whole olives the luteolin content varies from 66 to 218 mg/kg during the experimental period, whereas in the ethanol solution from 83 to 287 mg/kg. The solutions containing crushed and pitted olives in pure water showed a higher content of luteolin during the first days of the experimentation; these values will not vary significantly over the period of the debittering process. In fact, the content of luteolin for crushed olives varies from 126 to 202 mg/kg whereas for pitted olives from 110 to 190 mg/kg. The ethanol solutions have a different pattern with respect to the previous ones examined. In fact, from day one the solutions are very rich in luteolin and increases during all the debittering process. At the end of the experimentation almost 700 mg/

kg were found in the solution containing crushed olives and almost 800 mg/kg in the solution containing crushed ones (Figure 4).

Rutin

The ESI mass spectrum was characterized by an intense deprotonated molecular ion at m/z 609 and a fragment at m/z 301 which is diagnostic of quercetin derivatives (Figure 2). Many biological effects have been attributed to this flavonoid, which shows anti-oxidant, anti-inflammatory, anti-thrombotic, cytoprotective, vasoprotective and anti-microbial activity [37]. This glycoside flavone is stable in aqueous solution at neutral or alkaline pH; in an acid or by the action of enzymes specific hydrolase, the glycosidic bond is broken with the formation of anomeric hemiacetals. In the aqueous solutions analysed the concentration of rutin, from the first to the last day of experimentation, does not change much and remains constant when whole olives are used (from 40 to 44 mg/kg). A decrease in the concentration can be observed for crushed olives (from 110 to 31 mg/kg) and for pitted olives (from 179-101 mg/kg). The difference that can

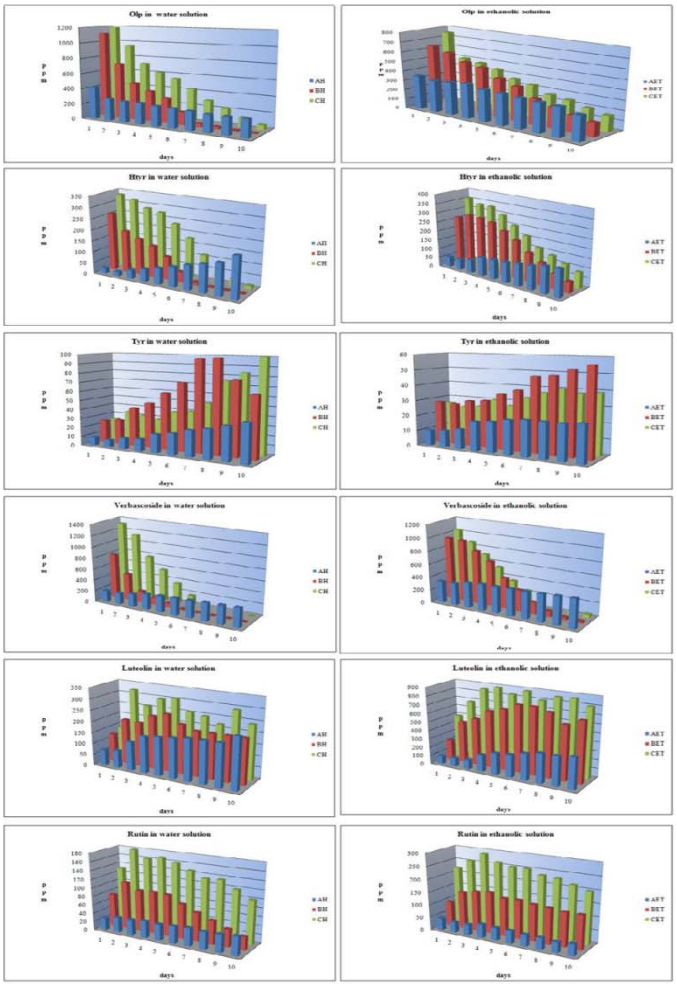

Figure 4: Trend of the debittering process in whole olives, crushed olives and pitted olives. Bar charts show the decay of active antioxidant compounds in the three thesis with particular emphasis on the eluent phases utilised: water and water/ethanol (80/20). The letters A, B and C are for whole olives, crushed olives and pitted olives respectively. H and ET are for water solution and 20% ethanolic solution respectively. The numbers 1 to 10 indicate the day of the experimentation.

be noticed when ethanol is added is that the content of rutin released is far greater, but constant throughout the experimentation period. For whole olive the content of rutin is the same as in the aqueous solution (from 41 to 49 mg/kg). The solutions containing pitted olives showed an increased content of the compound than those containing crushed olives (from 144 to 132 mg/kg and from 221 to 200 mg/kg, respectively) (Figure 4).

Conclusion

The results discussed above show, from a chemical point of view that, as expected, the glycosidic antioxidants are less stable in water than the other detected active principles, even in the absence of chemical catalysts that could affect the process. The observed behaviour is, therefore, probably driven by those deglycosylation enzymes which are extracted from the drupes together with the active species listed in Table 1 [24]. This observation allows the choice of the experimental conditions to be applied to favour the recovering of a particular family of nutraceuticals from olive drupes.

In conclusion, the results presented above provide evidences on the possibility of devising new strategies in the recovering of active principles from olives. They can be obtained either by mild procedures applied to the debittering of table olives or by treatment of wild olive drupes widespread in the Mediterranean area.

Acknowledgements

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Grazie, Grazie, Grazie

Ilaria

UNIVERSITÀ DELLA CALABRIA
CENTRO INTERDIPARTIMENTALE
FUNCTIONAL FOODS AND PET THERAPY
(FOODPET)

Arcavacata 28 Febbraio 2018

Dottorato di Ricerca in Medicina Traslazionale, indirizzo Progettazione Molecolare,
(XXIX CICLO, progettazione Molecolare)
Giudizio del Supervisore sull'attività della dottoranda SANTORO ILARIA

La dott.ssa ***Ilaria Santoro*** ha conseguito la laurea magistrale in Chimica ad indirizzo Organico, nel Dicembre 2012, con la votazione 107/110, discutendo la tesi sperimentale **La spettrometria di massa tandem nell'identificazione e reattività di microelementi algali: vitamina B12, omega 3 e omega 6**, svolta sotto la direzione del prof. Giovanni Sindona presso il Dipartimento di Chimica e Tecnologie Chimiche dell'Università della Calabria, in collaborazione con il centro CRA- Oli di Rende
Durante lo svolgimento della tesi la candidata ha avuto la possibilità di approfondire le metodologie che possono essere riassunte nel seguente schema:

- Metodi di estrazione *soft* ed ecosostenibile di principi attivi con proprietà sia nutraceutiche che utilizzabili in ulteriori trasformazione chimiche, per esempio nel settore energetico;
- Metodi di analisi a livello molecolare basati sulla spettrometria di massa *tandem* per la determinazione della struttura e della quantità relativa dei principi attivi direttamente dalla miscela;
- Caratterizzazione molecolare ed isolamento dei principi attivi dalla miscela.

L'oggetto della tesi di dottorato di ricerca rappresenta l'implementazione delle conoscenze e metodologie che avevano caratterizzato la formazione precedente, nella messa a punto, con notevole successo, dei composti bioattive presenti nei tessuti dell'olivo e di eucarioti unicellulari presenti nelle alghe.
In tale attività, svolta sotto la supervisione del relatore prof. Giovanni Sindona, è stata importante il contributo che la dott.ssa Monica Nardi nella formazione della candidata nelle metodologie *eco friendly*.
La figura scientifica della dott.ssa Santoro Ilaria si ricava fondamentalmente dal contributo scientifico descritto dalle pubblicazioni su riviste internazionali e dalla partecipazione a convegni nazionali ed internazionali di seguito riportati

Lavori scientifici prodotti e pubblicati durante il periodo di dottorato

- ***Tandem mass spectrometry: a convenient approach in the dosage of steviol glycosides in Stevia sweetened commercial food beverages***. *J. Mass Spectrom.* **2017**, *52*(5), 290-295. Di Donna, L., Mazzotti, F., **Santoro, I.,** Sindona, G.
- ***Aromatherapy: composition of the gaseous phase at equilibrium with liquid bergamot essential oil.*** *Chem Cent J.* **2017**, *11*(1), 111.Leggio, A., Leotta, V., Belsito, E. L., Di Gioia, M. L., Romio, E**., Santoro, I**., Liguori, A..
- ***Nutraceuticals from Olives Plain Water Extraction, Identification and Assay by LC-ESI-MS/MS***. *J Anal Bioanal Tech* **2015**, *6*(6), 1. Benincasa, C., Perri, E., Romano, E., **Santoro, I.,** Sindona, G.

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Comunicazioni a Congresso

- ***A Virtuous Approach to Block Lipid Peroxidation in Algal Oil***. CIS6 6th Czech Italian Spanish Conference on Molecular sieves and Catalysis GIC 2015 XVIII National Congress on Catalysis AIZ 2015 XII National Congress of Zeolite Science and Technology. Amantea (CS) 14-17 giugno 2015. Nardi M., Perri E., Russo A., **Santoro I.,** Sindona G.;

- ***Catalytic Behavior in Propane Aromatization Using Ga-MFI Catalyst*** CIS6 6th Czech Italian Spanish Conference on Molecular sieves and Catalysis GIC 2015 XVIII National Congress on Catalysis AIZ 2015 XII National Congress of Zeolite Science and Technology. Amantea (CS) 14-17 giugno 2015M. Aloise A., **Santoro I.**, Catizzone E., MiglioriM., Nagy J. B., Giordano G.;

- ***Natural Antioxidants and Oxidative Stability of Algae Oil: A Tandem Mass Spectrometry Investigation***. VIII Convegno Congiunto della sezioni Calabria e Sicilia SCI. Arcavacata di Rende (CS), 6-7 Dicembre 2012, Russo A., **Santoro I.**, Perri E., Sindona G.

- ***Steviol Glycosides in Food Beverages by Tandem Mass Spectrometry***. Workshop delle Sezioni Sicilia Calabria SCI - Messina 2016-17 P29. **Santoro I.**, Di Donna *L.*, Mazzotti *F.*, Sindona *G.*

- ***Improvements of extraction and identification methodologies of PUFA from algae***. XXIV Congresso Nazionale della Società Chimica Italiana 10-14 Settembre 2017, Paestum (NA) MAS-OR13. **Santoro I.**, Sindona *G.*, Nardi *M.*, Benincasa *C.*

- ***Identification of new adducts of fatty acids by LC-MS/MS mass spectrometry***. 5th MS FOOD DAY October 11-13, 2017 Bologna-Italy. Benincasa *C.*, Nardi *M.*, **Santoro I.**, Sindona G.

- ***The use of green chemistry for the recovery of nutraceuticals from olive tissues***. 5th MS FOOD DAY October 11-13, 2017 Bologna-Italy. **Santoro I.**, Benincasa *C.*, Pellegrino *M.*, Cassano *A.*, Perri E., Sindona G.

In conclusione il sottoscritto prof. Giovanni Sindona, ordinario di Chimica Organica, noto nel mondo per il suo contributo all'approfondimento delle metodologie MS/MS in *Food Chemistry*, esprime parere pienamente positivo sulla professionalità acquisita dalla candidata ***Ilaria Santoro*** che potrà essere spesa opportunamente nel settore applicativo dei nuovi farmaci denominati nutraceutici, essendo in grado di progettare la loro identificazione in fonti naturali, il loro isolamento e la loro caratterizzazione strutturale.
Il prof Giovanni Sindona esprime parere decisamente positivo per il conferimento del Dottorato di Ricerca in Medicina Traslazione alla candidata SANTORO ILARIA.
Prof. Giovanni Sindona

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